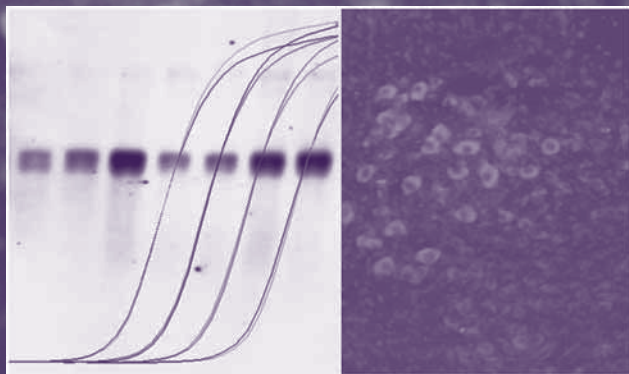


Protocols for Nucleic Acid Analysis by Nonradioactive Probes

Second Edition

Edited by

**Elena Hilario
and John Mackay**



**Protocols for Nucleic Acid Analysis
by Nonradioactive Probes, Second Edition**

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Preface

Since *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* was published in 1994, the use of techniques such as Southern and Northern blotting has continued unabated, despite the continuing explosion of polymerase chain reaction (PCR)-based techniques that have replaced many traditional molecular biology methods. Indeed, PCR is now frequently used with nonradioactive probe formats in applications such as real-time PCR that may be likened to an “online” Southern or Northern blot!

More often than is realized, radioactive techniques are not essential for the analysis of nucleic acids in molecular biology. Nonradioactive methods have been available for the scientific community for more than 20 years, providing excellent results. Scientists who rely on nonradioactive techniques have indirectly promoted the development of new nonradioactive haptens, substrates, means of detection, and a wide range of novel applications. These improvements on the detection limits of nonradioactive methods can easily compete with radioactive protocols, which are now seen as slow, cumbersome, and only required in very specific experimental designs.

Protocols for Nucleic Acid Analysis by Nonradioactive Probes, Second Edition aims to provide a firm background on the basic preparative protocols required for the analysis of nucleic acids by nonradioactive methods, as well as presenting the methodologies using these amazing new applications. This volume offers guide chapters on nucleic acid extractions, preparation of nucleic acid blots, and labeling of nucleic acids with nonradioactive haptens. There are two ways of detecting nonradioactive probes: by indirect methods using a labeled probe or by directly detecting the labeled nucleic acid. We have divided the contents accordingly. These are key examples of the extensive potential that nonradioactive detection provides to the molecular biology community. Our target audience is not limited to the research laboratory only; we hope that tertiary students and post-doctorates will find the content of this book a useful reference guide in their projects.

Elena Hilario
John Mackay

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I _____

NUCLEIC ACID EXTRACTIONS

Genomic DNA Isolation From Different Biological Materials

Duckchul Park

Summary

A comprehensive collection of different methods for extracting high-quality genomic DNA from Gram-positive and -negative bacteria and fungal mycelium and spores is described in this chapter. Special care has been taken in describing the ideal ratio of biological material to chemical reagents for an efficient extraction of genomic DNA, and in stating the appropriate application in molecular biology protocols (e.g., PCR or genomic DNA library-cloning quality).

Key Words: Fungal spores; fungi mycelium; genomic DNA isolation; Gram-negative bacteria; Gram-positive bacteria.

1. Introduction

Recently, genomic DNA isolation from living material, such as bacteria, fungi, plants, insects, animal cells, and blood, has become increasingly popular as the phylogenic or the population genetics research become increasingly important owing to environmental concerns. For these molecular genetic studies, it is important to isolate genomic DNA.

As a result, many molecular biology companies are producing specific genomic DNA isolation kits for a range of biological material. Most of these commercial products use columns and DNA-binding resin, which breaks the genomic DNA into small pieces. The cost of these products, however, can be prohibitive for large numbers of samples.

Conventional genomic DNA isolation from biological material involves three steps. The first step is lysis of the cell wall or membrane. The second step is the removal of any unwanted content, which may include proteins, polysaccharides, or cell wall debris. The third step is recovery of the pure DNA.

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There are many different protocols based on this procedure, differing in the type and quantity of reagent used. If researchers better understand the processes involved in DNA isolation, new and superior protocols can be specifically designed for their needs.

In the future, an automatic liquid handler or robotic arm will be one of the core laboratory instruments in the molecular biology laboratory, along with a sequencing machine and a real-time PCR instrument. Therefore, it makes sense to design new DNA isolation protocols suitable for automatic liquid handlers, in which time and labor are saved in the analysis of large quantities of samples.

Commercial genomic DNA isolation kits that are on the market follow the basic three-step DNA isolation procedure, but also involve the use of chaotropic salt and silica-based membranes. There are some exceptions to this (e.g., Prepman™ Ultra, Applied Biosystems).

In this chapter, several different DNA isolation protocols, which have consistently produced good results, are presented, along with detailed explanations and cautionary comments.

2. Materials

2.1. Genomic DNA Isolation From Gram-Negative Bacteria: CTAB Method

1. Tris-HCl-EDTA (TE) buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0. TE buffer can be made from 1 M Tris-HCl, pH 8.0, and 0.5 M EDTA, pH 8.0, stock solutions. EDTA powder will not dissolve until the pH of the solution reaches approx 8.0, after the addition of NaOH.
2. 20 mg/mL proteinase K (in H₂O). Proteinase K powder easily dissolves in H₂O without sterilization by filtration, because any contamination of enzyme can be digested with proteinase K itself. The stock solution of proteinase K can be stored at -20°C for long periods. Proteinase K may precipitate at -20°C, so ensure that it is fully dissolved before use.
3. 10% Sodium dodecyl sulfate (SDS). Autoclaving not required. **Caution:** always wear a protective mask while handling SDS powder.
4. 5 M NaCl.
5. Hexadecyl trimethyl-ammonium bromide (CTAB)/NaCl solution: 10% CTAB in 0.7 M NaCl. Add the CTAB powder slowly to the 0.7 M NaCl solution, while heating and stirring. The CTAB powder will take up a lot of the volume; therefore, add the powder to a volume of NaCl solution that is only 70% of the final volume.
6. Chloroform/isoamyl alcohol (24:1, v:v).
7. Phenol/chloroform/isoamyl alcohol (25:24:1). Melt the phenol on a hot plate or in a hot water bath. Equilibrate with an equal volume of sterile Tris-HCl-NaCl-EDTA (TNE) buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 1 mM EDTA) or TE buffer (pH 8.0). Incubate the mixture at room temperature for 2 to 3 h. Remove and discard the top layer. Add an equal volume of chloroform/isoamyl alcohol (24:1) to

the remaining layer. Mix thoroughly. Remove and discard the top layer. Store the bottom layer of phenol/chloroform/isoamyl alcohol at 4°C, away from light. It can be stored for up to 2 mo. **Caution:** phenol causes severe burns, so always wear gloves and safety glasses.

8. Isopropanol.
9. 70% Ethanol.
10. Heat block or water bath.
11. Microcentrifuge and vacuum microcentrifuge.
12. Spectrophotometer.

2.2. Genomic DNA Isolation From Gram-Negative Bacteria: Phenol Method

1. TNE buffer: 25 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, and 100 mM NaCl.
2. TE buffer (see **Subheading 2.1.**).
3. 100 µg/mL RNase A.
4. 100 µg/mL proteinase K.
5. 20% SDS.
6. Phenol/chloroform/isoamyl alcohol.
7. Chloroform/isoamyl alcohol.
8. 100 and 70% Ethanol.

2.3. Genomic DNA Isolation From Gram-Positive Bacteria

1. Sucrose–EDTA–Tris-HCl (SET) buffer: 20% sucrose, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.6.
2. 10 mg/mL RNase A solution. Dissolve 10 mg of RNase A powder in 1 mL of 10 mM Tris-HCl, pH 7.5, and 15 mM NaCl, in a microcentrifuge tube. Boil for 15 min and cool slowly to room temperature. Store at room temperature or at –20°C for longer-term storage.
3. 20 mg/mL proteinase K.
4. 5 mg/mL Lysozyme in water, freshly prepared.
5. 20% SDS; autoclaving not required.
6. 5 M NaCl.
7. 100% Ethanol.
8. Buffer-saturated phenol. Instead of buffer-saturated phenol, water-saturated phenol can be used.
9. Chloroform/isoamyl alcohol.
10. TE buffer (see **Subheading 2.1.**).

2.4. Genomic DNA Isolation From Fungi

DNA is isolated from *Mycelia* grown on agar media, using the DNeasy Plant Mini® kit (Qiagen).

1. Mortar and pestle: wrap with foil, and autoclave.
2. Liquid nitrogen. **Caution:** use protective glasses and clothing.

3. Vacuum centrifuge (Speed Vac™[Savant] or Freeze Dryer).
4. DNeasy Plant Mini kit.

2.5. DNA Isolation From Mycelium Grown in Liquid Media

1. Mira-Cloth® (Calbiochem).
2. Liquid nitrogen. **Caution:** use protective glasses and clothing.
3. Mortar and pestle: wrap with foil, and autoclave.
4. Vacuum centrifuge (Speed Vac or Freeze Drier).
5. Lysis buffer: 150 mM NaCl, 50 mM EDTA, and 10 mM Tris-HCl, pH 7.4.
6. 20 mg/mL proteinase K.
7. 20% SDS.
8. CTAB buffer: 10% CTAB, 500 mM Tris-HCl, and 100 mM EDTA, pH 8.0.
9. 5 M NaCl.
10. 100 and 70% Ethanol.
11. TE buffer (see **Subheading 2.1.**).
12. DNeasy Plant Mini kit.

2.6. Genomic DNA Isolation From Small Amounts of Fungal Spores

1. CTAB extraction buffer: CTAB 2% (w/v), 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, and 20 mM EDTA, pH 8.0.
2. β -Mercaptoethanol.
3. Polyvinylpyrrolidone (PVP) 360.
4. Glass beads (3-mm diameter; 1-mm diameter can also be used). Wash the glass beads with diluted HCl and water, and autoclave.
5. Chloroform/isoamyl alcohol (24:1 v:v).
6. 100 and 70% Ethanol.
7. TE buffer (see **Subheading 2.1.**).

3. Methods

3.1. Genomic DNA Isolation From Gram-Negative Bacteria: CTAB Method

DNA isolation from Gram-negative bacteria is fairly easy and straightforward. It follows the same three conventional steps as standard DNA isolation. SDS is used to lyse the cell walls, which are easily disrupted. Protein denaturation and polysaccharide removal vary for different protocols. Generally, CTAB and phenol are the two key components in protein denaturation (**1,2**). The DNA precipitation step is identical to all protocols that use ethanol precipitation.

1. Streak a single colony of bacteria onto appropriate agar media in a Petri dish (see **Note 1**).
2. Incubate the culture at the appropriate temperature until fully grown (cell density, $4\text{--}5 \times 10^8$ cells/mL).

3. Using a mini spatula, collect and resuspend the cells in 567 μL TE buffer, add 3 μL of 20 mg/mL proteinase K and 30 μL of 10% SDS, and incubate for 30 min at 37°C (see **Note 2**).
4. Add 100 μL of 5 M NaCl. Mix thoroughly (see **Note 3**).
5. Add 80 μL CTAB/NaCl solution. Mix thoroughly and incubate for 30 min at 65°C (see **Note 4**).
6. Extract with an equal volume of chloroform/isoamyl alcohol. Centrifuge for 15 min at 17,000g in a microcentrifuge (see **Note 5**).
7. Transfer the aqueous phase to a fresh microcentrifuge tube. Extract with an equal volume of phenol/chloroform/isoamyl alcohol. Centrifuge for 15 min at 14,000g in a microcentrifuge.
8. Transfer the aqueous phase to a fresh tube. Precipitate the DNA with 0.6 volumes of isopropanol.
9. Wash the DNA pellet with 1 mL of 70% ethanol.
10. Dry the DNA in a vacuum centrifuge for 5 min (see **Note 6**).
11. Resuspend the DNA pellet in 100 μL TE buffer (see **Note 7**).
12. Determine the DNA purity (A_{260}/A_{280} ratio) and concentration using a spectrophotometer (see **Note 8**).

3.2. Genomic DNA Isolation From Gram-Negative Bacteria: Phenol Method

This is a typical DNA isolation method for Gram-negative bacteria, and includes more phenol/chloroform steps than the CTAB method. It produces high-quality DNA and is particularly useful if the bacterial cells have a high quantity of polysaccharides.

1. Streak a single colony of bacteria onto appropriate agar media in a Petri dish.
2. Incubate the culture at appropriate temperature until fully grown (cell density, 4–5 $\times 10^8$ cells/mL).
3. Harvest the cells using a mini spatula.
4. Resuspend the cell pellet in 500 μL TNE buffer, and vortex.
5. Add 100 μL of 20% SDS and leave for 10 min at room temperature.
6. Add 400 μL of phenol/chloroform/isoamyl alcohol, and mix the tubes until the contents have been emulsified.
7. Separate the phases by centrifuging at 14,000g for 15 min at room temperature.
8. Using a wide-bore pipet tip, transfer the upper phase to a fresh tube.
9. Precipitate the DNA by adding 2 volumes of -20°C , 100% ethanol (see **Note 9**).
10. Dry the pellet in a vacuum centrifuge.
11. Add 100 μL TE buffer to the tubes.
12. Add 1 μL RNase A and incubate for 1 h at 37°C.
13. Add 1 μL proteinase K and incubate for 1 h at 37°C.
14. Add 250 μL TE buffer and 350 μL phenol/chloroform/isoamyl alcohol, mix, and centrifuge at 14,000g.
15. Recover the supernatant and place in a new tube.

16. Add 350 μL chloroform/isoamyl alcohol and centrifuge at 14,000*g*.
17. Recover the supernatant, add 2 volumes of 100% ethanol at -20°C , and mix.
18. Centrifuge at 14,000*g* for 15 min at 4°C . Discard the supernatant.
19. Add 1 mL of 70% ethanol, and mix.
20. Centrifuge for 10 min at 4°C . Discard the supernatant.
21. Dry the DNA in a vacuum centrifuge for 5 min (see **Note 10**).
22. Add 100 μL TE buffer.
23. Determine the DNA purity (A_{260}/A_{280} ratio) and concentration using a spectrophotometer (see **Note 8**).

3.3. Genomic DNA Isolation From Gram-Positive Bacteria

Gram-positive bacteria have relatively thick cell walls, which consist mainly of peptidoglycan (40–80% dry weight). This thick peptidoglycan layer contributes to the rigidity of the Gram-positive bacteria, making it difficult to break the cell walls. Special treatment using lysozyme and osmotic shock are, therefore, required. Once the cell wall is broken and the cytoplasm released, the remaining protocol is the same as for Gram-negative bacteria.

1. Streak a single colony of bacteria onto appropriate agar media in a Petri dish (see **Note 1**).
2. Incubate the culture at appropriate temperature until fully grown (cell density $4\text{--}5 \times 10^8$ cells/mL).
3. Collect the cells with a mini spatula, resuspend in 500 μL TE buffer, and centrifuge at 14,000*g* for 1 min at room temperature (see **Note 11**).
4. Resuspend the cell pellet in 500 μL SET buffer and add 50 μL of lysozyme. Incubate at 37°C for 30 min (see **Note 12**).
5. Divide the cell suspension into two microcentrifuge tubes. Add 200 μL TE buffer and 30 μL of 20% SDS solution to each tube. Immediately mix the contents by inverting the tubes several times (see **Note 13**).
6. Add 100 μL of 5 *M* NaCl and instantly mix (see **Note 13**).
7. Add an equal volume of saturated phenol and mix the tubes until the contents have been emulsified. Vortex for a short time (see **Note 14**).
8. Separate the phases by centrifuging at 14,000*g* for 15 min at room temperature.
9. With a wide-bore pipet tip, transfer the upper phase to a fresh tube.
10. Add an equal volume of chloroform/isoamyl alcohol, and mix by inversion.
11. Repeat **steps 8 to 10** until no white visible layer is present between the phases.
12. Precipitate the DNA by adding 2 volumes of -20°C , 100% ethanol.
13. Spool the DNA out of the solution with a pipet tip, and dip the DNA into a tube of 70% ethanol. Remove the 70% ethanol, leaving the DNA pellet at bottom (repeating this step reduces the viscosity of the final DNA solution, see **Note 15**).
14. Dry the DNA in a vacuum centrifuge for 5 min or air-dry for 1 h on the bench.
15. Resuspend the DNA pellet in 100 μL TE buffer.
16. Determine the purity (A_{260}/A_{280} ratio) and DNA concentration using a spectrophotometer (see **Note 8**).

3.4. Genomic DNA Isolation From Fungi

DNA is isolated from *Mycelia* grown on agar media, using the DNeasy Plant Mini kit. Traditionally, fungi were grown on agar media, for identification purposes, and liquid media was used for genomic DNA isolation. However, growing fungi in liquid media is time consuming and is not generally necessary.

If only small amounts of DNA are required (e.g., for PCR), agar media can be used to grow the fungi. The DNeasy Plant DNA isolation kit is then used to extract the DNA. This method gives good-quality DNA, suitable for PCR and most molecular biology work; however, the yield is sometimes low and the DNA may be sheared. If a higher DNA yield is required, a CTAB method, similar to that used for bacterial DNA isolation, is recommended, although it is a longer procedure and involves a number of phenol/chloroform steps.

After collecting the mycelium, there are several methods of drying the mycelium. The most favorable method is the freeze dryer. If this machine is not available, a vacuum centrifuge (Speed Vac) can be used. If the temperature of the vacuum centrifuge is set at 50°C, the actual temperature inside the machine, when under vacuum, will be lower than 20°C. Mycelium can be dried for up to 2 h without any damage to the DNA. This method only applies to small quantities of mycelium (up to 500 mg dry weight). For larger quantities of mycelium, a freeze dryer should be used. An acetone drying method can be used if neither machine is available in the laboratory (3).

1. Inoculate a plate with a small cube (3- to 5-mm square) of culture on agar.
2. Incubate for 4–7 d at the appropriate temperature (see **Note 16**).
3. Peel the mycelium from the surface of the agar with a scalpel (see **Note 16**).
4. Put the mycelium in a 1.5-mL microcentrifuge tube.
5. Dry the mycelium in a vacuum centrifuge or freeze dryer for 2 h.
6. Put liquid nitrogen in the mortar before adding the dried mycelium and grinding with a pestle.
7. Follow the DNeasy Plant Mini kit protocol.

3.5. DNA Isolation From Mycelium Grown in Liquid Media

This protocol is modified from Kim's method (4), using CTAB and a high salt concentration.

1. Inoculate fungal spores into 20 mL of the appropriate liquid media in a Petri dish (see **Note 17**).
2. Incubate for 4–7 d at the appropriate temperature.
3. Place the Mira-Cloth (10 × 10 cm) onto a pile of paper towels and pour the entire liquid media over the Mira-Cloth. Press the Mira-Cloth between the paper towels to remove as much liquid as possible.
4. If only a small quantity of mycelium is collected, it can be stored in a 1.5-mL microcentrifuge tube. Larger amounts can be wrapped in foil (see **Note 18**).

5. Put the sample in a beaker and cover with liquid nitrogen to freeze the mycelium, and store at -80 or -20°C until ready to be dried.
6. Dry the mycelium overnight in a freeze dryer (see **Note 19**).
7. Put the liquid nitrogen and dried mycelium in a mortar (see **Note 20**).
8. Transfer the powder to an microcentrifuge tube (see **Note 21**).
9. At this point, you may follow this protocol, or, alternatively, use the Qiagen Plant DNA purification mini kit protocol.
10. Add 200–500 μL ice-cold lysis buffer, followed by proteinase K, to a final concentration of 30 $\mu\text{g/mL}$. Vortex briefly.
11. Add 20% SDS solution to a final concentration of 2%.
12. Incubate at 65°C for 30 min.
13. Centrifuge at 14,000*g* for 15 min.
14. Transfer supernatant to a new tube.
15. Measure supernatant volume and add 5 *M* NaCl, to a final concentration of 1.4 *M*.
16. Add 1/10 volume of 10% CTAB buffer.
17. After thorough mixing, incubate at 65°C for 10 min.
18. Extract with an equal volume of chloroform/isoamyl alcohol. Centrifuge for 15 min at 14,000*g*, in a microcentrifuge.
19. Repeat **step 18** until the interface is clear.
20. Add 2.5 volumes of 100% cold ethanol, and mix by inverting.
21. Centrifuge at 14,000*g* for 15 min at 4°C .
22. Wash the DNA pellet with 1 mL of 70% ethanol.
23. Dry the DNA in a vacuum centrifuge for 5 min or air-dry for 1 h on the bench.
24. Resuspend the DNA pellet in 100 μL TE buffer.
25. Determine the purity (A_{260}/A_{280} ratio) and DNA concentration with a spectrophotometer (see **Note 8**).

3.6. Genomic DNA Isolation From Small Amounts of Fungal Spores

The general method of genomic DNA isolation in fungi requires the grinding of mycelia, either in frozen or lyophilized form, before extraction with phenol. This method requires a relatively large amount of mycelium. It is difficult to isolate DNA from very small quantities of mycelium (especially <5 mg dry mycelial weight). The plastic pestle is routinely used to grind animal tissue in an microcentrifuge tube. However, this crushing method can only be applied to very soft tissue. Dried fungal mycelium or spore samples are very hard, and are impossible to crush in a plastic tube with a plastic pestle. Alternatively, the mechanical force of glass beads can be used to break the cell walls of fungal mycelium or spores. Commercial products, such as BeadBeater[®], can be used instead of glass beads and vortexing (5). This little machine uses screw-capped vials containing 0.5 g of 0.1- to 3.0-mm silica–zirconium beads.

1. Put 10 pretreated glass beads into an microcentrifuge tube containing the spore sample.
2. Add 0.2% β -mercaptoethanol and 1% PVP-360 to the CTAB buffer. Add 200 μL hot (65°C) CTAB buffer mixture to each tube (see **Note 22**).

3. Vortex for 1 min at high speed.
4. Incubate at 65°C for 30 min.
5. Add 200 μ L chloroform/isoamyl alcohol (24:1).
6. Centrifuge at 14,000g for 15 min.
7. Precipitate with 2 volumes of 100% ethanol and leave sample at -20°C for at least 3 h. Repeat **step 6**.
8. Wash with 2 volumes of 70% ethanol, and repeat **step 6**. Discard supernatant.
9. Dry in a vacuum centrifuge.
10. Add 10 μ L TE buffer. Store at 4 or -20°C.

4. Notes

1. Liquid media (1–5 mL) can also be used for growing bacteria. Carefully choose the media to minimize the formation of polysaccharides. Very enriched media tends to produce more polysaccharides than minimal media.
2. Always add proteinase K before adding the SDS solution because SDS causes the bacterial suspension to become viscous. The cell suspension will become clear after addition of SDS. If it remains cloudy, the bacteria may not be Gram-negative, but a contaminant. SDS is a strong detergent that breaks cell walls and denatures proteins enhancing the activity of proteinase K.
3. A high concentration of NaCl will denature and precipitate protein and inhibit coprecipitation of the polysaccharides and DNA.
4. The optimum time and temperature for proteinase K activity is 65°C, and the activity can last only for approx 30 min, therefore, longer incubation times are not required. CTAB and NaCl will bind to proteins, making them insoluble.
5. This step removes CTAB–protein–polysaccharide complexes. In cases of very high polysaccharide content in cells, it is difficult to remove only the aqueous layer without interfering with the white interface. In this case, higher and longer centrifuge force and time will be needed. A wide-bore pipet tip should be used to remove the aqueous layer. These tips are available from commercial suppliers or can be made by cutting the end off the pipet tip with a scalpel.
6. DNA can be dried at room temperature for 1 to 2 h or in a heat block at 65°C for 15 min, without any damage to the DNA.
7. This CTAB method does not remove all of the polysaccharides and, therefore, sometimes it is difficult to resuspend the DNA. In this case, add 50 μ L of 8 mM NaOH to aid the resuspension of the DNA, followed by ethanol precipitation to remove the polysaccharides.
8. One A_{260} unit equals 50 μ g/mL of DNA; pure *Escherichia coli* DNA has an A_{260}/A_{280} ratio of 1.95.
9. Instead of ethanol, 0.6 volumes of isopropanol can be substituted. Ethanol precipitation is preferable, however, because it gives a more visible pellet and evaporates more efficiently from the pellet. One advantage of isopropanol is that a smaller volume is used, which is beneficial if tube space is limiting. In both cases, the DNA recovery rate will be the same. For genomic DNA isolation, different DNA precipitation temperatures and incubation times have little effect on DNA recovery rates.

One can directly centrifuge after adding ethanol without the -20°C incubation, and, if -20°C ethanol is not available, room temperature ethanol can be used.

10. DNA can be dried at room temperature for 1 to 2 h or in a heat block at 65°C for 15 min without any damage to the DNA.
11. Try not to take too many cells, to avoid a high polysaccharide content and poor DNA quality in the final DNA solution. Approximately two samples the size of a match head will be adequate.
12. The high sucrose content of the SET buffer will make the cells change to a spheroplast form after the lysozyme is added to the cell suspension. Sometimes 50 μL lysozyme will not be sufficient to break the cell wall. In this case, add a small amount of powdered lysozyme directly to the cell suspension and extend the incubation time.
13. The SDS solution will make the spheroplast cells burst, and the cell suspension will become clear. A high concentration of NaCl will denature and precipitate protein, and inhibit the coprecipitation of polysaccharides and DNA.
14. Most genomic DNA isolation protocols do not recommend vortexing because of danger of shearing the DNA. However, brief (up to 5 s) vortexing will help denature the protein bound to the DNA without causing damage.
15. In most cases, visible DNA will be formed instantly after adding ethanol. This spooling method gives better DNA quality than centrifugation. However, for very low DNA content or for sheared DNA, centrifugation at $14,000g$ at 4°C for 15 min is recommended.
16. The best results are obtained from very young mycelium. Some fungal mycelium tend to penetrate into the agar. In this case, just take mycelium with agar, which can be removed in a further purification step. Do not take too much mycelium, because the Qiagen Plant DNA purification mini kit column can only bind up to 50 μg of DNA. Ten to 100 mg of dried mycelium can be obtained using this method. If more than 50 mg of dried mycelium is obtained, more than one column will be needed. The final DNA quantity will be 5 to 20 μg , which is sufficient for most molecular biology applications.
17. All of the media are formulated for growing mycelium during short times. If there is a problem with too many polysaccharides, minimal media is recommended. Nutrient-rich media for *Ganoderma*: 10 g peptone, 10 g yeast extract, and 10 g glucose, add water to bring final volume to 1 L. Vogels media (used especially for *Pythomyces* and *Collototrichum*): 20 mL Vogels plus N, 10 g sucrose, 5 g yeast extract, and 980 mL water. *Schizopora* media: 5 g malt extract, 5 g yeast extract, 2 g glucose, and 500 mL water. Liquid Nobles media: 6.25 g malt extract and 500 mL water.
18. Approximately 100 to 200 mg of dried mycelium can be obtained from 15 to 20 mL liquid culture. Twenty to 100 μg of genomic DNA can be isolated from this amount of mycelium. Up to 1 g of dried mycelium can be obtained under optimal conditions. Transfer to a 30-mL conical tube and follow the instructions for the DNeasy Plant Maxi kit (Qiagen).
19. Instead of a freeze drier, a vacuum centrifuge can be used for small amounts of mycelium. Ten to 100 mg of wet mycelium needs approx 2 h of drying time. The temperature in the vacuum centrifuge can be as high as 55°C .

20. Approximately 5 to 10 mL of liquid nitrogen is put into the mortar containing the pestle, and left for 30 s. Add the dried mycelium and break into small pieces. Wait for the liquid nitrogen to evaporate before grinding to a fine powder.
21. If the quantity of powder is very small, lysis buffer can be added directly to the mortar. The lysis buffer may freeze because of the low temperature of the mortar. Wait for the lysis buffer to melt before transferring it to a microcentrifuge tube, with a wide bore tip.
22. PVP binds polyphenolic compounds (6). β -mercaptoethanol is reducing agent that helps to break cell walls.

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Extraction of Plant RNA

Elsbeth MacRae

Summary

Optimal sampling procedures for sampling plant tissue for RNA extractions are outlined in this chapter. To extract RNA, kits supplied from biotechnology companies are appropriate, but some procedures will not work with particular plant tissues. Two alternative methods are supplied for troublesome material. Basic methods to check RNA quantity and, more particularly, RNA quality before use are supplied.

Key Words: *Arabidopsis*; fruit; guanidine; plant; plant tissue sampling CTAB; polysaccharides; RNA.

1. Introduction

Plants are diverse, and individual species and organs or tissues of plants can behave differently during extraction of RNA (and DNA) for use in molecular studies. Hence, a range of extraction methods has also been devised, depending on the tissue or genotype being extracted. Problems encountered include the presence of large quantities of polysaccharides; high levels of RNases; various different kinds of phenolics, including tannins; low concentrations of nucleic acids (high water content); tissue, such as lignin (wood), that is difficult to break up; and so on. In addition, sampling techniques can have an effect on yield and lack of degradation, recognizing also that most tissues extracted are generally composed of a range of cell types and, hence, functions. In some instances, kits obtained from biotechnology supply companies are sufficient to perform the task, but, in other instances, especially with a new plant or tissue that has not had RNA extracted from it before, methods may need to be modified to suit the particular characteristics of that material. There is no simple indication that a tissue will be difficult. Depending on the use of the RNA extracted, further purification of messenger RNA (mRNA) may be required. In

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general, commercial kits will perform this job more than adequately, and any of a range of methods of quantification will be successful. The biggest problem encountered in RNA extraction usually originates from the initial sampling and extraction protocols, and from personal technique and care taken. Three extraction protocols will, therefore, be outlined that have had widespread success in plant RNA extractions in our laboratory.

2. Materials

2.1. Sampling

1. Liquid nitrogen (and container). **Caution:** wear protective clothing and gear.
2. Polystyrene box and/or second liquid nitrogen-proof container.
3. Sharp knife, scalpel, razor blade, tweezers, cork borer, metal needle/probe, and flame source.
4. Eppendorf tubes, tinfoil, and plastic bottles of various sizes.
5. Analytical balance.
6. Plant material.
7. -80°C freezer or liquid nitrogen storage container and/or dry ice.

2.2. Arabidopsis Extractions: Trizol™ or Guanidine Isothiocyanate-Based Method (see Note 1)

1. Trizol reagent (Invitrogen). Refer to the manufacturer's instructions and guidelines for stability and storage, and handle with eye and glove protection.
2. Chloroform.
3. Isopropyl alcohol.
4. 75% ethanol in RNase-free water.
5. RNase-free water (made by adding 0.01% DEPC [v/v], standing or stirring overnight, then autoclaving; or made by using Barnstead™ Ultrapure RNase-free water).
6. 0.1 M NaOH-washed and UV-treated plasticware, oven-baked sterile glassware, sterilized Eppendorf tubes, or clean sterile Falcon tubes (conical bottom).
7. Liquid nitrogen and mortar and pestle.
8. Benchtop centrifuges (refrigerated or access to cold room).
9. mRNA purification kit (Amersham Biosciences, now GE).

2.3. Extractions From Problem Tissues: CTAB Method (see Notes 1 and 2)

1. Sterile Falcon tubes (conical bottom, 25 or 50 mL).
2. Oakridge tubes (round bottom, sterile, and RNase-free).
3. Mira-Cloth® (Calbiochem).
4. Liquid nitrogen and mortar and pestle.
5. Benchtop centrifuge, vortex machine, refrigerator, and freezer.
6. RNase-free water in a baked storage bottle (in an oven at $>150^{\circ}\text{C}$, for >4 h).
7. Chloroform:isoamyl alcohol (24:1).
8. 12 M LiCl (use RNase-free water and autoclave or filter through a Nalgene™ 50-mm kit with 0.2- μm pore size).

9. Extraction buffer: 2% hexadecyl trimethyl-ammonium bromide (CTAB); 2% polyvinylpyrrolidone K30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, sodium form, pH 8.0; 2 M NaCl, 0.5 g/L spermidine, and 2% β -mercaptoethanol. Use RNase-free water for dissolving, and autoclave before using.
10. Sodium dodecyl sulfate–Tris-HCl–EDTA (SSTE) buffer: 1 M NaCl; 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, sodium form, pH 8.0. Use RNase-free water and autoclave before using.

2.4. Extractions From Problem Tissues: Non-CTAB-Based or Non-Guanidine-Based Method (see Notes 1 and 2)

1. Eppendorf and/or Falcon tubes (sterile and RNase-free).
2. Liquid nitrogen and mortar and pestle.
3. Polytron™ homogenizer.
4. Oakridge tubes (sterile and RNase-free) and Corex tubes (sterile and RNase-free).
5. Benchtop centrifuge, vortex machine, refrigerator, and freezer.
6. RNase-free water in a baked storage bottle (in an oven at $>150^{\circ}\text{C}$, for >4 h).
7. Preheated (65°C) lysis buffer: 150 mM Tris-HCl, 50 mM EDTA, 4% SDS, pH 7.5 titrated with boric acid, 1% β -mercaptoethanol, and 1% w/w polyvinylpolypyrrolidone (PVPP). Use RNase-free water and autoclave before use.
8. 5 M potassium acetate; use RNase-free water and autoclave or filter as in **Subheading 2.3., item 8**.
9. Cold absolute ethanol.
10. Chloroform:isoamyl alcohol (24:1).
11. Tris-HCl-equilibrated phenol, pH 8.0. Keep phenol in dark bottles in cold room (or -20°C); do not use old phenol that has been opened for a long time and is discolored. Make the Tris-HCl buffer RNase-free by adding DEPC to make buffer in a baked (or sterile) bottle, do not autoclave buffer; otherwise, filter as in **Subheading 2.3., item 8**. Equilibrate by melting 500 mL phenol at 65°C and adding 100 mL of RNase-free water, mixing, and leaving to partition overnight (can last for 4–6 wk). Discard the top, aqueous phase. Repeat two more times, but with 0.5 M Tris-HCl, pH 8.5, the first time, and 0.1 M Tris-HCl, pH 8.5, the second time. Phenol can now be used (some buffer can be left on top, but prevent carrying it over while pipetting). Store in the dark at 4°C for up to 2 to 3 wk.
12. 12 M LiCl (use RNase-free water and autoclave or filter as in **Subheading 2.3., item 8**).

2.5. mRNA Extraction

Use a kit from a biotechnology supplier. We have found the Amersham Biosciences (now GE) kit to work well, but other kits work equally well.

2.6. Quantification, Degradation, and Storage (see Note 3)

1. RNase-free water.
2. Two UV-capable glass spectrophotometer cuvetts.

3. 10X stock Tris-base–boric acid–EDTA buffer: 108 g Tris-base, 55 g boric acid, and 40 mL of 0.5 M EDTA, pH 8.0, in 1 L deionized water.
4. 37% formaldehyde.
5. Ultrapure™ agarose (Invitrogen).
6. 10X MOPS buffer: 0.2 M MOPS (3-[N-morpholino] propanesulfonic acid, 50 mM Na acetate, and 10 mM EDTA, as in **Subheading 2.6., item 3**).
7. Loading buffer (store in aliquots at -20°C): 0.75 mL deionized formamide, 0.15 mL of 10X MOPS buffer (autoclaved or filtered), 0.24 mL formaldehyde, 0.1 mL RNase-free water, 0.1 mL glycerol (autoclaved), and 10% w/v bromophenol blue dye. Add 3 μL ethidium bromide to 300 μL loading buffer before using.
8. Ethidium bromide as a 10% solution (**Caution:** ethidium bromide is toxic, handle with gloves; see **Note 4**).
9. RNase-free electrophoresis gel boxes, beds, and combs.
10. -80°C freezer.
11. Agilent™ chip.

3. Methods

Extraction of excellent quality plant RNA starts with good practice tissue sampling and storage. To reflect mRNA present in a snapshot moment in the growing intact plant, tissues need to be treated in a manner very similar to that for analysis of metabolic intermediates or active enzymes. Partial or complete degradation of mRNA can occur because of tissue sampling and storing techniques. Successful extraction may require alternative techniques, and we outline:

1. A standard method now used for *Arabidopsis* (the model plant in which the genome has been fully sequenced), tomato, maize, tobacco, and other commonly researched plants.
2. Two methods for use in more difficult tissues in which guanidine-based methods result in zero yield—the CTAB (1) and the hot phenol/chloroform methods (2)—which have had much greater success in tissues with low yields and/or high polysaccharide, secondary product, or RNase contents.

The need for care and for use of RNase-free solutions and equipment, including during quantification and storage, is common across all methods.

3.1. Sampling

Ample liquid nitrogen supply is essential. In general, tissue from a plant is sampled by plucking and covering immediately with liquid nitrogen in a polystyrene container, such as those in which chemicals are dispatched on dry ice by laboratory suppliers. For example, with leaves, whole leaves, rapidly hand-shredded leaves, or cork borer discs of leaves can be sampled and killed in a short time, equilibrating to liquid nitrogen temperatures within less than 1 min. It is essential to have a minimal time between removal from the plant and

immersion in liquid nitrogen, to minimize the expression of new mRNAs because of tissue wounding or detachment from the plant. Some mRNA has been shown to be upregulated within 5 min of tissue detachment from a plant. Other tissues are more bulky, e.g., fruit or tubers; these tissues take longer to equilibrate to liquid nitrogen temperatures if immersed whole. Bulky tissues hold more heat, and exchange is slower with liquid nitrogen. This leads to tissue damage (altering osmoticum leading to leaky cells) and degradation of the mRNA present, because RNases gain access to the mRNA. Hence, for bulky tissues, it is better to rapidly remove the tissue from the plant and to subsample quickly (preferably a minute between detachment and immersion of subsamples in liquid nitrogen). Slicing and/or dicing with cork borers is very effective. Take care to sample the tissue in which you are interested in a representative manner. Tissue samples can be added directly to preweighed Eppendorf tubes or storage containers or to homemade tinfoil pouches immersed in the larger liquid nitrogen container. If using Eppendorf tubes, prepare the tubes with a small hole (heat a needle over a flame and pierce the lid) to prevent explosions because of the remnants of liquid nitrogen inside the sealed tube when the lids are closed. This can also be done with other containers, or else the container can be drained before placing the lid on the container. The amount of tissue can be calculated if the containers or tubes are preweighed, then weighed again after the tissue has been killed and the liquid nitrogen evaporated from the container. Care needs to be taken, however, that the tissue does not thaw during weighing.

After the tissue has been sampled, store the sample in a liquid nitrogen storage container or a -80°C freezer. Take care not to remove tissue or allow it to reach subzero temperatures, by keeping the tissue in liquid nitrogen as much as is practical during subsampling and weighing before grinding (if not performed before storage) to extract the mRNA. Repeated removal from storage and subsampling of tissues has often led to reduced quality of mRNA. To extract the RNA, grind the weighed material in a mortar and pestle, under liquid nitrogen, to a fine powder and transfer the powder to the extraction buffer. **Caution:** do not ever let the plant tissue thaw after killing the tissue in liquid nitrogen and before complete mixing in extraction buffers after grinding the tissue to a powder.

The amount of tissue required to achieve acceptable yields of RNA varies according to the material. Tissues with a high water content require higher amounts of tissue to be extracted. For example, 2 g *Arabidopsis* leaf tissue yields approx 60 to 200 μg RNA (Trizol method); and 5 to 8 g fruit tissue (high water) yields approx 400 μg RNA (non-CTAB/non-guanidine-based method).

3.2. *Arabidopsis* Extractions

1. Grind approx 0.1 g tissue in liquid nitrogen.
2. Add 1 mL of Trizol reagent to the ground powder (see **Note 5**).

3. Transfer into Eppendorf tubes.
4. Centrifuge at 12,000*g* for 5 min at 2 to 8°C.
5. Remove supernatant to new Eppendorf tube.
6. Add 200 μ L of chloroform and shake vigorously by hand for approx 15 s.
7. Let stand at room temperature (~20–25°C) for 3 min.
8. Centrifuge at 12,000*g* for 15 min at 2 to 8°C.
9. Carefully transfer the upper aqueous phase to a new Eppendorf tube (ensure no interface debris is transferred, see **Note 1**).
10. Add 0.5 mL of isopropyl alcohol. Mix.
11. Let stand at room temperature for 10 min.
12. Centrifuge at 12,000*g* for 10 min, at 2 to 8°C.
13. Carefully discard supernatant (tip Eppendorf with the pellet position angled up and away from you and pipet out the supernatant). The pellet may be slightly glassy and transparent or may not be clearly visible at all.
14. Add 1 mL of 75% ethanol.
15. Vortex briefly and centrifuge at 12,000*g* for 5 min at 2 to 8°C.
16. Discard the supernatant as in **step 13** and allow pellet to air-dry for 10 min.
17. Dissolve the pellet in 20 μ L of RNase-free water by very gently sucking the liquid up and down with a pipet.
18. Quantify the RNA, check the purity and degradation, and either store at –20 or –80°C until used, or extract the mRNA using commercial kits (see **Subheadings 3.4.** and **3.5.**).

3.3. Extractions From Problem Tissues

3.3.1. CTAB-Based Method

1. Pipet 15 mL of extraction buffer (minus β -mercaptoethanol) into an RNase-free Falcon tube and add 300 μ L of β -mercaptoethanol. Warm in a water bath to 65°C (see **Note 6**).
2. Grind the tissue in liquid nitrogen and add the tissue gradually to the heated buffer so that no powder coagulates (freezes into a lump) and, therefore, thaws before mixing fully with the buffer. Vortex after each small addition to ensure that the powder is fully dispersed and thawing in the heated buffer.
3. Leave the sample sitting at room temperature while processing the next samples.
4. Mix the samples using the Polytron homogenizer for approx 1 min at full speed until the sample foams close to the top of the tube. Wash the Polytron homogenizer with distilled water after each sample.
5. Add an equal volume of chloroform:isoamyl alcohol, mix (vortex), transfer to an RNase-free Oakridge tube, balance the samples with buffer, and centrifuge (Sorvall SS34 rotor; 11,984*g*) for 10 min at room temperature to separate the phases.
6. Filter the upper aqueous phase through an autoclaved Mira-Cloth into a new RNase-free Oakridge tube (or carefully pipet off the top aqueous phase, ensuring no transfer of any interface material to a new tube). **Caution:** it is better to leave some aqueous phase behind than to transfer contaminants.

7. Add an equal volume of chloroform:isoamyl alcohol, mix, and centrifuge as in **step 5** to separate the phases.
8. Remove the top aqueous phase to an RNase-free Falcon tube and estimate the volume to the nearest milliliter. Add an appropriate volume of LiCl solution to give a final concentration of 2 M LiCl (1 volume of 4 M, 0.5 volumes of 6 M, 0.33 volumes of 8 M, 0.25 volumes of 10 M, or 0.2 volumes of 12 M).
9. Leave at 5°C (refrigerator) overnight.
10. Centrifuge at 4°C and 11,984g for 20 min (Sorvall SS34 rotor).
11. Pour off the supernatant, and invert the tubes to drain onto a tissue.
12. Preheat SSTE buffer to 65°C. Dissolve the pellet in 200 µL of heated SSTE, and transfer to a 1.5-mL, RNase-free Eppendorf tube.
13. If the SDS in the SSTE buffer precipitates (a white cloudiness), place the Eppendorf with the sample in a heating block (37°C) until it has dissolved again before continuing.
14. Add an equal volume of chloroform:isoamyl alcohol. Vortex immediately before adding and immediately after to mix completely.
15. Add 2 volumes of absolute ethanol to precipitate the RNA (>30 min at -70°C, or >2 h at -20°C).
16. Centrifuge the tube in a microcentrifuge in a cold room (or in a temperature-controlled microcentrifuge) for 20 min at maximum speed.
17. Discard the supernatant, allow the pellet to air-dry, and resuspend in 20 µL RNase-free water, as in **Subheading 3.2., step 17**.

3.3.2. Non-CTAB-Based or Non-Guanidine-Based Method (see **Note 2**)

1. Very slowly add 5 g of ground powder to 15 mL of preheated lysis buffer containing PVPP and freshly added β-mercaptoethanol, and vortex between additions (do not allow powder to thaw or form lumps).
2. Homogenize the suspension using the Polytron homogenizer at maximum speed for 20 s, or until the froth reaches the top of the tube.
3. Add 0.1 volumes (1.5 mL) of 5 M potassium acetate and 0.25 volumes (4 mL) of cold absolute ethanol to the tube and vortex for 30 s.
4. Put 1 volume of chloroform:isoamyl alcohol into each of two Oakridge tubes and put half of the homogenate into each tube, vortex, and centrifuge at 2000g for 10 min at room temperature.
5. Remove the top aqueous phase to an RNase-free Falcon tube and add 10 mL of buffered phenol and 10 mL of chloroform:isoamyl alcohol.
6. Vortex to mix, and centrifuge at 2000g for 10 min to separate the phases.
7. Repeat **steps 5 and 6**.
8. Remove the top aqueous phase to an Oakridge tube and add one-third volume of 12 M LiCl. Incubate overnight at -20°C.
9. Centrifuge at 20,000g for 20 min to precipitate the pellet.
10. Pour off the supernatant and resuspend the pellet (by vortexing) in 10 mL of 3 M LiCl (12 M LiCl diluted with RNase-free water). Centrifuge at 20,000g for 20 min.

11. Pour off the supernatant, and resuspend the pellet (by vortexing) in 2 mL of RNase-free water, transfer to a 30-mL Corex tube.
12. Add 180 μ L of 5 M potassium acetate and 6 mL of cold absolute ethanol. Cover with Parafilm and leave at -20°C for 1 h.
13. Centrifuge at 12,000g for 10 min.
14. Pour off the supernatant and dry pellet in air for 10 min.
15. Dissolve the pellet in 200 μ L of sterile RNase-free water. Store at -20°C until use, with or without aliquoting into different tubes.

3.4. mRNA Extraction

Use a manufactured kit. We find the Amersham Biosciences kits (now GE) effective.

3.5. Quantification, Degradation, and Storage (3)

1. To quantify and assess the degree of purity, take a 2.5- μ L aliquot (or larger or smaller) and dilute with 1 mL of water. Scan in a scanning spectrophotometer from 190 nm to 320 nm. Alternatively, read in a spectrophotometer at 260 and 280 nm. Discard the 1 mL sample—it will now be degraded.
2. Calculate the concentration of RNA by the formula: $\text{OD}_{260} \times \text{dilution factor}/25$; $1 \times \text{OD}_{260} = 40 \mu\text{g/mL RNA}$ (see **Note 7**).
3. To assess whether extracted RNA is degraded and to confirm the quantification, run an aliquot on an agarose gel. Place between 1 and 2.5 μ L of RNA in an Eppendorf tube and add 10 μ L loading buffer.
4. To prepare the gel apparatus, soak the apparatus for at least 1 h in water plus SDS (~10%) to denature any RNases. Rinse in RNase-free water.
5. Prepare a 1% formamide agarose gel. For a 30-mL gel, take 3 mL of 10X MOPS buffer, add 25.3 mL RNase-free water and 0.3 g agarose; heat in a microwave oven for 35 s (**Caution:** do not close the container; see **Note 4**), and add 1.7 mL of 37% formaldehyde.
6. Pour the solution from **step 5** into the gel apparatus and wait until set (see **Note 4**). Remove the combs. Add 200 mL of 1X MOPS running buffer to cover the gel and wells.
7. Pre-equilibrate gel by running at 80 V for 10 min.
8. Add 2 volumes of RNA loading buffer to 1 volume of sample. Heat at 65°C for 10 min. Rinse wells with buffer, and load the RNA samples into lanes. Load one lane with 5 μ L or the recommended quantity of an RNA standard. Run the gel for approx 1.5 h at 80 V. Visualize under UV light. Wear UV-protective goggles.
9. RNA degradation (or contamination) can be detected by:
 - a. A blob at the running edge end of the gel—the RNA is totally degraded.
 - b. A smear with indistinct bands present (this can also mean there is a lot of polysaccharide in the sample).
 - c. The two main ribosomal bands are equal in intensity, or the lower band is higher than the upper band.

- d. There is a bright band at the top of the gel near the loading wells indicating the presence of DNA in the sample. Depending on the use of the RNA, this may not be a problem. It can be removed by digesting with an RNase-free DNase.
10. RNA can, alternatively, be both quantified and assessed for degradation using an Agilent Chip and Agilent Technologies 2100 Bioanalyzer. The samples are run on a prefabricated gel associated with the chip, including a specific RNA ladder. The chip is scanned and the ladder is used to quantify the RNA in each sample, which can also be examined visually for degradation of RNA.

4. Notes

1. Generally, the method used by *Arabidopsis* researchers is adequate for a wide range of plant tissues. However, many plant tissues also contain other compounds that interfere with the extractions. In particular, some tissues (e.g., algae, some fruits, some leaves, and woody material) have high concentrations of polysaccharides, derived either from the plant cell wall or present as mucilages. These generally entrap the RNA during extraction and, if they are not removed in the first steps and partitioned into a discarded phase, they will remain through the rest of the extraction. Hence, it is important not to take any debris or interface material during the chloroform partitioning. Heat is a good way of removing polysaccharides, by making them more soluble. Many plant tissues also have high levels of RNases and, generally, the best way to remove these RNases is to increase the SDS present in the extraction buffer. We have gone as high as 8% for a fruit that also had high polysaccharide content. The result of insufficient SDS is partially or fully degraded RNA. The PVPP helps to bind polyphenolics, which can also be a problem in some tissues that have high levels of polyphenolics. With very low RNA/high water-containing tissue, more material to extract in a given volume of buffer is usually required. Otherwise, there is insufficient RNA present to partition properly during purification.
2. For reasons we do not understand fully, both CTAB and guanidine can cause precipitation problems or other mixing problems when extracting some tissues. We have not found a way to predict this occurrence (other than the presence of polysaccharides).
3. To minimize the presence of RNases, it is important to keep equipment aside for use only with RNA extractions. Pipet tips and Eppendorf tubes should be used only for RNA work and not mixed. Gloves should be used at all times. Keep one set of gel electrophoresis equipment for use just with RNA. Keep solutions RNase free by not using the solutions in other procedures. Always use RNase-free water for solutions. The solid chemicals are NOT RNase free, therefore, buffers should be autoclaved. Tris-HCl buffers cannot be autoclaved.
4. RNA gel matrices can be prepared in advance by dissolving the agarose in buffer in a Sorvall or similar bottle (able to be autoclaved). Melt the aliquot and add formaldehyde and continue as indicated. Take care to fill the bottles only partially full (e.g., 500 mL in a 1 L bottle). This can be stored with the lid on until use. If you have a microwave oven, loosen the lid so that air can escape and heat in a microwave oven until melted. Remove with care (it will be hot) and pour out whatever is

required for the gel. Leave the remainder to resolidify in the bottle and store again. Take care when handling ethidium bromide, it is a mutagen and toxic, and gloves should always be used and surfaces wiped down after use.

5. Do not let ground (or intact) plant tissue thaw without being in the presence of extraction buffer. Small amounts of material can thaw when taking samples in and out of the freezer, weighing out aliquots, or during grinding in liquid nitrogen. It can be particularly important to ensure that the tissue does not form a lump, where the outside is in contact with the buffer, but the inside is thawing directly. When adding tissue to a hot extract, add only a little at a time, using a spoon or spatula that has also been precooled in liquid nitrogen.
6. Always add β -mercaptoethanol fresh on the day of extraction. It will become ineffective in solutions within 12 to 24 h.
7. A ratio of approx 1.8 to 2.0 (A_{260}/A_{280} nm) means that the RNA is sufficiently pure and without polysaccharide contamination for use in most applications and is soluble. A lower ratio generally means polysaccharide contamination and/or insolubility. A high reading at 240 nm also suggests polysaccharide contamination.

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II

INDIRECT DETECTION

Overview of Hybridization and Detection Techniques

Elena Hilario

Summary

A misconception regarding the sensitivity of nonradioactive methods for screening genomic DNA libraries often hinders the establishment of these environmentally friendly techniques in molecular biology laboratories. Nonradioactive probes, properly prepared and quantified, can detect DNA target molecules to the femtomole range. However, appropriate hybridization techniques and detection methods should also be adopted for an efficient use of nonradioactive techniques. Detailed descriptions of genomic library handling before and during the nonradioactive hybridization and detection are often omitted from publications. This chapter aims to fill this void by providing a collection of technical tips on hybridization and detection techniques.

Key Words: Bacterial artificial clone libraries; chemiluminescence; digoxigenin; high-density membrane; hybridization.

1. Blotting and Hybridization

1.1. Colony Blots

Arrayed bacterial colonies harboring cloned DNA allow for high-throughput screening either by PCR amplification of the desired cloned gene, or by growing the bacteria on membranes for hybridization. Although PCR screening is a cheap and quick method for screening a few hundred bacterial colonies, complete genomic DNA libraries require the preparation of clone DNA pools by robotic methods; or, if the library is cloned in a bacteriophage vector, producing lytic plaques to be picked and analyzed.

Colony blots produced from a genomic DNA library have several advantages. Nowadays, most genomic DNA libraries are arrayed in multititer plates, with either 96 or 384 wells, contained in tens (prokaryotes) to hundreds (eukaryotes) of plates to ensure several-fold coverage of the genome. The arrayed clones

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grow to saturation in liquid medium with selective antibiotics and can be used to produce either plate DNA pools or colony blots. Robotic systems can grid several multititer plates per membrane in duplicate and in a unique pattern, to prevent identifying false positives and to allow the production of several copies of these high-density membranes in one session. The membranes can be stored at -20°C for several years. Another advantage is that heterologous DNA probes can be used for screening the high-density membranes several times under different stringency conditions, because the membranes can be stripped and reprobed several times.

Critical parameters involved in making and screening colony blots are bacterial culture density, inoculation volume, bacterial growth medium, type of membrane, methods for denaturing and fixing DNA to membranes, and interference of bacterial debris.

1.1.1. Bacterial Culture Density

A key parameter of the successful production of colony blots is a healthy bacterial culture. Each bacterial host used for storing DNA libraries requires specific growing media and selective antibiotics, but common requirements for all of them are stable temperature conditions and aeration, for the bacteria to reach its maximum density. Each bacterium will harbor different cloned DNA; some of these clones will be toxic and will slow the bacterial growth. However, regardless of the type of host or the cloned DNA, the easiest way to standardize the growth of the entire library is to prepare working microtiter plates. A working microtiter plate is inoculated from the master plate and is allowed to grow to saturation for up to 24 h. Growing the bacteria on working microtiter plates allows the bacteria to recover from freezer storage. It is tempting to inoculate directly from library master plates to save on plasticware and time, but this shortcut often yields poor growth of the bacterial colonies on membranes, and increases the chances of contaminating the library master plate set.

Only half of the volume capacity of a microtiter plate well should be filled with liquid medium, because the gridding tool will displace some liquid during inoculation, and spilling over into the neighboring wells should be avoided. Some evaporation will occur while filling the working plate, inoculating, incubating, and membrane gridding.

Working microtiter plates can serve one more purpose: after being used to produce high-density membranes, their contents can be collected by centrifuging the plate (inverted over a container, e.g., the top of a 96-filter tip box) at 62g for 2 min. The pooled contents can be extracted to obtain a pool of DNA plasmids by standard alkaline lysis.

1.1.2. Tools for Arraying Colony Blots

Arraying a few hundred bacterial colonies on membranes can be performed manually, but in the era of automatization, most institutions have access to

robotic systems, either simple liquid handling systems adapted to handle pin tools, or sophisticated multitask robots. The two robotic systems differ in their ability to produce high-density membranes. Most liquid handling systems with pin tool adaptors only duplicate the working copy onto either liquid media or over membranes or a solid medium. Multitask robotic workstations allow the user to program the exact spacing between colonies, to fit as many plates per membrane as desired. The low throughput systems are recommended for secondary screenings, in which only a few selected clones are screened, whereas high throughput systems are required for gridding complete genomic libraries. Care should be taken regarding the diameter and the composition material of the pin. The diameter will determine the amount of bacterial culture to be inoculated; therefore, the size of the colony expected to grow during a certain time. The material used for making the pins also determines the amount of liquid able to cling to its surface: if it is too hydrophobic, a small volume of bacterial culture will be inoculated. Pin tips for bacterial inoculations do not need to be modified by slots or grooves, as is necessary for other types of arraying. Simple sharp pin tips are the only necessary tools for library gridding.

1.1.3. Types of Membrane for Colony Blots

Because this book is dedicated to nonradioactive methods of detection, only membranes compatible with this procedure are discussed. Positively charged supported nylon membranes where the active material is immersed onto an inert matrix is ideal for all nonradioactive nucleic acid detection experiments: bacterial colony, plaque lifts, and DNA and RNA blots. The supporting inert matrix gives the membrane a high tensile strength needed for re-probing. These membranes have a high protein-binding capacity and empty areas need to be completely blocked before applying antibody solutions for the detection. If properly blocked, positively charged nylon membranes show very low background signal by nonradioactive methods. A wide variety of membrane formats is commercially available, to suit many different blot requirements.

1.1.4. Bacterial Solid Medium and Incubation Time

Bacteria colonies growing over a membrane absorb nutrients from a solid medium underneath. Diffusion of nutrients and localized bacterial overpopulation (i.e., a colony) are critical for producing uniform colony blots. There is no universal recipe for preparing solid medium, because each bacterial host has special needs, and should be considered when producing colony blots. However, most solid media require at least twice the amount of nutrients compared with the liquid medium used for the same bacterial host.

Two critical parameters should be considered when preparing the solid media: plate pouring and surface tension on the plate walls. Pouring plates may seem trivial to any molecular biologist. Once the agar setting point has been

mastered, anyone can produce smooth and bubble-free plates. However, high-density membranes produced by robotic systems require evenly leveled agar plates containing the same volume, because the replicating pin tool will be lowered only to a certain height: shallow plates will not be inoculated with enough bacterial culture, overloaded plates can damage the pin tool or make the pins perforate the membrane, and plates that are not level will have bacteria inoculated only in certain areas of the membrane.

The surface tension exerted by the plate walls over the medium produces a meniscus, and it is necessary to ensure that most of the printed bacterial colonies stay as far as possible from the meniscus. However, in high-density membranes produced by robotic pin tools, the pins are not fixed to the holding platform and are allowed to adjust their height after reaching the surface. Avoiding bubbles on the solid medium and air pockets trapped between the solid medium and the membrane helps to produce uniform bacterial colonies. To squeeze out any air pockets, a sterile plastic spreader should be slid over the membrane before inoculation.

The time required to grow bacterial colonies on membranes depends on the nature of the cloned DNA harbored by the bacterial host, the amount of liquid bacterial culture used for inoculation, and the incubation temperature. Assuming that all of these parameters are optimal, overnight incubation (at least 16 h) is enough to observe colonies of approx 0.5 mm. If you can see the colony with the naked eye, you can assume there will be enough plasmid DNA bound to the membrane after fixation. For some bacterial hosts, a 24-h incubation under optimal conditions is necessary. To avoid drying out the solid medium, store the plates in a plastic container that is loosely closed, or put a beaker filled with water in the incubator to create a humidified atmosphere.

1.1.5. Methods to Fix Total DNA From Colonies Onto Membranes

Bacteria growing on surfaces produce complex molecules to build up films and scaffolds. These molecules bind the bacteria to the solid medium very tightly. Older methods to extract the plasmid DNA from these colonies denatured the bacteria by capillary absorption of solutions through the membrane, sometimes with mixed results: not enough lysis and too much bacterial debris.

The same solutions used for denaturing and neutralizing DNA resolved by electrophoresis in agarose gels are used for lysing the bacteria and denaturing the DNA from colony blots. A good practice is to use 0.2 L of solution for an approx 700-cm² membrane area (either in one piece or a set of three membranes of 7 × 11 cm). The membrane with colonies is peeled off of the solid medium and placed bacteria-side down in the denaturing solution. It should be completely immersed for 5 min. The membrane is transferred to neutralizing solution, also immersed for 5 min, with gentle manual shaking using forceps. Most

of the bacterial debris will come off at this stage. After immersion in the neutralizing solution, the membrane is transferred to 3X standard sodium citrate (SSC) buffer (450 mM NaCl, and 45 mM sodium citrate tribasic, pH 7.0), and gently shaken with the forceps for 2 to 3 min. The membrane is placed on a sheet of Whatman™ 3MM paper, DNA side up, and air-dried for 30 min to 1 h. The DNA is permanently fixed to the positively nylon membrane by placing it with the DNA side down over a UV transilluminator for 2 min. DNA crosslinking to positively charged nylon membranes can be performed with a regular UV transilluminator, the same kind used for photographing ethidium bromide-stained agarose gels; although there are specific UV crosslinkers available in the market that achieve the same result. The membranes can be processed immediately, or stored in individual zip-lock plastic bags at -20°C for several years.

1.1.6. Bacterial Debris on Membranes

The most frustrating and common problem with colony/library screening is nonspecific binding of the probe to solid particles bound to the membrane. Labeled probe molecules cling to this mass of debris like cotton threads to a tumbling cardoon, producing false-positive results. To avoid this problem, a prewash step before hybridization can remove the debris. A prehybridization treatment prewash solution (see Chapter 6) for 1 h with vigorous shaking will remove most of the debris. After the incubation, the membrane is wiped off using a lint-free tissue soaked in prewash solution, until no debris is visible. The permanently crosslinked DNA is not harmed.

1.2. Nucleic Acid Blots

General guidelines for resolving nucleic acid fragments on agarose gels by electrophoresis can be found in any molecular biology laboratory manual (1–3). However, some basic principles should always be considered: the nature of the nucleic acid, the degree of resolution required according to the size of the fragment to be resolved, the method for transferring the nucleic acid molecules from the agarose matrix into a solid support (i.e., positively charged nylon membrane), the inclusion of a nonradioactive prelabeled DNA ladder, and time restrictions.

Special semidry transfer blotters are commercially available that speed up the transfer by applying an electric current across the gel/membrane sandwich placed between two plate electrodes. Care should be taken not to squash the gel or leave the transfer unit on for longer than recommended, because this will dry out the gel completely. Overall, the easiest way to transfer nucleic acids onto a membrane is by capillary action overnight. The liquid medium for neutral transfers (i.e., Southern DNA blots) is usually 20X SSC buffer (3 M NaCl and 0.3 M sodium citrate tribasic, pH 7.0); however, this high concentration of buffer is not required, a 3X SSC buffer ensures an even transfer without compromising

the amount or size of the material incorporated onto the membrane. Two hundred milliliters of transfer buffer on each side of the filter paper bridge is sufficient to transfer a large agarose gel.

There is no need to mask the edges of the gel with plastic film to avoid a shortcut of the transfer buffer across to the blotting paper without going through the gel if the membrane, filter paper, and blotting tissue paper are cut to exactly the same size as the gel. A stack of blotting tissue paper 6- to 7-cm high is sufficient for driving the transfer buffer upward. There is no need to place a glass plate and a weight on top of the stack of blotting tissue paper to make sure that the transfer will be even; this procedure will usually flatten the gel even before the transfer is completed, distorting the location of the nucleic acids on the membrane (i.e., the gel photo will not be comparable to the blot results). If care is taken to avoid all air bubbles between layers by rolling a glass rod every time a layer is placed on the pile, the transfer will be smooth without the need of too many gadgets.

After the transfer, the membrane has to be dried out completely before crosslinking with UV light (see **Subheading 1.1.5.**, and check with supplier's instructions). The efficiency of UV crosslinking is dependent on the amount of water contained in the membrane; wet membranes require more energy to efficiently crosslink the nucleic acids to the matrix, compared with dried membranes. Long exposures to UV light may produce thymidine dimers, which will hinder the annealing of the probe. To speed the drying process, place the membrane on a plastic box loosely covered inside a 37°C incubator for 20 to 30 min. The membrane will be bone dry when the edges start curling up, make sure to place a sheet of filter paper on top to flatten it on the UV transilluminator before crosslinking, for 2 min on the DNA side only.

Store dried and fixed membranes in zip-lock plastic bags at -20°C until needed.

2. Detection Techniques

2.1. Membrane Hybridizations Set-Up

The typical system for membrane hybridization experiments is a hybridization oven. As long as the temperature can be controlled between 20 and 80°C ($\pm 0.5^\circ\text{C}$), any oven provides a good service. Ovens with shaker modules save laboratory space, but are not essential. Hybridization ovens vary in the number and type of bottles the rotisserie can hold: 6, 8, 10, or 12; small, medium, and extra-long narrow-mouth bottles (~4-cm internal diameter), or medium-size wide-mouth bottles (~7-cm internal diameter). The most convenient way to handle a membrane during hybridization is by placing it on a nylon mesh (DNA side up) the same diameter and length as the bottle. If only one membrane is used per bottle, there is no need to place another nylon mesh on top of the membrane.

If several membranes are hybridized in one bottle, a multiple layered sandwich can be assembled on a plastic tray, with warm prehybridization solution, squeezing all of the air bubbles out with a glass rod, and then rolling the sandwich into the bottle. Regardless of whether there are one or several membranes, shifting and overlapping of the membrane edges will occur during hybridization. It is very important to fold the membrane/mesh in the same direction as the rotation of the rotisserie; in this way you can ensure that some hybridization solution will go through the small gap on the overlapping membrane edges while rotating. Even if the membrane(s) overlap, if properly set up, hybridization still occurs.

If hybridization ovens are not available, membrane hybridizations can be performed in plastic bags, placed on shaker/incubators or in water baths with shaking platforms. Very good results can also be achieved with this equipment, even with multiple membranes, if needed. The membrane is placed in a specially designed, commercially available hybridization bag, although these bags can be expensive. However, some food freezing bags available in supermarkets can be used, but plastic composition, thickness, and quality vary among brands, plus, you may need to purchase their own bag sealer. Perhaps the safest and most readily available plastic bag in any molecular biology laboratory is the common autoclave bag for disposing of used biological and plastic material. Most laboratories use yellow bags with the biohazard label, but there are clear autoclave bags with no printed label available from the same suppliers. A standard plastic bag sealer with several heating settings can seal autoclave bags.

Plastic bags should be large enough to accommodate the membrane plus a margin of approx 2 cm around the membrane, to allow for circulation of the hybridization solution and to leave enough space to cut and seal the corners when the prehybridization solution is replaced by the hybridization solution. To ensure even hybridization in a plastic bag, all bubbles must be removed. This task may prove difficult because of the high concentration of denaturing detergent, but a safe way to do this is to seal the bag completely, then cut an opening of approx 1 cm on a corner. Place the bag on a flat surface at the same level as the bag sealer, lift the cut corner slightly and push the air bubbles through the opening with your free hand. When finished, seal the bag immediately, and dry the outside seams.

To ensure good circulation of the hybridization solution inside the bag, tape the bag down to a flat surface (e.g., a plastic box with a lid) and securely attach the box to the floor of the shaker/incubator or water bath/shaker. Using a plastic box helps to keep the hybridization temperature more stable, because the plastic bag is so thin that when the shaker or water bath is opened, the temperature inside drops quickly.

Prehybridizations are required to block the empty spaces on the membrane with denaturing detergent to avoid the probe binding to the empty spaces. It

usually takes 1 h at the desired temperature. To avoid uneven wetting, soak the membrane in water or 3X SSC buffer before prehybridization (this is only necessary for dry membranes). Some laboratories that process membranes infrequently do not realize that the prehybridization solutions can be reused; this is of critical importance for laboratories that perform membrane hybridizations in a high throughput manner. Reusing the prehybridization solution, up to five to seven times, or until debris accumulates, can save a considerable amount of preparation time and money. Storage at -20°C after use is required.

Hybridization of the probe to the target DNA depends on many factors that are beyond the scope of this chapter. However, some good habits should be adopted. To avoid localized hybridization of the probe to the membrane, never add the probe (a few microliters) to the prehybridization solution while the solution is still in the bottle or plastic bag. Prepare the hybridization solution separately, denature the solution by incubating at 95 to 98°C for 10 min, quench on ice, and then add the hybridization solution to the membrane. Never assume that more probe means higher signal, often it is the opposite. It is reported that most background problems with nonradioactive systems are caused by excess probe that is unable to be removed with the stringency buffers. Always quantify your random-labeled probe or riboprobe by testing on a dot-blot against control reactions with known amounts of labeled DNA/RNA. The incubation time depends on the nature of the target nucleic acid, the amount of the target, and the amount of the probe. Southern blots of plasmid DNA require the shortest hybridization incubation times (1–2 h), even if the amount of plasmid DNA is 1 to 2 pg per lane. The number of target molecules/weight ratio is high compared with genomic DNA Southern blots, or Northern blots, which require longer than 16 h of incubation. Hybridization solutions can also be reused; probes labeled with digoxigenin, biotin, or fluorescein can be stored at -20°C after use for longer than 1 yr. Depending on the amount bound to the membrane in each experiment (either because of clean signal or just general background), it is recommended to add one-tenth of the original amount of probe used for preparing the hybridization solution to keep the concentration stable. Before adding the hybridization solution to the membrane, bring the solution up to the hybridization temperature.

2.2. Stringency Washes

The hybridization solution is a low stringency medium containing a high concentration of salts, which allows the probe to bind to the target but also to other nontarget molecules. To remove the probe from nontarget molecules, the membrane is washed with low-salt buffers with denaturing detergent. The exact salt concentration depends on the nature of the probe. As a general rule, oligonucleotide probes require high concentrations of salt (i.e., low stringency);

highly homologous probes require low concentrations of salt (i.e., high stringency). This is a process of trial and error until the optimum signal to background ratio is obtained. However, we have found that, by using large volumes of wash buffers and replacing them every 15 to 30 min (depending on the selected protocol), improvements in probe removal can be readily obtained. The membrane should never be allowed to stick to the bottom of the plastic box used for the washes, and the DNA side should always be facing upward. Adding the wash buffer prewarmed to the required temperature ensures that the washing incubation time is actually what the protocol states. To prewarm, microwave the buffer 1 min per 0.5 L at maximum power in a standard microwave; otherwise set up a separate water bath. The plastic boxes used for detection should be used for this purpose only, and should be washed out with hot water, without scrubbing or brushing, because this will scratch the plastic surface and could lead to accumulation of probe or other debris, which may increase the background signal.

2.3. Antibody Detection of Nonradioactive Labeled Probe

After the stringency washes, the detection of nonradioactive probes follows a standard Western blot procedure: an antibody–hapten reaction, in which the antibody is covalently bound to an enzyme (e.g., alkaline phosphatase or horseradish peroxidase). The enzyme cleaves the substrate and generates either a product that precipitates near the target molecule, or produces light.

The membrane should be blocked with a proteinaceous solution made up either with 1% Hammerstein-grade casein (4) in a suitable buffer, or with another commercially available blocking buffer. Always ensure that the casein solution is dissolved completely by heating the required buffer to 65°C and adding the casein stepwise, stirring constantly until it is fully dissolved. The solution should then be autoclaved. Regardless of the protocol selected, the pre-incubation of the membrane in the blocking buffer requires 30 min, and can be extended overnight at room temperature; however, the incubation with the antibody/blocking buffer mixture should be for no longer than 30 min, otherwise unspecific binding will occur even after blocking the empty spaces with the casein-based solution. Sometimes a spotty background is observed; this is usually caused by precipitated or undissolved blocking powder, or by denatured antibody. The antibody stock should always be centrifuged at 9391g, 5 min before each use and before adding the antibody to the buffer. Discard the last 10 µL of the antibody stock, which is denatured protein only. Do not add the antibody stock to the blocking buffer in which the membrane is incubated, prepare a fresh mixture with blocking buffer, discard the used blocking buffer, and then add the antibody/blocking solution. This will prevent localized binding of the antibody to the membrane.

2.4. Conjugated Enzyme Detection

Developing the enzymatic activity of the coupled enzyme–antibody–target complex will depend on the nature of the conjugated enzyme. The vendor has usually optimized the substrate concentration; however, some improvements on the signal to background ratio can be achieved by using less substrate. Colorimetric reactions only require enough substrate solution to cover the membrane, and be incubated in the dark without shaking.

In chemiluminescent methods, the membrane is packed between two plastic sheets (i.e., a standard clear plastic bag) and sealed to contain the substrate solution. If the membrane is small (e.g., 7×11 cm), the substrate can be added by placing the drop on the plastic bag and laying the membrane DNA side down gently, without trapping air bubbles. For larger membranes, the substrate can be applied with a small thin-layer chromatography sprayer. The sprayer should have a working capacity of 5 to 25 mL, because the minimum volume that can be sprayed with confidence is 2 mL (consider the volume wasted in the inner tube). A thorough rinse with deionized water before and after use is enough to keep the sprayer clean, although the sprayer can be autoclaved if necessary. Ensure that the source of compressed air is constant to generate an even mist, and place a cotton wool filter along the path of the compressed air to the sprayer to avoid debris sprayed on the membrane. Next, the top plastic sheet is laid on the sprayed membrane, and the excess liquid can be squeezed out before sealing the membrane, avoiding trapping air bubbles. Too much substrate does not enhance the signal and it usually provides extra background.

Washing off the substrate solution with a buffer containing a chelating agent stops the colorimetric reaction when the desired high signal to background ratio has been achieved. Chemiluminescent reactions do not need to be stopped. Several exposure times can be accomplished with the same membrane, or if a weak signal is obtained after a 3 h exposure, a new membrane/substrate sandwich can be assembled and exposed to an X-ray film overnight. Specially designed X-ray films for chemiluminescence are available from several suppliers, and all achieve comparable results. The main feature is that they provide a clear background that enhances the signal above any other interference. Developing the X-ray film should be performed as specified by the vendor, either manually or in automatic developers.

2.5. Miscellaneous Considerations

This section can be considered as a collection of tips of the trade; however, other miscellaneous hints are provided here.

To handle membranes, use a stainless steel forceps without a grip, standard point. Avoid touching the membrane with gloves and bare hands. Instead, handle

the membrane with the aid of a clean glass or plastic rod; it will prevent the membrane to curl up over itself.

Hybridization bottles, nylon mesh, storage boxes, washing boxes, and any other containers should be washed with mild detergent and hot water, without scrubbing to avoid abrasion. If required, autoclave hybridization bottles and mesh between experiments to avoid cross contamination.

The surface of some standard plastic bags is slightly charged, and can produce electrostatic discharges when rubbed onto a dry surface (e.g., tissue paper). It will produce an interesting, although undesirable, web-like pattern on the X-ray film. To quench the electrostatic charge, wipe the plastic bag with ethanol, and let it dry completely.

If the development of a Southern or Northern blot results in a spotty background, even under optimized conditions, the spots can be removed by wiping the surface of the membrane with a lint-free tissue soaked in stripping solution, similar to the debris removal performed on colony blots or high-density library membranes discussed in **Subheading 1.1.6**. The stripping solution composition varies, although most membranes require a solution containing 0.2 M NaOH and 0.1% SDS, at 37°C. Check the vendor specifications.

After stripping, membranes should be stored in 3X SSC buffer at 4°C. Seal the membranes in individual bags with sufficient buffer, or place several membranes in an airtight plastic box. Examine the membranes regularly for fungal growth. Some fungi can be removed by wiping the membrane with prewash or stripping solution, as described in **Subheading 1.1.6**. If the growth is not too extensive, the membrane may be used again, but discard the prehybridization and hybridization solutions after use to avoid contaminating other membranes. If the starting material is available in great quantities, do not waste time cleaning, but instead, prepare a new membrane.

DNA ladders can be easily labeled using 3'-end labeling nonradioactive methods. Using digoxigenin-11-ddUTP as the hapten, 1 µg of 1-kbp ladder can be easily labeled according to the manufacturer's protocol (Roche Applied Science). Up to 12-kbp fragments can be labeled efficiently with this method. It is advisable to purchase prelabeled ladders if larger fragments are required.

As with many methodologies in molecular biology, it is recommended that 10X stock solutions be prepared and stored at room temperature or frozen. Hybridization buffers should always be prepared in large volumes, to avoid variation from batch to batch.

Acknowledgments

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Checkerboard DNA–DNA Hybridization Technology Using Digoxigenin Detection

Lisa S. Gellen, Glenn M. Wall-Manning, and Chris H. Sissons

Summary

Checkerboard DNA–DNA hybridization (CKB) is a technique that provides a simultaneous quantitative analysis of 40 microbial species against up to 28 mixed microbiota samples on a single membrane; using digoxigenin (DIG)-labeled, whole-genome DNA probes. Developed initially to study the predominantly Gram-negative dental plaque microorganisms involved in periodontitis, we modified the probe species composition to focus on putative pathogens involved in the development of dental caries. CKB analysis is applicable to species from other biodiverse ecosystems and to a large number of samples. The major limitations are that high-quality DNA is required for the preparation of DIG-labeled probes and standards, and that probe specificity requires careful evaluation. Overall, CKB analysis provides a powerful ecological fingerprint of highly biodiverse microbiota based on key cultivable bacteria.

Key Words: Checkerboard DNA hybridization; dental caries; dental plaque; microbial ecosystem; species fingerprinting.

1. Introduction

With the advent of molecular technologies, it has become clear that analysis of microbial ecosystems by culture techniques yields a misleading appreciation of their biodiversity (1). For example, in the 1970s, dental plaque was thought to be composed of approx 20 species and its role in dental disease was thought to be well understood. With the advent of ribosomal DNA (rDNA) sequencing, the number of species-level taxa has now increased to 500 or more—approximately half cultivated and speciated. The other half is still uncultivated, and detectable only by cloning and sequence analysis (2–5). A similar situation is true of many environmental microbial systems, especially complex biofilm systems, in which spatial heterogeneity maximizes available habitats (6).

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To characterize these extraordinarily complex systems, microbiota fingerprinting can be applied. This does not characterize the system in complete detail, but, rather, gives an overall characterization of its composition. For bacteria, predominately rDNA PCR-based techniques are used. These mostly involve gel separations, and include terminal-restriction fragment length polymorphism analysis (7) and denaturing gradient gel electrophoresis (8) as major options. Denaturing gradient gel electrophoresis also allows subsequent cloning and sequence-based identification of species present. An inherent limitation for such PCR-based techniques is the bias against high G + C DNA regions in templates (9). Metabolic characterization of ecosystems using substrate utilization techniques (e.g., Biolog™ plates) is also possible (10).

Checkerboard DNA–DNA hybridization (CKB) analysis is a particularly powerful non-PCR technique that is based on simultaneous hybridization of 40 digoxigenin (DIG)-labeled (11) whole-genome DNA probes. These probes are chosen from cultivatable microbes that are thought to be either significant species or ecological indicators of the ecosystem (Fig. 1; refs. 12–14). CKB analysis provides a quantitative analysis of these 40 species simultaneously in microbial ecosystem samples and was developed for the study of dental plaque pathogens by Drs. S. S. Socransky and A. Haffajee at The Forsyth Institute (Boston, MA; ref. 12). DNA standards and DIG-labeled whole-genome probes of the target species are prepared and mixed. DNA standards equivalent to 10^5 and 10^6 cells per target species, and up to 28 alkali lysates of dental plaque, are fixed on a membrane in thin lanes using a specialized “checkerboard” MiniSlot system (Fig. 2A) from Immunetics Inc. (Cambridge, MA; ref. 12). The membranes are then hybridized simultaneously with 40 DIG-labeled whole-genome probes in a perpendicular axis, using a specialized checkerboard Miniblotter system (Fig. 2B) from Immunetics, as illustrated in Fig. 1. The hybrids are then detected quantitatively. A flow chart of the steps in the procedure is shown in Fig. 3.

Using this technology and community ordination analysis, Socransky et al. (13) demonstrated that a particular set of clusters of species were related to the development of periodontitis. Socransky et al. (13) also detected a cascade of pre-pathogen microbiota complexes, which enabled the establishment of the cluster of major pathogens that cause periodontitis. A series of major studies illuminating the plaque microbiota and its relationship to health and disease followed (see reviews in refs. 15 and 16). We have modified the species panel to focus more on the Gram-positive putative pathogens of dental caries (17). CKB analysis has considerable potential for characterizing

the behavior

of other complex microbial ecosystems, because it provides a powerful ecological fingerprint of highly biodiverse microbiota based on key cultivable bacteria (14).

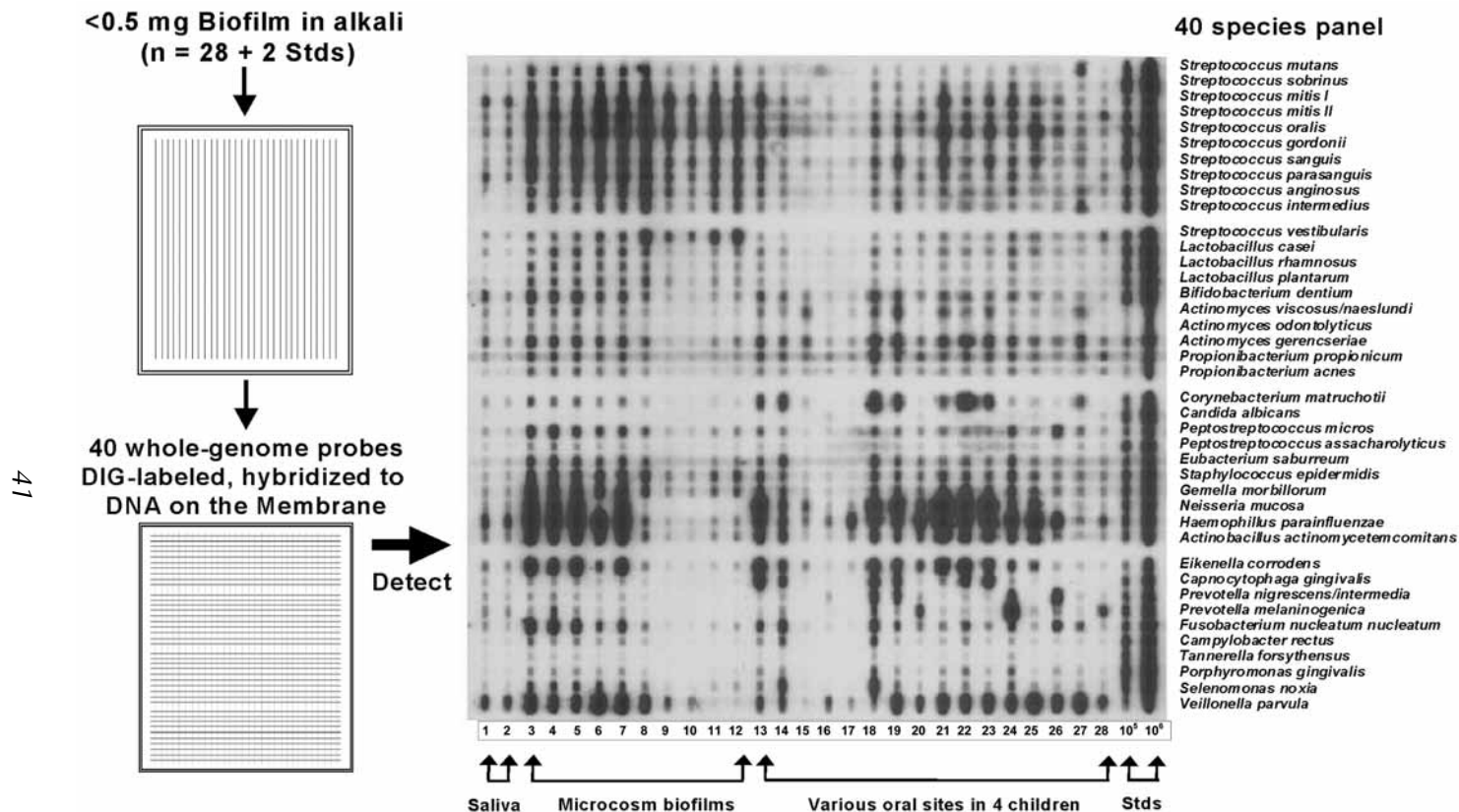


Fig. 1. Principle of CKB analysis with a membrane X-ray film image showing various dental plaques and plaque microcosms. The plaque microcosms were cultured in our Multiplaque Artificial Mouth under different conditions (15–17). The intra-oral plaques came from similar clinical sites in four children. Of note are the similarities between the clinical and microcosm plaques, reinforcing plaque microcosm biofilms as a valid model system to study plaque growth and development.

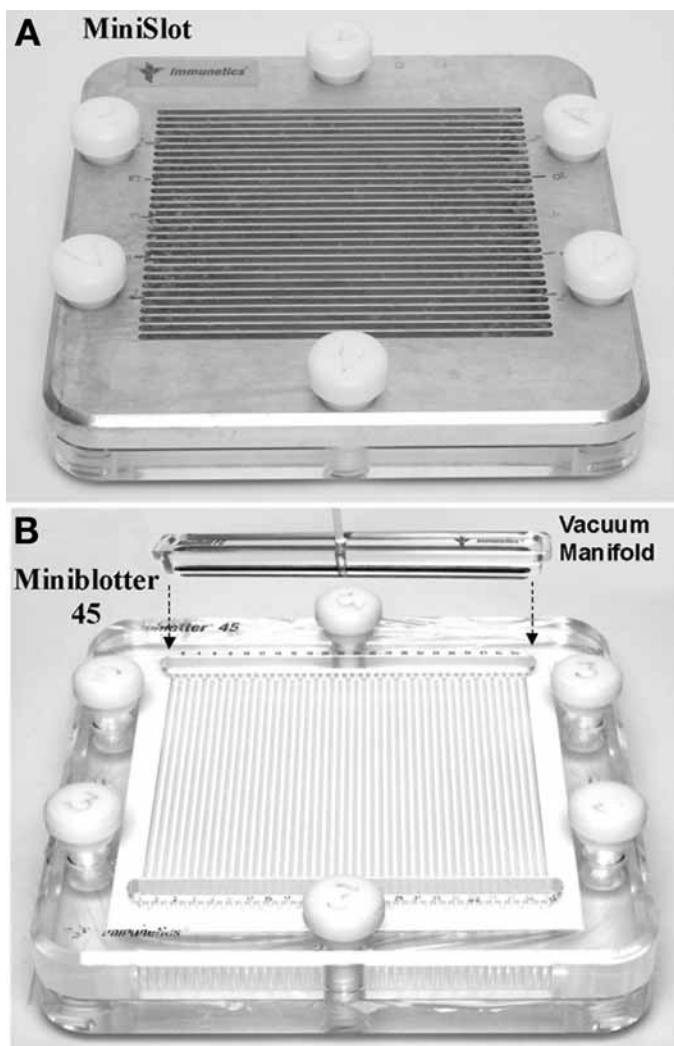


Fig. 2. Checkerboard MiniSlot and Miniblotter apparatus (Immunetics). **(A)** Assembled MiniSlot apparatus used to deposit the sample in alkali onto the membrane in thin (800- μ m wide) lanes. **(B)** The assembled Miniblotter 45 apparatus that forms the chambers for the 40 probes during hybridization, together with the vacuum manifold that is used to remove the hybridization mixtures by aspiration.

2. Materials

2.1. Preparation of Plaque Samples for Analysis

1. 1.5-mL Screw cap tubes with O-rings (see **Note 1**).
2. Disposable brush applicators (see **Note 2**).

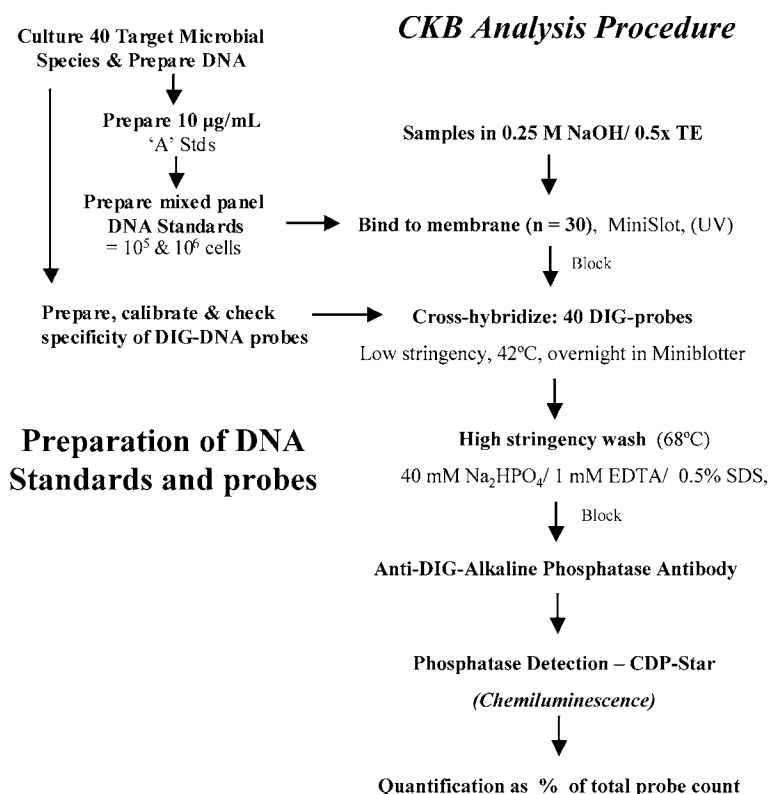


Fig. 3. CKB analysis flow schematic.

3. Tris-HCl-EDTA (TE) buffer (1X TE), pH 8.0: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0. 1 M Tris-HCl and 0.5 M EDTA stocks are prepared separately and stored at room temperature.
4. 0.25 M NaOH (freshly prepared)/0.5X TE (5 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA, pH 8.0) in aliquots of 250 µL per tube.

2.2. Preparation of Genomic DNA From Bacteria to Use in Checkerboard Standards and Probes

2.2.1. Growth of Cultures

1. TSBYK broth (1 L): 15 g tryptic soy broth, 18.5 g brain heart infusion, 1% yeast extract, 10 mL of hemin stock (50 mg hemin and 1.74 g K₂HPO₄ in 100 mL of H₂O). Boil to dissolve, and store at -20°C). Autoclave the media and add 1 mL of vitamin K stock (4 mg of water-soluble menadione in 10 mL of H₂O. Filter sterilize [see **Note 3**], and store at -20°C). For TSBYK/blood agar (1 L): add 1.5% agar to TSBYK broth, autoclave, and supplement with vitamin K and 5% defibrinated sheep blood.

2. Alternative media used for particular species include: *Actinomyces* broth and agar (*Actinomyces* broth + 1.5% agar); potato dextrose agar; brain heart infusion (BHI) broth and agar; Rogosa SL agar; fastidious anaerobe agar (Lab M Ltd., Lancashire, UK); and chocolate agar (place TSBYK/blood agar plate, medium side facing down, at 65°C for 90 min, until it is a uniform chocolate color).
3. The supplements used include: *N*-acetyl muramic acid (Sigma; 100 mg in 10 mL of H₂O, filter sterilize, and store at -20°C); formate/fumarate (6% sodium formate and 6% fumaric acid, pH 7.0; filter sterilize and store at 4°C).
4. 10 mg/mL Penicillin G (PEN-NA, Sigma) in H₂O, filter sterilize and store at -20°C.

2.2.2. DNA Extraction

1. Lysozyme.
2. 20 mg/mL Proteinase K in H₂O, store in 0.5-mL aliquots at -20°C.
3. 10 mg/mL RNase A (see **Note 4**) in 1X TE buffer: add 0.1 g RNase A to 10 mL of 1X TE buffer, boil for 15 min, cool slowly, and store in 0.5-mL aliquots at -20°C.
4. 20% Sodium dodecyl sulfate (SDS).
5. 5 M NaCl.
6. 10% CTAB/0.7 M NaCl: dissolve NaCl in H₂O, slowly add hexadecyl trimethylammonium bromide (CTAB) while heating to 65°C and stirring. Adjust to final volume with H₂O and autoclave.
7. Chloroform:isoamyl alcohol, 24:1 (v/v).
8. Isopropanol (Propan-2-ol).
9. 100% Ethanol (-20°C).
10. 70% Ethanol.
11. Phase Lock Gel Heavy, 3-mL syringe (Eppendorf).
12. 3 M Na-acetate, pH 5.2.
13. Buffer-saturated phenol: stored under 0.1 M Tris-HCl buffer, pH 8.0, at 4°C.

2.2.3. Evaluation of Quality and Quantity of DNA

1. Scanning spectrophotometer.
2. Gel electrophoresis tank, tray, comb, and power pack.
3. 50X Tris-base-acetate-EDTA (TAE) buffer stock (1 L): 242 g Tris-base, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA, pH 8.0. Autoclave. Dilute 1:50 for a 1X working solution.
4. 6X Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400, Amesham Pharmacia) in H₂O. Store at room temperature.
5. DNA Molecular Weight Ladder (λ DNA digested with *Hind*III).
6. 0.5 μ g/mL Ethidium bromide: 50 μ L of 10 mg/mL ethidium bromide stock (1 g per 100 mL of H₂O stirred for several hours) in 1 L of H₂O. Store in light-proof bottle at room temperature. **Caution:** ethidium bromide is a powerful mutagen and moderately toxic.

2.3. Preparation of Standards and Calibrated Probes

1. DIG-High Prime (Roche Applied Science; [ref. 11](#)).

2.4. CKB Technique

1. MiniSlot™ device (SB-30, Immunetics; **Fig. 3A**).
2. Miniblotter® (MN-45, Immunetics; **Fig. 3B**).
3. Manifold (F2, Immunetics; **Fig. 3B**).
4. Plastic cushions (PC2, Immunetics).
5. Nylon membranes, positively charged (Roche Applied Science).
6. Chemiluminescent detection film (e.g., Lumi-Film, Roche Applied Science).
7. Alkaline phosphatase (AP)-linked antibody (Anti-DIG-AP, Fab fragments, Roche Applied Science).
8. CDP-Star (Roche Applied Science).
9. UV crosslinker.
10. Hybridization oven/shaker.
11. Flat-ended forceps.
12. Reciprocating shaker.
13. Heat sealer.
14. Vacuum pump (e.g., water pump).
15. 72- μ m Homogenizer bags (Guest Medical Ltd, Edinbridge, Kent, UK).
16. Whatman 3MM chromatography paper.
17. Saran wrap or equivalent.
18. Disk Wisk apparatus (Schleicher & Schuell, Dassel, Germany; see **Note 5**).
19. Reaction folders (e.g., R-F, Schleicher & Schuell).
20. X-ray cassette, developing equipment, and chemicals.
21. DIG detection imaging system for hybridized membranes; either a charge-coupled device (CCD) camera capable of recording chemiluminescence or, after use of a fluorescent AP substrate, a laser scanning fluorescent detection system.
22. 5 M NH_4 acetate.
23. Formamide (molecular biology grade).
24. 50X Denhardt's solution: 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin (Fraction V), add H_2O to bring to 500 mL. Filter to clarify, and store in 10-mL aliquots at -20°C .
25. 20X sodium citrate sodium chloride: 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0. Sterilize by autoclaving.
26. Prehybridization buffer stock solution: 88 g NaCl, 44 g Na_3 citrate, and 6.0 g Na_2HPO_4 , adjust pH to 6.5 with HCl, bring volume up to 500 mL with H_2O , and autoclave.
27. Hybridization stock solution: 30.7 g NaCl, 16.1 g Na_3 citrate, and 3.4 g Na_2HPO_4 , adjust pH to 6.5 with HCl, bring volume up to 500 mL with H_2O , and autoclave.
28. 10X Maleic acid buffer stock (1 L): 116 g maleic acid (1 M), 175.4 g NaCl (3 M), adjust pH to 7.5 with NaOH, bring volume up to 1 L with H_2O , aliquot into 100-mL bottles, and autoclave.
29. 10X Antibody blocking buffer stock (1 L): Stir 100 g casein (Sigma) in 1 L of 1X maleic acid buffer, with gentle heating (to 60°C), for approx 1 h or until totally dissolved. Aliquot into 100-mL bottles, and autoclave.

30. 10X Detection buffer stock (1 L): 121.1 g Tris-base (1 M), 58.44 g NaCl (1 M), adjust pH to 9.5 with HCl, bring volume up to 1 L with H₂O, aliquot into 100-mL bottles, and autoclave.
31. Prehybridization solution: 25 mL of formamide, 5 mL of 50X Denhardt's solution, 5 mL of 10% casein, bring volume up to 50 mL with prehybridization stock solution. Make fresh.
32. Hybridization solution: 500 mL formamide, 20 mL of 50X Denhardt's solution, 100 g dextran sulphate, 430 mL hybridization buffer stock solution, and 100 mL of 10% casein (see **Note 6**). Store at -20°C.
33. Phosphate/SDS buffer: 175 mL of 20% SDS (0.5%), 2.6 g EDTA (1 mM), 24.5 g Na₂HPO₄ (40 mM), bring volume up to 7 L with H₂O. Make fresh.
34. 1X Antibody blocking buffer: 100 mL of 10X antibody blocking buffer stock, 900 mL of 1X maleic acid buffer. Store at 4°C.
35. 1X Washing buffer: 100 mL of 10X maleic acid buffer stock, 3 mL of Tween-20, 897 mL of sterile H₂O. Make fresh.
36. 1X Detection buffer: 100 mL of 10X detection buffer stock and 900 mL of sterile H₂O. Make fresh.

2.5. Data Processing

1. Software for quantifying membranes (e.g., proprietary software by Dr. S. S. Socransky and Dr. A. D. Haffajee, The Forsyth Institute, Boston, MA).
2. Data processing software, Microsoft Excel, and statistics programs suitable for community ordination and related ecological analyses (see **Subheading 3.5.2.**).

3. Methods

3.1. Preparation of Plaque Samples for Analysis (see Note 7)

Plaque samples and standards containing mixed DNA of all of the probe-panel oral microbes are prepared. Plaque samples can be derived from dental plaque microcosms (**18,19**), or from clinical plaque or oral biofilm samples taken with small disposable brushes (SDI Points applicators). The plaque samples are labeled and stored at -20°C until use. Each microcosm plaque sample from the Multiplaque Artificial Mouth (**20**) is given a unique number, which is recorded in the checkerboard sample log, along with the sample details. Clinical sample details are similarly kept in a separate log.

3.1.1. Saliva

1. For saliva, centrifuge 1 mL of saliva at 12,000g in a tared microcentrifuge tube to give a salivary sediment pellet.
2. Weigh the pellet and make a 20 mg/mL suspension by adding an appropriate volume of 1X TE.
3. Transfer 100 µL of the 20 mg/mL suspension (2 mg) to a sterile tube.
4. Add 100 µL of 0.5 M NaOH immediately before boiling (see **Subheading 3.4.1.**).

3.1.2. Plaque

For a clinical plaque, the sample size should be between 0.25 mg and 2 mg (see **Note 8**).

1. Plaques are resuspended in 250 μ L of 0.25 M NaOH/0.5X TE. They can be stored for short times at room temperature, or long term at -20°C , or indefinitely at -80°C .
2. For all plaque samples, the target amount to be applied to the membrane is 0.25 mg. For solid plaque samples that have not been suspended in alkali (e.g., microcosm plaques), make a 2.5 mg/mL suspension by adding an appropriate volume of 1X TE to the plaque pellet.
3. Resuspend the pellet by vortexing, and transfer 100 μ L of the 2.5 mg/mL suspension (0.25 mg) to a sterile tube.
4. Add 100 μ L of 0.5 M NaOH immediately before boiling (see **Subheading 3.4.1.**).

3.1.3. Layout

Our standard layout includes up to 25 plaque samples, flanked on each side by a set of two DNA standards equal to 10^5 and 10^6 cells. *Eschericia coli* DNA (10 ng) is also included as a negative control.

3.2. Preparation of Genomic DNA From Bacteria to Use in Checkerboard Standards and Probes

High-quality whole-genome DNA is required from all of the oral microbes used for preparing checkerboard probes and standard mixtures.

3.2.1. Growth of Cultures (see **Note 9**)

Oral bacteria are generally grown in TSBYK media (**21**). *Tannerella forsythensis* (previously, *Bacteroides forsythus*) cultures are supplemented with 10 mg/L *N*-acetyl muramic acid and *Campylobacter* spp. cultures are supplemented with 5% formate/fumarate. *Lactobacillus* spp. can also alternatively be grown in either Rogosa or BHI media, and *Streptococcus* spp. can, alternatively, be grown in BHI media. *Actinomyces* spp. and *Bifidobacterium dentium* are grown in *Actinomyces* media, *Candida albicans* in potato dextrose agar media, *Eubacterium* spp. in fastidious anaerobe agar or TSBYK broth, and *Haemophilus parainfluenzae* in chocolate agar. All cultures are grown under appropriate atmospheric conditions.

3.2.2. DNA Extraction (see **Note 10**)

Generally, DNA is isolated using this protocol, which is a modification of the method described by Smith et al. (**22**). This procedure uses 250-mL broth volumes (see **Note 11**). All centrifugation steps are performed at room temperature

unless otherwise noted. Centrifuge times from **step 8** onward can be shortened by increasing the centrifugal force.

1. Inoculate 5 mL of TSBYK broth in a tube from a single colony on an agar plate and incubate overnight at 35°C in a shaking incubator, with anaerobes in an anaerobic hood.
2. Inoculate 1:50 into the 250 mL of broth and incubate at 35°C to the early exponential phase (for most bacteria, ~3 h). Add 250 µL of 10 mg/mL penicillin G to the Gram-positive organisms (see **Note 12**) and continue growing cultures for approximately three further doubling times.
3. Centrifuge for 10 min at 3290g, carefully pour off supernatant, resuspend the cells in 20 mL of 1X TE buffer, and re-centrifuge in 50-mL tubes.
4. Pour off the supernatant and resuspend the pellet in 5 mL of 1X TE buffer (see **Note 13**).
5. Place approx 10 mg of lysozyme into the resuspended pellet, and incubate at 37°C for 1 h.
6. Add 300 µL of 20% SDS to Gram-positive organisms (150 µL of 20% SDS to Gram-negative organisms) and 60 µL of 10 mg/mL Proteinase K, and incubate at 37°C for 1 h or until the solution is clear.
7. Add 1.5 mL of 5 M NaCl, mix by inversion several times, add 850 µL of CTAB/NaCl solution (prewarmed to 65°C), mix well by inversion, and incubate at 65°C for 20 min.
8. Add an equal volume of chloroform:isoamyl alcohol (24:1), mix by inversion to emulsification, and centrifuge for 30 min at 3290g (or 5 min at 12,000g).
9. Carefully transfer the aqueous layer, with a sterile pipet, to a clean, sterile 15-mL centrifuge tube, add an equal volume of isopropanol, and gently invert the tube several times to precipitate the DNA.
10. Centrifuge for 30 min at 3290g and discard the supernatant by careful decantation.
11. Add 5 mL of 70% ethanol to the pellet, centrifuge for 10 min at 3290g, carefully decant the 70% alcohol to discard, and place the tubes upside down on a clean tissue to drain and dry.
12. Dissolve the pellet in 2 mL of 1X TE buffer at room temperature overnight (or at 65°C for 1 h), add 20 µL of RNase A (10 mg/mL) and incubate at 37°C for 1 h.
13. Add 1 mL of Phase Lock Gel to tube and centrifuge for 2 min at 3290g.
14. Add 1 mL of phenol and 1 mL of chloroform:isoamyl alcohol (24:1), mix well and centrifuge for 10 min at 3290g. Repeat this extraction twice by adding 1 mL of phenol and 1 mL of chloroform:isoamyl alcohol (24:1) into the same tube.
15. Add 2 mL of chloroform:isoamyl alcohol (24:1), mix well, and centrifuge for 10 min at 3290g. Repeat this extraction once.
16. Decant the aqueous layer into a clean 15-mL centrifuge tube, add 1/10 volume of 3 M sodium acetate, mix well, and slowly add exactly 2 volumes of cold (–20°C) 100% ethanol. Slowly invert several times to precipitate the DNA and place at –20°C for at least 1 h.
17. Centrifuge for 30 min at 4°C at 3290g, pour off the supernatant, and add 5 mL of 70% ethanol.

18. Centrifuge for 10 min at 3290g, pour off the supernatant, and place the tubes upside down on tissue to drain and dry.
19. Resuspend in 1X TE buffer, between 50 μ L and 200 μ L, depending on the apparent yield of DNA. Incubate at 37°C for several hours or overnight to dissolve the DNA.

3.2.3. Evaluation of the Quantity and Quality of DNA (see **Note 14**)

High-quality DNA (i.e., no RNA or protein contamination) for standard and probe preparation is a key requirement of this technique. DNA quality is evaluated by UV spectrophotometry and agarose gel electrophoresis.

1. Scan a UV spectrum of a 1:10 dilution of the DNA extracts.
2. Calculate the concentration (ng/ μ L) of DNA ($A_{260} \times 50$ [for double-stranded DNA] $\times$ dilution factor) and the A_{260}/A_{280} ratio.
3. Also check for DNA degradation and purity (negligible RNA or protein contamination) by electrophoresing 2 μ L of each sample against λ DNA/*Hind*III or a similar DNA marker on a 0.7% agarose gel in 1X Tris-base–acetate–EDTA buffer, at approx 10 V/cm, until the bromophenol blue dye front approaches the far end of the gel.
4. Stain the gel in an ethidium bromide solution for 30 min, destain in H₂O for 30 min, visualize on a transilluminator, and photograph using an appropriate camera and filter set-up.
5. When the extracted DNA is adequately pure (i.e., within the correct ratio and free from RNA contamination) enter the details into an “Entire DNA Stock” list and archive the analytical results.

3.3. Preparation of Standards and Calibrated Probes

3.3.1. Preparation of Standardized DNA at 10 μ g/mL (A Std) Stocks (see **Note 15**)

Calculate the appropriate dilution and make up 1 mL of 10 μ g/mL A Std in 1X TE buffer in a 1.5-mL microcentrifuge tube.

3.3.2. Preparation of CKB Standards

CKB standards are mixed-species, whole-genome DNA standards of 1 ng and 10 ng, taken to be equivalent to 10^5 and 10^6 cells/mL, respectively (**23**).

1. For a 10 mL volume, mix 100 μ L of the A Std for each of the bacterial species in the panel. Bring the volume up to 10 mL with 1X TE buffer in a disposable polypropylene tube. For *Candida* spp., use 500 μ L (five times the amount for bacteria) to allow for its greater genome size. Vortex briefly to mix well, and label this mix “ 10^6 .” Dilute 10-fold to prepare the 10^5 standards.
2. Dispense 100- μ L aliquots of each mix into sterile 1.5-mL microcentrifuge tubes and label appropriately. Store at -20°C .
3. To use, thaw at room temperature and add 100 μ L of 0.5 M NaOH. Incubate at room temperature for 5 min to denature the DNA, and add 800 μ L of freshly prepared 5 M NH₄ acetate to neutralize the solution. Lay the standards on the membrane with the

PROBE PREPARATION WORKSHEET				
Date:	16-Feb-04			
Prepared by:	Lisa G			
Organism:	Streptococcus vestibularis LM-1			
<u>1µg prep</u>				
DNA used:	G75			
DNA conc (ng/µL):	789			
Probe no:	P241	DNA vol (µL): 1.3	Water vol (µL): 14.7	
<u>2.5µg prep</u>				
DNA used:	G75			
DNA conc (ng/µL):	789			
Probe no:	P242	DNA vol (µL): 3.2	Water vol (µL): 12.8	
<u>Calibration</u>				
Membrane no:	04029			
Use probe no:	P241	at	20 µL	
Use probe no:	P242	at	10 µL	

Fig. 4. Example of probe preparation worksheet. This simple spreadsheet provides a reliable and robust method to calculate the DNA and water volumes required to prepare the two probes in a labeling experiment. The calibration procedure and results for these *Streptococcus vestibularis* probes are shown in Fig. 5. After the calibration procedure has been completed, the optimal volume of each probe to use is recorded on this worksheet.

experimental samples. The 10⁶ standard is laid in lanes 2 and 30, with the 10⁵ standard in lanes 1 and 29.

3.3.3. Preparation and Calibration of DIG-Labeled Probes (see **Note 16**)

Genomic DNA from each species is labeled with DIG to create the probe used for detection of each species. Two preparations, one using 1 µg DNA and one using 2.5 µg DNA, are performed at the same time, because DIG-labeling reactions can vary in efficiency. The calculation is performed and documented using a Probe Preparation Worksheet (see Fig. 4). The newly prepared probes are checked against the previous probe preparation for that organism, and hybridized against the DNA standards, to determine the optimal volume for use. Each newly prepared probe is tested at volumes of 40, 20, 10, 5, and 2.5 µL (Fig. 5).

1. Using the volumes calculated on the Probe Preparation Worksheet, add the DNA and H₂O volumes together in a microcentrifuge tube (see Note 17).
2. Dilute this amount of DNA to 16 µL with H₂O, and incubate for 5 min in a boiling waterbath. Cool in an ethanol/ice bath for 5 min, and centrifuge briefly to return the condensation to the bottom of the tube. Add 4 µL of DIG–High Prime, mix well, and incubate overnight at 37°C.
3. Add 2 µL of 0.2 M EDTA to stop the reaction and add 1 mL of 1X TE buffer for the final probe solution.
4. Prepare a membrane to test the probes by laying 10⁵ and 10⁶ standards in the usual lanes. In addition, using the 10-µg/mL A Std DNA solutions, lay 100 ng, 10 ng,

Date Laid: 11.3.04

By: LG

Membrane Number: 04029

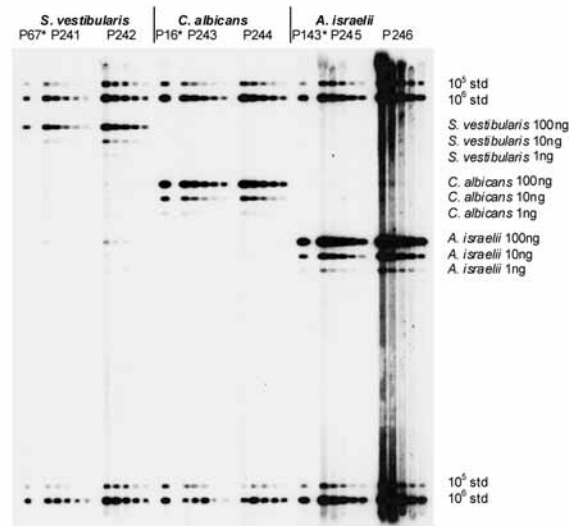
Date Probed: 22.3.04

By: LG

Probe Calibration

Membrane sample #	Sample name	Strain ID	A Std. number	Probe Amount
1	10 ⁷ std			
2	10 ⁶ std			
3				
4	<i>S. vestibularis</i>	LM-1	A64	100ng
5				10ng
6				1ng
7				
8	<i>C. albicans</i>	A72	A80	100ng
9				10ng
10				1ng
11				
12	<i>A. israelii</i>	12102	A89	100ng
13				10ng
14				1ng
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30	10 ⁷ std			
	10 ⁶ std			

1	Probe	Strain	Probe #	Vol
2				
3	<i>S. vestibularis</i>	LM-1	P67	2.5
4				
5			P241	40
6				20
7				10
8				5
9				2.5
10				
11			P242	40
12				20
13				10
14				5
15				2.5
16				
17	<i>C. albicans</i>	A72	P16	5
18				
19			P243	40
20				20
21				10
22				5
23				2.5
24				
25			P244	40
26				20
27				10
28				5
29				2.5
30				
31	<i>A. israelii</i>	12102	P143	20
32				
33			P245	40
34				20
35				10
36				5
37				2.5
38				
39			P246	40
40				20
41				10
42				5
43				2.5
44				
45				

A Membrane Worksheet

* Optimal probe previously used.
 Each probe is a 2-fold dilution series - see (a)

B Membrane Image

Fig. 5. Example of probe calibration. **(A)** Membrane worksheet (04029), filled in detailing the target DNA to be laid, in this case corresponding to 10⁷, 10⁶, and 10⁵ bacterial cells (100, 10, and 1 ng of purified DNA). The usual standards are also laid. The membrane is probed with the current probe for each species, (e.g., P67 for *Streptococcus vestibularis* LM-1) and with dilutions of the freshly prepared probes. **(B)** Corresponding membrane X-ray film image. Comparisons between the dilutions and the current probe determine the optimal volume to use, with the highest dilution (lowest volume) that gives an equivalent signal selected. Note how overloading with probe (40, 20, and 10 μ L of P246) causes “streaking” along the lane.

and 1 ng of genomic DNA of the appropriate species, using the same denaturation and neutralization methods used for the standards. Prehybridize using standard conditions (see **Subheading 3.4.2.**).

5. Dilute each newly prepared probe to be tested and compared with a total volume of 200 μL in hybridization solution (e.g., for a required probe volume of 40 μL , use 53.3 μL of probe + 146.7 μL of hybridization solution). This extra volume allows for losses of the highly viscous solution.
6. Boil the probes for 5 min, and cool on ice for 5 min.
7. Prepare the Miniblotter with the test membrane prepared in **Subheading 3.3.3., step 4** apply the probes to the membrane, and hybridize overnight (see **Subheading 3.4.2.**).
8. Wash, expose, and develop X-ray film, and visually compare the newly prepared probes against the current probe. Probes that match the intensity of the current probe against the DNA for that organism and the 10^5 and 10^6 standards, without streaking, can be used. Further refinement of the volumes may be necessary, in which case, the calibration procedure needs to be repeated. When the final optimal probe volume has been determined, enter this value into the Probe Stock List.

3.3.4. DNA and Probe Stock Maintenance Procedures

Because of the number of species involved, it is crucial to maintain detailed records of all of the DNA preparations, DNA standards, and probe preparations, to monitor stock depletion and to identify when stocks will run out. Our procedures for this are outlined in **Notes 18** and **19**.

3.4. CKB Technique

CKB samples are managed using log records, and each membrane is recorded in a CKB “Membrane Worksheet” (e.g., **Fig. 6A**; the corresponding membrane image is shown in **Fig. 6B**; see **Note 20**). The samples and standards are laid onto a nylon membrane using the MiniSlot device. Our usual layout includes two sets of 10^5 and 10^6 standards, an *E. coli* control, and up to 25 samples (see **Fig. 6B**). The next step consists of hybridizing 40 DIG-labeled probes at low stringency in thin channels at right angles to the sample lanes using a Miniblotter. The membranes are then washed at fixed high stringency, and the DIG-labeled hybrids are detected.

3.4.1. Application of Samples Onto Membrane (see **Note 21**)

1. Freshly prepare 3 mL of 0.5 M NaOH and 24 mL of 5 M NH_4 acetate for each membrane.
2. Assemble the MiniSlot: cut 12 pieces of 15 $\times$ 15-cm blotting paper, lay the stack over the lower base, and cover with one piece of 15 $\times$ 15-cm Whatman 3MM chromatography paper (see **Note 22**).
3. Lay the membrane on the Whatman paper and, with a soft (2B) pencil, make registration marks next to the small plastic alignment pegs on the lower board. Label the membrane with the unique membrane number in the lower right-hand corner.

A

Membrane Number: 02094 Date Laid: 29.8.02 By: LG					Date Probed: 2.9.02 By: LG				
SUMMARY Clinical samples from Christchurch caries study									
SAMPLES									
Membrane sample #	Sample name	Sample details	Sample No.	Sample conc/amt	LANE	PROBE	STRAIN	PROBE #	VOL.
1	10 ⁵ std				1	-----			
2	10 ⁶ std				2	<i>Strep. mutans</i>	ATCC 25175	P60	2.5
3	E.coli				3	<i>Strep. sobrinus</i>	OIH	P99	5.0
4	Base plaque				4	<i>Strep. mitis I</i>	ATCC 49456	P57	2.0
5	Patient 1024	U ant	1	100µL	5	<i>Strep. mitis II</i>	SK149	P119	7.0
6		L ant	2	100µL	6	<i>Strep. oralis</i>	ATCC 35037	P83	3.0
7		U right P	3	100µL	7	<i>Strep. gordonii</i>	ATCC 10558	P55	2.5
8		U left P	4	100µL	8	<i>Strep. sanguinis</i>	ATCC 10556	P64	2.5
9		L right P	5	100µL	9	<i>Strep. parvaesanguinis</i>	M1021	P63	2.5
10		L left P	6	100µL	10	<i>Strep. anginosus</i>	ATCC 33397	P102	10
11		Tongue	7	100µL	11	<i>Strep. intermedius</i>	ATCC 27335	P56	5.5
12					12	-----			
13	Patient 1025	U ant	1	100µL	13	<i>Strep. vestibularis</i>	LM-1	P67	2.5
14		L ant	2	100µL	14	<i>Lactobacillus acidophilus</i>	ATCC 4356	P24	5
15		U right P	3	100µL	15	<i>Lactobacillus casei</i>	ATCC 393	P26	3
16		U left P	4	100µL	16	<i>Lactobacillus rhamnosus</i>	ATCC 7649	P79	20
17		L right P	5	100µL	17	<i>Lactobacillus fermentum</i>	ATCC 14931	P29	2
18		L left P	6	100µL	18	<i>Lactobacillus plantarum</i>	SA-1	P34	3
19		Tongue	7	100µL	19	<i>Actinomyces gerencseriae</i>	ATCC 23860	P105	30
20					20	<i>Actinomyces israelii</i>	ATCC 12102	P109	30
21	Patient 1026	U ant	1	100µL	21	<i>Actinomyces naeslundii</i>	ATCC 12104	P7	10
22		L ant	2	100µL	22	<i>Actinomyces odontolyticus</i>	ATCC 17929	P103	2.5
23		U right P	3	100µL	23	-----			
24		U left P	4	100µL	24	<i>Candida albicans</i>	A72	P15	5
25		L right P	5	100µL	25	<i>Neisseria mucosa</i>	ATCC 19696	P41	1.5
26		L left P	6	100µL	26	<i>Haemoph. parainfluenzae</i>	III1	P23	5
27		Tongue	7	100µL	27	<i>A. actinomycetemcomitans</i>	ATCC 29523	P2	2.5
28					28	<i>Bifidobacterium dentium</i>	ATCC 27534	P71	7
29					29	<i>Subacterium salinarum</i>	ATCC 33271	P94	10
30					30	<i>Gemella morbillorum</i>	ATCC 27824	P70	5
31					31	<i>Corynebact. matruchotii</i>	ATCC 14266	P18	2.5
32					32	<i>Peptostreptococcus micros</i>	ATCC 33270	P81	40
33					33	<i>Pepto. cassacharolyticus</i>	ATCC 14963	P43	10
34					34	-----			
35					35	<i>Eskenella corrodens</i>	ATCC 23834	P78	10
36					36	<i>Capno. gingivalis</i>	ATCC 33624	P117	10
37					37	<i>Fuso. nucleatum mc.</i>	ATCC 25586	P114	40
38					38	<i>Campylobacter rectus</i>	ATCC 33238	P13	5
39					39	<i>Leptotrichia buccalis</i>	ATCC 14201	P90	2.5
40					40	<i>Prevotella melaninogenica</i>	ATCC 25845	P47	4
41					41	<i>Pr. nigrescens intermedia</i>	ATCC 32563/25611	P48/46	3/3
42					42	<i>Porphyromonas gingivalis</i>	ATCC 33277	P45	2.0
43					43	<i>Selenomonas noxia</i>	ATCC 43541	P52	2
44					44	<i>Veillonella parvula</i>	ATCC 10790	P116	20
45					45	-----			

Fig. 6. (Continued)

- Carefully place the upper part of the MiniSlot on top of the membrane, ensuring that the membrane does not move during assembly. Place the screws in the holes and screw down hand-tight, tightening the screws evenly in diagonally opposite pairs.
- Label 1.5-mL microcentrifuge tubes according to the membrane worksheet. Resuspend the plaque samples to the required concentration in these tubes. For samples, standards, and *E. coli* in 1X TE buffer to 100 µL, add 100 µL of 0.5 M NaOH. For samples in 0.25 M NaOH/0.5X TE, transfer 200 µL to a fresh tube.
- For plaque analysis, we also include an *E. coli* negative control, prepared by making a 1:100 dilution of a 10 µg/mL A Std in 1X TE buffer.

B

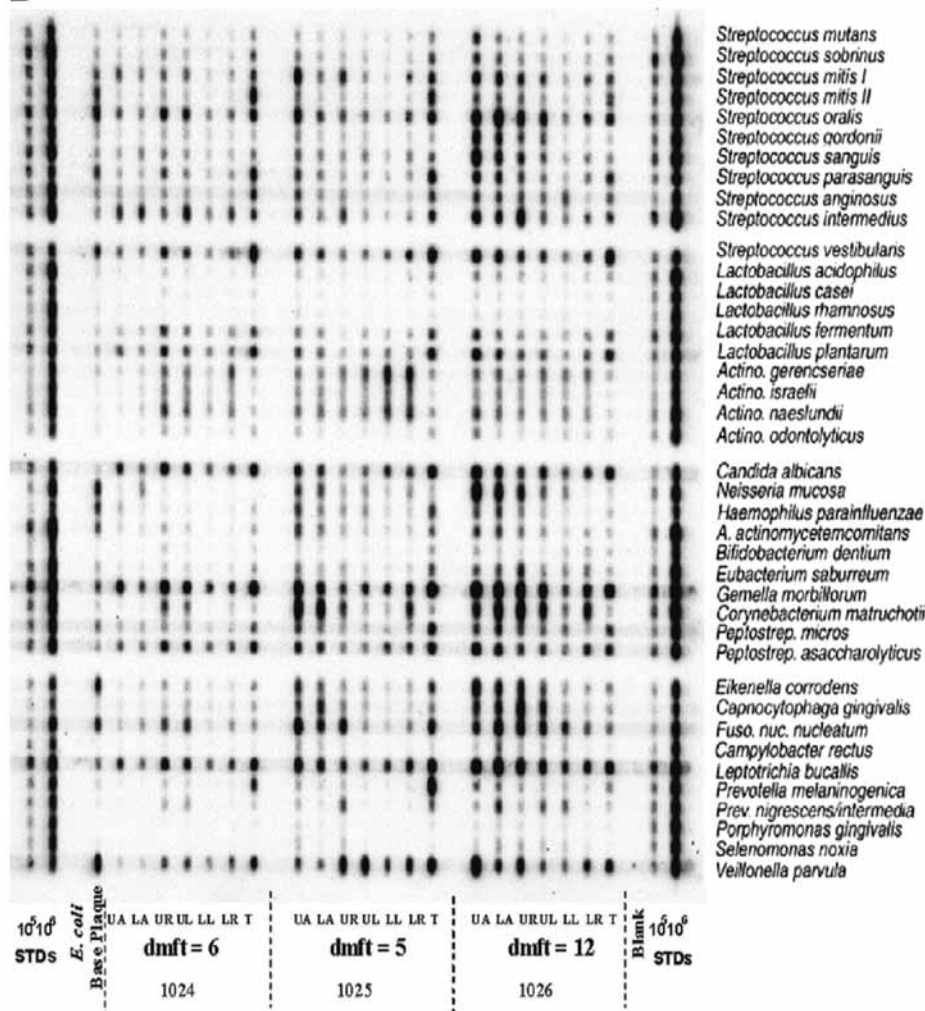


Fig. 6. Example of typical hybridization layout: caries-related probes and analysis of plaque from seven different oral sites of three 5-yr-old children with high-dental caries, including flanking standards and *E. coli* control. **(A)** The Membrane Worksheet (02094) detailing the samples laid along with the probes and volumes used. The “Base Plaque” loaded in lane 4 is a positive control. U, upper; L, lower; Ant, anterior; P, proximal sites. **(B)** Typical checkerboard image (02094). The digitized image is quantified, with the individual spots in each lane converted to numerical values by comparison with the 10^5 and 10^6 equivalent standards.

7. Boil the samples for 5 min (to lyse the cells and extract the DNA). Denature the 10^5 and 10^6 standards and the *E. coli* by incubation at room temperature for 5 min. Then add 800 μL of 5 M NH_4 acetate to all samples to neutralize the pH.
8. To apply the samples to the MiniSlot: vortex each tube, apply all of the sample into the appropriate slot of the MiniSlot, and rock the apparatus from side to side four times to ensure even coverage along the slot.
9. After all of the samples have been applied, wait for complete absorption of the samples onto the membrane (a few minutes). It is possible to see the liquid being drawn through the membrane, and when the surface liquid has disappeared.
10. When the samples have been absorbed, disassemble the MiniSlot (rinse as soon as possible after use), and fix the DNA to the moist or dry membrane using a UV crosslinker on an energy setting of 70 mJ/cm^2 . Membranes may be stored at room temperature until required for hybridization.

3.4.2. Prehybridization and Hybridization

The prehybridization procedure blocks areas on the membrane that contain no DNA to prevent nonspecific probe binding.

1. Place the membrane (or two membranes back-to-back) in a homogenizer bag, add 50 mL of freshly prepared prehybridization solution, remove significant air bubbles, and seal the bag using a heat sealer.
2. Incubate the membranes at 42°C with gentle rocking (e.g., 15 strokes/min) in the hybridization oven. Leave for at least 1 h, or a maximum of 8 h. Ensure that the membranes are completely exposed to the prehybridization solution.
3. Label the probe microcentrifuge tubes with the appropriate lane number, because this minimizes errors when loading.
4. Add the appropriate amount of hybridization buffer and probe (as determined by the calibration procedure; see **Subheading 3.3.3.**) to the labeled tube. The hybridization solution and probe volume should equal a total volume of 155 μL per membrane, plus an extra 45 μL for up to four membranes to allow for pipetting losses (see **Note 23**).
5. Vortex to mix, and boil probes for 5 min.
6. Cool rapidly on ice for 5 min to delay re-annealing of the single DNA strands.
7. Prepare the membrane for probing: remove the membrane (in prehybridization solution) from the homogenizer bag and blot on a piece of Whatman filter paper until the excess prehybridization buffer has been removed (for a standardized 15 s only, see **Note 24**).
8. Place the upper part of a Miniblotter face-down and place the membrane face-down on the Miniblotter, aligning the pencil registration marks with the plastic locating pegs and with the right-hand membrane number on the bottom left.
9. Place Saran wrap over the membrane, a PC2 plastic cushion over the cling film, add the lower part of the Miniblotter, and turn the whole apparatus over.
10. Pierce the Saran wrap in the screw holes and firmly hand-tighten the screws evenly (see **Fig. 3B**).
11. Pipet 155 μL of each probe into the appropriate lane of the Miniblotter.

12. Fill lanes 1, 12, 23, 34, and 45 with 155 μ L of hybridization solution. These blank lanes aid the alignment and analysis of the membrane.
13. Wrap the whole apparatus with cling film, and incubate face-up, overnight, at 42°C in the hybridization oven, keeping conditions humid by placing a container of water in the oven. Set the shaker to a gentle rock (15 strokes/min) oriented along the length of the lanes.

3.4.3. Stringency Wash of Membrane: To Remove Excess Probes From the Membrane and Set the Stringency Condition

1. Heat 7 L of phosphate/SDS buffer to 68°C in the Disk Wisk. Heat another 7 L to 68°C on hot plates.
2. Set up a vacuum suction apparatus by attaching the checkerboard manifold to the vacuum pump, and start the vacuum.
3. Remove the checkerboard apparatus from the hybridization oven.
4. Attach the manifold to the Miniblotter, and remove all of the liquid from the lanes. Remove the manifold assembly and disassemble the checkerboard.
5. Place the membrane in the basket already in the Disk Wisk, using flat-ended forceps. Two membranes can be placed back-to-back in each section of the basket.
6. Wash for 20 min at 68°C (see **Note 25**).
7. Remove the basket from the Disk Wisk, and tip the used phosphate buffer into a sink. **Caution:** the liquid will be hot.
8. Immediately refill the Disk Wisk with fresh 68°C phosphate buffer, replace the basket, and incubate the membrane for another 20 min.
9. Remove the membranes from the Disk Wisk, and wash once with approx 250 mL of 1X wash buffer for 1 min in a clean, shallow, square, plastic dish.

3.4.4. Coupling of DIG-Specific AP-Linked Sheep Antibody

1. Incubate the membrane in a homogenizer bag containing 40 mL of 1X antibody blocking buffer (see **Note 26**), after removal of significant air bubbles, for 1 h on a reciprocating shaker set at 50 rpm. Two membranes may be placed back-to-back in a bag.
2. Centrifuge the anti-DIG-AP conjugate for 1 min at 12,000g at room temperature to sediment any precipitate, and dilute 4 μ L of antibody solution into 40 mL of fresh 1X antibody blocking buffer (1:10,000 dilution).
3. Cut the top off the bag, drain the used antibody blocking buffer from the bag, add the freshly prepared antibody/1X antibody blocking buffer solution, again remove significant air bubbles, reseal the bag, and return to the shaker (50 rpm) for an additional 1 h at room temperature.
4. Remove the membrane from the bag and transfer into a clean, shallow, square, plastic dish. Wash the membrane with approx 250 mL of fresh 1X wash buffer for 15 min with shaking (~75 rpm). Drain off the buffer.
5. Replace with fresh 1X wash buffer, wash for a further 15 min, and drain off the used wash buffer.
6. Equilibrate the membrane with excess (>100 mL) 1X detection buffer for 1 min.

3.4.5. Detection and Quantification of DIG-AP DNA Hybrids

There are several options for detecting and quantifying the AP-linked hybrids, depending on facilities available. The simplest set of options involves reaction with a chemiluminescent AP substrate, such as CDP-Star, and detection by exposing a chemiluminescent detection film. Alternatively, a sensitive CCD digital camera system or chemifluorescent system may be used (*see Note 27*). Our procedure includes exposure of chemiluminescent detection film to obtain a quick permanent image of the membrane, followed by a 1-h exposure (Syngene darkroom) to acquire a digital image (*see Note 28*).

1. Dilute CDP-Star 1:500 (*see Note 29*) in detection buffer: for a single membrane, dilute 40 μL of CDP-Star into 20 mL of detection buffer in a clean, shallow, square, plastic dish.
2. Carefully place the membrane onto the CDP-Star solution, DNA side up, and rock the container so that the entire membrane becomes covered with solution. Incubate for 1 min while continuing to rock the container.
3. Remove the membrane from the CDP-Star solution and drain. Important—do not let the membrane dry out!
4. Place the membrane in a Reaction Folder (*see Note 30*), and seal the edges with the heat sealer.
5. Incubate the reaction folder in a development cassette for approx 30 min at room temperature.
6. To activate a second membrane using the same solution, add an extra 5 μL of CDP-Star. Use fresh solution for each pair of membranes.

3.4.6. Hybrid Exposure, Development, and Quantification of Film

1. To expose the blot to X-ray film to obtain a permanent image, load a sheet of chemiluminescent detection film into a film cassette with the blot, DNA side closest to the film (*see Note 31*). Wear gloves and handle the film by the corners only.
2. Expose the film for approx 4 min, remove, and develop using appropriate X-ray film developers and procedures.
3. Expose and process a second film for a shorter period, approx 1 to 2 min. This is to allow adequate resolution of high concentration hybridization signals.
4. Archive the films, membrane worksheet, and any relevant results in a “Checkerboard Membrane Details” folder.

Simple semiquantification is possible by ranking each hybrid spot on the film with respect to the 10^5 and 10^6 standards, i.e., 10^4 (approximate lower limit of detection), 5×10^4 , 10^5 , 5×10^5 , 10^6 , and $>10^6$.

3.4.6.1. DIGITIZATION AND ANALYSIS OF X-RAY FILM

Scan the film using a conventional scanner. Transfer the image to an image analysis program (e.g., Adobe Photoshop), and process as described in **Subheading 3.4.6., step 3**. The major limitation of this method is the small effective dynamic

range of the X-ray film (<250 gray scales), resulting in saturation of the major spots and, hence, an underestimation of the number of cells present.

3.4.6.2. DIRECT IMAGE ACQUISITION IN THE SYNGENE CHEMIGENIUS II DARKROOM (SEE **NOTE 31**)

This requires a very sensitive cooled CCD camera, with a high photo-efficiency and planar lens. The advantage is a much greater dynamic range than film, of greater than 10^3 gray scales for 12-bit cameras and greater than 10^4 gray scales for 16-bit cameras. For the Syngene ChemiGenius II using GeneSnap software, membranes are exposed for 1 h, and an unmodifiable .sgd image file is archived. A TIFF image is exported to Photoshop for optimization of the image and background by rotation and cropping, with further adjustment primarily made by using the levels adjustment. A permanent TIFF image record of the optimized membrane image is archived, and a 16-bit image is transferred to the image analysis program (see **Subheading 3.4.6., step 3**).

3.4.6.3. ANALYSIS OF THE DIGITAL CKB IMAGE

A permanent TIFF image record of the membrane is archived after any further needed rotation, cropping, and other manipulation is completed. To accurately quantify each spot, a Unix program has been developed by Dr. S. S. Socransky and Dr. A. D. Haffajee. This program is used to analyze checkerboard chemiluminescence or fluorescence images, to quantify the CKB hybridization signals, and to configure the resulting data into a useable form. In the program, a 16-bit 1.3-Mb version of the membrane image is processed. The CKB technique is based on a precise grid, but involves a flexible substratum (membrane). Hence, to establish a repeatable location and area for quantifying each spot, an alignment process to fit a modifiable grid in the software is necessary. The first step in processing is to carry out this spot location procedure. The program essentially scans the spots relating to one probe, pixel by pixel, recognizes a user-defined area locating each spot, quantifies the pixels in the area, and subtracts a background determined from the between-spot pixels. The density of each hybridization signal is converted to an equivalent cell number by comparison with the DNA standards. The total equivalent cell number of all 40 probes for each sample is summed, each is expressed as a percentage of the total, and the data is transferred to Excel for further analysis (see **Note 32**). This relative pattern of probe-specific cell quantity is the primary data used for comparative analyses of samples.

3.5. Data Analysis and Interpretation

3.5.1. Basic Data Analysis

Further data processing depends on experimental design and grouping of samples being processed, and on the type of statistical analyses planned. For the simplest

design, in which samples within groups are being compared, descriptive statistics can be calculated for percentages of the total probe count for each species in each group and the groups compared, for example, by analysis of variance. If the sampling biomass is known or approximated, absolute values of each species can be used. Graphical display of changes or comparisons of 40 species is a challenge. Group comparisons can be displayed on a single graph, with a vertical list of species and multiple bar graphs for each group (e.g., [Fig. 7](#)). A double graph of the frequency of each species, with points connected, also displays changes in relative composition between two groups ([15](#)). Time series pose particular problems for graphical display. For these, it may be best to group related species to reduce the 40 species data sets to 10 or less aggregated groups of species, such as the color groups of Socransky et al. ([13](#)), and then display pie charts reflecting each time point. An alternative is to focus on particular species or on groups of species and display them as a time series.

3.5.2. Ecological Analysis

For ecological comparisons, community ordination clustering and dimensioning techniques are required ([24](#)). These techniques condense large data sets to reveal the ecological relationships among species. Clustering allows the degree of similarity between individual samples of complex ecosystems to be evaluated. Cluster analysis is usually performed by the unweighted pair-group method, using arithmetic averages (UPGMA procedure). Principal Component analysis identifies axes of maximum variation from the data matrix with derived variables (principal components) that measure distances in the principal component space between samples and which, applied to species, exposes underlying bacterial community patterns (see [Note 33](#)).

3.5.3. Strengths and Limitations

CKB analysis is robust, reliable, efficient, economical, and sensitive (detection threshold $\sim 10^4$ cells; [ref. 14](#)). It also provides a valuable complement to PCR-based techniques with their inherent bias in analyzing complex microbial systems ([9](#)). Multiple membranes can be processed simultaneously (in our case, up to four). Hence, large numbers of plaque samples can be analyzed. The major technical limitations are:

1. The technique is confined to cultivable species.
2. The technique requires the isolation of very high-quality DNA from a range of usually intractable species.
3. The use of one set of stringency conditions, irrespective of G + C content of the genome.
4. Nonspecific binding occurs if too much target sample or too much probe is present.
5. Careful reaction optimization and cross-reaction checks are required.
6. Very high-quality DNA for preparation of probes and standards is needed ([14](#)).

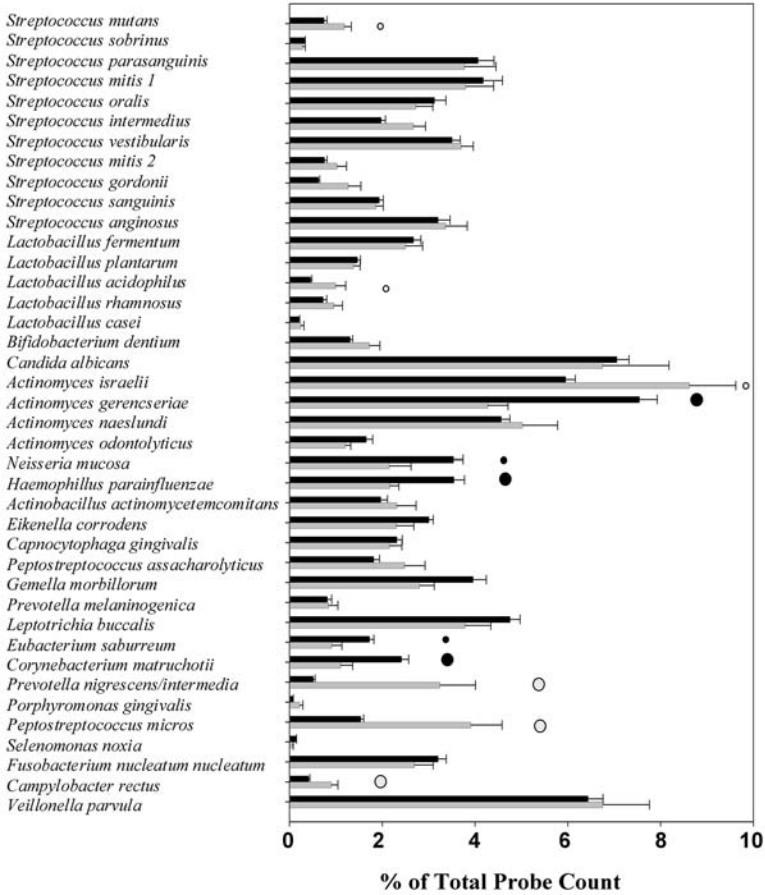


Fig. 7. Stacked bar graph comparing the lower proximal (embrasure) plaque of a small group of 5-yr-old children showing no caries, with plaque from children having high caries. The low-carries group ($n = 8$) had no decayed, missing, or filled teeth (dmft = 0) and are shown in gray, the high-carries group ($n = 15$), shown in black, had an average dmft of 8.1. Significant differences in level of species are indicated with small dots ($p \leq 0.05$) and larger dots ($p \leq 0.01$), with black dots associated with caries, and gray dots absence of caries. These results suggest that the presence and level of “health compatible” species are as important to the study of dental caries as they are to the study of periodontitis (20,21).

Probe discrimination can be improved by use of subtraction hybridization of the probe DNA with DNA of closely related species (see Note 34; refs. 25 and 26). As with all direct DNA-based techniques, it also measures the ecosystem content of species DNA, irrespective of viability state.

3.5.4. Interpretation Caveat

Some cross-reaction between probes and closely related and perhaps unknown species/strains is inevitable. A major theoretical complication is a lack of consensus regarding how species are to be defined (1,27). Differing and disputed criteria are provided by analysis of DNA genome relatedness (by hybridization), gene sequence (e.g., of rDNA), phenotype, and ecotype. Considerable caution is needed in ascribing any particular hybridization reaction to some other definition of species. Basically, the CKB technique sets up a probe-specific taxonomy. Each probe will hybridize with varying efficiency to a closely related group of strains (14). For example, a strain that has 5% cross-reaction with the probe species DNA and is present at 20 times the probe strain will contribute an equal amount of hybridization signal. Hence, for any sample, potentially different mixtures of cross-reacting species can achieve a given level of probe hybrids; what you see is what you get. The technique yields an ecological fingerprint of a highly complex microbiota and is not a species identification technique. A distinctive specificity range is true of all DNA-based characterization techniques.

3.5.5. Developments of the Technique

Stripping the probes from the membrane with alkali and re-probing the membrane with a different probe panel is also a potential option that doubles the species being quantified to 80 species, and can be repeated (14). Use of oligonucleotide probes from the rDNA region, for example, is one option, but will accentuate differences in the G + C content of probes and, hence, the stringency of hybridization and crossreactions. It is also less robust than whole-genome probes (S. Socransky, personal communication). In a reverse CKB technique, such oligonucleotides can be bound to the membrane and probed by the mixed ecosystem DNA (28). Discrimination in this instance depends on the particular specificity of the DNA region chosen. However, this technique has the advantage that probes to uncultivated species can be included (3). In many respects, CKB analysis is a more accurate semi-microarray technique. Probably any technique based on hybridization can be adapted to use the checkerboard system.

An important paper from the laboratory that developed the CKB technique reviews its advantages and limitations and its potential applicability to complex microbial ecosystems (29). It contains a description of important features needed to carry out the technique, including an extensive discussion of sensitivity and specificity issues. Procedures to increase specificity in species where this is a significant problem are also described.

4. Notes

1. To prevent sample leakage during transport and storage, tightly sealed 1.5-mL screwcap microcentrifuge tubes with O-rings are needed. The tubes that we use

are from Scientific Specialties Inc., Lodi, CA. Leakage will occur with standard flip/push-cap microcentrifuge tubes, even with parafilm.

2. We use brush applicators from Points, SDI Ltd., Victoria, Australia. The brushes are made from nonabsorbant fibers, which hold the sample in suspension until used. Do not use foam tips.
3. Filter sterilize solutions using a 0.2- μ m syringe filter and syringe.
4. We use RNase A from Roche Applied Science, Mannheim, Germany. Other RNase A may require a different buffer to dissolve.
5. This is a water bath to contain 7 L of buffer with a basket to vertically support and immerse the membranes.
6. After a few hours, a floating precipitate can form, which must be removed. Separate the clear solution using a separating funnel. Repeat this separation if the precipitate re-forms.
7. The boiling step can also be performed in a dry heating block set at 100°C.
8. If the sample is too concentrated, this can result in blocked and/or smeared checkerboard spots. There should be no visible cloudiness in the sample. If there is, dilution is required.
9. Risk level II bacteria should either be handled in a class II biohazard cabinet or an Anaerobic Hood. Aseptic techniques must be used at all times when handling bacteria, and all reagents, pipet tips, and microcentrifuge tubes must be sterilized before use.
10. Generally, this procedure is used because bacteria are preferentially grown in broth culture. Some bacteria (e.g., *Actinomyces gerencseriae* and *A. israelii*), however, may prove recalcitrant to the lysis procedure. It may be necessary to harvest bacteria directly from plate cultures. In this case, we also use the method described by Smith et al. (17), with components from a MasterPure DNA Purification Kit from Epicentre Technologies. Lysozyme and achromopeptidase (and penicillin) are used to weaken the bacterial cell wall. SDS and Proteinase K assist in the complete lysis of the cell, by further degrading the cell wall. To ensure complete lysis, the cells can be kept overnight in the lysing solution. For *C. albicans*, we use the method based on that of Cryer et al. (4).
11. When making 250 mL volumes of TSBYK broth, omit the hemin solution because this interferes with the DNA extraction.
12. The tough composition of the outer cell wall of Gram-positive bacteria can prove problematic in obtaining good lysis and, therefore, good DNA yields. To combat this, these microbes are grown for a time in sublethal concentrations of penicillin to weaken the bacterial cell wall and aid lysis.
13. Cells must be totally resuspended. Clumps of cells will result in poor lysis, a poor yield, and a low-quality DNA extraction.
14. DNA extractions are measured spectrophotometrically, and the concentration (OD_{260} 1.00 = 50 ng/mL) and ratio (A_{260}/A_{280}) are calculated. The ratio should fall between 1.7 and 2.0. Ratios lower than 1.7 indicate protein contamination, and the preparation should be re-extracted with phenol/chloroform. Ratios above 2.0 indicate RNA contamination, which can be confirmed by gel electrophoresis. Smearing of the DNA in the gel can indicate degradation. If RNA is present, the

extract will need a further RNase treatment, followed by re-extraction with phenol/chloroform. Each DNA preparation is assigned a "G" (genomic DNA) accession number and entered in a DNA Log.

15. An A Std is DNA that has been standardized to a concentration of 10 µg/mL. In our procedure, whenever an A Std is used, or a new A Std is prepared, detailed records are maintained. Hardcopy records of all A Std stocks are kept, and consist of an A Std Stock List and an A Std Log. Electronic versions consist of an Excel worksheet with two workbooks, a Stock List and a Log, and updated hardcopies are printed out and filed. Information in each of the sections contains a Stock List (all A Std preparations, including the approximate volume and total amount [ng] of A Std in stock) and an A Std Log (all A Std preparations, in sequential order of their unique "A" number). Each new A Std preparation is entered into the A Std Log, assigning an accession number carrying on in sequential order from the last A Std preparation to be logged. A Std preparations are prefixed with the letter "A." Enter the "G" number of the genomic DNA to be used to make the A Std into the "DNA used" column of the log, and the concentration of the DNA (ng/µL). In our worksheet, the volumes of DNA and 1X TE buffer to be used to make the A Std will be calculated and displayed in the next two columns. When the A Std has been prepared, it is entered into the Stock List, listing the organism, strain, current panel status, unique number, and volume. The amount (ng) in stock is calculated automatically. Each time an A Std is used, the volume used plus an estimate of pipetting loss needs to be deducted from the volume in stock. The amount (ng) in stock is automatically updated.
16. Enter the probe to be prepared into the Probe Log. Record the name of the organism and the strain number, and assign an accession probe number, beginning with "P." This number follows in sequential order from the last probe logged, irrespective of species. Each organism involves probe preparations with two different concentrations of DNA and probe numbers. An electronic Probe Preparation Worksheet is prepared, recording: date, prepared by, organism, DNA used (record the "G" number obtained from the DNA Stock List), original DNA concentration (found in the DNA Stock List), probe number (as designated in the Probe Log), and the DNA and H₂O volumes that are to be used in the probe preparation procedure, as calculated. Subtract the volume of DNA used from the original volume in the DNA Stock List.
17. Print out the worksheet and log the membrane into the membrane log. Lay the dilution series of DNA of each organism from A Stds (10 mg/mL), on the membrane. Subtract the volume of the A Std used from the volumes listed in the A Std Stock List.
18. Whenever genomic DNA is used or a new DNA extraction is prepared, the records of DNA stocks are updated to ensure that the status can be monitored. Hardcopy records comprise: Depleted DNA Stocks List, Entire DNA Stock List, and DNA Log; also DNA Measurements, DNA gels, and A Stds Stock List. Electronic versions consist of an Excel worksheet with three workbooks: Entire Stock List (all DNA preparations and relevant information), Depleted Stocks (stocks of 20 µg or

less), and DNA Log (all DNA preparations in sequential order of their unique “G” number). For newly prepared DNA (the stocks prefixed “G”): enter the “G” number into DNA log when setting up the DNA preparation, and assign a number sequential to the last DNA preparation logged. Once the DNA extracts have been measured and checked for RNA contamination, their concentrations and A_{260}/A_{280} ratios are entered into the DNA log. An Entire DNA Stock List is kept, recording: the organism in alphabetical order, the strain, whether or not the species is a member of the current probe panel, the unique “G” number, the date extracted, the A_{260} reading, the A_{280} reading, and the approximate volume. The concentration (ng/ μ L), the A_{260}/A_{280} ratio, and the total amount of DNA (μ g) is calculated. Each time the DNA is used, the volume used is deducted from the volume in stock. Once the total amount of DNA remaining is 20 μ g or less, the stock is flagged as indicating that a new DNA preparation is needed for this organism. The information is copied to a “Depleted DNA Stock” list, which also contains the total volume of the A Std and probe stocks remaining, allowing an estimate of the number of membranes that may be analyzed before the stock is totally exhausted and, hence, the urgency of the need for a new DNA preparation.

19. Whenever a probe is used or a new probe is prepared, the records are updated to reflect the status of the probe stocks. Hardcopy records of all probes comprise: a Probe Stock List (a list of calibrated probes in alphabetical order according to the name of the organism and including the volume of probe to be used for each membrane, volume in stock, and the number of membranes analyzable with that volume), a Probe Log (list in sequential probe accession “P” numbers), a Low Probe Stock List (less than the equivalent of 20 membranes remaining), and completed Probe Preparation Worksheets. Electronic versions consist of an Excel worksheet with three workbooks: a Low Stock List, a Probe Log, and a Probe Stock List. If changes are made to the electronic version, updated versions are printed out and filed.
20. For the efficient flow and management of samples being processed by the checkerboard technique, membrane data worksheets are used to fully describe the samples laid on each membrane. Each worksheet corresponds to one membrane, is identified by the membrane number, and allows the easy identification of the samples on that membrane.
21. Aseptic techniques should be used, and powder-free examination gloves must be worn at all times when handling membranes, to avoid contaminating the membrane with nucleases and bacteria. Avoid touching the sample areas of the membrane; handle the membranes by the edges and underside only, preferably using clean, blunt forceps.
22. The use of an acrylic base instead of the standard stainless steel vacuum base is recommended. Immunetics will substitute the metal base with an acrylic base if this is requested at the time of purchase.
23. The probe preparation material volume of the probes used is recorded on the membrane worksheet.
24. This timing of 15 s was standardized because the blotting of liquid occurs quickly and timing is crucial. Too much blotting will cause the membrane to dry out and

too little blotting will dilute the probe as it moves across the membrane, changing the efficiency of hybridization.

25. This sets a single stringency condition for all hybrids, irrespective of G + C content, and is a key step. Minor changes in buffer temperature (e.g., $\pm 2^{\circ}\text{C}$) affected the sensitivity of detection but did not seem to modify specificity.
26. The blocking buffer is slightly cloudy, but make sure that there is no precipitate (indicating contamination), because this results in a dark background on the exposed film.
27. Another option is to use a fluorescent AP substrate, such as Attophos, and a laser-scanning fluorescent detector, such as the Storm Fluorimager system (14).
28. Ensure that there is no precipitate in the CDP-Star after warming to 37°C , because this results in a speckled background on the exposed film. If this occurs, open a fresh bottle of CDP-Star.
29. After placing the membrane in the reaction folder, it is essential to expel any air bubbles that might be present between the membrane and folder. Air bubbles appear as light circular areas on the film image and distort the CKB results.
30. Many X-ray films, although able to produce an image with the chemiluminescence emissions, result in a poorer image than specialized chemiluminescent films.
31. Although we use the Syngene ChemiGenius II, it is 12-bit for chemiluminescence and has the minimum usable specifications needed in terms of sensitivity and pin-cushioning of the lens. There are now 16-bit camera systems that are more sensitive on the market, including models from Syngene.
32. The converted data is in numerical form and is transferred into Excel Worksheet (with species labels and sample description added), using the Text Import Wizard and omitting columns 2, 3, 5, 6, 7, and 8; and, finally, saved with the filename being the membrane number. The data is copied to a previously saved worksheet template containing ordered species probe labels.
33. For this type of statistical processing, we use BioNumerics v.3 (Applied Maths, Kortrijk, Belgium) and SAS release 8.0 (SAS Institute Inc., Cary, NC), or SPSS v.12.0.1 for Windows (SPSS Inc., Chicago, IL).
34. Analysis of complex samples, such as plaque, seems to yield more results that are specific than analysis of a few species in consortia. We speculate that this could be caused by some sort of blocking, for example, of highly conserved DNA regions, by related bacteria in complex samples.

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Nonradioactive Northern and Southern Analyses From Plant Samples

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Summary

Several specific problems are encountered when nonradioactive detection methods are used in conjunction with plant nucleic acids. In this chapter, we describe protocols for the isolation of DNA and RNA from plant leaves and the preparation of probe molecules by either PCR or in vitro transcription with different haptens. Furthermore, standard conditions and possible modifications for hybridization and detection of probes are given.

Key Words: Biotin; digoxigenin; DNA; fluorescein; Northern; plant; RNA; Southern.

1. Introduction

Hybridization-based detection remains a commonly used technique to determine the presence of a given sequence within a population of DNA (Southern) or RNA (Northern) fragments. Nonradioactive methods have proven to be superior to radioactive analyses because of their improved sensitivity and their more convenient handling procedures (1,2). Although these analyses are, in principle, similar when comparing plant and animal samples, several specific problems have to be addressed when using plants as a starting material. These problems include the high content of nucleolytic activities as well as phenolic compounds and polysaccharides, and the presence of plastid DNA and RNA in the samples (3,4). In addition, the methylation pattern of plant DNA is different from animal DNA (5). These specific questions will be addressed, particularly in **Heading 4**. In this chapter, we describe the nucleic acid preparation, the electrophoretic separation on agarose gels, the generation of DNA and RNA probes, as well as recommended conditions for hybridization, washing steps,

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and detection. The protocols we provide have been established with barley leaf material, but have also been used for a set of different species, including both monocots and dicots.

2. Materials

1. DNA extraction buffer: 100 mM Tris-HCl, pH 7.5, 700 mM NaCl, 10 mM EDTA, and 1% w/v cetyl trimethyl-ammonium bromide (CTAB; add freshly).
2. Alternative DNA extraction buffer: 100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; and 2% sodium dodecyl sulfate (SDS).
3. Trizol™: 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, pH 5.0, 5% glycerol, and 38% phenol (water equilibrated).
4. Phenol/chloroform (1:1, v/v), pH 8.0, and chloroform.
5. 3 M sodium acetate, pH 5.0.
6. 96 and 70% Ethanol.
7. 1X Tris-HCl-EDTA (TE) buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.
8. Microcentrifuge (13,000g).
9. Standard PCR equipment.
10. Digoxigenin (DIG)-11-dUTP (Roche Applied Science).
11. DIG RNA labeling kit (Roche Applied Science).
12. Positively charged nylon membrane.
13. 1X Tris-acetate-EDTA (TAE) buffer: 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA.
14. Formaldehyde.
15. DNA loading buffer: 30% (v/v) glycerol, 0.02% bromophenol blue, and 0.02% xylene cyanol.
16. 0.25 M HCl.
17. DNA transfer buffer: 0.5 M NaOH and 1.5 M NaCl.
18. 1X MOPS: 20 mM morpholinopropanesulfonic acid, 2 mM sodium acetate, 1 mM EDTA, pH 7.0.
19. RNA loading buffer: 60% (v/v) formamide, 20% (v/v) formaldehyde solution, 13% (v/v) 10X MOPS, 7% (v/v) glycerol, and 0.1% bromophenol blue.
20. 20X standard sodium citrate (SSC): 3 M NaCl and 0.3 M citric acid, pH 7.0.
21. DIG Easy Hyb (Roche Applied Science).
22. 2X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; and 0.1X SSC, 0.1% SDS.
23. Wash buffer: 100 mM maleic acid, 150 mM NaCl, and 0.3% Tween-20, pH 7.5 (NaOH).
24. Blocking buffer: wash buffer plus 1% (w/v) blocking reagent (Roche Applied Science).
25. Anti-DIG-alkaline phosphatase conjugate (Roche Applied Science).
26. Alkaline phosphatase buffer: 0.1 M Tris-HCl pH 9.5, 0.15 M NaCl.
27. CDP-Star (Roche Applied Science).
28. X-ray film and developer, or charge-coupled device (CCD) camera.
29. Heat-labile transparencies and heat sealer.

3. Methods

3.1. Nucleic Acid Preparation

For nucleic acid preparation from plants, kits are provided from several suppliers that yield a good quality and sufficient amounts of DNA or RNA, respectively. However, here, we would like to recommend two hand-made protocols that work well in our hands and are easy to perform. Both protocols are based on organic extraction of the sample material.

3.1.1. DNA Preparation

The DNA preparation is based on the CTAB protocol, as described previously (6). The isolated DNA still contains RNA, which is usually separated by adding 100 ng/mL RNase A to the gel-loading buffer before electrophoresis.

1. Harvest approx 100 mg of leaf material and grind to a fine powder in liquid nitrogen (see **Note 1**).
2. Resuspend in 5 volumes of prewarmed DNA extraction buffer and incubate at 65°C for 1 h (mix by inverting from time to time).
3. Add 1 volume of phenol/chloroform (1:1) and mix gently for 10 min. Separate phases by centrifugation at 13,000g for 10 min.
4. Transfer the upper phase to a new tube and extract with 1 volume of chloroform.
5. Transfer the upper phase to a new tube, add 0.1 volume of 3 M sodium acetate, pH 5.0, and 2 volumes of 96% ethanol, and mix gently but carefully.
6. Fish the precipitated DNA from the solution with a hook made from a Pasteur pipet (see **Note 2**).
7. Wash by dipping the DNA into 70% ethanol and dry briefly (DNA should just become transparent).
8. Resuspend in 1X TE buffer (e.g., 50 µL) by heating to 65°C for 10 min and incubating at 4°C overnight.
9. Determine the DNA concentration by UV spectrophotometry or gel electrophoresis (see **Note 3**).

3.1.2. RNA Preparation

The RNA preparation protocol is based on the Trizol method introduced by Chomczynski in 1993 (7).

1. Harvest approx 100 mg of leaf material and grind to a fine powder in liquid nitrogen (see **Note 1**).
2. Add 10 volumes of Trizol and mix by vortexing for 10 min.
3. Incubate at room temperature for 10 min and repeat **step 2**.
4. Add 0.2 volumes of chloroform and mix by vortexing for 10 min.
5. Separate phases by centrifugation at 13,000g for 10 min.
6. Transfer the upper phase to a new tube and extract with 1 volume of chloroform, as in **Subheading 3.1.1., steps 3 and 4**.

7. Repeat **step 6** (optional).
8. Add 2 volumes of ethanol (96%) and mix by vortexing for 2 min.
9. Incubate for 1 h at -20°C and centrifuge at 13,000g for 15 min (4°C).
10. Wash the pellet with 70% ethanol and dry briefly.
11. Dissolve the pellet in an appropriate volume of 1X TE buffer (e.g., 30 μL).
12. Determine the concentration by UV spectrophotometry or gel electrophoresis (see **Note 3**).

3.2. Electrophoresis and Transfer

The electrophoretic separation of DNA after restriction is performed in non-denaturing agarose gels, whereas RNA is separated under denaturing conditions. In both cases, we transfer the nucleic acids to a positively charged nylon membrane by capillary blotting, using standard methods (8). After transfer, the nucleic acids are fixed by UV crosslinking (254 nm, 125 mJ; see **Note 4**).

3.2.1. DNA Electrophoresis

DNA electrophoresis is performed in 0.8% to 1.5% (w/v) agarose gels (depending on the average fragment size), in 1X TAE buffer, preferably at 20 V, overnight. Depending on the size of the analyzed genome, 1 μg (e.g., *Arabidopsis*) to 20 μg (e.g., maize) of restricted DNA is sufficient for the detection of single-copy genes. Before transfer, gels are first shaken for exactly 15 min in 0.25 M HCl and afterwards for at least 30 min in DNA transfer buffer (see **Note 5**).

3.2.2. RNA Electrophoresis

RNA electrophoresis is performed in 1.5% (w/v) agarose gels in 1X MOPS buffer containing 4% (v/v) formaldehyde solution (37%) at 80 V for approx 3 h. One hundred nanograms to 1 μg of total RNA are mixed with 3 volumes of RNA loading buffer and denatured (at 68°C for 10 min) before loading. After electrophoresis, the gel is shaken for at least 30 min in 20X SSC, and capillary transfer is performed in the same buffer (see **Note 6**).

3.2.3. Probe Labeling

Both Southern and Northern analyses can be performed with DNA or RNA probes, respectively. RNA probes, in general, show a high affinity to the target sequence and are strand specific, but DNA probes are easier to prepare. Usually, we obtain good results with DNA probes in Southern analyses, but we highly recommend the use of RNA probes for Northern analyses. By using the three available haptens, DIG, fluorescein, and biotin, in subsequent experiments, membranes can be re-probed without any complicated stripping procedures between the hybridizations (2).

3.2.4. PCR Labeling

PCR labeling of specific sequences can be directly performed from genomic templates or from cloned sequences. The DNA polymerase integrates the modified nucleotide into the probe. In contrast to random-primed labeling, PCR labeling will generate full-length probes with enhanced binding affinity and specificity. PCR conditions should be optimized for the individual reaction, using nonlabeled nucleotides. Amplification products should usually be 300 to 800 bp in length, but there is no absolute upper or lower size limit. The ratio of labeled nucleotide (usually DIG-11-dUTP) to nonlabeled nucleotide (dTTP) must be determined empirically. We usually obtain good results with a ratio of 1:10 (DIG-dUTP:TTP), but ratios between 1:3 and 1:20 are possible. This especially depends on the A/T content of the probe sequence, because integration of several haptens in spatial proximity can lead to steric hindrance of the polymerase reaction. Integration of the hapten can be also monitored by gel electrophoresis, because the higher molecular weight of the modified nucleotides leads to a reduced electrophoretic mobility of the labeled PCR product (see [Fig. 1](#)). Modified nucleotides carrying fluorescein or biotin haptens can be used accordingly (see **Note 7**).

3.2.5. In Vitro Transcription

RNA probes are synthesized from phage RNA polymerase promoters, such as the T7, T3, or SP6 promoters. These are present in the polylinker region of most commercially available cloning vectors. RNA probes are best synthesized using commercial in vitro transcription kits (e.g., DIG RNA labeling kit, Roche Applied Science). To avoid labeling of vector sequences, the DNA template should be terminated behind the insert sequence. This is mostly obtained by linearization of the vector with restriction endonucleases that generate a 3' overhang, to prevent template strand switching. We prefer amplifying the insert region from vectors by PCR using primers specific for the T7 and T3 promoter regions. After purification, the PCR product can be directly used as a template for in vitro transcription (see **Note 8** and [Fig. 1](#)).

3.3. Hybridization

We strongly recommend using DIG Easy Hyb™ as a denaturing hybridization buffer for both Northern and Southern analyses. Hybridizations are performed overnight, with 50 μ L buffer/cm² membrane, using a hybridization oven and glass tubes. If possible, use adequately sized tubes, in which the membranes do not overlap. The following temperatures are recommended as a first estimate, and can be optimized empirically (see **Note 9**): DNA:DNA hybridization, 37°C; DNA:RNA hybridization, 50°C; and RNA:RNA hybridization, 68°C. For in vitro

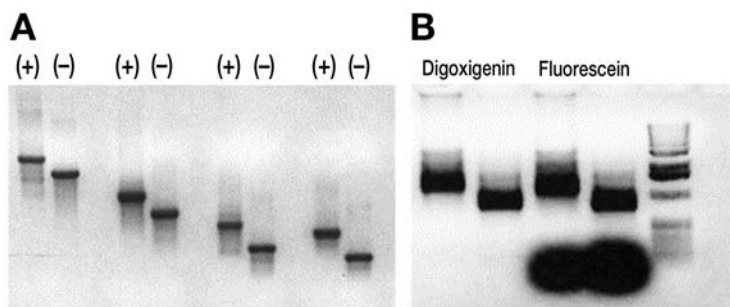


Fig. 1. Labeling of DNA and RNA probes. **(A)** PCR labeling of DNA probes. Four different PCR products were generated either with unlabeled nucleotides (–) or in the presence of DIG-11-dUTP (+). Note the electrophoretic mobility shift caused by integration of the modified nucleotide. **(B)** Labeling of RNA probes. Two different PCR products were used as templates for in vitro transcription in the presence of either DIG-UTP or fluorescein-UTP, as indicated. The templates are hardly visible, just above the strong RNA product. Note the free fluorescein-labeled nucleotides that are also excited by the UV light.

transcripts, 100 ng of denatured probe per milliliter of hybridization buffer can be used. For DNA probes, the optimal probe concentration should be determined empirically. This is easiest performed by “mock” hybridizations, in which empty membrane pieces are hybridized with different concentrations of the probe. Often, unspecific background appears at higher probe concentrations. The highest concentration at which no background is visible should be used. DNA probes should be denatured extensively before hybridization (at 95°C for 15 min).

3.4. Washing Steps and Detection

After hybridization, the membrane is washed in low-salt buffers to remove nonspecifically bound probe molecules. Afterwards, the membrane is incubated with an antibody–reporter enzyme conjugate against the chosen hapten, and excess antibody is removed by washing. The probe is detected by addition of a substrate. We describe the detection of a DIG-labeled probe using a chemiluminescent substrate (see **Note 10**).

1. Wash the membrane in 2X SSC and 0.1% SDS once for 5 min at room temperature, and then in 0.5X SSC and 0.1% SDS for three times 10 min each at 68°C (for RNA:RNA hybridizations 0.1X SSC and 0.1% SDS may be used). Washing in a water bath in large containers is recommended because the use of excess volumes of wash buffer clearly reduces background problems.
2. All subsequent reactions are performed at room temperature.
3. Incubate in wash buffer for 1 min and then in blocking buffer for 30 min (see **Note 11**).
4. Add anti-DIG–alkaline phosphatase conjugate (1:5000–1:20,000 final dilution; centrifuge precipitates before use for 5 min in a microcentrifuge at maximum speed).

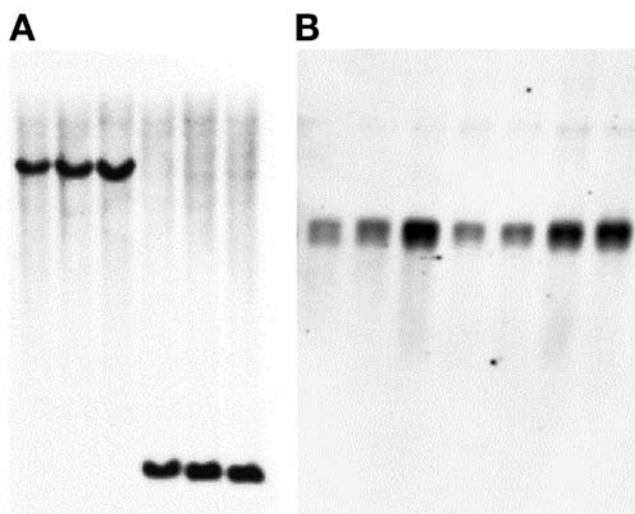


Fig. 2. Examples for typical results from Southern and Northern analyses. **(A)** Detection of a single-copy gene from genomic DNA isolated from six different barley cultivars (each 10 μ g DNA template). **(B)** Detection of the rare actin messenger RNA from different samples of barley total RNA (each 1 μ g RNA template). The experiments were performed as described in **Heading 3.**, and the membranes were exposed to an X-ray film for 60 min (Southern) and 30 min (Northern), respectively.

5. Wash the membrane twice for at least 15 min each in wash buffer.
6. Briefly rinse in alkaline phosphatase buffer.
7. Add a 1:100 dilution of CDP-Star in alkaline phosphatase buffer using the “transparency technique” (see **Note 12**).
8. Pre-incubate for 5 min, preferably at 37°C, and expose the membrane for 2 min to 2 h (strongly depending on the experiment) to an X-ray film. Develop the X-ray film as recommended by manufacturer. Alternatively, chemiluminescence can be detected using a CCD camera. For typical examples, see **Fig. 2**.

4. Notes

1. In general, the best yield and purity is obtained from young leaf material. The older the tissue, the more rigid is the cell wall, and the higher the content of phenolic substances and polysaccharides. For tissues with exceptional concentrations of these substances, specific protocols have been published elsewhere (**3,4**). It often helps to use the alternative DNA extraction buffer, as given in **Heading 2**. The RNA obtained by the protocol provided is free from DNA, as judged by gel electrophoresis. This quality is sufficient for any Northern application.
2. Briefly hold the end of the pipet over an open flame until a hook is formed. In our hands, the quality of the isolated DNA is much better using this procedure instead of centrifuging the nucleic acids. Seemingly, less co-purifying components

are precipitated. The precipitate is also much easier to re-dissolve using this technique.

3. Spectrophotometric measurements are often inaccurate, and nucleic acid contents are overestimated because of co-purifying agents. We usually separate the RNA on an agarose gel and estimate the intensity of the 27S ribosomal RNA band. This is performed most accurately using fluorescence quantification software, as provided with many gel imaging systems. Genomic DNA concentrations can be determined accordingly.
4. Make sure that the membrane is exposed to a defined UV irradiation, because excessive energy will irreversibly damage the membrane-bound DNA. Alternatively, the DNA may be fixed to the membrane by baking (for 30 min at 120°C).
5. We generally run gels for Southern analyses without ethidium bromide. For unknown reasons, this clearly reduces background problems. The HCl incubation can be skipped if the average fragment size is lower than 2 kbp. However, do not incubate agarose gels for longer than 15 min in the HCl solution, because this will lead to very short DNA fragments, to which the probe will no longer bind in an efficient manner.
6. Always pour and run denaturing formaldehyde gels under a hood. Add 4% (v/v) formaldehyde solution (37%) slowly only after boiling the agarose and cooling the solution to approx 70°C.
7. A 1:10 ratio of DIG-dUTP to dTTP can be used as a standard. The sensitivity of the probe is not drastically increased by lower ratios, and higher ratios usually do not lead to a clearly better PCR efficiency. In special cases, in which the A/T content of the probe sequence is very low or very high, it might be worth titrating the concentration of the modified nucleotide. We obtain equal results with DIG and fluorescein as a hapten, but sometimes encounter background problems when using biotin-11-dUTP. Therefore, the former two are recommended for standard use and re-probing. Probes can be easily stripped from Southern membranes if the hapten is linked to the nucleotide by an alkali-labile bound. However, this technique is not applicable for Northern analyses because the template RNA is hydrolyzed under alkaline conditions.
8. For Northern analyses, make sure that the antisense strand is labeled in the *in vitro* transcription reaction. It is essential that the labeling of vector sequences be avoided as far as possible. Even small homologies, e.g., to ribosomal RNA, might lead to unspecific binding of the probe. Therefore, the use of PCR products is strongly recommended, although, even here, the homology of polylinker sequences to plant RNA sequences might result in the appearance of unexpected additional bands (2). Using a gene-specific reverse primer for PCR amplification might circumvent this problem.
9. The correct hybridization temperature depends on several parameters: RNA:RNA hybrids are much more stable than DNA:DNA hybrids. The melting temperature increases with the length of the probe, however, this is negligible if probes are more than a few hundred base pairs in length. The GC-content of the probe and the identity of probe and target also have a positive impact on the stability of the

hybrid. The buffer recommended in this protocol contains denaturing agents. In nondenaturing buffers, the hybridization temperature should be significantly increased, by 10 to 30°C.

10. The washing conditions depend on the stability of the hybrid between the probe and target and, therefore, on the parameters, as given in **Note 9**. The stringency of washing buffers is usually controlled by the salt concentration. The lower the concentration of monovalent ions, the higher the stringency. We also obtain good results with colorimetric substrates, such as NBT/BCIP, instead of chemiluminescent substrates. Especially, this often leads to a reduction of background signals. Incubation times can be up to 24 h at room temperature in the dark. However, colorimetric signals are hardly stripped from membranes.
11. The concentration of the blocking reagent may be increased to up to 5% (w/v) in case of background problems. Casein might be used instead of the commercially available blocking solution, at least for the more robust Southern applications, but the use of nonfat milk powder often leads to increased background signals. For beginners, the use of the Wash and Block Buffer Set (Roche Applied Science) can be helpful.
12. The “transparency technique” was described by Rüger et al. (9) and works the following way: the membrane is placed between two heat-labile transparency foils. The upper transparency is lifted, and approx 1 mL of detection solution for a 200-cm² membrane is added at one edge of the membrane. The transparency is rolled over the membrane, and excess buffer is squeezed out. The transparency foils are sealed around the membrane, using a heat sealer. By this method, it is possible to work with very low amounts of detection solution and to avoid any contamination with detection reagent again leading to background signals.

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Screening a BAC Library With Nonradioactive Overlapping Oligonucleotide (Overgo) Probes

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Summary

Comparative mapping has been the primary approach for analyzing genomes of divergent species because gene linkage is often conserved over evolutionary time, a phenomenon known as synteny. Comparisons are based on computational detection of orthologous sequences between species. Small overlapping oligonucleotide (overgo) probes, consisting of two self-annealing oligonucleotides at the 3'-end, are filled in with labeled deoxynucleotides. Overgo probes are designed against highly conserved regions between two genomes, at regular intervals (30–40 kbp). All methodologies reported to date have used radioactive dATP and dCTP to label overgo probes. We report a nonradioactive labeling protocol to produce digoxigenin-labeled overgo probes for high-throughput screening of the *Actinidia chinensis* bacterial artificial chromosome library.

Key Words: Bacterial artificial chromosome (BAC); chemiluminescence; digoxigenin; overgo; overlapping oligonucleotide.

1. Introduction

Synteny is the foundation of comparative mapping. Gene linkage between closely related species is often conserved over a long evolutionary time. However, two related genomes can also have unique regions not shared between them. Numerous events of chromosome rearrangements, duplications, the presence of retrotransposons, and polyploidy complicate the comparison between two related genomes. Overall, orthologous gene content and order in divergent species are enough to establish regions of microsynteny. The main goal of many genetic mapping enterprises is to anchor the genetic markers into a physical map. Whole-genome sequencing approaches rely on genetic maps to solve the position of conflicting regions during genome assembly (1). Both approaches

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are extremely demanding and, often, a research group cannot tackle both at the same time because of time and financial restrictions. Comprehensive gene-order data only exists for a relatively small number of model species, the genomes of which have now been sequenced and assembled. It is prohibitively expensive to generate an equivalent level of information from each species of interest. Comparative approaches use the level of synteny that remains between a model species and the species of interest. In cases in which the relationship between these species is relatively distant, a fair degree of microsynteny may still remain and be useful for accelerating genome projects in nonmodel species.

The development of stable DNA cloning systems to propagate large fragments of genomic DNA, and the decreasing costs of DNA fingerprinting and sequencing methods have provided valuable tools for comparative mapping projects. Large genomes have been cloned into bacterial artificial chromosome (BAC) vectors to produce arrayed libraries. PCR or DNA hybridizations can be used to anchor genetic markers and expressed sequence tags to produce an integrated genetic map. PCR screening of pooled BAC clones from a library is effective for locating a small number of genetic markers. Hybridization of pooled DNA probes against an arrayed BAC library allows for large-scale anchoring of genetic markers in a high-throughput manner; however, probe size, amount of label, and heterogeneous GC content may discourage a mapping team from attempting to establish these techniques because each probe may need individual optimization.

Overgo probes are short overlapping oligonucleotides consisting of two complementary oligonucleotides annealed at the 3'-end to generate a double-stranded labeled probe approx 40-bp long (2). Overgo probes are designed to anneal at regular intervals (30–40 kbp) to conserved regions between genomes. The criteria to select such regions depend on stringent cut-off values of pairwise comparisons. Because overgo hybridizations require a high-throughput approach, the GC content of the probes has to be similar (~0.5) to allow the probes to remain annealed to their true targets during the stringency washes after hybridization. Overgo hybridizations have successfully anchored probes to BAC clones, allowing the construction of BAC sequence-ready contigs in mouse chromosome 11 (3), human chromosomes 16q (4) and 19 (5), rice (6,7), maize (8), fungal pathogen *Cryptococcus neoformans* (9), chicken (10), horse (11,12), and comparative mapping among mammals (13).

The argument for using radioactivity has been that nonradioactive detection will be hindered by the short double-stranded probe with only few radioactive deoxynucleotides incorporated hybridizing to a low-copy plasmid (e.g., a BAC plasmid). All of the examples cited in the previous paragraph (3–13) use radioactive probes labeled with ^{32}P -dATP and ^{32}P -dCTP. Advances in non-radioactive methods using haptens, such as digoxigenin (DIG), attached to the

3'-end of an oligonucleotide 24 to 30 bases long, can detect up to 10 fmol of target DNA by chemiluminescence detection (14). If we assume that an average bacterial colony of *Escherichia coli* at the stationary phase contains 1×10^8 cells, each carrying two BAC plasmid molecules, we have approx 33 fmol of BAC DNA target, which should be able to be detected by nonradioactive methods, without compromising the results. The level of detection offered by the nonradioactive method described in **Note 1** has been optimal for screening a genomic DNA BAC library from *Actinidia chinensis* using overgo probes (Kiwifruit Mapping Team at HortResearch Ltd., unpublished results, 2006).

2. Materials

1. 10X DIG-dNTP stock: 1 mM each of dATP, dCTP, and dGTP; 0.35 mM DIG-11-dUTP; and 0.65 mM dTTP.
2. Designed forward and reverse pairs of overgos, each 24 bases long, as dried pellets.
3. 4.6 U/ μ L Klenow DNA polymerase (large fragment of DNA polymerase I).
4. Klenow DNA polymerase 10X reaction buffer: 500 mM Tris-HCl, pH 8.0; 100 mM MgCl_2 ; and 500 mM NaCl.
5. 1X Tris-HCl-EDTA (TE) buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, sterile.
6. Positively charged nylon filter, precut to 73×115 mm.
7. UV transilluminator or UV crosslinker.
8. Plastic boxes, with tight seal, several sizes.
9. Prewash solution: 5X standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and 1 mM EDTA, sterile.
10. Lint-free tissues.
11. Large Petri dishes.
12. Stainless steel forceps with flat tip, and one flat-edge spatula.
13. Autoclave bags: clear and with no labels, medium (slightly bigger than 30×40 cm) and small size.
14. 0.5 M Na_2HPO_4 , pH 7.5—this solution is 1 M Na^+ ions (see **Note 2**; ref. 15).
15. Church buffer: 1 mM EDTA, 0.25 M Na_2HPO_4 , 7% SDS, pH 7.5—this solution contains 0.5 M sodium ions (see **Note 3**; ref. 15).
16. 0.5 M EDTA, pH 8.0.
17. 100 mg/mL Salmon sperm DNA, sheared and denatured (e.g., DNA MB grade, Roche Applied Science).
18. 20X SSC: 3 M NaCl and 0.3 M trisodium citrate, pH 7.0; sterile.
19. 20% SDS, sterile.
20. Low-stringency buffer: 2X SSC, 0.1% SDS (no need to autoclave).
21. High-stringency buffer: 0.5X SSC, 0.1% SDS (no need to autoclave).
22. 24 Omni-trays (rectangular Petri dish).
23. Maleic buffer: 100 mM maleic acid and 150 mM NaCl, pH 7.5, titrated with NaOH (see **Note 4**).
24. Wash buffer: sterile maleic buffer with 0.3% Tween-20 added after autoclaving.

25. 1% blocking solution: maleic buffer with 1% Hammerstein-grade casein (see **Note 5**).
26. Anti-DIG–alkaline phosphatase conjugate Fab fragment (Roche Applied Science).
27. Color substrate buffer: 0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5; sterile.
28. 25 mM CDP-*Star* stock (12.38 mg/mL; Roche Applied Science).
29. Thin-layer chromatography reagent sprayer, 25-mL working capacity, with standard ground joint.
30. High-speed detection X-ray film for chemiluminescent signals, cassettes, and developing facilities.
31. Temperature-controlled platform shakers (or water baths with platform shakers).
32. Hybridization ovens with glass bottles (narrow, 30 or 35 cm long) and nylon mesh cut exactly to wrap the inside of the bottle in one layer.
33. Water baths, microplate centrifuge, and thermocycler.
34. UV transilluminator, low-energy setting.
35. Propylene plastic bags and bag sealer.
36. Four rectangles (3 × 9 cm) of plastic transparency painted with glow-in-the-dark fluorescent paint (available in toy stores).
37. Stripping solution: 0.2 M NaOH and 0.1% SDS, store in plastic container and do not autoclave.

3. Methods

The methods outlined here have been designed for screening the *A. chinensis* BAC library, printed onto 23 high-density filters (positively charged nylon filter, 73 × 115 mm), in a 4 × 4 pattern (eight 384-well plates spotted in duplicate on each filter) with a pool of 96 overgo probes. Each filter is labeled with the plate numbers on the same side that the DNA is printed. An overview on how to produce and care for high-density filters from a BAC library can be found in Chapter 3 of this book.

The temperature for hybridization and stringency washes reported in this chapter is the same as reported by others (**3–13**). If the background becomes a problem, increase the stringency from 1X SSC and 0.1% SDS to 0.5X SSC and 0.1% SDS, but this is usually not necessary. We do not recommend higher stringency or temperatures because the overgo probes are only 40 bp long and may detach from their specific targets.

3.1. Labeling Overgo Probes With DIG

1. Add 400 μ L deionized water to each overgo pair contained in the 96-well daughter plate (see **Note 6**). The final concentration of each oligonucleotide is 10 pmol/ μ L. Seal the plate.
2. To anneal the overgo pairs, place the 96-well daughter plate in a water bath at 80°C for 5 min, transfer to a water bath at 37°C and incubate for 10 min, and place on ice for longer than 10 min. Centrifuge at maximum speed for 1 min. Alternatively, use a thermal cycler programmed at the temperatures mentioned.

3. With a multichannel pipet, transfer 10 μL from each well of the annealed overgo daughter plate to a sterile plastic reservoir (e.g., plastic trays used for dispensing liquid with a multichannel pipet). Collect the contents of the reservoir and transfer them to a sterile 1.5-mL centrifuge tube and place the tube on ice. The total volume should be 960 μL .
4. Add 192 μL of Klenow DNA polymerase 10X reaction buffer, 96 μL of 10X DIG-dNTP stock, and 10 μL of Klenow DNA Polymerase (final volume 1258 μL ; see **Note 7**). Mix contents by gentle inversion, and centrifuge briefly.
5. Incubate at 37°C overnight (~16 h).
6. Store tube at 4°C for immediate use, or keep at -20°C until needed.

3.2. Dot-Blot Preparation

To assess the labeling efficiency of the overgo probes, a dot-blot of the unlabeled overgos should be hybridized at the same time as the library filters.

1. With a multichannel pipet, aliquot 1 mL of TE buffer into a 96-deep well plate (dilution plate). Transfer 10 μL of the annealed overgo pairs (10 pmol/ μL) from the daughter 96-well plate to the dilution plate. The dilution plate now contains 0.1 pmol/ μL of each overgo per well.
2. Label the right-hand side border of the positively charged filter with a sharp pencil with the dot-blot no., the overgo set no., the date, and initials.
3. Spot 1 μL of each diluted overgo from the dilution plate onto the positively charged nylon filter using the multichannel pipet or any robotic equipment available, allowing even spacing between the spots, without overlapping. Let the filter dry at room temperature for 30 min. Each spot contains 0.1 pmol of each oligonucleotide.
4. Place the filter with the DNA side facing the UV transilluminator. Expose the filter to UV light for 2 min. Alternatively, use a specialized UV crosslinker, under the conditions specified by the vendor.
5. Store the filter in a zip-lock plastic bag at -20°C until needed.

3.3. Prewash BAC Library High-Density Filters

1. Keep prewash solution at 69°C during the procedure. The prewash solution precipitates at room temperature.
2. Take one complete set of library filters from the -20°C freezer. Each filter must be stored in an individual zip-lock bag, which should be slightly larger than the filter.
3. Add 20 mL of prewash solution. Seal the plastic bag, avoiding trapping air bubbles.
4. Place all of the filled plastic bags inside a plastic box, in three or four layers. Place the plastic container in a water bath with a flat shaker platform (or dry-shaker incubator) at 69°C. Shake vigorously for 1 h. Do not worry if some prewash solution leaks out of the plastic bag.
5. Remove plastic boxes from the water bath. Open the plastic bag, discard liquid, and cut out the sides of the bag to pull out the filter. Place the filter on the inverted lid of a large Petri dish, DNA side up.

6. Hold the filter with forceps or the flat edge of the spatula (see **Note 8**).
7. Soak one or two lint-free tissues in prewash solution, and gently wipe the surface of the filter several times, until no debris is visible. Use more tissues if needed. No DNA is stripped in this procedure.
8. Transfer the filter to a plastic container filled with 0.5 L of 3X SSC buffer.
9. Repeat **steps 5–8** for each filter.
10. After wiping all filters, discard the 3X SSC buffer and add another 0.5 L of 3X SSC. Store at 4°C (see **Note 9**).

3.4. Hybridization

1. Prewarm the Church buffer and 0.5 L of 3X SSC at 58°C.
2. Set the hybridization oven to 58°C.
3. Place the dot-blot filter in 3X SSC to hydrate.
4. Remove the nylon mesh from the hybridization bottle and place it on a clean surface. Arrange four filters per bottle. Roll up each filter over itself, with all filters in the same direction (see **Note 10**). Return the nylon mesh with the filters to the bottle. Remember to include the dot-blot together with the BAC library filter set.
5. Add 50 mL of 3X SSC to each bottle and place in the hybridization oven for 30 min.
6. Discard the 3X SSC solution, and add 50 mL of Church buffer (prehybridization solution). Incubate for 1 h. When using a new library filter set, extend the incubation time to 2 to 3 h.
7. While prehybridizing, prepare the hybridization solution. Transfer 629 μ L of DIG-labeled, pooled, 96 overgo probe (**Subheading 3.1., step 6**) to a screw-cap, sterile, 1.5-mL tube. Boil the probe for 5 min, then cool the probe on ice for longer than 10 min. Centrifuge for 1 min at maximum speed.
8. Transfer 300 mL of Church buffer to a 0.5-L glass bottle. Add the DIG-labeled pooled 96 overgo probe and mix very well. Keep at 58°C.
9. Recover the prehybridization solution, and store it in a 0.5-L glass bottle at –20°C (see **Note 11**).
10. Add 50 mL of hybridization solution per bottle. Place bottles back in the hybridization oven in the same orientation as in **step 4**. Incubate overnight (~16 h).

3.5. Stringency Washes

1. Use a water bath with a platform shaker, or an oven with a shaker set at 58°C. All of the washes are performed at 58°C.
2. Recover the hybridization solution, and store at –20°C.
3. Set up two plastic boxes, each containing 0.5 L of low-stringency buffer at 58°C. Remove the nylon mesh from each bottle, place it on the plastic tray, and transfer 12 filters to the first box (see **Note 12**). Transfer the remaining filters to the second box. Close the boxes tightly.
4. Place the boxes in the water bath or oven. Shake vigorously (~150–180 rpm) for 5 min. Take care that the filters do not stick to the top of the lid. If this occurs, peel them off using the forceps and return them to the buffer.

5. Pour off the buffer, and replace it with fresh 0.5 L of low-stringency buffer per box. Shake vigorously (~150–180 rpm) for 5 min.
6. Pour off the second low-stringency buffer solution and add 0.5 L of high-stringency buffer to each box. Shake vigorously (~150–180 rpm) for 15 min. Repeat this step once more.
7. All of the steps beyond this point are performed at room temperature.
8. Transfer the 24 filters to a plastic box with 0.5 L maleic buffer. Rinse briefly, discard the buffer, and add another 0.5 L of maleic buffer.

3.6. Chemiluminescence Detection

1. Pipet 8 mL of 1% blocking buffer into each omni tray.
2. Place one filter per omni tray, DNA side facing up.
3. Shake the omni trays very gently for 30 min at room temperature.
4. While blocking the filters, mix and centrifuge the anti-DIG–alkaline phosphatase conjugate Fab fragment for 5 min at approx 10,600g, in a microcentrifuge.
5. Transfer 200 mL of 1% blocking buffer to a clean glass bottle. Add 20 μ L of anti-DIG–alkaline phosphatase conjugate Fab fragment. Mix well.
6. To avoid drying out the filters, work in sets of four omni trays. After 30 min of incubation in blocking reagent, discard the blocking reagent of four omni trays, pipet 8 mL of antibody solution (**step 5**) to each plate. Repeat this step until all of the filters have the antibody solution.
7. Shake gently for 30 min.
8. Transfer the filters to the wash buffer: 12 filters per box containing 0.5 L wash buffer each, as outlined for the stringency washes (**Subheading 3.4., step 2**).
9. Shake vigorously (~150–180 rpm) for 15 min at room temperature. Replace the buffer with another 0.5 L of wash buffer for each box, and shake for another 15 min.
10. Transfer all filters to one box containing 0.5 L of color substrate buffer. Rinse briefly and replace the solution with another 0.5 L of color substrate buffer. Let the filters stand at room temperature for at least 5 min.
11. Set up the sprayer in a fume hood, with the sprayer connected to a compressed air source by plastic tubing. Place a small cotton wool ball along the path of the compressed air to catch any debris. Turn off the fume hood. Clean the sprayer by spraying approx 4 mL of sterile deionized water.
12. Prepare a 1:200 dilution of CDP-Star: pipet 8 mL of color substrate buffer into a sterile, disposable, 15-mL plastic tube. Add 40 μ L CDP-Star stock. Mix well and protect from light (see **Note 13**). Transfer contents to sprayer.
13. Cut the sides of the propylene plastic bag. Open the bag and place 12 filters in a 3 $\times$ 4 array, DNA side up. Cover the filters and transfer them to the fume hood using the plastic tray.
14. Turn on the compressed air, and adjust the airflow to obtain a fine mist. Test it briefly.
15. Uncover the filters and spray them with approx 4 mL of CDP-Star solution. Cover the filters with the plastic sheet. Repeat **step 13** with the remaining 12 filters.

16. Seal the long sides of the plastic bag, and with a gentle, circular motion, spread the CDP-Star solution evenly over the entire bag. Squeeze out excess liquid through the remaining bag opening. Avoid any air bubbles (see **Note 14**). Repeat this step for the other 12 filters. Seal the remaining side of each bag.
17. Make bag labels: write down the filter order in each bag using any text editor. Type in a text box (3 × 9 cm), with white font against black background. Create another text box with all of the details of the experiment: date, author, library, probe, hybridization temperature and time, stringency washes (number and incubation time), blocking and antibody incubation, CDP-Star dilution, and exposure time. Print out the labels, and cut to exact size.
18. Overlay the plastic transparency rectangle painted with glow-in-the-dark paint with the bag label. Place it inside a small plastic bag and seal. Repeat for the other label.
19. Tape the filter bag to an X-ray film cassette. Wipe off with ethanol (see **Note 15**), and push any remaining air bubbles to the sides. Tape the appropriate bag label approx 1 cm away from the filter bag. Repeat this step for the other filter bag and its label.
20. In the dark room, before turning off the white light, open the X-ray film cassettes, and expose the glow-in-the-dark labels for 1 to 2 min. Turn off the lights (except the safety red lights), and place 1 sheet (30 × 40 cm) of X-ray film, ensuring that all filters and labels are covered. Close the cassette and expose the film for 2 h.
21. Develop the X-ray film as recommended by vendor.
22. If another exposure is necessary, repeat **steps 11–21**, but expose the X-ray film overnight (see **Note 1**). Typical results are shown in **Fig. 1**.
23. Transfer 12 filters to a plastic box containing 0.5 L stripping solution, prewarmed at 37°C. Do the same with the other 12 filters.
24. Shake vigorously in the water bath with platform, for 15 min at 37°C. Repeat once more.
25. Rinse each box twice with 0.5 L deionized water, each time, at room temperature. Rinse one time with 3X SSC buffer, and store all filters in one box with 0.5 L of 3X SSC, at 4°C (see **Note 16**). For long-term storage, refer to **Note 8**.

4. Notes

1. Under the conditions described in this protocol, good signal should be detected after 2 h of exposure. If too much background and unspecific hybridization is observed, dilute the hybridization solution with an equal volume of Church buffer and repeat the experiment under the same conditions. If unspecific binding or high background persists, make sure that the antibody dilution is correct and/or reduce the CDP-Star solution to 1:100, and repeat the experiment. The last resort is to increase the hybridization temperature by 5°C increments, but use the same low- and high-stringency buffers, at the new selected temperature. Do not reduce the salt concentration too drastically; remember that you are using a 40-bp oligonucleotide pair probe. The signal detected in the control dot-blot may seem uneven. A possible explanation is that because 1 µL of unlabeled overgo spotted

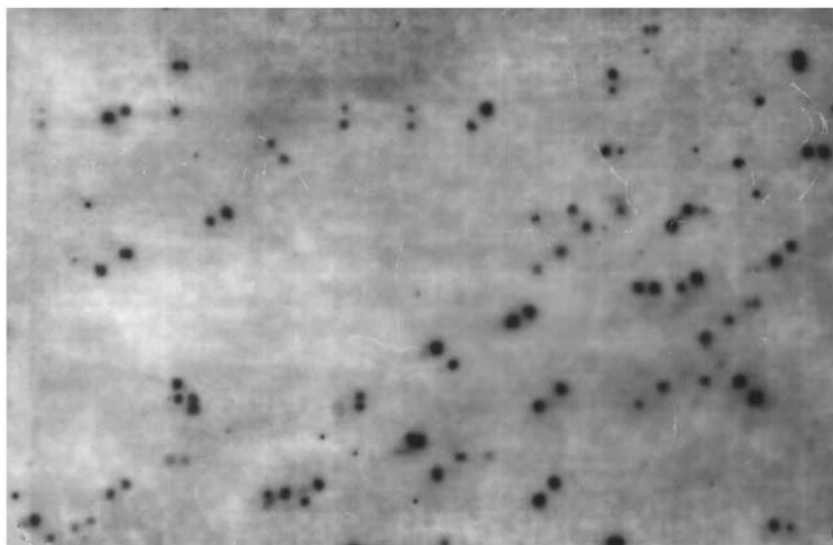


Fig. 1. Kiwifruit BAC high-density filter hybridized with a pool of 96 overgo probes labeled with DIG and detected by chemiluminescence. Hybridization, stringency washes, and detection are described in **Heading 3**.

on the filter spreads over a wide area compared with the compact bacterial colony, this effect produces a faint signal at 58°C. To confirm that each DIG-overgo probe hybridizes to its unlabeled counterpart, perform the dot-blot hybridization and stringency washes at 45°C, with the stringency buffers described in this chapter.

2. The original citation from Church and Gilbert (15) on how to prepare the popular “Church” was a little unclear, because the molarity reported in the final solution reflects the total moles of sodium ions, not the molarity of the dibasic sodium phosphate solution. To prepare 1 L of 0.5 M Na_2HPO_4 , pH 7.5, buffer, first add 4 mL H_3PO_4 (85% v/v) to the deionized water, then add 134 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71 g anhydrous Na_2HPO_4 , with vigorous stirring. Adjust the pH to 7.5 and bring the volume to 1 L. Split the solution into two 1-L bottles, and autoclave.
3. To prepare 1 L of Church buffer, add 200 mL deionized H_2O to the sterile 0.5 L of 0.5 M Na_2HPO_4 , pH 7.5, mentioned in **Note 1**. Dissolve 70 g SDS. **Caution:** use protective gear. Add 2 mL of 0.5 M EDTA, pH 8.0. Bring the volume to 1 L. Split solution into two 1-L bottles, and autoclave. After cooling the solution to room temperature, add 5 mL of 100 mg/mL salmon sperm DNA to each 0.5 L of sterile Church buffer, mix well, and store at -20°C . If you are not a frequent user of Church buffer, make aliquots in 50-mL sterile propylene tubes.
4. The pH for the maleic buffer is between its two pKa, i.e., is outside of the buffer capacity of maleic acid. To prepare 1 L of maleic buffer, add 11.6 g maleic acid, 8.8 g NaCl, and 8.0 g NaOH to 750 mL deionized H_2O . Dissolve completely. The

pH should be close to 7.0. To adjust the pH to 7.5, add a few drops of 1 M NaOH. If too many drops are added, the pH will shoot up to 10 or more. Bring up the volume to 1 L, and autoclave.

5. Do not replace Hammerstein-grade casein for regular casein or dry milk. This special casein formulation does not produce background (16). To prepare the blocking solution, in a microwave, heat 0.5 L of maleic buffer in a 1-L glass beaker for 2 min at maximum power. Transfer the beaker to a magnetic stirrer at full speed; add 10 g Hammerstein-grade casein, stepwise, until completely dissolved. Heat another 0.5 L maleic buffer, and add to the blocking solution. Split the solution into two 1-L bottles, and autoclave. Store protected from light. Once open, store the bottle at -20°C .
6. Order the forward and reverse oligonucleotides for making the overgo probes in sets of 96. The forward and reverse oligonucleotides are stored in separate plates, but in the same orientation, i.e., plate 1, row A, column 1 has its overgo in plate 2, row A, column 1, and so on. Ask your provider to perform the following operations:
 - a. Normalize the concentration to 12 nmols and resuspend each pellet in 120 μL deionized water.
 - b. Take an aliquot of 40 μL from each plate, and combine in a daughter plate (96-deep well plate).
 - c. Ship all of the plates as dried pellets.

On arrival, store the forward and reverse plates as backups at -20°C . Resuspend each pellet of the daughter plate in 400 μL sterile deionized water, which gives a final concentration of 10 pmol/ μL for each oligonucleotide. Store the daughter plate at -20°C .

7. To label one overgo pair, you only need 0.35 nmol of DIG-11-dUTP and 0.4 U of Klenow DNA polymerase. Each labeling reaction contains 100 pmol of forward overgo and 100 pmol reverse overgo; 200 pmol of total oligonucleotides.
8. Never use a forceps with serrated tips, it will make prints on the filter that will accumulate debris and produce unexpected spurious signals. Keep one set of forceps and spatula dedicated for this task only. Never handle the filters with bare hands or even with gloved hands. Use powder-free gloves only.
9. Once the BAC library filters have been hydrated and washed, they should always be kept in a buffered solution. If the filters dry out, unexpected patches of dark background will start appearing. Nothing can be done at this point, and the filters must be discarded. For short-term storage (1–2 mo), keep them in 3X SSC at 4°C . For long-term storage, rinse the filters thoroughly with sterile TE buffer, transfer them to an autoclave bag, fill the bag with sterile TE, and seal the bag as tightly as possible without leaving any air bubbles. Store the bagged filters in a plastic container at 4°C .
10. Use large plastic trays as working surfaces. This will make cleaning easier, prevent contamination of the filters, and allow for easy transportation of the filters around the laboratory. The filters should be rolled up in the same direction as the rotisserie of the hybridization oven, i.e., the overlapping lip of each filter should be pointing in the same direction as the rotation of the rotisserie. Whatever convention you adopt, be consistent with all of the filters. The reason for leaving the

overlapping lip pointing in the rotation direction is that it will ensure that some hybridization solution covers the overlapping end of the filter.

11. The first time a library filter set is prehybridized, it will leave a yellowish color in the prehybridization solution. This should not be a problem for future hybridizations, but if you are concerned, discard the solution. However, keep in mind that the prehybridization solution can be reused five to eight times without problems. The hybridization solution can also be reused, but has to be spiked with fresh probe every two rounds of hybridization. In this case, add 60 to 70 μ L of DIG probe to the hybridization solution. Boil all of the solution (300 mL) for 10 min, and cool on ice completely by swirling the bottle for approx 5 min. Prewarm again to 58°C before adding it to the filters. The hybridization solution can be used approximately five times, or until debris start to accumulate or the signal is too low.
12. Prewarm the stringency wash buffers 1 min at maximum power in a standard microwave. Alternatively, incubate 2 L of each stringency wash buffer in a large water bath at 58°C overnight (while the filters are being hybridized).
13. To extend the shelf life of CDP-*Star*, store it in 200- μ L aliquots at -20°C. Store the working stock at 4°C, protected from light. Centrifuge the tube briefly to collect the contents from the lid. If you find a white pellet in the CDP-*Star* stock after centrifuging, resuspend it by vortexing or pipetting. A white suspension will be visible, but it does not interfere in the chemiluminescent detection. You can use this stock without any problems.
14. Excess CDP-*Star* substrate left in the bag may produce blotchy exposures. Do not worry if the bag seems empty, enough substrate has already reached the enzyme.
15. If you experience electrostatic sparks while rubbing the plastic bags, quench them by wiping the plastic bag with ethanol. Not all propylene bags give problems; it varies from brand to brand.
16. Document the number of times the BAC library has been used, the probe, the date, and the number of times the filters have been stripped. In our hands, an *A. chinensis* BAC library filter set stripped seven times still produced reliable signal, although faint compared with the first round of screening. When the signal is too weak, discard the complete filter set. Do not mix filters from different sets, because you may lose track of the number of times the filters have been stripped. We recommend always working with filters of the same age.

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Direct In-Gel Hybridization of DNA With Digoxigenin-Labeled Probes

Saeed A. Khan and Mohamed S. Nawaz

Summary

In-gel hybridization with digoxigenin (DIG)-labeled probes has been shown to detect complementary DNA sequences in dried agarose gels. Gels dried at room temperature or at 55°C in an oven do not show detectable changes in the sensitivity of detection. However, gels dried under vacuum seem to lose the sensitivity by approx 8- to 10-fold. In-gel hybridization after blotting high molecular weight T7 DNA (40 kbp) onto nylon membranes has been demonstrated to transfer the DNA to the membrane inefficiently. In-gel hybridization of DIG-labeled probes with the complementary DNA sequences has been determined to detect as little as 0.05 ng of 40-kbp T7 DNA and single copies of the erythromycin resistance marker gene *ermA*. Nonradioactive in-gel hybridization provides better quantitation of nucleic acids than filter hybridization in Southern and Northern blot analysis.

Key Words: Colorimetric; digoxigenin; direct in-gel hybridization.

1. Introduction

Several in-gel hybridization methods that involve the use of radio-labeled probes are widely used and provide better sensitivity than Southern or Northern blotting methods, because of inefficient transfer of nucleic acid molecules from gels to nylon membranes (1–11). However, the use of radioactivity is not always desirable because of environmental concerns, government regulations regarding the use and disposal of radioactive materials, and safety issues related to radiation effects on human health. Development of nonradioactive hybridization procedures has minimized the use of radioactivity, but most of the non-radioactive procedures are limited to filter or *in situ* hybridization (12–20). In-gel hybridization procedures (21–23), using nonradioactive probes, are rapid, less

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expensive, less time consuming, have no known consequences to human health, and are more quantitative than Northern and Southern blots. This chapter focuses on the detection of complementary nucleic acid sequences with digoxigenin (DIG)-labeled probes, using in-gel hybridization and color detection. As little as 0.05 ng of DNA and a single copy of a gene can be detected using this procedure. The disadvantage of in-gel hybridization is that, unlike filter hybridization, the gel cannot be stripped of the probe and reused. However, the advantages outweigh the disadvantages. Safety, ease of use, no blotting requirements, better quantitation than Southern and Northern blots, longer probe life, and improvements in the sensitivity of signal detection make in-gel hybridization an appropriate technique for routine use in molecular biology laboratories.

2. Materials

1. PCR reagents (polymerase, buffer, and dNTPs).
2. DIG-oligonucleotide 3'-end labeling kit (Roche Applied Science).
3. PCR DIG probe synthesis kit (Roche Applied Science).
4. DIG-DNA labeling and detection kit (Roche Applied Science).
5. Oligonucleotide primers.
6. Agarose and electrophoresis equipment.
7. Gel documentation system, GDS 8000 (UVP, Inc., Upland, CA).
8. VacuGene blotter (Amersham Biosciences).
9. Hybridization oven, model 136500 (Boekel Scientific, Feasterville, PA).
10. UV Stratalinker 1800 (Stratagene, La Jolla, CA).
11. Nylon membrane (Millipore, Inc.).
12. Whatman 3MM filter.
13. Transparency film XTR 650S (Labelon, Corp, Canandaigua, NY).
14. Hyperfilm™ X-ray film (Amersham Biosciences).
15. Tris-HCl-EDTA (TE) buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.
16. Electrophoresis buffer: 0.089 M Tris-acetate, pH 8.4, and 0.001 M EDTA.
17. 1 mg/mL Ethidium bromide solution.
18. Denaturation buffer: 0.5 M NaOH and 1.5 M NaCl.
19. Neutralization buffer: 0.5 M Tris-HCl, pH 7.5, and 1.5 M NaCl.
20. Prehybridization solution (5X standard sodium citrate [SSC]): 0.075 M sodium citrate; 0.75 M NaCl, pH 7.0; 0.1% (w/v) *N*-lauroylsarcosine, 1.0% (w/v) sodium dodecyl sulphate (SDS), and 1.0% blocking solution (Roche Applied Science).
21. Posthybridization washing solution I: 2X SSC and 0.1% SDS.
22. Posthybridization washing solution II: 0.2X SSC and 0.1% SDS.
23. Gel/membrane washing solution: 0.1 M maleic acid, 0.15 M NaCl, and 3% Tween-20 (v/v), pH 7.5.
24. Dilution buffer for blocking solution: 0.015 M sodium citrate, 0.15 M NaCl, and 1.0% SDS, pH 7.0.
25. Anti-DIG-alkaline phosphatase (AP) conjugate (Roche Applied Science).
26. Gel equilibration buffer: 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 50 mM MgCl₂.

27. Color detection solution: 0.33 mg/mL nitro blue tetrazolium (NBT) and 0.17 mg/mL 5 bromo-4 chloro-3-indolyl phosphate (BCIP) dissolved in gel equilibration buffer.

3. Methods

The methods described in this section focus on the DIG-labeling of probes, step-by-step gel processing for in-gel hybridization, in-gel hybridization under different conditions.

3.1. DIG-Labeling of Probes

Bacteriophage T7 DNA (250 ng) was labeled by nick translation. For PCR amplification of the *ermC* gene, the forward primer 5'-AGTACAGAG GTG-TAATTTTCG-3' and the reverse primer 5'-AATTCCTGCATGTTTTAAGG-3' were used. For the detection of a single copy of *ermA*, a 3'-end-labeled internal oligonucleotide probe, 5'-TAGCAGTAAGTGCATCGACTCATT-3', was used. The labeling was performed, per manufacturer's instructions, by using individual DIG-labeling kits for chromosomal DNA, PCR fragments, and 3'-end labeling of oligonucleotides.

3.2. Step-By-Step Gel Processing for In-Gel Hybridization

1. Dry the gel on a transparency in an oven at 55°C, store the gel in a box at room temperature and, when needed, process for hybridization as follows.
2. Submerge the gel in distilled water for 10 to 15 min.
3. Denature the gel in denaturation buffer for 30 min.
4. Rinse the gel twice with distilled water (see **Note 1**).
5. Neutralize the gel in neutralization buffer for another 30 min.
6. Crosslink the DNA in agarose gel by setting the crosslinker to "auto."
7. Transfer the gel in a clean container with prehybridization buffer (10–15 mL/100 cm²) and incubate at 45°C with constant shaking for 2 h.
8. Replace the prehybridization buffer containing denatured DIG-labeled probe and leave it overnight (16–24 h), at 10°C below the melting temperature (T_m) of the probe. Make sure that the probes were properly denatured (see **Notes 2** and **3**).
9. Wash the gel twice with posthybridization washing solution I at room temperature for 5 to 10 min.
10. Wash the gel twice with posthybridization washing solution II at 5 to 8°C below the T_m of the probe for 5 to 10 min.
11. Rinse the gel in gel washing solution for 5 min at room temperature, with gentle shaking.
12. Incubate the gel in blocking solution for 30 min at room temperature, with gentle shaking (**Note 4**).
13. Briefly centrifuge anti-DIG-AP conjugate at 1300 rpm for 1 min in an Eppendorf centrifuge, even if the solution looks clear. Dilute to 1:5000 in blocking solution.

14. Incubate the gel in diluted anti-DIG–AP conjugate for 30 min, with constant shaking.
15. Wash the gel twice, for 15 min each, with gel washing solution.
16. Equilibrate the gel in gel equilibration buffer for 5 to 10 min at room temperature.
17. Incubate the gel in freshly prepared color development solution (NBT/BCIP in equilibration buffer) and leave the gel at room temperature in the dark without shaking (see **Note 5**).
18. Wait until color develops (usually 2 h to overnight for in-gel detection).
19. When the color develops to sufficient intensity, stop the reaction by washing the gel with TE buffer for 5 min.
20. The gel, once used for in-gel nonradioactive hybridization, cannot be stripped of the probe and reused for hybridization (see **Note 6**). A schematic drawing of probe labeling and in-gel hybridization is presented in **Fig. 1**.

3.3. In-Gel Hybridization Under Different Conditions

3.3.1. Effect of Salts on In-Gel Hybridization

The dried gels can be stored for several months to years, rehydrated when needed, and processed for hybridization. It is normal for the gels to either stick to the transparency or to be crinkled after drying. After rehydration, the gels never acquire the original thickness, but they do retain their physical size and shape, which makes it easier for alignment after hybridization. Minor distortions can be corrected with imaging software that can be obtained from Scion Corp., Fredrick, MD (<http://www.scioncorp.com>). It is important to remove salts from the gel before it is dried (see **Note 7**). If the salts are not removed properly, the dried gel looks opaque and the salts may cause background after hybridization and signal detection (**Fig. 2B,C**). Gels with small amounts of salts are transparent, with scattered opaque areas, and do not have much of a background problem (**Fig. 2D,E**). If the salts are removed properly, the gel looks clean and transparent after drying. The background is also significantly reduced (**Fig. 2F,G**).

3.3.2. Vacuum vs Non-Vacuum-Dried Gels

Gels can be dried at room temperature, in an oven at 55°C, or under vacuum. It is difficult to notice a qualitative change in hybridization pattern whether the gel is dried at 55°C or room temperature (**Fig. 3A,B**). However, if the gel is dried under vacuum, a loss in sensitivity is observed (**Fig. 3C**), because DNA is lost during vacuum drying. Therefore, for quantitation, it is important to dry the gels either at room temperature or at 55°C in the oven.

3.3.3. In-Gel vs Membrane Hybridization

In-gel hybridization is similar to membrane hybridization. However, for quantitation, in-gel data is more reliable than filter hybridization, because

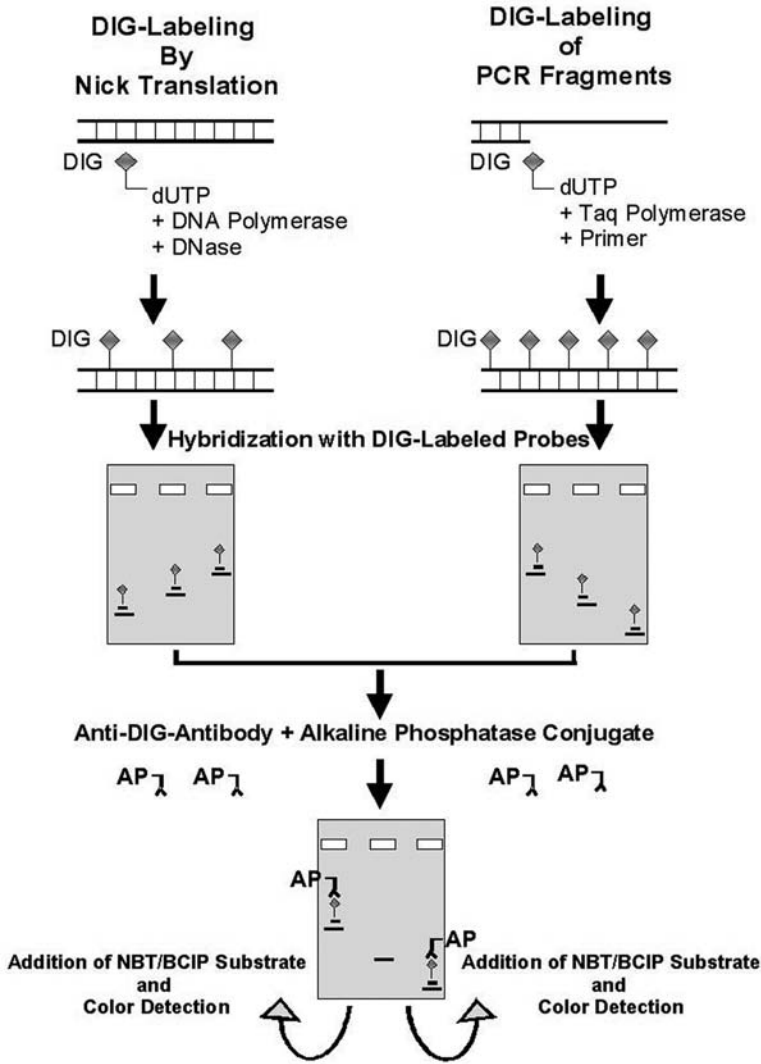


Fig. 1. Schematic drawing of probe-labeling and in-gel hybridization procedure. The drawing shows the major steps involved in in-gel hybridization.

nucleic acid molecules larger than 20 kbp are not efficiently transferred to nylon membranes during blotting. Furthermore, because DIG does not bind to agarose, blocking and/or prehybridization is not necessary (23). It is, however, advisable to include this step (see **Note 4**). To test the efficiency of DNA transfer, we tried to transfer DNA molecules ranging from 0.52 to 40 kbp onto nylon membranes. We noticed that, even after 1 h of blotting, some of

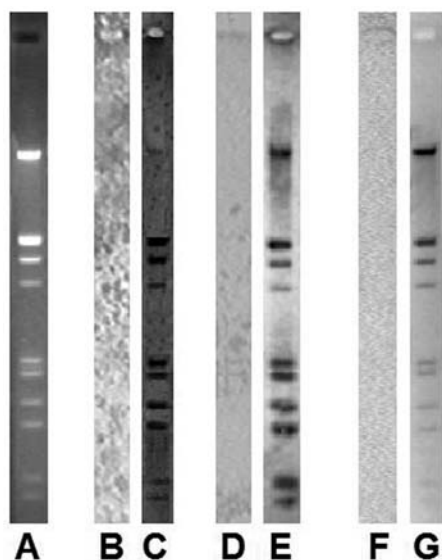


Fig. 2. Effect of salts on the quality of signal in dried agarose gels. DIG-labeled molecular weight markers were separated on a 1.0% agarose gel in three separate lanes. The lanes were cut, photographed, washed with water, dried at 55°C in an oven, and processed for hybridization. (A) Ethidium bromide stained gel, (B) unwashed and dried gel, (C) gel B after hybridization, (D) dried gel after 5 min of washing with water, (E) gel C after hybridization, (F) dried gel after two washes of 10 min each with water, and (G) gel F after hybridization.

the 40-kbp DNA remained on the gel (Fig. 4A,B). There was no problem with the transfer of low molecular weight DNA, which was transferred efficiently. Filter hybridization, therefore, seems to be less quantitative than in-gel hybridization.

3.3.4. Detecting Single-Copy Genes

It is possible to detect a single copies of genes by nonradioactive in-gel hybridization. In our experience, the technique works as long as all of the necessary precautions are taken (see **Notes 1–7**). For example, we digested the chromosomal DNA of two *Staphylococcus aureus* strains with *EcoRI* and, after electrophoresis and gel drying, probed the gel with an internal probe for the erythromycin resistance gene *ermA*. The probe was designed in such a way that it had no *EcoRI* site. Therefore, if the probe hybridizes with one or more bands on the gel, it will indicate that many copies of the gene in the chromosomal DNA. Using this strategy, we detected two to three bands in *S. aureus* strains (Fig. 5A,B), each band indicating one copy of the gene.

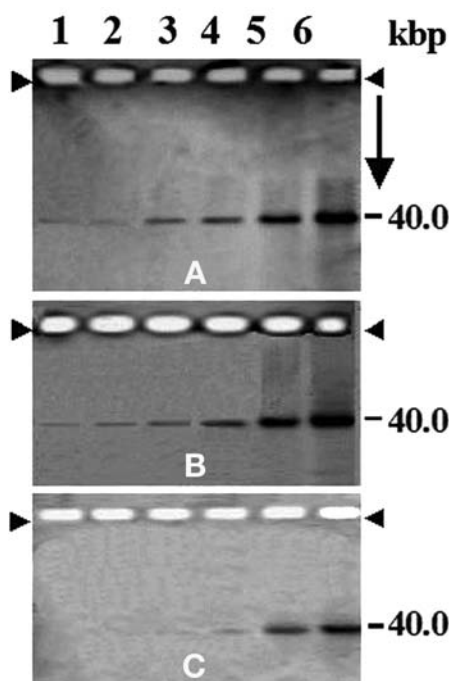


Fig. 3. Effects of different gel-drying procedures on the sensitivity of signal detection. Three gels were run for 3 h at 1 V/cm, dried, and probed with 100 nM DIG-labeled probe. The gels were dried under the following conditions: **(A)** dried at 55°C in an oven overnight, **(B)** dried in air overnight at room temperature, and **(C)** dried under vacuum at room temperature for 1 h. The concentrations of bacteriophage T7 DNA loaded were as follows: lane 1, 0.045 ng; lane 2, 0.135 ng; lane 3, 0.405 ng; lane 4, 1.215 ng; and lane 6, 3.645 ng. The least amount of DNA that could be detected in vacuum-dried agarose gel was 0.405 ng, which is almost 10-fold less than the amount observed in an oven- or room temperature-dried gel.

4. Notes

1. The presence of salts in the gels is one of the causes of background in nonradioactive in-gel hybridization. It is important to remove salts from the gels before drying. In addition, gels need to be handled with powder-free gloves to avoid finger marks.
2. Rinsing the gel twice with distilled water, after denaturation and before neutralization steps, results in better signal quality and low background.
3. It is reported that blocking is not necessary (23) for in-gel hybridization, but we have observed cleaner results after a blocking step is included. If some background is observed, increasing the concentration of blocking reagent (1–5%) will help, but care should be taken not to use excess blocking solution to avoid non-specific blocking of the target.

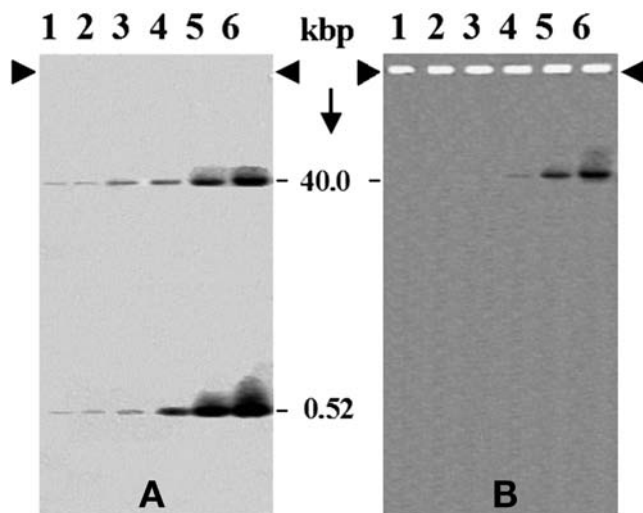


Fig. 4. Efficiency of high (40.0-kbp) and low (0.52-kbp) molecular weight DNA transfer on nylon membrane. A 1.0% agarose gel was run, as described in Fig. 3 along with a partially amplified 0.52-kbp *ermC* gene. The gel was blotted on a nylon membrane in a VacuGene blotter at 100-millibar suction pressure for 1.5 h at room temperature. The leftover gel and the nylon membrane were subjected to hybridization with T7- and *ermC*-specific probes. (A) Filter hybridization, and (B) in-gel hybridization using the gel in (A) after blotting.

4. We have found that hybridization at 10°C below the T_m and washing at 5°C below the T_m [$T_m = 2(A+T) + 4(G+C)$] always works better if higher specificity is desired.
5. One of the causes of failure is improper or incomplete denaturation of the probes. As a rule of thumb, denature the probes by heating the probe in a boiling water bath or in a PCR machine for 10 min at 97°C and immediately transferring it to ice. The boiling of oligonucleotide probes is not necessary, but if secondary structures are suspected, it is important to denature the probes by boiling.
6. Color development reagent should always be prepared fresh because of its light sensitivity. It is important not to shake the gel during color development, because the development of color is caused by the precipitation of AP during reaction with its substrate (NBT/BCIP). Shaking the gel during this process would cause improper use of the substrate by the enzyme and, hence, would result in a less intense signal.
7. Do not try to strip the gel off the probe, because heating the agarose gel in dimethyl formamide solution dissolves the gel. This is the only known disadvantage of nonradioactive in-gel hybridization. In contrast to membrane hybridization, the gels cannot be reused.

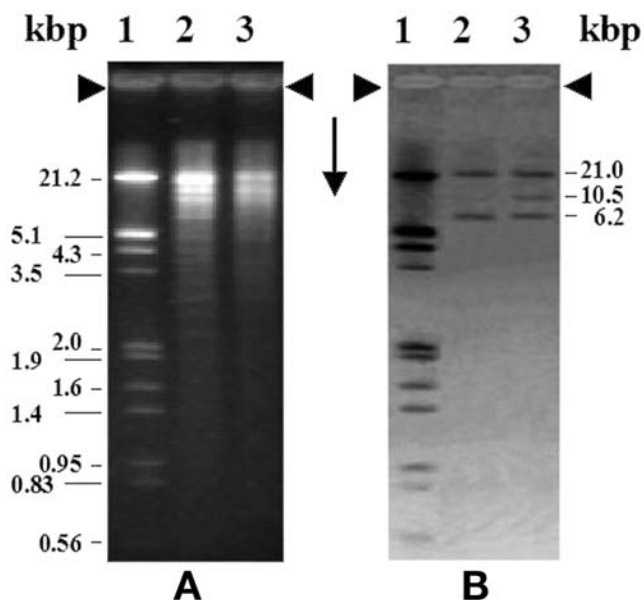


Fig. 5. Detecting a single copy of the gene. Chromosomal DNA from two *S. aureus* strains, one from poultry (p58) and another from a clinical source (c18), was digested with *Eco*RI. The digests were separated on 1.0% agarose gel and probed with an internal *ermA* gene probe. **(A)** Lane 1, DIG-labeled molecular weight markers; lane 2, p58/*Eco*RI digest; and lane 3, c18/*Eco*RI digest. **(B)** In-gel hybridization with an *ermA* gene-specific probe. Hybridized bands represent the number of times this gene was present in the chromosome, clearly demonstrating that if a single copy of the gene were present, it would be detectable by nonradioactive in-gel hybridization.

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***In Situ* Hybridization of Termite Microbes**

Shigeharu Moriya, Satoko Noda, Moriya Ohkuma, and Toshiaki Kudo

Summary

In situ hybridization is one of the most direct and reliable ways to ascertain the origin of the gene from complex mixed cellular systems. This method is essential for studying communities of uncultured microorganism in their natural ecosystem. In this chapter, we introduce our protocols for the *in situ* hybridization of the messenger RNA of uncultured symbiotic protists of termite hindgut and the ribosomal RNA of the symbiotic bacteria of the protists using nonradioactive labeling protocols. We hope that you will find these protocols useful in your own work to unravel the complex functions and to discover new organisms in the ecosystem.

Key Words: *In situ* hybridization; protists; symbiotic bacteria; symbiotic microorganisms; termite.

1. Introduction

Although axenic cultivation has revealed thousands of microbial strains, recent advances in culture-independent molecular microbial ecological techniques now reveal that the information of the vast majority of microorganisms in the natural ecosystem remains to be analyzed (1–3). To understand natural microbial ecosystems, we need to analyze the diversity and phylogenetic character of community members. For these purposes, ribosomal RNA (rRNA) and functional gene sequences amplified from environmental DNA need to be analyzed in diverse environments. Extracting taxonomic marker genes to identify community members and retrieving key functional genes from the diverse environments and analyzing their expression patterns can accomplish this task. Two of the most important and difficult experimental procedures in this type of investigation are identifying the origin of the obtained gene sequences and the

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exact localization of the marker genes in the community. *In situ* hybridization is one of the most direct and reliable ways to ascertain the origin of such genes. Further, *in situ* hybridization with rRNA-targeted probes for specific microorganisms yields phylogenetic information regarding the detected cells, and the probes for functional genes can identify the expression of the “key” gene of an individual microorganism.

Our uncultured target is a microbial community of termite symbionts (4). Termites harbor symbiotic protists (single-cell eukaryotes) in their hindgut. These protists are indispensable for the digestion of cellulose, and carry endobiotic and/or ectobiotic bacteria. Because most members of the symbiotic community in the termite gut are difficult to cultivate, we have applied *in situ* hybridization techniques to understand both the function and community structure of this unculturable community. Here, we introduce our protocols for the *in situ* hybridization of the messenger RNA (mRNA) of the symbiotic protists (5,6) and the rRNA of the symbiotic bacteria of the protists.

2. Materials

1. Silane-coated slide glass.
2. CoverWell Incubation Chamber (GRACE Bio-Labs).
3. Solution U: 0.216% NaCl, 0.0773% NaHCO₃, 0.1509% trisodium citrate, 0.1784% KH₂PO₄, 0.0083% CaCl₂, and 0.0048% MgSO₄ (w/v).
4. PBS (-): 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄.
5. PBS-T: 0.05% Tween-20 in PBS.
6. 10% NP-40 solution in water.
7. Fix solution: 4% paraformaldehyde in PBS (-).
8. 50, 80, and 100% Ethanol.
9. 0.25 N HCl.
10. 20X SSC: 3 M NaCl and 300 mM sodium citrate, pH 7.0.
11. Hybridization buffer 1: 5X SSC, 0.1% N-lauroyl sarcosine, and 0.02% sodium dodecyl sulfate.
12. Blocking reagent (Roche Applied Science), 5 µg/mL of a random 9-mer oligonucleotide.
13. Hybridization buffer 2: 0.1 M Tris-HCl, pH 8.0, and 0.9 M NaCl.
14. Washing buffer 1: 20 mM Tris-HCl, pH 7.2, 180 mM NaCl, 0.01% sodium dodecyl sulfate, and 5 mM EDTA.
15. Washing buffer 2: 0.2 M NaCl and 0.1 M Tris-HCl, pH 8.0.
16. Antifading solution: 0.5% triethylene diamine and 90% glycerol in PBS (-).
17. Alkaline phosphatase-conjugated antibody to FITC.
18. 3% sheep serum and 5 mg/mL heparin.
19. Appropriate oligonucleotide probe labeled with FITC, Texas Red, or FAM.
20. Vector RED alkaline phosphatase substrate kit (VECTOR lab).

3. Methods

3.1. Whole Cell In Situ Hybridization of the mRNA of the Protists

If using mRNA from a mixed population of termite symbionts as a starting material for reverse transcriptase-PCR, it is important to determine the origin of the obtained sequence. Several methods can be used to determine the origin of the sequences for the corresponding microorganisms. One effective method, the direct cloning of specific genes from micromanipulated cells by PCR, makes it possible to obtain the sequences from a single cell. With this method, however, there always remains a chance that contamination will generate amplification products. In contrast to reverse transcriptase-PCR, *in situ* hybridization furnishes simple, direct, and certain evidence of the origin of the sequence. This is why it is so important to use *in situ* hybridization for the identification of the obtained sequence. For whole-cell *in situ* hybridization, usually, rRNA is targeted because of its high copy number and stability (7). Because the mRNA of a structural gene has a lower copy number and is less stable than the rRNA, the mRNA is more difficult to detect than the rRNA.

When our group applied the ordinary protocol of whole-cell *in situ* hybridization using fluorescently labeled oligonucleotide probes, we failed to detect signals. In a subsequent effort to detect mRNAs with relatively low copy numbers, we tried to amplify the signal from probe-hybridized cells using FITC-labeled probes together with an alkaline phosphatase-conjugated FITC antibody (5,6). Using this strategy, we succeeded in detecting the mRNAs of several structural genes of symbiotic protists of the termite *Reticulitermes speratus*. This method will help us collect information of great value and importance for our understanding of the biological functions of uncultured environmental microorganisms.

3.1.1. Fixation and Immobilization

The duration of the fixation step is a crucial factor in detecting mRNA. If the fixation is too brief, the mRNA will easily diffuse because of its relatively poor interaction with proteins. To avoid this, we usually fix the cells overnight. Although this is somewhat too long in the case *in situ* hybridization targeting rRNA, it has proven appropriate for mRNA.

1. Remove the entire termite gut by forceps, place it in a 1.5-mL tube, and add 100 μ L of Solution U. Crush the gut with forceps, gently resuspend, and add another 500 μ L of Solution U. Centrifuge at 50g for 3 min, and discard the supernatant.
2. Resuspend the hindgut contents of 10 termites in the fix solution overnight at 4°C, wash in PBS (–), and store in 50% ethanol in PBS (–) at 4°C.
3. Add 1/10 volume of 1% NP-40 to the suspension of fixed cells, then spot 10 μ L of the suspension onto a silane-coated slide glass. Be sure never to spot all of the

suspension at once. Spot gradually in 1 to 2 μL portions and air-dry between each portion. The addition of too much suspension at once will disperse the specimen over too wide an area on the slide glass, resulting in a very sparse cell density.

4. Dehydrate the dried slide in 50, 70, and 100% ethanol for 3 min at each step. Dry the slide and spot 300 μL of 0.25 *N* HCl solution onto the slide. Keep the slide at room temperature for 30 min for deproteinization. Rinse the specimen with water, and dehydrate again with 70 and 100% ethanol for 3 min in each step.
5. The fixed sample can be stored at two points during the entire procedure: after **step 1** (store at -20°C) and after **step 4** (store in a dry chamber). Storage for up to several months is acceptable in both cases.

3.1.2. Hybridization

Although the actual reason remains unclear, a high hybridization temperature may lead to poor results in *in situ* hybridization targeting mRNA. In our experience, 37°C has usually proven to be the most suitable temperature for detecting mRNA. We strongly recommend using this hybridization temperature in your first trial, even if your probe has a relatively high melting temperature (T_m) value. Here, we used three different oligonucleotides that were 5'-labeled with FITC. Probe A-5[540] (5'-GTTCGTAGATCGATCGAAG-3'; **ref. 6**) is complementary to a region of the specific sequence of the elongation factor-1 α of *Dinenympha exilis*, a symbiotic protist in the hindgut of the lower termite *R. speratus*. A-14[540] (5'-GTTAGGCGAACGCTCCAT-3'; **ref. 6**) is complementary to a region of the specific sequence of the elongation factor-1 α of *Pyrsonympha grandis*, another symbiont in *R. speratus*. To evaluate permeability, we also used EF-UNIV (5'-CCXGTDATCATRTTYTT-3'), a probe that can hybridize to known eukaryotic elongation factor-1 α sequences. This type of control experiment is necessary for all *in situ* hybridizations. We also recommend that a control experiment be performed with an antisense probe against all target sequences, as a negative control. This helps identify the false-positive signals that sometimes appear because of the very low hybridization temperature (see **Note 1**).

1. For prehybridization, spot 80 μL of hybridization solution 1 onto the specimen in a CoverWell Incubation Chamber. Put the slide in the air incubator and incubate for 30 min at 42°C .
2. After prehybridization, apply 80 μL of hybridization solution 1 with 100 pmol/mL of FITC-labeled probe oligonucleotide into the CoverWell Incubation Chamber, and incubate overnight at 37°C (see **Note 1**).
3. To remove the unbound probes, wash the specimen twice with washing buffer 1 for 15 min at 42°C .

3.1.3. Immunological Detection and Enzymatic Amplification

The signal amplification is the most important factor in detecting the mRNA signal by *in situ* hybridization. Our group uses an alkaline phosphatase-conjugated

antibody to FITC and an FITC-labeled target gene-specific oligonucleotide for this step. If this step is skipped, the signal will usually be absent. The suitable duration of the enzymatic signal amplification depends on the copy number of the target gene. If the target gene is a rarely expressed gene, the incubation time should extend overnight.

1. Wash the specimen with PBS-T and block with PBS-T containing 3% sheep serum with 5 mg/mL heparin for 30 min at room temperature.
2. Dilute the antibody with the same solution, apply it to the specimen in the CoverWell Incubation Chamber, and incubate for 1 h at 37°C. Be sure to keep the sample shaded from light from this step onward (until the final microscopic observation). FITC decays easily under light.
3. Soak and gently agitate the slide in PBS-T, three times for 5 min.
4. Apply VECTOR RED substrate solution onto the slide, and incubate at room temperature until a pink color appears (see **Note 2**). Do not agitate the sample. Agitating the sample will diffuse and weaken the signal (see **Note 3**).
5. Detect the signal by washing the specimen with water, mounting it in antifading solution, and observing it by light microscopy.

3.2. In Situ Hybridization of the rRNA of Symbiotic Bacteria in Termite Gut

Because of the relatively small number of microorganisms so far isolated from termites, identification methods that depend on the culturing of microorganisms may provide limited information on the microbial diversity. Moreover, culture-independent analyses based on comparisons of PCR-amplified 16S rRNA genes have revealed that most members of the symbiotic community in the termite gut have yet to be cultivated (8–11).

Spirochetes are one of the most abundant and morphologically distinct groups of bacteria that are commonly found in the termite gut (12,13). Diverse spirochetes have been discovered in termite species (14,15), but only a few strains have been isolated from the termite gut (16). The spirochetes of the termite gut either exist freely in the gut fluid or attach to the cell surfaces of the gut protists as ectosymbionts (17,18). In this section, we introduce the method for *in situ* hybridization with the rRNA-targeted probes that detected the spirochetes in the termite gut.

3.2.1. Preparation of Fixed Cells

At the outset, the specimen must be fixed to preserve the morphology. Crosslinking fixatives, such as formalin or paraformaldehyde, are usually used for bacteria in natural environments. The duration of fixation must be carefully set, excessive fixation will remarkably decrease the permeability of the probe, whereas insufficient fixation will lead to poor morphology after the hybridization.

The cells must be pretreated to ensure that the probes penetrate the cells. Incubation in hydrochloric acid increases the signal intensity. The precise action of the acid is not known, but extraction of proteins and partial hydrolysis of the target sequences may help improve the signal-to-noise ratio. The method used for pretreatment depends on the type of specimen and fixation methods. It is important to optimize both the fixation and pretreatment steps.

1. Remove the entire termite gut by forceps, place it in a 1.5-mL tube, and add 300 μ L of Solution U.
2. Crush the gut with forceps, gently suspend, add another 500 μ L of Solution U, centrifuge at 23g for 15 s, and decant the supernatant.
3. Add 1000 μ L of 4% paraformaldehyde in PBS (–), suspend, and incubate on ice for 4 h.
4. Centrifuge at 23g for 15 s, discard the supernatant, and add 1 mL of Solution U. Repeat this step once more.
5. Suspend the fixed cells in 200 μ L of Solution U, spot the sample onto a silane-coated slide glass, and air-dry.
6. Spot 300 μ L of 0.25 N HCl solution on the specimen, and incubate for 30 min.
7. Wash the specimen with PBS (–), dry with air, and dehydrate the samples with 50, 80, and 100% ethanol.

3.2.2. Hybridization

The most important points in this process are the design of the probe and the hybridization temperature. Both will influence the specificity in the detection of target cells. The probes used in this section were EUBAC (5'-GCTGCCTCC CGTAGGAGT-3') and TT1-732 (5'-CTCAGCGTCAGTCATTGGCT-3'). EUBAC labeled at the 5'-end with Texas Red binds to most eubacterial cells, and is used as a control for the permeability of the cells. The target sequences of TT1-732 are conserved for the spirochetes in the termite *Treponema* cluster I (15) and labeled at the 5'-end with 6-FAM. The optimal hybridization temperature for a specific probe depends on the length of the probe and the G plus C content. Sum up 4°C for each G or C and 2°C for each A or T (EUBAC: $12(G + C) \times 4^\circ\text{C} + 6(A + T) \times 2^\circ\text{C} = 60^\circ\text{C}$). Hybridize at 2 to 10°C below the obtained T_m .

1. Apply hybridization buffer 2 with fluorescently labeled probe (50 pmol/100 μ L), sealed in a CoverWell Incubation Chamber, and incubate at 54°C for 1 h (see Note 4).
2. Wash the specimen for 15 min in washing buffer 2 at 54°C (see Note 5).
3. Mount with antifading solution.

3.2.3. Signal Detection

The maximum absorption and emission of Texas Red are near 596 and 490 nm, respectively. Those of FAM are near 620 nm and 520 nm, respectively.

Before starting the observation, an optimized fluorescence cube should be selected for each microscope (see **Note 6**).

1. Place a prepared slide on the microscope stage, turn on the transmitted light, and focus on the specimen image, using the coarse- and fine-adjustment knobs.
2. Using epifluorescence, scan the specimen for red fluorescence derived from Texas Red. Next, check the permeability of the cells by comparing the fluorescence signal and the image of the phase contrast.
3. Change the filter set for FAM and scan the specimen.

4. Notes

1. If a false-positive signal appears, increase the hybridization temperature by 2 to 5°C. Make sure not to forget the positive controls, such as the EF-UNIV probe. If the EF-UNIV signal resembles that of control, the condition is too stringent, and the hybridization temperature can be increased.
2. Nitro blue tetrazolium (NBT)/5-Bromo-4-chloro-3-indolyl phosphate (BCIP) can also be used. In this case, add 20 μ L of NBT/BCIP stock solution (18.75 ng/mL NBT chloride, 9.4 mg/mL BCIP, toluidine salt in 67% DMSO [v/v]) into 1 mL of 0.1 M Tris-HCl, pH 9.5, and 0.1 M NaCl. Next, apply the diluted NBT/BCIP solution to the sample by the procedure described in **Subheading 3.1.3., step 4**. When staining with NBT/BCIP, a dark blue signal will appear. When a weak signal is obtained with the use of either VECTOR RED or NBT/BCIP, add freshly prepared substrate solution and incubate for one more night. If no improvement is observed, decrease the hybridization temperature.
3. The appearance of a brownish signal with the use of VECTOR RED or NBT/BCIP is cause for doubt. If the entire signal is brownish, perform a negative control experiment without probe or with antisense oligonucleotides.
4. The hybridization and washing steps should be performed under dark conditions. The fluorescently labeled probe is weak under light and should be handled with care.
5. Posthybridization washes at increased stringencies ensure dissociation of mismatched hybrids and nonspecifically bound probe. Various wash stringencies are achieved by changing the salt concentration or wash temperature. These stringencies should be determined empirically for each probe.
6. A strong autofluorescence derived from the wood particles or cellulose in the termite gut has been observed. If you use a wide-band excitation filter, you can easily distinguish the signal from the FAM (green) and the autofluorescence from the wood particle (yellow).

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III

FLUORESCENT LABELING AND DETECTION

RNA Electrophoretic Mobility Shift Assay Using a Fluorescent DNA Sequencer

Yukinori Eguchi

Summary

The RNA electrophoretic mobility shift assay is a simple and rapid method for visualizing the existence of specific RNA–protein interaction. We have developed a useful method for the detection of mRNA-binding proteins using fluorescence-labeled synthetic RNA. In this method, RNA was prepared by *in vitro* transcription using Texas Red-labeled nucleotides. This method has higher resolution than the conventional method using slab gels, is safer, and provides results rapidly because of the use of fluorescence detection.

Key Words: Fluorescence-labeled oligonucleotide; fluorescent DNA sequencer; *in vitro* transcription; poly(A) tail linker; RNA electrophoretic mobility shift assay; RT-PCR.

1. Introduction

DNA-binding proteins play essential roles in the regulation of gene expression, replication, recombination, and in diverse processes, such as specific modification and cleavage of DNA. Recently, not only DNA-binding proteins but also RNA-binding proteins have been shown to have roles in the regulation of gene expression, for example, in posttranscriptional control (1–4). The RNA electrophoretic mobility shift assay has been used to search for RNA-binding proteins (5–10). The conventional method used a radiolabeled synthetic RNA. The fluorescent method is more advantageous than the conventional method because it is a simple, rapid, and safe technique using nonradiolabeled synthetic RNA.

For example, in a study on the stability of human α -globin messenger RNA (mRNA), the stability of mRNA *in vivo* was determined by the ability of its 3′-untranslated region (UTR) to assemble a messenger ribonucleoprotein. The poly(A) tail length is important for mRNA stability because the affinity of the

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messenger ribonucleoprotein is related to the number of poly(A)-binding proteins bound to the poly(A) tail (1,2). These binding sites in α -globin 3'-UTR are universally conserved across diverse species.

2. Materials

1. Moloney murine leukemia virus reverse transcriptase (RT; Gibco-BRL).
2. T7 RNA polymerase (from *Escherichia coli*).
3. T4 RNA ligase.
4. *Taq* DNA polymerase.
5. DNase I (RNase-free).
6. Shrimp alkaline phosphatase (Amersham Biosciences).
7. Oligonucleotide primer and linker: poly(A) tail linker (p-CCCGGATCC GGG-OH), 3'-terminal-specific primer (5'-CCCGGTACCGGGTTTTTTTTTTTTT-3'), chicken α^A -globin exon 3 primer (5'-TTCCCTGGACAAGTTCTTGT-3'), chicken 3'-terminal-specific primer (5'-CCCGGTACCGGGTTTTTTTTTTTTT-3'), universal primers (reverse, 5'-TGTGGAATTGTGAGCGG-3'; and forward, 5'-TGTAACACGACGGCAGT-3' primer for M13), and Texas Red-labeled M13 forward primer (5'-TexasRed-TGTAACACGACGGCCAGT-3').
8. RNA extraction solution: 4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 1% β -mercaptoethanol.
9. 2 M Sodium acetate, pH 4.0.
10. Water-saturated phenol.
11. chloroform/isoamyl alcohol mixture (49:1).
12. Isopropanol.
13. 75% Ethanol.
14. RNase-free deionized water.
15. Shrimp alkaline phosphatase (Roche Applied Science).
16. T4 RNase ligase or DNA ligation kit (Takara Bio, Japan).
17. RT stock 5X buffer: 250 mM Tris-HCl buffer, pH 7.5, 375 mM KCl, 15 mM $MgCl_2$, and 50 mM DTT.
18. dNTP stock solution: 2.5 mM ddATP, ddCTP, ddGTP, and ddTTP.
19. NTP stock solution: 2.5 mM ATP, CTP, GTP, and UTP.
20. PCR stock 10X stock buffer: 100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$.
21. Mineral oil.
22. Transcription 5X stock buffer: 200 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 10 mM spermidine, and 25 mM DTT.
23. Extraction 5X stock buffer: 50 mM Tris-HCl buffer, pH 7.4, 7.5 mM $MgCl_2$, 50 mM KCl, and 2.5 mM DTT.
24. RNA-protein-binding 5X stock buffer: 50 mM Tris-HCl buffer, pH 7.4, 750 mM KCl, 1.5 mM $MgCl_2$, 100 mM DTT, 50 mM PMSE, and 5 mg/mL aprotinin.
25. Adjusting buffer: 1.5 M KCl, 200 mM DTT, 100 mM PMSE, and 10 mg/mL aprotinin.
26. Electrophoresis buffer: 44.5 mM Tris-HCl buffer, pH 8.4, 44.5 mM boric acid, and 1 mM EDTA (0.5X TBE buffer).

27. pT7Blue T-vector (Novagen).
28. *E. coli* strain DH5 α .
29. Kits: DNA Ligation kit II (Takara), QIAquick Plasmid purification kit, and QIAquick PCR purification kit (QIAGEN).
30. Nucleo-spin cartridge (Clontech).
31. Oligotex-dT30 (Takara).
32. Glass-Teflon homogenizer and Dounce homogenizer.
33. Agarose gel electrophoresis equipment and NuSieve GTG agarose (FMC BioProduct).
34. Thermal cycler, model PC-700, Astec.
35. Fluorescent DNA sequencer equipment (model SQ-5500, Hitachi; see **Note 1**).
36. 25 mg/mL Ampicillin stock solution, sterile.
37. LB media: 1% bacto-tryptone, 0.5% yeast extract, and 1% NaCl.
38. Phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄/KH₂PO₄, pH 7.2.
39. Acrylamide solution: 5% acrylamide, 0.83% *N,N*-methylene-bis acrylamide (ratio is 60:1) in electrophoresis buffer.
40. *NNN'*-tetramethylethylenediamine.
41. 10% Ammonium peroxodisulfate.

3. Methods

The methods described outline (see [Fig. 1](#)):

1. RNA extraction.
2. RT reaction for poly(A) tail-containing mRNA.
3. Amplification of target complementary DNA (cDNA).
4. Cloning of target cDNA.
5. Synthesis of target RNA from cDNA clone.
6. Fluorescence labeling of target RNA.
7. Nuclear protein extraction.
8. RNA electrophoretic mobility shift assay.

The RNA for the mobility shift assay can be synthesized by conventional methods. If you need poly(A) tail-containing mRNA, it can be synthesized by the procedure described in **Subheading 3.5. (11)**. The target RNA was produced by in vitro transcription using T7 RNA polymerase (**12**).

3.1. RNA Extraction

Total RNA was isolated by a one-step method from bone marrow cells (**Note 2**; **ref. 13**).

1. Immediately after removal from the animal, the tissue was minced on ice and homogenized with 1 mL of RNA extraction solution in a glass-Teflon homogenizer and subsequently transferred to a 15-mL polypropylene centrifuge tube.
2. Sequentially, 0.1 mL of 2 *M* sodium acetate, 1 mL of water-saturated phenol, and 0.2 mL of chloroform/isoamyl alcohol mixture (49:1) were added to the

homogenate. After addition of each reagent, the mixture was thoroughly mixed by inversion.

3. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min.
4. Samples were centrifuged at 10,000*g* for 20 min at 4°C.
5. The aqueous phase was transferred to a fresh tube, mixed with 1 mL of isopropanol, and then placed at –20°C for at least 1 h to precipitate the RNA (see **Note 3**).
6. Centrifugation at 10,000*g* for 20 min was again performed, and the resulting RNA pellet was dissolved in 300 µL of RNA extraction solution, transferred into a 1.5-mL microcentrifuge tube, and precipitated with 1 volume of isopropanol at –20°C for 1 h.
7. After centrifugation for 10 min at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried for 5 min, and dissolved in 50 µL of RNase-free water at 65°C for 10 min.

3.2. RT Reaction of Poly(A) Tail-containing Region

3.2.1. Ligation of Linker to mRNA Poly(A) Tail

The first step was ligation of the anchor linker to the poly(A) tail of mRNA.

1. The extracted total RNA (0.1–0.5 µg) was dephosphorylated at the 5′-terminal using 10 U of shrimp alkaline phosphatase at 37°C for 60 min to allow linker attachment. Reaction conditions were as specified by vendor.
2. The mixture was incubated at 85°C for 15 min for inactivation of the shrimp alkaline phosphatase.
3. The dephosphorylated RNA was ligated with a poly(A) tail linker using T4 RNA ligase at 16°C for 2 h or a DNA ligation kit II at 16°C for 60 min. Reaction conditions were as specified by vendor.

3.2.2. RT Reaction

The second step was a conventional RT reaction using a 3′-terminal-specific primer.

1. Total RNA (0.1 µg) connected to linker was supplemented with 50 pmol of 3′-terminal-specific primer, 10 µL RT 5X stock buffer, 12.8 µL dNTP stock solution, and 1000 U of Moloney murine leukemia virus RT. The final reaction volume was 50 µL.
2. The mixture was incubated at 37°C for 90 min.

3.3. Amplification of Target cDNA

The next step was a conventional PCR using a 3′-terminal-specific primer with an mRNA-specific primer (14). The estimated poly(A) tail length was calculated by deducting the sizes of the primer and the 3′-UTR region from that of the PCR product (see **Fig. 2**).

1. The PCR mixture contained 0.1 volumes of the RT reaction solution, 8 µL dNTP stock, 50 pmol of each primer (target gene-specific primer; chicken α^A -globin

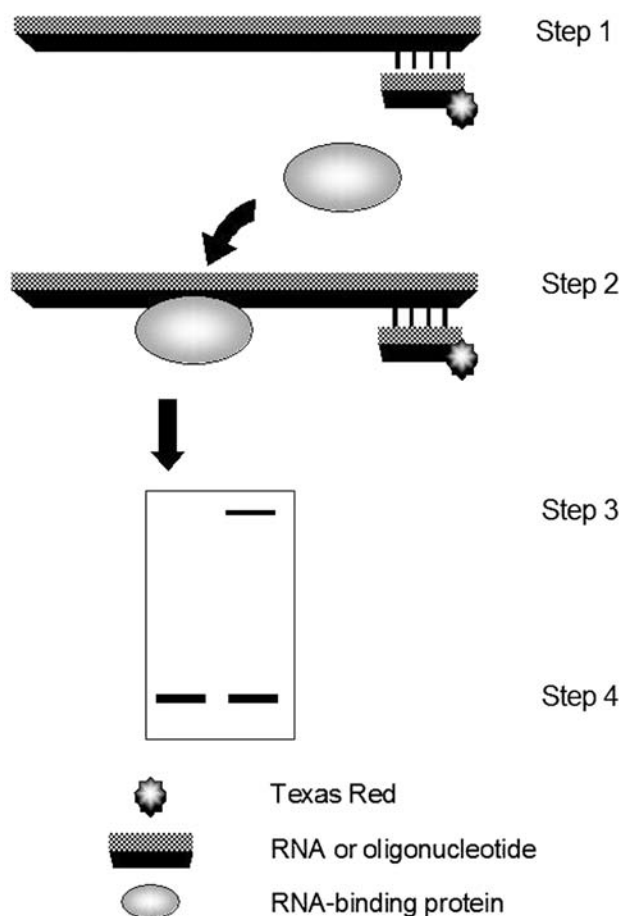


Fig. 1. Schematic diagram of fluorescence-labeled mRNA-binding assay. Step 1: the synthetic mRNA 3'-UTR region is annealed with Texas Red-labeled nucleotides for 85°C at 5 min. Step 2: 0.3 µg of nuclear extract protein fraction is added. Step 3: incubation is performed for 1 h at room temperature. Step 4: electrophoresis is performed in an automatic DNA sequencer.

- exon 3, and 3'-terminal-specific primer), 10 µL PCR reaction stock buffer, and 2 U of *Taq* DNA polymerase in a total volume of 100 µL.
2. All specimens were overlaid with approx 30 µL of mineral oil (not required if the thermal cycler has a heated lid).
 3. Amplification was performed using a programmable temperature-control system (PC-700) for 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min.
 4. The PCR products were analyzed by electrophoresis in 2.5% NuSieve GTG agarose gels.

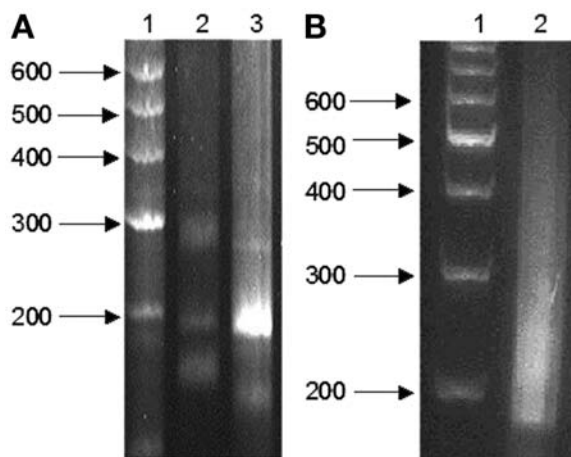


Fig. 2. Electrophoresis to determine the poly(A) tail length of the PCR product. The PCR products were analyzed by agarose gel electrophoresis. **(A)** Electrophoresis of amplified poly(A) tail fragments of human α -globin mRNA. Lane 1 contains the 100-bp size markers; lane 2, standard bands of 0, 20, and 120 nucleotide poly(A) tail products from chicken α^A -globin mRNA; and lane 3, poly(A) tail product of human α -globin from peripheral blood cells. **(B)** Electrophoresis to determine the sized of amplified poly(A) tail fragments of chicken α^A -globin mRNA. Lane 1, 100-bp size marker; and lane 2, poly(A) tail product of chicken α^A -globin from chicken erythrocyte mRNA.

3.4. Cloning of a Target cDNA

The target cDNA contained poly(A) tails with some differences of length. A single clone of target cDNA was selected by the conventional cloning technique. The selected single clone was useful for reproducing the same results in separate experiments (15).

1. The fragment of cDNA containing the poly(A) tail region was ligated into a pT7Blue T-vector using a DNA ligation kit II, and the DNA was transformed into *E. coli* DH5 α cells, as recommended by the vendor.
2. The *E. coli* DH5 α cells were then plated on LB plates containing 100 μ g/mL ampicillin, and incubated overnight at 37°C.
3. Single colonies were selected and incubated overnight in LB with ampicillin.
4. The plasmid DNA was extracted by a conventional method (15) or by using a plasmid purification kit.
5. The purified plasmid DNA was checked for the presence of the insert, which was amplified by PCR using the universal primer.
6. The PCR mixtures contained 50 pg of plasmid, 50 pmol of universal primers (reverse and forward for M13), 12.5 μ L dNTP stock solution, 10 μ L PCR stock buffer, and 2 U of *Taq* DNA polymerase in 100 μ L.
7. The PCR conditions consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min.

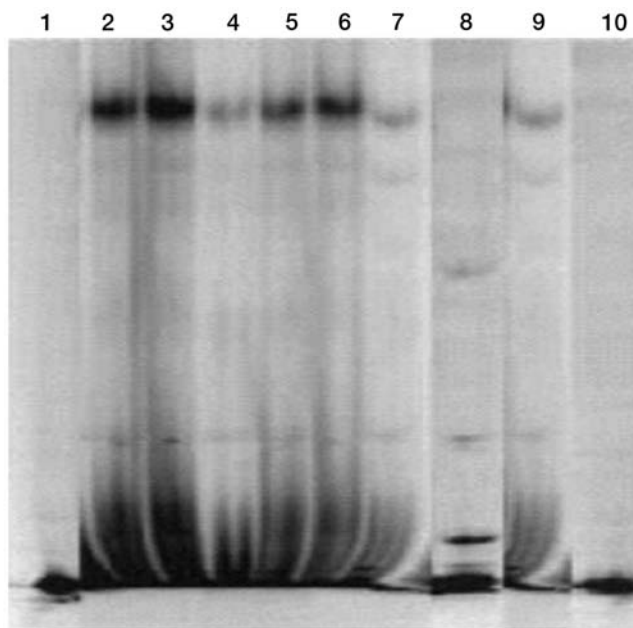


Fig. 3. Electrophoretic mobility shift assay using extracts from erythrocyte nuclei and a RNA fragment from the chicken α -globin 3'-UTR as a probe. Binding reaction, electrophoresis, and detection were performed as described in **Subheading 3.8**. RNA (50 fmol) was used for each reaction. Lane 1, synthetic target RNA labeled only with Texas Red. Lanes 2 and 3, binding reaction using different amounts of protein nuclear extracts (lane 2, 1 μ g; and lane 3, 3 μ g). Lane 4, binding reaction using 3 μ g of protein nuclear extract in the presence of 100-fold molar excess of specific nonlabeled competitor. Lanes 5 and 6, binding reactions using different amounts of nuclear extracts in the presence of 100-fold molar excess of nonspecific competitor (lane 5, 1 μ g; and lane 6, 3 μ g of protein nuclear extract). Lane 7, 100-fold molar excess of poly(C), which is a specific nonlabeled competitor of the α -complex. Lanes 8 and 9, reactions supplemented with proteinase K and RNase, respectively, after 30 min of reaction with 3 μ g of protein nuclear extract. Lane 12, excess of 0.5 pmol of probe with RNase without nuclear extract protein.

8. The amplified DNA fragments were analyzed by agarose electrophoresis in 2.5% Nusieve GTG agarose.

3.5. Synthesis of Target RNA Containing a Poly(A) Tail

The RNA for the mobility shift assay was synthesized by in vitro transcription using T7 RNA polymerase. The in vitro transcription was performed in a conventional manner (16):

1. The amplified target product that was checked by the agarose gel electrophoresis was purified using Nucleo-Spin cartridges.

2. In vitro transcription was carried out with 1 µg of the amplified fragment as the template, 12.5 µL of NTP stock solution, 20 µL of transcription 5X stock buffer, and 500 U of T7 RNA polymerase in 100 µL.
3. The synthetic target RNA was purified using 5 U of RNase-free DNase I at 37°C for 15 min, or purified using Oligtex dT30 (see **Note 4**).

3.6. Fluorescence Labeling of Target RNA

1. Approximately 20 pmol of transcript synthetic RNA was incubated at 85°C for 5 min, with 25 pmol of Texas Red-labeled M13 forward primer, which annealed to a universal forward sequence site located at the 3'-terminus of the transcripts (see **Note 5**).

3.7. Nuclear Protein Extraction

Cytoplasmic extracts were prepared from chicken bone marrow cells by a modification of Dignam's method (17,18). The entire procedure was performed at 4°C:

1. The cells were collected in the 15-mL centrifuge tube by centrifugation (1000g) for 10 min at room temperature. The cells were washed with 10 volumes of phosphate-buffered saline. The cells were resuspended at a concentration of 6×10^7 cells/mL in homogenization buffer.
2. The harvested cells were lysed with 20 strokes in a Dounce homogenizer.
3. The homogenate was adjusted to the RNA-protein-binding buffer conditions by adding 0.2 volumes of adjusting buffer and centrifuged for 10 min at 2000g to pellet the nuclei.
4. The supernatant from this step was harvested and centrifuged at 100,000g for 60 min at 4°C.
5. The protein concentration of this high-speed S-100 supernatant was determined by a assay based on Bradford's method (17,18).
6. The final protein concentration of the S-100 from this procedure was adjusted to approx 3 mg/mL.
7. The extracted nuclear protein was stored at -80°C until use in the assay (see **Note 6**).

3.8. RNA Electrophoretic Mobility Shift Assay Using a Fluorescent DNA Sequencer

The mRNA that annealed to the Texas Red-labeled M13 oligomer was allowed to cool to room temperature. The RNA-protein-binding reaction was performed at room temperature.

1. To determine the binding site for the S-100 extract in the respective 3'-UTR, the oligonucleotides complementary to the 3'-UTR were added in 100:1 ratio to the target RNA, and incubated for 30 min at room temperature before the addition of the S-100 extract.
2. Labeled RNA (50 fmol) was incubated with 1 to 3 µg of S-100 extract for 1 h in 5 µL of RNA-protein-binding buffer.

3. After the binding reaction, 2 to 3 μL of the reaction mixture was applied to the gel and electrophoresed through a 5% nondenaturing polyacrylamide gel (27 cm height $\times$ 20 cm width) in electrophoresis buffer, and the fluorescence-labeled RNA was detected 17 cm from the top of the gel (see **Note 7**).
4. Electrophoresis was performed at a voltage of 1 KV for 1.5 h, and the results were analyzed using the appropriate software packages (see **Note 8**).

4. Notes

1. We used fluorescence detection and a slab-type DNA sequencer (SQ-5500, Hitachi Co., Tokyo, Japan). This method makes it possible to use the same type of DNA sequencer used for fluorescence detection in DNA/RNA electrophoretic mobility shift assays. Other DNA sequencer systems can also be used for the detection.
2. Recently, we obtained a satisfactory yield of extracted RNA using a commercially available RNA extraction kit (RNeasy Total RNA system, QIAGEN). We recommend using RNA extraction kits for high yield and good quality of extracted total RNA.
3. After centrifugation, RNA was present in the aqueous phase, whereas DNA and proteins were present in the interface and phenol phase.
4. Purification of the synthetic target RNA using oligo dT30 gave more satisfactory results than the digestion of template DNA by DNase I. The purification using oligo dT30 has the disadvantages of requiring several steps of manipulation and a synthetic target RNA with a poly(A) tail region. However, oligo dT30 purification has the advantage of yielding synthetic target RNA for which it is possible to adjust the concentration of the RNA solution, and for which the buffer can be changed to a different kind of buffer.
5. This method had sufficient sensitivity. If you need better sensitivity in the RNA-binding assay, you can use two kinds of fluorescence-labeled primers, which are attached to the synthetic target RNA. Usually, the synthetic target RNA is produced from a cDNA clone amplified with universal M13 primers that are linked with double Texas Red-labeled forward and reverse primers.
6. The stored protein solution is stable for approx 1 yr.
7. Polyacrylamide gel was polymerized by adding 5 μL of TEMED and 50 μL of 10% ammonium peroxodisulfate in 20 mL of 5% acrylamide solution.
8. We extracted the fluorogram image from the raw data files. The SQ-5500 software was used to display and extract the requisite area from the fluorogram image. The extracted file was saved in the PICT file format.

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Comparative Quantitation of mRNA Expression in the Central Nervous System Using Fluorescence *In Situ* Hybridization

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Summary

In situ hybridization to messenger RNA (mRNA) in complex tissues, such as the brain, allows the localization of gene expression to functionally distinct regions. It has been difficult to measure relative changes in gene expression within these regions because of the poor cellular resolution afforded by radioactively labeled probes and problems associated with densitometric analysis by counting silver grain deposition. Fluorescence *in situ* hybridization, using probes directly labeled with dyes that exhibit high quantum yield, provides both high-resolution localization of mRNA and high sensitivity for detection of hybridized probe. Digital image capture of fluorescence is readily amenable to densitometric analysis, thereby allowing relative quantification of mRNA expression in single cells or discrete brain nuclei. In this chapter, we describe protocols suitable for measuring relative changes in gene expression within individual cells of brain sections mounted on glass slides.

Key Words: Central nervous system; fluorescence *in situ* hybridization; gene expression; mRNA FISH; opioid receptors; relative quantitation; riboprobes.

1. Introduction

Estimation of messenger RNA (mRNA) expression levels of genes can be readily achieved using techniques such as Northern blotting or quantitative real-time PCR. These techniques, however, require dissection and homogenization of tissue and are not suited for single-cell analysis of gene expression in topologically complex tissues such as the brain. Fluorescence *in situ* hybridization (FISH) offers the distinct advantage of allowing gene expression to be related to tissue topology and has sufficient resolution to allow analysis of mRNA expression in individual cells. This is particularly important in tissues, such as

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the brain, in which cells are organized into specialized fiber tracts and nuclei, such that the topological location of cells correlates with their function and their characteristic pattern of gene expression.

Nonradioactive *in situ* hybridization protocols are nonquantitative because enzymatic or immunological amplification steps that are used for probe visualization typically show a poor correlation (nonlinearity) between signal intensity and the abundance of mRNA. This is in contrast to *in situ* hybridization with radiolabeled probes (RISH), in which signal intensity is usually accepted to be proportional to the amount of mRNA present in the sample. In this chapter, we describe the use of directly labeled fluorescent riboprobes to detect mRNA for a comparative quantitation of gene expression of neurotransmitter receptors in the rat brain. As with RISH, use of directly labeled fluorescent riboprobes allows visualization of hybridized probe without signal amplification, thus, ensuring that the detected fluorescence is proportional to the mRNA content. This relationship holds true if the tissue sections are the same thickness and are processed and photographed under identical conditions.

2. Materials

1. Designed oligonucleotide primers.
2. DEPC.
3. Tris-HCl-EDTA (TE) buffer: 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA.
4. 200 μ M dNTPs: an equimolar mixture of dATP, dCTP, dGTP, and dTTP.
5. Reagents for Deep Vent exo^- DNA polymerase reaction: 2 mM MgSO_4 , 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.1% Triton X-100.
6. Deep Vent exo^- DNA polymerase (New England Biolabs, Beverly, MA).
7. Submarine agarose gel equipment, water baths, and thermal cycler.
8. Transcription buffer: 40 mM Tris-HCl buffer, pH 8.0, containing: 8 mM MgCl_2 , 2 mM spermidine- $(\text{HCl})_3$, 25 mM NaCl, 0.5 mM each of CTP, GTP, and TTP, 0.4 mM UTP; 0.1 mM of either fluorescein-UTP, Cy3-UTP, or Cy5-UTP, and 5 mM DTT.
9. Tris-acetate-EDTA (TAE) buffer: 40 mM Tris-acetate, pH 8.0, containing 2 mM EDTA.
10. 3 M Sodium acetate, pH 5.2.
11. Carrier solution: 1 mg/mL linear acrylamide.
12. 2 mg/mL Ethidium bromide.
13. Restriction enzymes, RNase inhibitor, *Taq* polymerase, and T7 RNA polymerase.
14. Fluorescein-labeled UTP, Cy3-labeled UTP, and Cy5-labeled UTP (Amersham Biosciences).
15. Standard sodium citrate (SSC): 15 mM sodium citrate, pH 7.0, and 150 mM NaCl (prepared from a 20X concentrated stock).
16. Hybridization buffer: 4X SSC buffer containing 50% formamide (v/v); 40 μ g/mL sheared, denatured salmon sperm DNA; and 10 mM DTT.
17. DAPI.

18. Antifade solution (Vectorshield, Burlingame, CA).
19. *In situ* frames (Thermo Hybaid, Middlesex, UK).
20. Cryostat.
21. Superfrost Plus slides and cover slips.
22. OCT compound (Miles Scientific, Naperville, IL).
23. 1 M NaOH.
24. Phosphate-buffered saline (PBS) buffer: 150 mM phosphate and 150 mM NaCl, pH 7.4 (prepared from a 10X stock).
25. Paraformaldehyde.
26. Formamide.
27. 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride.
28. 70, 80, 95, and 100% ethanol solutions.
29. Coplin jars.
30. Photomicroscope with digital camera.
31. Computer and software for image analysis.

3. Methods

The methods described detail the design considerations for a FISH experiment, the production of RNA probes (riboprobes) complementary to (antisense) and identical to (sense) the target mRNA, the preparation of brain tissue for *in situ* hybridization, the hybridization and stringent washes of tissue sections on glass slides, and image analysis.

3.1. Design Considerations for FISH Experiments

Correctly designing an experiment and ensuring adequate controls are of the utmost importance if meaningful results regarding the location and relative abundance of transcripts are to be obtained. **Subheadings 3.1.1.** and **3.1.2.** describe considerations for choosing the region of mRNA to target, determining the probe length and choice of fluorophores, and ensuring that suitable controls are included to guarantee specificity for the target.

3.1.1. Riboprobe Design

FISH experiments designed to localize and compare the expression of receptors involved in signal transduction pathways need to ensure that the probes are specific for the target mRNAs and will not cross-hybridize to mRNA from other related receptors. In our laboratory, we designed and produced riboprobes for analyzing opioid receptor mRNAs in the rat and mouse brain (**1**). The opioid receptors belong to the family of G protein-coupled receptors that are highly conserved and share significant sequence identity in their transmembrane helical domains (**2,3**). Alignment of the mRNA sequences for the μ -opioid receptor (MOR), δ -opioid receptor, and κ -opioid receptor shows that the greatest sequence divergence exists in the regions encoding the carboxy and amino terminals,

thereby making these regions of the mRNA the most suitable for designing riboprobes that will discriminate between these three highly conserved opioid receptors (4–6). The use of probes prepared by the limited hydrolysis of full-length complementary RNA generates some fragments capable of hybridizing to all opioid receptor types (7). Although this approach enhances sensitivity (detection of signal) by increasing the size of the targeted area, it reduces the specificity of the probe by increasing the extent of cross-hybridization with related targets. We have found that short riboprobes of 200 to 300 nucleotides (nt) give sufficient sensitivity and specificity for localizing opioid receptor mRNAs in the brain. Similar considerations apply to detecting other G protein-coupled receptors and neurotransmitter transporter mRNAs. Even shorter, directly labeled riboprobes can be used; for example, we have used riboprobes as short as 53 nt for localizing expression of MOR exons involved in alternative mRNA splicing; however, the reduced sensitivity makes these short probes less suitable for detecting low-abundance transcripts (1).

The choice of fluorophore for labeling FISH riboprobes for use in brain tissue is not constrained by high background autofluorescence caused by endogenous pigments, as is the case for some tissues. Fluorescein-, Cy3-, and Cy5-modified UTPs are commercially available and have good spectral separation and high quantum yields, making them ideal for FISH experiments designed for multiple-probe labeling. The dye-modified nucleotides are readily incorporated into the riboprobe by RNA polymerases, allowing probes of high specific content to be prepared. The use of fluorescein-labeled probes may be advantageous for some applications, because antibodies to fluorescein conjugated to a variety of reporter enzymes are commercially available, should enzymatic amplification of signal be required.

3.1.2. Hybridization Specificity Controls

To be able to adequately assign visualized signal as originating from riboprobe hybridized to cognate mRNA, a number of essential controls are required (8).

1. Hybridization performed on adjacent sections using identical conditions with either an equal amount of sense-labeled riboprobe (same sequence as the mRNA strand) or the antisense-labeled riboprobe (complementary to the mRNA) should give minimal signal for the sense probe compared with the antisense probe. Nonspecific interactions between labeled probes and tissue can result in signal if too low a hybridization temperature or insufficiently stringent washes are performed, or if the tissue has not been adequately blocked. The use of a sense-labeled riboprobe control hybridization is of the utmost importance (Fig. 1), and studies that omit this control should be interpreted with caution.
2. Co-localization of the expressed protein, detected by immunohistochemistry, in the same cell as the mRNA detected by FISH strongly supports the conclusion that

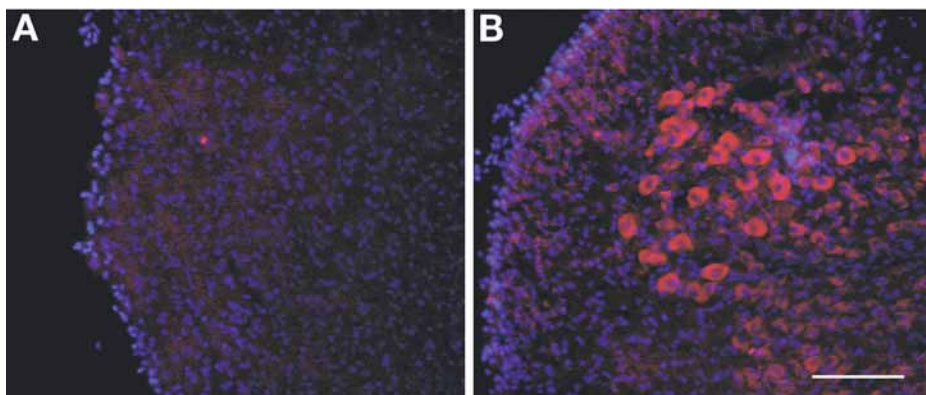


Fig. 1. Hybridization with sense and antisense complementary RNA (cRNA) probes. mRNA FISH staining in the spinal cord using sense and antisense MOR exon 1 CY3-labeled cRNA probes (red). **(A)** Staining with the sense probe and **(B)** with the anti-sense probe. Equivalent amounts of each probe were used, and sections were processed and photographed under identical conditions. Cell nuclei are counterstained with DAPI (blue). Bar = 100 μ m.

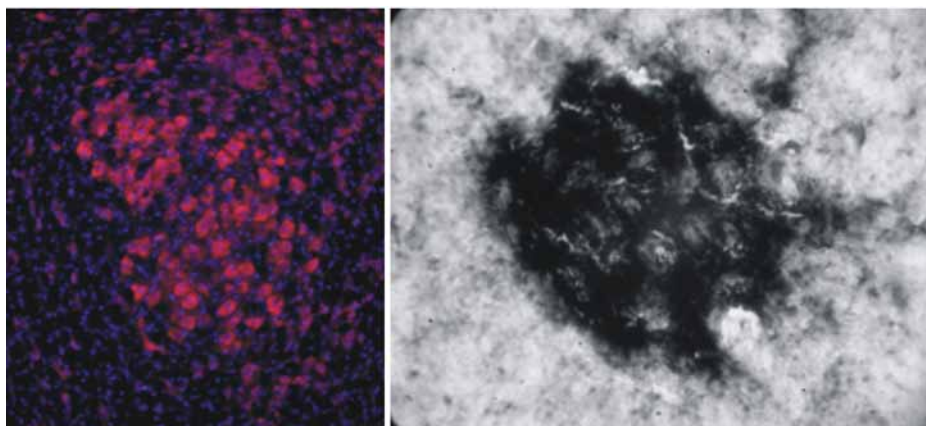


Fig. 2. Co-localization of MOR mRNA and protein in the nucleus ambiguus. The nucleus ambiguus is a brain region that has been well-documented as expressing MOR mRNA and protein, and, thus, provides a good positive control for riboprobe specificity. **(A)** mRNA staining for MOR using a CY3-labeled riboprobe (red). Cell nuclei are stained with DAPI (blue). MOR protein is localized to the same region of a matched slide by immunohistochemistry staining using a MOR-specific antibody **(B)**.

the antisense riboprobe has correctly hybridized to its target (**Fig. 2**). Care must be taken, however, in interpreting immunohistochemistry data in conjunction with FISH staining, because it is possible that staining of mRNA in the cell body may

be a considerable distance from staining of the translated protein, as can be the case for some receptors and transporters that are synthesized in the cell body of the neuron but expressed as protein at the synapse or dendritic spines.

3. Co-localization of two different riboprobes targeting the same transcript is also a good indication that the probes are specific for their cognate target. The use of different fluorophores for the two probes facilitates confirmation of their co-localization (**Fig. 3**). Once again, care must be taken in interpreting the data because alternate splicing, use of alternative promoters, or changes in mRNA processing can lead to nonconcordance in staining.
4. Localization of mRNA by FISH to tissue regions previously identified by *in situ* hybridization, or shown to express the target mRNA by other techniques, such as reverse transcription (RT)-PCR or Northern blotting, is a good positive control, particularly if documenting expression in particular cells for the first time (**Fig. 4**).

Other, less important, controls include:

1. Ensuring that signal from labeled riboprobe can be out-competed by addition of a 50-fold molar excess of unlabeled probe, the rationale being that there are a limited number of specific sites for hybridization compared with a vast excess of nonspecific sites.
2. Elevation of the hybridization temperature and increasing stringency of posthybridization washes results in the probe melting from the target in a manner consistent with the riboprobe length and buffer composition. Estimates of the temperature and stringency conditions for melting are based on probe length and GC content of the RNA. Suitable starting temperatures can be calculated by a number of commercially available software packages for probe design, such as Oligo 6.0 (Molecular Biology Insights; **ref. 9**).
3. Complete alkaline hydrolysis of the riboprobe results in no hybridization signal.
4. Digestion of RNA in the tissue with RNase or alkali results in loss of hybridization signal. The use of RNase in a laboratory working with mRNA is, however, not recommended.

3.2. Synthesis of Directly Labeled Riboprobes for mRNA FISH

All solutions and glassware used for mRNA FISH should be RNase-free and treated with 0.1% DEPC to minimize residual RNase activity. Standard procedures for working with RNA and using DEPC should be followed at all times (**10,11**).

The preparation of riboprobes suitable for FISH involves:

1. Preparing and purifying a suitable DNA template containing a promoter sequence for an RNA polymerase.
2. Transcription from the RNA polymerase promoter in the presence of fluorescently labeled ribonucleotides.
3. Purification and analysis of the riboprobe.

3.2.1. DNA Template

The construction of the template used for transcription is readily achieved by PCR amplification. The template used for PCR can be either complementary

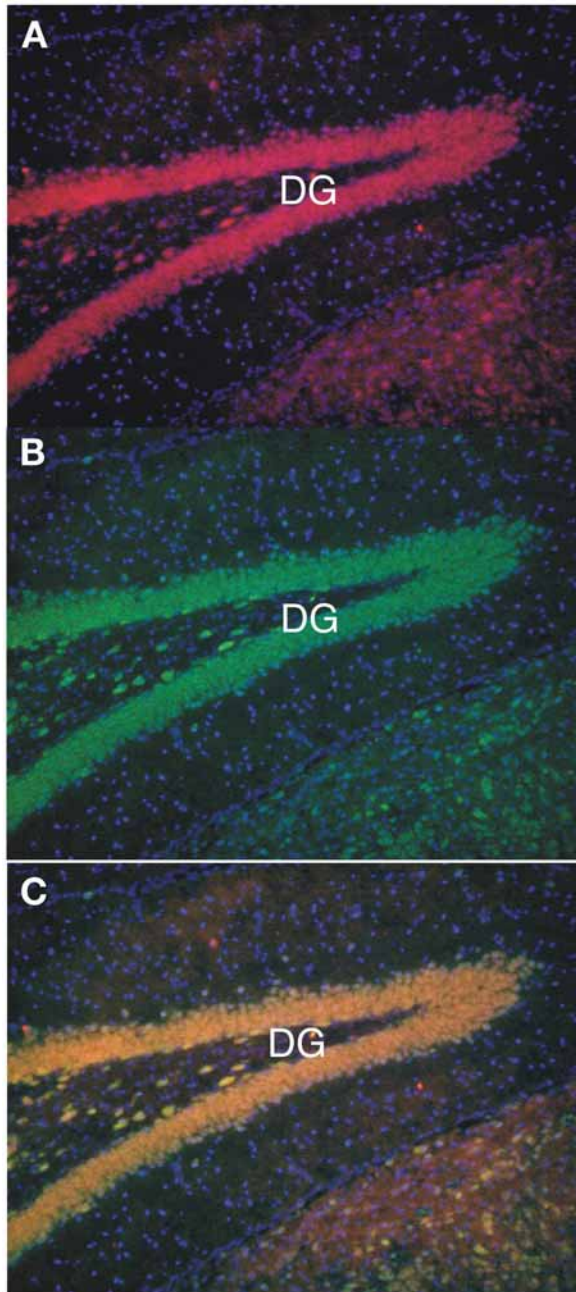


Fig. 3. Co-localization of riboprobes targeting different regions within the same mRNA molecule. Antisense riboprobes that target exon 4 (**A**, red) and exon 1 (**B**, green) of the MOR show co-localization of staining (**C**, A and B superimposed) within the dentate gyrus (DG) of the rat brain. Cell nuclei are counterstained with DAPI (blue).

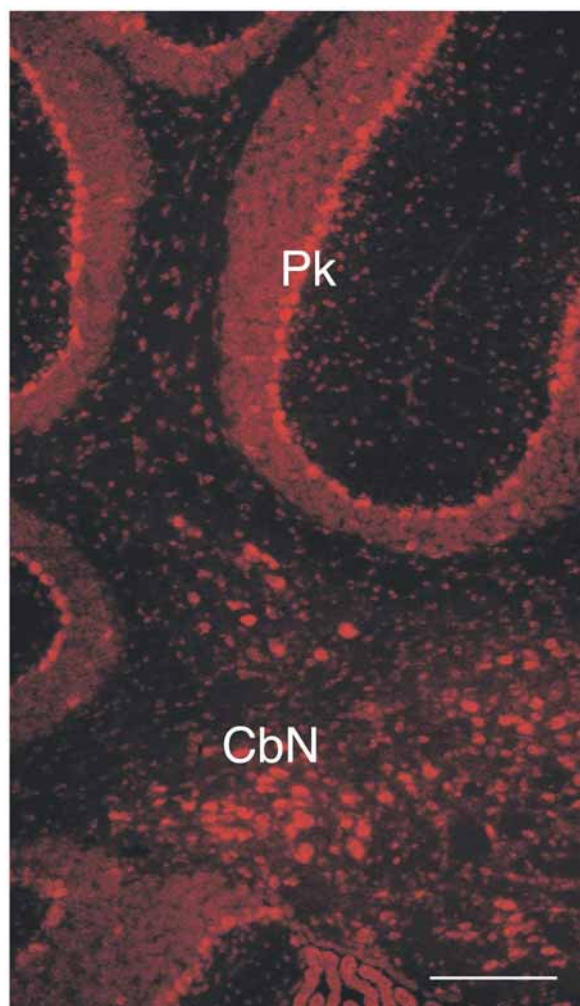


Fig. 4. Staining for MOR mRNA within a deep cerebellar nucleus and in the Purkinje cells of the adult rat cerebellum. MOR had previously been identified within deep cerebellar nuclei (CbN) but not in Purkinje cells (Pk) of the Purkinje layer in the rat cerebellum. Staining for MOR using an exon 1 specific MOR CY3 riboprobe (red) shows intense staining within a deep cerebellar nucleus. This staining serves as an internal positive control for identification of MOR mRNA in Purkinje cells in the same section. Bar = 200 μ m.

DNA (cDNA) prepared from a total cellular RNA extraction, or a plasmid clone of the target gene. It is beyond the scope of this chapter to detail procedures for isolation of total RNA and generation of cDNA from brain, or for the construction and propagation of plasmid clones. General guidelines and considerations

are, however, given in **Note 1** and can be found in *Molecular Cloning: A Laboratory Manual* (10).

T7 RNA polymerase is suitable for making RNA run-off transcripts from double-stranded DNA that contains the T7 RNA polymerase promoter sequence. The promoter sequence can be introduced into any piece of DNA to allow transcription by PCR amplification using an oligonucleotide that contains the promoter sequence as a noncomplementary 5'-tail. Introduction of the sequence 5'-TAATACGACTCACTATAGGG-3' to the 5'-end of the reverse PCR primer will allow synthesis of antisense riboprobe that will be complementary to the target mRNA (12); whereas introduction of the promoter sequence to the 5'-end of the forward primer will allow synthesis of sense riboprobe for use as a hybridization control.

3.2.2. Introduction of T7 RNA Polymerase Promoter Sequence for Riboprobe Synthesis From an RT-PCR Product

1. Perform an RT-PCR amplification from total cellular RNA using thermal cycling parameters that give maximum specificity for the amplification product. Confirm the yield and purity of an aliquot of product by agarose gel electrophoresis using standard methods (10).
2. Dilute the PCR product 100-fold in TE buffer for use as template in a second round of PCR.
3. On ice, prepare the following amplification mixture: 20 mM Tris-HCl, pH 8.8, containing: 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100 (1X Deep Vent buffer), 200 μM dNTPs, and 400 nM each of forward and reverse primers. The reverse primer should contain the T7 RNA polymerase promoter sequence if making antisense probe. The final reaction volume should be 50 μL and should include 1 μL of the diluted RT-PCR product as template (see **Note 2**).
4. Program a thermal cycling protocol using an appropriate annealing temperature for the primers (as for the initial amplification by RT-PCR). A suitable cycle for amplifying a 100- to 500-bp product would be similar to the following protocol: initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at between 55 and 65°C for 30 s, and extension at 72°C for 30 s.
5. Initiate the amplification using a manual hot-start by pausing the thermal cycler during the initial 2-min denaturing step, and carefully adding 1 U of Deep Vent exo⁻DNA polymerase without removing the tube from the heating block. Work quickly to prevent loss by evaporation, and resume the thermal cycle as soon as practicable. Dispersal of the enzyme by convection is sufficient to ensure a successful amplification.
6. Prepare an agarose gel of suitable density (between 1 and 2%, depending on the fragment size) in TAE buffer and load the entire amplification reaction in a single well. Load a suitable amount of size markers next to the sample to facilitate correct

identification of the desired product, and stain the nucleic acid in the gel with ethidium bromide (10).

7. Using a UV transilluminator on “preparative setting” (or another short-wave UV source), illuminate the gel and excise the product from the gel using a clean scalpel or razor blade. Minimize the exposure time of the DNA to the UV light source to prevent UV-induced damage of the DNA.
8. Extract and purify the product from the agarose using any one of a number of commercially available kits designed for the purification of PCR products from agarose gels (13). Follow the manufacturer’s instructions up to the step in which the PCR product is eluted from the matrix to which it was bound. Check that the elution buffer provided in the kit does not contain EDTA, because EDTA is a potent inhibitor of T7 RNA polymerase. If it does, replace it with 10 mM Tris-HCl, pH 8.0, then elute as directed by the manufacturer’s instructions, but using the EDTA-free buffer.
9. Run 10% of the recovered material on an agarose gel to estimate the yield and purity by comparison with suitable size markers. The gel-purified PCR product is the template for probe synthesis by *in vitro* transcription.

3.2.3. Introduction of T7 RNA Polymerase Promoter Sequence for Riboprobe Synthesis From Plasmid Clones

1. Using standard techniques (10), digest between 0.5 and 1.0 µg of plasmid in a volume of 20 µL, with a restriction enzyme that does not cut internal to the riboprobe sequence.
2. Dilute an aliquot of the restriction digest to a concentration of approx 1 ng/µL in TE buffer. Use this diluted DNA as template for PCR and continue from **Subheading 3.2.2, step 3** to amplify and purify the T7 promoter-tailed PCR product.

3.2.4. Synthesis of Fluorescently Labeled Riboprobes by T7 RNA Polymerase Via *In Vitro* Transcription

All steps that use fluorescently labeled nucleotides or labeled riboprobes should be protected from bright lights, particularly sunlight, to avoid photo bleaching of the dyes. Complete darkness, however, is not necessary.

1. Prepare the following transcription mixture in a plastic reaction tube in a final volume of 20 µL, using the concentrated (10X) reaction buffer that is usually provided with the T7 RNA polymerase. Transcribe approx 100 to 200 ng of the purified PCR product (**Subheading 3.2.2.**) in transcription buffer, 20 U of RNase inhibitor, and 50 U of T7 RNA polymerase.
2. Incubate in a water bath at 37°C for 2 to 6 h (see **Note 3**) to transcribe the riboprobe from the PCR product used as template (**Subheading 3.2.2.**).
3. Digest the template DNA by adding 150 U of DNase I (1 µL) directly to the transcription mixture, and continue the incubation at 37°C for a further 15 min.
4. The riboprobe is purified immediately after synthesis, typically by ethanol precipitation (see **Subheading 3.2.5.**), or, occasionally, by using a commercially available

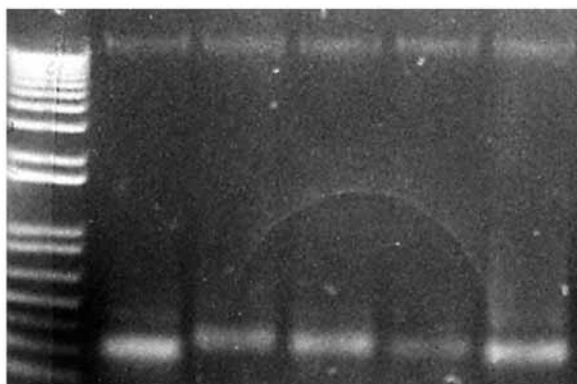


Fig. 5. Agarose gel electrophoresis of riboprobes. Riboprobes prepared and purified as in **Subheadings 3.2.4.** and **3.2.5.** were analyzed by agarose gel electrophoresis to assess the yield of product. From left to right, the lanes contain: lane 1, molecular size markers (1 Kb+ ladder, Invitrogen); lane 2, antisense probe, 310 nt; lane 3, sense probe, 355 nt; lane 4, antisense probe, 355 nt; lane 5, sense probe, 310 nt; and lane 6, sense probe, 287 nt.

RNA purification kit, such as the RNeasy kit (Qiagen), and following the manufacturer's instructions.

5. Resuspend the RNA in 50 μ L of 2X SSC and check the reaction yield by electrophoresis of a 1 μ L aliquot on a 1.8% agarose gel (see [Fig. 5](#) and **Note 4**).

3.2.5. Purification of Labeled Riboprobe by Ethanol Precipitation

1. Add 2 μ L of 3 M sodium acetate buffer, pH 5.2, to the 20 μ L T7 transcription mixture, with 1 μ g of linear acrylamide as a carrier (see **Note 5**).
2. Add 60 μ L of ice-cold absolute ethanol and mix well. Leave the RNA to precipitate for at least 30 min at -20°C .
3. Centrifuge the ethanolic mixture at 12,000g at 4°C in a refrigerated microcentrifuge for 10 min to pellet the RNA. Carefully aspirate and discard the supernatant. A colored pellet should be clearly visible.
4. Gently run 100 μ L of ice-cold 70% ethanol down the side of the tube to rinse the pellet, and then centrifuge at 12,000g for 3 min at 4°C .
5. Discard the supernatant and air-dry the RNA pellet to remove the last traces of ethanol. Resuspend the RNA in 50 μ L of 2X SSC buffer and store at -80°C until required. Do not over-dry the pellet by using a vacuum centrifuge (SpeedVac), because it can be difficult to rehydrate an over-dried pellet.

3.3. Preparation of Unfixed Brain Tissue for FISH

Successful preparation of tissue for mRNA requires working as rapidly as possible and maintaining all surfaces that contact the tissue nominally RNase-free and as cold as practicable to minimize RNase activity.

3.3.1. Embedding the Brain for Sectioning

1. All experiments involving animals should be in accordance with the appropriate animal ethics procedures and protocols.
2. Adult rats or mice should be euthanized by CO₂ asphyxiation followed by rapid decapitation and dissection of the brain from the skull while maintaining the integrity of the brain structures.
3. Rinse the brain briefly in ice-cold PBS to remove blood and other loose tissue and either slice along the midline (for sectioning in the sagittal plane) with a single clean cut using a sterile hard-backed razor blade, or cut in the coronal plane into blocks of sufficient dimension to fit the chuck of the cryostat microtome.
4. Line a wax-embedding histology tray with aluminum foil, ensuring that there are no creases on the floor of the block. Place the brain onto the aluminum foil, with the cut face flush against the flat bottom surface of the block.
5. Liberally cover the brain in embedding medium (OCT compound, Miles Scientific, Naperville, IL) and quickly place the embedding tray containing the brain in an ice bucket filled with dry ice. Sprinkle the brain with finely powdered dry ice to freeze the OCT compound and embed the brain for sectioning in the cryostat.
6. When the brain has frozen, remove the aluminum foil lining from the wax-embedding tray and store the brain wrapped in its aluminum foil at -80°C until required for sectioning.

3.3.2. Sectioning the Tissue

Sagittal and/or coronal sections of approx 12- μ m thickness should be prepared using a cryostat microtome. The best settings for the temperature of the tissue, the chamber temperature, and the angle of the blade, and so on, will have to be found empirically using the manufacturer's recommendations as a guide. A starting point is to have the cryostat chamber at -25°C and the tissue at -15°C. Ensure that a sharp blade is used and replace the blade frequently.

1. Chill a suitable number of Superfrost Plus glass slides in the cryostat cabinet. Using fine clean paintbrushes, position the still frozen 12- μ m tissue section on the slide immediately after slicing.
2. Thaw-mount the section by warming the underneath of the slide by rubbing with a finger in a circular motion to melt the section onto the slide.
3. As soon as the section has melted, re-freeze the section and store in the cryostat chamber before transferring to storage at -80°C.
4. The slides can be stored at -80°C for up to several months before hybridizing with riboprobe.

3.4. Fluorescence In Situ Hybridization

The sections below describe the necessary steps for fixing and blocking the tissue sections, hybridizing and washing away unbound probe, and visualizing and photographing the slides.

3.4.1. Fixation and Blocking Tissue Sections

1. Tissue sections stored at -80°C should be thawed then dried in an oven at 42°C for 15 min before processing to ensure that the tissue adheres to the slide sufficiently to withstand hybridization and washing.
2. A few hours before required, prepare a solution of 4% paraformaldehyde in PBS. The paraformaldehyde will need to be heated with stirring to dissolve. A few drops of 1 M NaOH may help the process. Chill the paraformaldehyde solution to 4°C before using.
3. All of the tissue sections that are to be used for a comparative FISH experiment **must** be fixed at the same time.
4. Using a suitable slide rack, immerse all slides together into the 4°C paraformaldehyde solution for 5 min, ensuring that the solution completely covers the slides.
5. After fixation, briefly rinse the slides in two changes of PBS at room temperature to remove all of the paraformaldehyde to prevent overfixation.
6. Acetylate the tissue sections by immersing the slides for 5 min in a freshly prepared solution of 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride. Change the solution and continue acetylating for a further 5 min. Acetylation helps block nonspecific binding of the riboprobe.
7. Rinse the slides in two changes of PBS to remove excess acetylating reagent.
8. Dehydrate the slides through a cold (-20°C) ethanol series by sequentially placing the slides in 70, 80, 95, and 100% ethanol solutions for 2 min each.
9. Air-dry the slides after the last wash and adhere an adhesive “*in situ* frame” around the tissue (see **Note 6**).

3.4.2. Hybridization and Stringency Washes

It is essential for comparative studies that all slides are processed under identical conditions. This is best achieved by batch processing all of the slides simultaneously.

1. Prepare sufficient hybridization solution for all of the slides as a master mix to ensure consistency of the hybridization solution between slides.
2. Place the appropriate volume of hybridization solution in the hybridization chamber (65 μL for a 2.40 cm^2 area, 125 μL for a 4.75 cm^2 -encased area) and seal with the flexible plastic cover slip provided with the *in situ* frame. Avoid introducing any air bubbles (see **Note 6**).
3. Denature the probe and target mRNA by placing the slides on an 80°C aluminum block for 4 min.
4. Transfer the slides to a hybridization chamber equilibrated to the desired temperature (see **Note 7**). Incubate for 12 to 16 h (overnight).
5. Remove the adhesive frames with forceps and wash the slides in two changes of 2X SSC for 15 min each wash.
6. Wash twice in 1X SSC for 15 min each (see **Note 8**).
7. Equilibrate a Coplin jar filled with 4X SSC containing 50% formamide in a water bath set to the same temperature as that used for hybridization (see **Note 9** for considerations regarding the stringent wash). Wash the slides for 20 min.

8. Terminate the stringent wash by briefly washing the slides in 2X SSC at room temperature (1 min) to cool them and remove the formamide.
9. Counterstain the cell nuclei by washing for 10 min in 2X SSC containing 20 ng/mL DAPI.
10. Wash the slides for 5 min in 2X SSC to remove excess DAPI. Shake off excess liquid and air-dry the slides.
11. Mount the tissue in antifade solution under no. 0 cover slips. For a 22 × 22-mm cover slip, 20 μ L of antifade solution is sufficient. Seal the edge of the cover slip with vulcanizing solution (rubber solution used to repair bicycle tire punctures) or a silicone adhesive.

3.4.3. Visualization and Photography

Stained sections on the slides should be viewed with a photomicroscope fitted with appropriate narrow band filters specific for DAPI, fluorescein, Cy3, and Cy5. Image capture is best achieved using a digital charge-coupled device (CCD) camera with at least a 3-megapixel array. To prevent photobleaching, avoid exposing the slides to bright lights and minimize the illumination time on the microscope stage, particularly if using high-power objectives.

1. For comparative studies, identify the slide that has the greatest fluorescence intensity for the hybridized riboprobe. Use this slide to determine the required exposure for this and all other slides.
2. Set an exposure such that the brightest region approaches the maximum grayscale value for the CCD camera. Typically, this is an intensity value of 256 (see **Note 10**).
3. Repeat the process for all other fluorophores.
4. Use the just-determined exposures to photograph areas of interest, using the appropriate filter for each fluorophore. Do not move the stage or adjust the magnification/zoom while photographing the same area or you will not be able to make digital overlays of your images.

3.5. Image Analysis

Relative expression levels between matched (same region of the brain) and identically processed slides can be determined by densitometric analysis of the digital images. A number of commercially available software packages specifically designed for densitometry are available.

To ensure the best possible resolution for data analysis, images should be captured as suggested, even though this leads to apparently high background fluorescence. This background fluorescence should then be subtracted from the images before analysis. The background fluorescence should be determined for each image by taking multiple measurements of the fluorescence intensity areas of surrounding tissue that does not express the mRNA. The matrix between cells is ideal if the cell density permits such a measurement to be made. Exposure as suggested, such that the maximum grayscale value for the camera

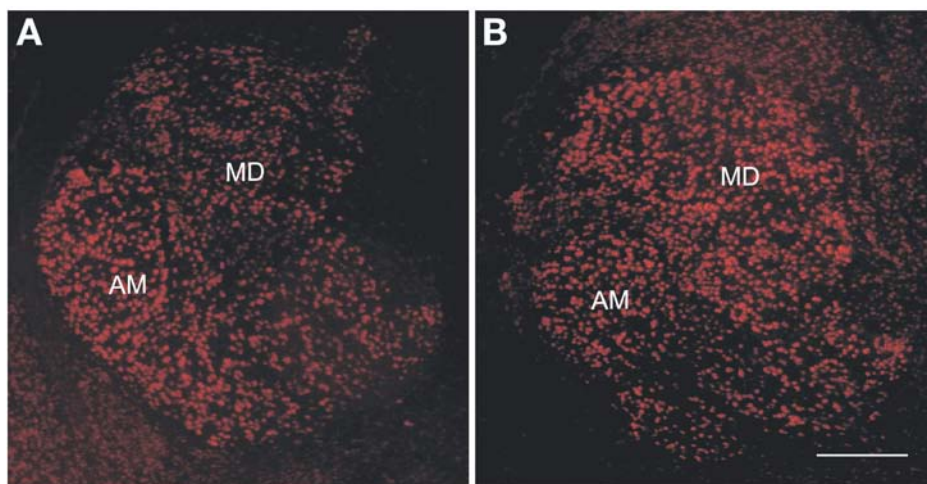


Fig. 6. Quantitative analysis of MOR expression in the thalamus of a wild-type and preproenkephalin-knockout mouse. Quantitative FISH to MOR mRNA was performed using a CY3-labeled cRNA probe for exon 1 of MOR on matched sagittal sections from a wild-type (**A**) and preproenkephalin-knockout mouse (**B**). The anteriomedial (AM) and mediodorsal (MD) thalamic nuclei are as indicated. There is a 25% increase in the mean level of MOR mRNA in the MD of the knockout mouse compared with the wild-type mouse, but no significant change was detected within the AM. Bar = 200 μ m.

is achieved, ensures that the detected fluorescence is spread over as large a range of grayscale values as possible. This maximizes the quality and resolution of the data by increasing the dynamic range. A good quality data set is one in which the fluorescence density is spread over a large range of grayscale values and the background fluorescence is low and differs considerably from the mean fluorescence density. The fluorescence density is calculated by calculating the product of the grayscale value and the number of pixels at that grayscale value, then summing all of the values.

Regions of brain that express the target mRNA can be more easily delineated from the background by setting a threshold to select a fraction of the data significantly above the background and then viewing this data set. This will provide information for preliminary decisions on which regions of the section to analyze. We have found that a good criterion for selecting a threshold grayscale value to identify signal significantly above the background is to determine which image has the greatest fluorescence density from all those in the experiment, then calculate what grayscale value will select 95% of the fluorescence density. Use of this threshold value for all images in the experiment will identify all fluorescence significantly above the background. The value of 95% total fluorescence density for a selection threshold is arbitrary and can be altered as

required. We have found a value of 95% density a useful criterion for identifying low-quality data in which the fluorescence density is spread over a narrow range of grayscale values and is close to that of the corrected background. We strongly recommend that the above criteria be applied before any image enhancement using software such as Adobe Photoshop. Image enhancement, if the researcher is not careful, can lead to inadvertent boosting of contrast and brightness levels, such that poor-quality data look respectable as a printed image, but are, however, unsuitable for quantitative analysis. **Figure 6** shows the results of this analysis applied to expression of MOR mRNA in a wild-type and preproenkephalin-knockout mouse.

4. Notes

1. All reagents and equipment used for RNA extraction should be RNase-free. The purchase of RNase-free disposable plasticware and use of equipment and facilities dedicated to RNA work are advised. Extraction of RNA using Trizol™, following the manufacturer's instructions, gives high-quality RNA suitable for RT-PCR. We have found that the use of gene-specific primers or oligo-dT for reverse transcription with superscript II reverse transcriptase for making cDNA, then PCR with a hot-start thermophilic polymerase, such as Platinum *Taq*, consistently gives good results with a variety of templates and primers. Guidelines for working with RNA and performing mRNA *in situ* hybridization are described in a number of freely available "Application Manuals" (11,14).
2. The buffer for PCR is supplied as a 10X concentrate when the Vent exo⁻ polymerase is purchased. The use of *Taq* polymerase is not advised because the non-templated addition of 3'-dA overhang catalyzed by the terminal transferase activity of *Taq* polymerase can induce a double-stranded hairpin RNA when transcribed by T7 RNA polymerase. Removal of the 3' overhang, by brief incubation with Klenow fragment and/or T4 DNA polymerase, as used for "polishing PCR products" for blunt cloning (10), generates a suitable template for transcription. The use of an alternative thermophilic DNA polymerase, lacking terminal transferase activity, would also be satisfactory. We have found that good amplification with Deep Vent exo⁻ is achieved using the same thermal cycling parameters as used for amplifications with *Taq* polymerase, making amplification with Deep Vent exo⁻ the easiest method for preparing suitable DNA template for transcription.
3. The incubation time required depends on the molar amount of template in the transcription reaction and whether or not dye-modified ribonucleotides are being incorporated. If an unlabeled probe is prepared for a competitive hybridization experiment (see **Subheading 3.1.2.**), a 2-h incubation is sufficient, fluorescein-UTP labeling is typically complete within 3 to 4 h, and Cy3-UTP or Cy5-UTP labeling in longer than 4 h.
4. It is not necessary to analyze the transcription products on MOPS/formamide agarose gels as is usual for electrophoresis of RNA-containing samples. A normal agarose gel stained with ethidium bromide, as used for sizing double-stranded

- DNA molecules, is sufficient to determine the approximate yield of transcription reaction product. The RNA products may run as doublets because of the presence of alternate conformations and stable secondary structures.
5. Linear acrylamide is an inert carrier used to facilitate precipitation of small amounts of nucleic acids. The addition of a carrier, however, is unlikely to be necessary, because of the large amounts of RNA transcribed, but it does aid in visualizing the pellet after centrifugation.
 6. The use of an *in situ* hybridization chamber formed directly on the slide by use of an “*in situ* frame” such as the EasiSeal frame, is highly recommended. Alternatives, such as sealing reagents under a glass cover slip, are generally inferior, more difficult to use, and prone to leakage. The larger reagent volume applied with the *in situ* frames also helps achieve results that are more consistent, by minimizing changes in the hybridization buffer caused by salts or liquids in the tissue, as well as by minimizing depletion of riboprobe by hybridization to its target.
 7. A covered water bath with aluminum blocks submerged just below the water surface is a cheaper, and, in many ways, superior, alternative to a commercial hybridization oven. Placing the slides directly on the aluminum blocks ensures good heat transfer and accurate temperature control that is not adversely affected by entry and exit to the hybridization bath, as can be the case for hybridization ovens. The *in situ* frames that form the reaction chambers provide water- and gas-tight seals, even at temperatures as high as 97°C. The optimum temperature for hybridization is dependent on the probe length, the buffer composition (concentration of salts and percentage formamide used), and the GC content of the probe, but is typically in the range 45 to 65°C. For initial experiments, start with a low hybridization temperature, such as 45°C, then apply increasingly stringent washes and view the slide after each stringent wash (see **Note 8**) to determine the temperature at which the probe is washed off. Hybridization is best performed approx 5°C below the temperature at which a significant reduction in fluorescence of the hybridized probe is observed. The slides will need to be counterstained with DAPI again after each stringent wash.
 8. If first optimizing hybridization parameters, it is advisable to jump to **Subheading 3.4.2., step 9** to counterstain and mount the slides before performing a stringent wash, to ensure that fluorescence signal can be easily seen. After viewing, a stringent wash can be performed by removing the rubber seal formed by the vulcanizing solution, and washing the slide with gentle rocking twice in 2X SSC for 5 min to remove the cover slip and antifade solution. After stringent washing, counterstain and mount as in **Subheading 3.4.2., step 11** for viewing. This process can be repeated as many times as required.
 9. If you have identified a suitable hybridization temperature, as detailed in **Note 7**, then a suitable temperature for the stringent wash will be approx 5°C higher than that used for hybridization. It is worth confirming that the selected temperature is suitable (achieves maximum selectivity) by raising the temperature of the stringent wash a further 5°C (i.e., to 10°C above the hybridization temperature) and confirming that most of the probe is washed away. See **Note 8** for how to perform

repeated stringent washes. The ability to be able to rapidly assess the consequences of alternative stringent washing conditions is a major advantage of FISH compared with RISH, because the latter requires long exposures and multiple experiments to achieve the same result.

10. Some cameras that are more advanced offer more grayscale levels. The tenet of ensuring exposure close to the maximum grayscale value remains unchanged.

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Visualization of Gene Expression by Fluorescent Multiplex Reverse Transcriptase-PCR Amplification

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and José Luis Micol

Summary

Many developmental and physiological analyses, population studies, and diagnostic tests can be performed by simply determining the presence or absence of a limited number of gene products. Here, we describe a rapid and sensitive procedure, based on the reverse transcription of total RNA samples followed by the co-amplification of specific complementary DNA molecules, for the simultaneous detection of different transcripts. Multiplex PCR amplification products are obtained in a single reaction mix containing several primer pairs, each of which includes a fluorescently labeled oligonucleotide; the amplification products are finally electrophoresed in an automated DNA sequencer controlled by fragment analysis software. The electropherograms obtained in this way allow a semiquantitative and efficient visualization of gene expression.

Key Words: Differential gene expression; fluorescent labeling; multiplex polymerase chain reaction; transcription detection.

1. Introduction

The assessment of gene activity by means of messenger RNA detection is an important tool for the understanding and manipulation of living organisms as well as for medical purposes. For many years, the laborious and time consuming Northern analysis (1,2) was the technique of choice for this purpose, but it has been almost completely replaced by PCR-based techniques, usually termed reverse transcriptase (RT)-PCR (3), and by microarray analyses (4). Detection of a single gene product is usually performed by RT-PCR procedures, in which total RNA is first extracted from the cells or tissues under study and then reverse transcribed into complementary DNA (cDNA) before being amplified

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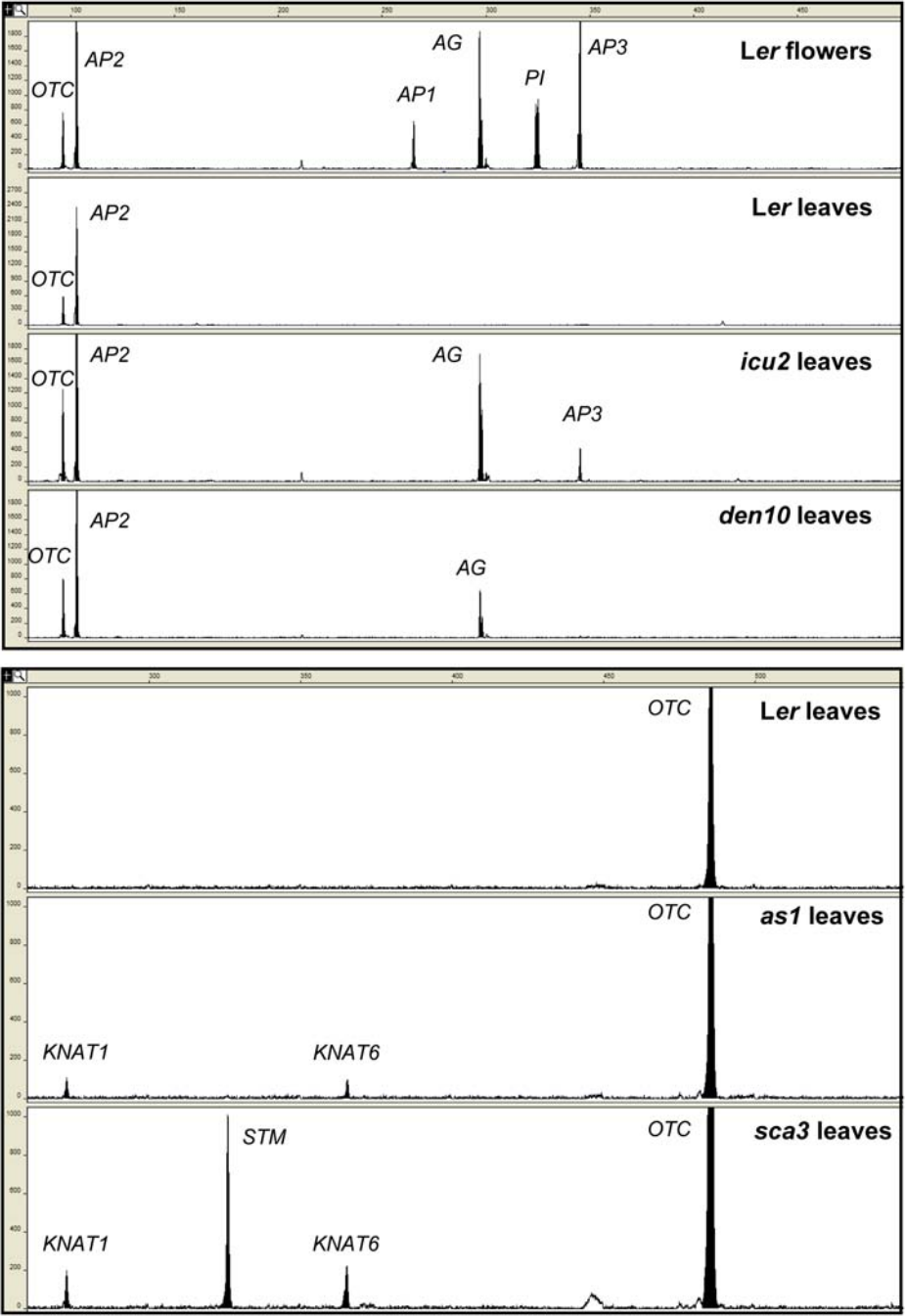


Fig. 1.

by PCR. Microarray analysis is the best choice for the simultaneous detection of a large set of gene products, although it is not a simple or very sensitive procedure.

The procedure detailed here allows the simultaneous PCR co-amplification of different messenger RNAs in a single tube containing several gene-specific primer pairs, each including a fluorescently labeled oligonucleotide. The electrophoresis and detection of the fluorescently labeled amplification products in a DNA sequencer controlled by fragment analysis software means that it makes the use of radiolabeling or ethidium bromide staining unnecessary. The method is semiquantitative, rapid, sensitive, and reproducible, and has been shown to be efficient for analyzing differential gene expression in a number of reaction mixes containing distinct primer combinations and using cDNA obtained from RNA extracts as a template. As with many other multiplex RT-PCR procedures, the one detailed in this chapter makes it possible to determine only the presence or absence of transcripts, and should not be considered quantitative.

Here, we present two examples of fluorescent multiplex PCR analyses of the activity of several genes in different organs of the plant model system, *Arabidopsis thaliana* (5). Several of the genes studied here encode transcription factors that, in the wild-type plants, are spatially restricted to some flower organs: AGAMOUS (AG), PISTILLATA (PI), APETALA (AP)1, AP2, and AP3 (6,7). On the other hand, the Knotted-like *A. thaliana*1 (KNAT)1, KNAT6, and SHOOT MERISTEMLESS (STM) genes are expressed only in the shoot apical meristem of wild-type plants (8,9). The AG gene is ectopically derepressed in the leaves of the denticulata10 (*den10*; ref. 10) mutant, together with the AP3 gene in the incurvata2 (*icu2*; ref. 11) mutant (Fig. 1A–D). The KNAT1 and KNAT6 genes are ectopically expressed in the leaves of the asymmetric leaves1 (*asl*; ref. 12) mutant, together with the STM gene in the leaves of the scabra3 (*sca3*; ref. 10)

Fig. 1. Electropherograms illustrating results of analyses performed following the method presented in this chapter, on RNA extracted from flowers (A) or leaves (B–G) of the Landsberg *erecta* (Ler) wild-type (A, B, E) and the *icu2* (C), *den10* (D), *asl* (F), and *sca3* (G) mutants of *A. thaliana*, which display aberrantly shaped leaves, a phenotypic trait that correlates with the ectopic derepression of some floral (AP3 and AG) or shoot apical meristem (KNAT1, KNAT6, and STM) genes. The *OTC* housekeeping gene was used as an internal control (see Subheading 3.4.2.). The horizontal and vertical axes indicate, respectively, the size of the electrophoresed molecules (in nucleotides) and the intensity of fluorophore emissions (in arbitrary units of fluorescent signal strength). Each electropherogram contains peaks that represent the molecules obtained from the multiplex PCR amplification of complementary DNA samples obtained from leaves or flowers (a mixed sample of flower buds and mature flowers) excised from plants 21 d or 5 wk after sowing, respectively. The electropherograms were produced by the Genescan 3.7 software and were simplified by removing the peaks corresponding to the internal molecular weight standard. Every peak is denoted with the name of the gene to which it corresponds.

mutant (**Fig. 1E–G**). We used the ORNITHINE TRANSCARBAMYLASE (OTC) housekeeping gene, which is ubiquitously expressed in all plant organs and tissues (**13**), as an internal control.

2. Materials

All plastic materials and solutions for RNA extraction and cDNA synthesis should be RNase-free.

2.1. Equipment and Supplies

1. 1.5-mL Eppendorf tubes.
2. Autoclavable plastic pestles (Sigma).
3. Cordless motor grinder (Sigma).
4. Heat block.
5. Vortex.
6. Microcentrifuge.
7. Spectrophotometer.
8. Freezers (–20 and –80°C).
9. 0.2-mL thin-walled PCR tubes.
10. 96-Well optical reaction plate (Applied Biosystems).
11. 3100 Capillary Array 36 cm (Applied Biosystems).
12. ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).
13. GeneAmp PCR System 9700 (Applied Biosystems).
14. Genescan 3.7 software (Applied Biosystems).

2.2. Reagents and Buffers

1. Liquid nitrogen.
2. RNeasy Plant Mini Kit (Qiagen).
3. Hi-Di formamide (Applied Biosystems).
4. Ethanol.
5. RNase-free H₂O.
6. DNase I (RNase-free, Roche).
7. 10X DNase I buffer.
8. 3 M NaOAc, pH 5.2.
9. RNaseOUT (recombinant RNase inhibitor, Invitrogen).
10. 10 mM dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP), for reverse transcription.
11. 100 mM DTT (Invitrogen).
12. 5X first-strand buffer (Invitrogen).
13. SuperScript II (RNase H⁻ reverse transcriptase, Invitrogen).
14. Random primers (p[dN]6, Roche).
15. Taq DNA polymerase and 10X PCR buffer.
16. 2.5 mM dNTP mix (2.5 mM each, dATP, dCTP, dGTP and dTTP) for PCR amplifications.
17. Oligonucleotide mixes (see Primer mix 1 and Primer mix 2 in **Table 1**).

Table 1
Oligonucleotide Sets Used for Multiplex Reverse Trascriptase-Polymerase Chain Reaction Amplifications

Oligonucleotides sequences (5'→3')				Amplification product size (bp)	
	Gene	Labeled forward primer	Unlabeled reverse primer	Complementary DNA template	Genomic DNA template
Primer mix 1	<i>AG</i>	ACTCCAACAGGCAATTGATG	TCAAACGGTTGAGATTGCGTTT	299	700
	<i>AP1</i>	GGCTTAAGGCTAAGATTGAGC	CTGCTCCTGTTGAGCCCTAA	266	1100
	<i>AP2</i>	CGAGTATTTACGATGAGGAACT	CTTCGAATTAGCCGAATTTCCC	103	209
	<i>AP3</i>	CAAGAAACCAAGAGGAAACTGT	AAGAGCGTAAGCACGTGACC	346	1123
	<i>PI</i>	ACATGGCCTCGACAAAGTCC	CACACAGATTGATAAAGACAA	323	602
	<i>OTC</i>	TCCTTGCCAAATCATGGCCG	CATGTTGTTCCCATCTCCAAC	99	349
Primer mix 2	<i>STM</i>	AGGAATCAACCCTTGCTCCTC	ATTGACACTCGACACGTTGAAG	326	785
	<i>KNAT1</i>	TGAAGCTAGCTCCTCAAGAATC	GGCAGAGACAGACGGTGTTG	271	382
	<i>KNAT6</i>	GTGATTATTCAGATAAGTCGGTTC	CAACCTCTTGCTTATAAACATCAC	365	402
	<i>OTC</i>	TCCTTGCCAAATCATGGCCG	GCATGCATGCGATTCTCCGC	487	868

18. 50 mM MgCl₂.
19. 3100 POP-6 polymer (Applied Biosystems).
20. GeneScan-500 TAMRA size standard (Applied Biosystems).

3. Methods

3.1. RNA Extraction

Although several RNA extraction protocols have been described, we obtained very reproducible results using the RNeasy Plant Mini Kit (see **Heading 2.**). For RNA extraction, 80 to 100 mg of excised rosette leaves or flowers are collected in 1.5-mL Eppendorf tubes (see **Note 1**), which are then weighed and immersed in liquid nitrogen. RNA extraction is performed following the instructions of the kit manufacturer, the only exception being that frozen samples are homogenized using a cordless motor grinder fitted with a plastic pestle (see **Heading 2.**), instead of using a classic mortar and pestle. RNA is finally eluted into 86 μ L of RNase-free water.

3.2. DNase Treatment

To eliminate genomic DNA from the RNA samples:

1. Add 10 μ L of 10X DNase I buffer and 4 μ L of 10 U/ μ L DNase I to the RNA solution, mix gently, and incubate at 37°C for 30 min.
2. Inactivate DNase I by heating the tubes for 10 min at 70°C.
3. Add 100 μ L of RNase-free water; 20 μ L of 3 M NaOAc, pH 5.2; and 600 μ L of absolute ethanol.
4. Mix gently and precipitate RNA at -20°C for 1 h.
5. Centrifuge at maximum speed for 15 min at 4°C in a microcentrifuge.
6. Discard supernatant.
7. Wash the RNA precipitate with 300 μ L of 70% ethanol.
8. Centrifuge at maximum speed for 10 min at 4°C in a microcentrifuge.
9. Discard the supernatant and allow the precipitate to air-dry (usually 15 to 20 min).
10. Resuspend the precipitate in 40 μ L of RNase-free water, previously warmed to 55°C.
11. Ascertain RNA yield spectrophotometrically, and check RNA quality by electrophoresis (see **Note 2**).
12. Store the RNA samples at -80°C.

3.3. Reverse Transcription

1. Perform first-strand cDNA synthesis using 2.5 μ g of RNA as a template.
2. Add 400 ng of random primers (p[dN]6) and RNase-free water to reach a final volume of 11 μ L, and incubate at 65°C for 5 min (see **Note 3**).
3. Keep the tubes at room temperature for 10 min.
4. Incubate the tubes on ice for 2 min.

5. Add the following reagents in the following order (see **Note 4**):
 - a. 1 μ L of 40 U/ μ L RNaseOUT.
 - b. 1 μ L of 10 mM dNTP mix (10 mM each).
 - c. 2 μ L of 100 mM DTT.
 - d. 4 μ L of 5X first-strand buffer.
 - e. 1 μ L of 200 U SuperScript II.
6. Incubate the tubes at 42°C for 1 h.
7. Inactivate the reverse transcriptase by heating the tubes at 70°C for 15 min.
8. Return the tubes to ice.
9. Proceed with the PCR amplification or store the cDNA at -20°C until use.

3.4. Multiplex PCR Amplifications

3.4.1. Oligonucleotide Design

1. Oligonucleotides should be designed based on exonic sequences spanning at least one intron, which will allow RT-PCR products to be distinguished according to whether they have been amplified from cDNA or genomic DNA, because amplifications from the latter template yield longer products (**Table 1**; see **Note 5**).
2. Choose oligonucleotide pairs yielding PCR products of similar but not identical sizes, to avoid preferential product amplification.
3. All oligonucleotides should have a similar melting temperature (T_m), which can be easily calculated as $T_m = 4(C + G) + 2(A + T)$, known as the Wallace rule (**14**), where $G + C$ is the number of G or C residues in the oligonucleotide and $A + T$ is the number of A or T residues. Amplifications are usually performed using an annealing temperature 5°C lower than the calculated T_m .
4. If the transcription of structurally related genes is to be tested (such as the *AG*, *AP1*, *AP3*, and *PI* genes, all of which are members of the MADS-box family), non-homologous sequences should be identified and used for oligonucleotide design.
5. Individual gene amplifications can be optimized by using a series of increasing annealing temperatures. To perform the multiplex PCR amplifications, the highest common temperature found for all the successful individual amplifications should be chosen.

3.4.2. Preparation of Oligonucleotide Mixes

We use two oligonucleotide mixes, each of which allows the simultaneous detection of several transcripts in a single tube (see **Note 6**). Primer mix 1 is used for the detection of the activity of the *AG*, *PI*, *AP1*, *AP2*, and *AP3* genes, and primer mix 2 for detection of the activity of *KNAT1*, *KNAT6*, and *STM* genes (**Table 1**). By using two different primer pairs, both primer mixes allow, in addition, the detection of the *OTC* gene, which serves as a control (**Table 1**). All primer pairs include one oligonucleotide that was fluorescently labeled with HEX phosphoramidite (**Table 1**; see **Note 7**).

Each primer mix is prepared (see **Note 8**) in a volume of 250 μ L, reaching a final concentration of 250 nM for each oligonucleotide.

3.4.3. PCR Amplifications

PCR amplifications are performed in a final volume of 5 μL , to minimize the amount of labeled primers consumed. To avoid pipetting errors, a master mix for at least 10 reactions should be prepared (see **Note 9**).

1. Prepare PCR master mixes 1 and 2, both including the following components per sample to be analyzed: 1.6 μL H_2O , 0.5 μL of 10X PCR buffer, 0.2 μL of 50 mM MgCl_2 , 0.4 μL of 2.5 mM dNTP mix, 0.04 μL of 5 U/ μL *Taq* DNA polymerase (see **Note 10**), and 1.2 μL of primer mix (1 or 2).
2. Dispense 4- μL aliquots of the PCR master mix into 0.2-mL thin-walled tubes. Add 1 μL of cDNA to each tube.
3. Place the tubes in a thermocycler and run the following program: 3 min at 94°C, followed by 35 cycles (30 s at 94°C, 15 s at 55°C, and 1.5 min at 72°C) and a final 7-min incubation at 72°C (see **Note 11**).

3.5. Electrophoresis and Sizing of PCR Amplification Products

1. Dilute each PCR mix 1:5 by adding 20 μL of water, to avoid saturation of the Genetic analyzer (see **Note 12**).
2. Prepare a loading buffer by mixing 1 mL of Hi-Di formamide and 30 μL of GeneScan-500 TAMRA Size Standard and keep at 4°C.
3. Dispense 1 μL of the diluted PCR mix and 10 μL of loading buffer into each well of a 96-well optical reaction plate.
4. Immediately before electrophoresis in an ABI PRISM 3100 Genetic Analyzer (see **Note 13**), heat the 96-well optical reaction plate containing the samples at 94°C for 2 min in a thermocycler or a water bath, and keep on ice for at least 5 min.
5. Perform the electrophoresis of the amplification products using a Capillary Array of 36 cm, the POP-6 polymer, and the Data collection 3.1 program with the GeneScan36_POP6 default module and the Dye Set C option (see **Note 14**).
6. After the end of the electrophoresis, the electrophoresed molecules can be sized by using the Genescan 3.7 software.

4. Notes

1. The RNA isolation kit used in this work was designed for performing extractions from less than 100 mg of tissue, and is specific for plant and fungal material. An alternative procedure should be used for large-scale extractions for isolating RNA from other organisms (for example, see Chapter 2).
2. The RNA concentration should be assessed by determining the absorbance at 260 nm of a 40-fold dilution of the extract obtained. The RNA quality should be monitored in addition by gel electrophoresis (use 2 μL of the extract in a 1% agarose gel in 1X Tris-acetate-EDTA buffer), based on the presence of intact ribosomal RNA and the absence of genomic DNA.
3. Incubation of RNA molecules at 65°C helps to remove their secondary structures and increases the efficiency of their hybridization with the random primers used for reverse transcription.

4. To avoid degradation of RNA caused by ribonuclease contamination of the preparation, it is important to add the RNaseOUT ribonuclease inhibitor first.
5. Because the method described is very sensitive, even small amounts of contaminating genomic DNA present in the RNA preparation may be amplified along with the cDNA. This is why each primer set includes only exonic sequences and spans at least one intron, which makes it possible to differentiate between cDNA and genomic DNA amplification products. In addition, the amplification efficiency of primers spanning an intron can be tested using genomic DNA as a template.
6. Our method is semiquantitative in the sense that it allows the reproducible detection of the presence or absence of transcripts; however, it does not allow quantification, because the intensity of the signal for a particular amplification product may be different in different replicate analyses.
7. We selected HEX to label our forward primers because it produces narrower electropherogram peaks than other available phosphoramidites.
8. We recommend the acquisition of lyophilized primers, which can be dissolved in water to reach the required concentration. To avoid contaminations, we use filtered micropipet tips for the preparation and manipulation of oligonucleotide stock solutions.
9. A positive control should always be included in each experiment to ensure that the method works properly. In the experiments described here, as a positive control, we used wild-type flower cDNA for amplifications performed with primer mix 1 (**Fig. 1A**), and cDNA obtained from leaves of the *as1* mutant (**Fig. 1F**) for those performed with primer mix 2. The *as1* mutant is known to suffer ectopic derepression of several *KNAT* genes (**12**).
10. Although we used a polymerase provided by a local supplier (Ecotaq, Ecogen), any other *Taq* polymerase should yield efficient amplification.
11. Although we routinely use ABI thermocyclers, many others could serve to perform the amplifications described here, although some optimization of the annealing temperature might be necessary.
12. The dilution of the PCR products should be increased up to 1:15 if the signal intensity of any of them saturates the detector of the Genetic analyzer.
13. DNA sequencers other than those from ABI can be used with the appropriate software for data collection and analysis. Check the list of fluorophores compatible with your sequencer before ordering labeled oligonucleotides.
14. The Dye Set option of the Genescan software is fluorophore-specific. Follow the instructions of the manufacturer to choose the most appropriate dye set option for the fluorophores of your labeled oligonucleotides.

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Fluorescence *In Situ* Hybridization for the Identification of Environmental Microbes

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Summary

This chapter presents a protocol for the phylogenetic identification of microorganisms in environmental samples (water and sediments) by means of fluorescence *in situ* hybridization (FISH) with ribosomal RNA-targeted oligonucleotide probes and signal amplification (catalyzed reporter deposition [CARD]). The FISH probes are labeled with the enzyme, horseradish peroxidase (HRP). A subsequent deposition of fluorescently labeled tyramides results in substantially higher signal intensities of target cells than after FISH with probes directly labeled with fluorochromes. Sample preparation and cell permeabilization strategies for various microbial cell wall types are discussed. The custom labeling of tyramides with different fluorochromes is described. A sequential multicolor CARD-FISH protocol is outlined for the simultaneous detection of different phylogenetic groups.

1. Introduction

Fluorescence *in situ* hybridization (FISH) with ribosomal RNA (rRNA)-targeted oligonucleotide probes has developed into an invaluable molecular tool for the identification of microorganisms in the environment (1–8). However, its application is sometimes hindered by its restricted sensitivity. Most bacteria in aquatic habitats are small, slow growing, or starving, and therefore may contain a low number of ribosomes per single cell (9). Because the fluorescence intensities of hybridized cells depend on the concentration of the probe target, i.e., the rRNA, the hybridization intensities of cells in environmental samples are frequently below microscopic detection limits or lost in high background.

Recently, a novel FISH protocol has been presented for the reliable identification of bacterial cells with low rRNA content in marine plankton and benthos and in freshwater samples (5,10,11). This approach is based on the use of horseradish

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peroxidase (HRP)-labeled oligonucleotide probes and tyramide signal amplification, also termed catalyzed reporter deposition (CARD).

CARD was introduced more than a decade ago for immunoblotting and immunosorbent assays (12). Subsequently, Kersten et al. (13) and Raap et al. (14) reported a substantial increase of FISH signal intensity using this approach. CARD is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity. HRP reacts with hydrogen peroxide and the phenolic part of labeled tyramide to produce a quinone-like structure bearing a radical on the C2 group (15). This activated tyramide then covalently binds to tyrosine residues in the target cell. Each HRP conferred by a probe catalyzes the deposition of many labeled tyramides. This results in greatly enhanced FISH sensitivity compared with probes directly labeled with a single fluorochrome.

CARD in combination with nucleic acid probes is routinely used in histology and cytochemistry to localize specific nucleic acid sequences (DNA, RNA) in microscopic preparations of tissues, cells, and chromosomes (16,17). In such samples, it allows the detection of rare and even single-copy-number targets (messenger RNA, genes; ref. 18). To date, a wide variety of research and diagnostic applications have been described, making this technique an integral part of studies of gene mapping, gene expression, RNA processing and transport, the three-dimensional organization of the nucleus, tumor genetics, microbial infections, and prenatal diagnosis (19).

CARD-FISH is a multistep procedure including preparation of the biological material, nucleic acid probe selection, hybridization with a labeled probe, cytochemical probe detection, and microscopy.

Preparation of the specimen for CARD-FISH involves routine fixation to obtain maximum retention of target nucleic acid sequences and morphology. Such fixatives are, for example, buffered paraformaldehyde (PFA) and/or ethanol. Usually, cells need to be concentrated and/or immobilized. Plankton samples may be filtered onto polycarbonate membrane filters. Sediment bacteria are separated from particles by appropriate techniques (5,6), and are subsequently filtered onto membrane filters or directly attached to gelatin- or poly-L-lysine-coated glass slides. Depending on the cell walls of the target organisms and the fixation, different permeabilization strategies need to be applied, such as treatment with various enzymes, diluted HCl or detergents, thereby facilitating access of the labeled nucleic acid probes and cytochemical detection molecules.

Three types of probes are mainly used for FISH: double-stranded DNA probes, single-stranded RNA (or DNA) probes, and oligonucleotide probes. For use in combination with CARD, these probes are labeled with a hapten (such as fluorescein, digoxigenin, and biotin), which can then be detected immunochemically using an antibody to hapten conjugated to a peroxidase. Oligonucleotide probes can also be directly linked to HRP.

Several reviews and protocols are available on the principles of hybridization (2,7) as well as on methodological aspects and applications of FISH with rRNA-targeted oligonucleotides (1,3). Here, we focus on the use of oligonucleotide probes labeled with HRP. Those probes can either be purchased or custom-labeled by a procedure described elsewhere (20).

2. Materials

Use powder-free gloves for all procedures, because the powder may cause background fluorescence in FISH preparations.

1. HRP-labeled oligonucleotide probes (<http://www.biomers.net>).
2. White polycarbonate filters (pore size 0.2 μm , Millipore, Eschborn, Germany).
3. 0.1 (w/v) Low gelling point agarose, in deionized H_2O .
4. 30% H_2O_2 .
5. 0.01 M HCl.
6. 20X phosphate-buffered saline (PBS): 2.74 M NaCl, 54 mM KCl, 2 M Na_2HPO_4 , and 0.4 M KH_2PO_4 , pH 7.6.
7. 10% (w/v) Blocking reagent (Roche Applied Science, Mannheim, Germany).
8. DAPI.
9. Citifluor antibleaching mountant (Citifluor Ltd., London, England).
10. Liquid nitrogen or helium.
11. Lysozyme (from chicken egg white, Fluka, Taufkirchen, Germany).
12. Tris-HCl-EDTA (TE) buffer: 0.05 M EDTA and 0.1 M Tris-HCl, pH 8.0.
13. Achromopeptidase (Enzyme Commission Number: 3.4.21.50, Sigma-Aldrich, Seelze, Germany).
14. Proteinase K (*Tritirachium album*, 600 mAnson U/mL, Merck, Darmstadt, Germany).
15. 5 M NaCl.
16. 1 M Tris-HCl, pH 8.0.
17. 0.5 M EDTA, pH 8.0.
18. 20% (w/v) sodium dodecyl sulfate (SDS).
19. Formamide (molecular biology grade).
20. Dextran sulphate (powder).
21. Sonicator probe MS73 (Sonoplus HD70, Bandelin, Berlin, Germany).
22. Glass slides (e.g., Superfrost Plus, Menzel, Braunschweig, Germany).
23. 96 to 80% Ethanol (v/v).
24. Rotation shaker, temperature controlled.
25. Tyramide synthesis:
 - a. Active dye stocks: 1 mg succinimidyl ester (ALEXA546, ALEXA633, or ALEXA488) in 100 μL dimethylformamide; 5 mg succinimidyl ester (ALEXA350) in 500 μL dimethylformamide; or 100 mg succinimidyl ester (5- or 6-carboxyfluorescein) in 10 mL dimethylformamide (see **Note 1**).
 - b. Tyramine HCl stock: 10 μL triethylamine, 1 mL dimethylformamide (water-free), and 10 mg tyramine-HCl.

- c. Add active dye ester in 1.1-fold molar excess to tyramine HCl stock solution:
 - i. 100 μ L Alexa488 stock + 25.2 μ L tyramine HCl stock.
 - ii. 100 μ L Alexa546 stock + 14.7 μ L tyramine HCl stock.
 - iii. 100 μ L Alexa633 stock + 13.1 μ L tyramine HCl stock.
 - iv. 500 μ L Alexa350 stock + 193 μ L tyramine HCl stock.
 - v. mL (5- [and 6-] carboxyfluorescein) stock + 3.3 mL tyramine HCl stock.
 - d. Incubate for 6 to 12 h at room temperature in the dark.
 - e. Dilute reaction mixture with absolute ethanol to a final concentration of 1 mg active dye/mL. Dispense in aliquots of 20 μ L and desiccate in a freeze dryer or under vacuum at room temperature. Desiccated tyramides are stable for years if stored at -20°C .
 - f. For use, tyramides are reconstituted in 20 μ L of sterile deionized water or dimethylformamide containing 20 mg/mL *p*-iodophenylboronic acid (IPBA). In our lab, we dissolve tyramides labeled with Alexa546, Alexa488, Alexa633, or fluorescein in dimethylformamide (final concentration, 1 mg/mL) containing 20 mg/mL IPBA. The Alexa350-labeled tyramide should be dissolved in MilliQ water. Tyramides in dimethylformamide can be stored in the freezer; tyramides in aqueous solution should be stored in the refrigerator.
26. 20% (w/v) PFA solution.
- a. Add 20 g of PFA to 70 mL of sterile deionized water (see **Note 2**).
 - b. Heat to approx 60°C while stirring (must not boil!) and add solid NaOH until suspension is clear (approx 0.5 h).
 - c. Add 5 mL of 20X PBS, pH 7.6.
 - d. Adjust pH to 7.6 with fuming HCl and add deionized H_2O to 100 mL final volume.
 - e. Filter through 0.2- μm filter.
 - f. Bubble with N_2 or He for 2 to 5 min to remove oxygen, and close the bottle (airtight).
 - g. If kept oxygen-free in the dark at room temperature, the PFA fixative can be stored for more than 1 yr. PFA fixative not treated with N_2 or He should be used within days.
27. Enzyme solutions for cell permeabilization (prepare fresh when needed).
- a. Dissolve lysozyme in 0.05 M EDTA and 0.1 M Tris-HCl, pH 8.0 (final concentration, 10 mg/mL).
 - b. Prepare achromopeptidase solution (final concentration 60 U/mL) with 0.01 M NaCl and 0.01 M Tris-HCl, pH 8.0.
 - c. Dissolve proteinase K in TE buffer (final concentration ranging from 0.1 to 10 $\mu\text{g/mL}$) and mix gently.
28. Preparation of hybridization buffer and washing buffer.
- a. Hybridization buffer: mix in a 50-mL tube: 3.6 mL of 5 M NaCl, 0.4 mL of 1 M Tris-HCl, 20 μ L of 20% (w/v) SDS, x mL deionized H_2O (see **Table 1**), x mL formamide (see **Table 1**), and 2.0 mL of 10% (w/v) blocking reagent. Add 2.0 g of dextran sulphate. Heat (40 to 60°C) and shake until the dextran sulphate has dissolved completely. Aliquots of the buffer can then be stored at -20°C for several months.

Table 1
Volumes of Formamide and Water for 20 mL of Hybridization Buffer

Formamide in hybridization buffer (%)	Formamide (mL)	Water (mL)
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

Table 2
Volumes of 5 M NaCl in 50 mL of Washing Buffer, With Corresponding Formamide Concentration in the Hybridization Buffer^a

Formamide in hybridization buffer (%)	5 M NaCl (μL)
20	1350
25	950
30	640
35	420
40	270
45	160
50	90
55	30
60	0
65	0
70	0

^aThe Na⁺ concentration is calculated for stringent washing at 37°C after hybridization at 35°C.

- b. Washing buffer: (produce freshly when needed) mix in a 50-mL tube: 0.5 mL of 0.5 M EDTA, 1.0 mL of 1 M Tris-HCl, x μL of 5 M NaCl (see [Table 2](#)), deionized H₂O to a final volume of 50 mL, and 25 μL 20% (w/v) SDS. The NaCl concentration in the washing buffer, as well as the formamide concentration of the hybridization buffer determines the stringency of the hybridization at the selected temperature.

29. CARD amplification buffer: mix in a 50 mL tube: 2 mL of 20X PBS, 0.4 mL of 10% (w/v) blocking reagent, and 16 mL of 5 M NaCl. Add 4 g of dextran sulphate. Add sterile deionized H₂O to a final volume of 40 mL. Heat (40 to 60°C) and shake until the dextran sulphate has dissolved completely. The amplification buffer can be stored in the refrigerator for several weeks.

3. Methods

3.1. Quality Check of Probes

Purified probe stocks are frequently delivered lyophilized. To test whether the probe concentration corresponds to the concentration claimed by the manufacturer, the probe is reconstituted with 50 μ L of deionized H₂O. The absorbance of the 1:100 diluted stock solution should be measured at 260 nm. Because both the HRP and the probe contribute to the absorption maximum at 260 nm, the probe concentration has to be lowered by a correction factor (cf) of 0.276.

$$\text{OD}_{260} = \text{measured OD}_{260} - \text{measured OD}_{404} \times \text{cf}$$

After this correction, 1 OD₂₆₀ nm is equivalent to approx 20 ng/ μ L of single-stranded DNA oligonucleotide. Furthermore, the labeling of the oligonucleotide should be checked. The absorption of the peroxidase is measured at 404 nm (A_{404}). Presuming optimal labeling, the peak ratio (OD₂₆₀/OD₄₀₄) should be approx 3.

Probe working solutions are prepared at a concentration of 50 ng/ μ L DNA and stored in small portions (50 to 100 μ L) at -20°C . Once thawed, the HRP labeled probes should be stored in the refrigerator and must not be refrozen, because repeated freeze-thawing will damage the peroxidase. When stored at 4°C , the probe working solutions can be used for up to 6 mo.

3.2. Sample Fixation and Preparation

3.2.1. Water Samples

1. Add buffered particle-free PFA (final concentration 1 to 2% [v/v]) to the sample and fix at 4°C for 24 h, or at room temperature for 1 h. This works well for marine plankton and benthos. For freshwater samples containing high numbers of Gram-positive Actinobacteria, a fixation in 50% (v/v) ethanol for 24 h at 4°C is recommended (11).
2. Filter the samples gently (pressure of approx 5 mmHg) onto white polycarbonate filters and wash twice with 5 to 10 mL of particle-free deionized water.
3. The air-dried filters can then be stored at -20°C from several months to years.

3.2.2. Sediment Samples

1. Fix sediment as described in **Subheading 3.2.1**.
2. Wash the fixed samples three times in 1X PBS, with centrifugation at 16,000g for 10 min between washes, and store samples in PBS/ethanol (1:1) at -20°C until further processing.
3. Detach cells from sediment particles by pipetting 100 μ L of sediment into 900 μ L of 1X PBS, and sonicate at minimum power for 20 s with a sonication probe

MS73. Mix the sonicated sample with 0.001% SDS and 0.1% agarose in PBS at 55°C for 3 to 5 min.

4. Pipet 10 μ L of sample suspension onto glass slides and let dry at room temperature. Then dehydrate slides in ethanol (50% for 5 min, 80% for 1 min, and 96% for 1 min) and let dry at room temperature.

3.3. Attachment

Cell wall compositions of bacteria and archaea from different environments may be diverse. Therefore, one has to consider different permeabilization and fixation strategies for each cell type. Here, we describe protocols developed for CARD-FISH of marine planktonic archaea and bacteria (8,10), for marine benthic bacteria (5), and for freshwater samples dominated by Actinobacteria (11).

To prevent cell loss during the permeabilization procedure, the cells have to be attached onto polycarbonate filters, for example with agarose. Although this procedure did not cause cell loss in our samples, we recommend that cell densities be checked before and after pretreatment and hybridization.

1. Boil the low-gelling-point agarose (0.1%) before each use in a microwave oven.
2. Fill the agarose in a Petri dish and let it cool to 35 to 40°C.
3. Dip the filters with both sides into the agarose and put the filters face-down onto a clean glass plate or onto Parafilm.
4. Let the filters dry at 20 to 40°C for approx 10 to 30 min.
5. To remove the filters from the glass plate, pipet ethanol (96 to 80% [v/v]) onto the filters and carefully peel them off.
6. Let the filters air-dry on a paper tissue.

3.4. Inactivation of Endogenous Peroxidases (see Note 3)

1. Incubate the filter sections in 50 mL of 0.01 M HCl for 10 min at room temperature.
2. Wash filters in 50 mL of 1X PBS, then in 50 mL of deionized H₂O. Filter sections can then be further processed or air-dried and stored in a refrigerator.

3.5. Permeabilization

3.5.1. Permeabilization With Lysozyme

1. Incubate the filters in lysozyme solution for 60 min at 37°C.
2. Wash the filters twice in 50 mL of deionized H₂O, then in 50 mL of absolute ethanol. Let filters air-dry.

3.5.2. Permeabilization With Achromopeptidase

For CARD-FISH staining of Gram-positive freshwater Actinobacteria, cell walls need to be permeabilized with lysozyme, followed by a digestion with achromopeptidase (26).

1. Incubate filters in achromopeptidase solution for 30 min at 37°C.
2. Wash filters as described in **Subheading 3.5.1., step 2** (see **Note 4**).

3.5.3. Permeabilization With Proteinase K

For CARD-FISH of Archaea and other microbes that were not amenable to the permeabilization strategies given in **Subheading 3.5.**, the treatment with proteinase K is the best alternative (**8**). However, the digestion with proteinase K is critical, because cell lysis will occur at high concentrations of the enzyme or after very long incubation times. In addition, the specific fixation conditions (duration and PFA concentration), as well as the cell wall composition of the target microorganisms, will influence the degree of digestion. Therefore, the appropriate concentration of proteinase K and the optimal incubation period might have to be tested and modified for particular samples and target cells.

1. Incubate the filters or slides in proteinase K solution for 20 to 60 min at 37°C.
2. Wash filters three times in 50 mL of deionized H₂O, then once in 50 mL of absolute ethanol. Let the preparations air-dry.
3. After permeabilization, the filters can be stored at -20°C for several months.

3.6. Hybridization

1. Cut the filters in sections (for example, ~16 sections can be cut out of a filter with a diameter of 47 mm). Label sections with a lead pencil, because other markers might contain fluorescent compounds.
2. Mix hybridization buffer and probe (300:1). Place filter sections in a reaction vial (0.5 mL to 2 mL, depending on the number of sections) and pipet the hybridization mix onto the sections. At least two-thirds of the total volume of the reaction vial should be filled with buffer. Hybridize on a rotation shaker (~10 rpm) for 2 to 15 h at 35°C (see **Note 5**).
3. For stringent washing, prepare washing buffer and preheat at 37°C. Wash sections after hybridization for 5 min in 50 mL of washing buffer. Do not let the filter sections run dry during hybridization! This will reduce the activity of the HRP.

3.7. Catalyzed Reporter Deposition (Cytochemical Probe Detection)

1. Remove the filter sections from the washing buffer and incubate them in 50 mL PBS for 15 min at room temperature to equilibrate the probe-delivered HRP.
2. Prepare fresh 100X H₂O₂ stock by mixing 1000 µL of 1X PBS with 5 µL of 30% H₂O₂.
3. Mix 1000 µL of amplification buffer with 10 µL of the 100X H₂O₂ stock and 1 to 5 µL of fluorescently labeled tyramide.
4. Put the filter sections in a reaction vial and pipet the amplification buffer with the tyramide onto the sections. Incubate for 10 to 15 min in the dark, at temperatures between 37 and 46°C (FISH signal intensity increases with temperature).
5. Remove excess liquid by dabbing filters onto blotting paper. Wash sections in 50 mL of PBS for 5 to 15 min at room temperature in the dark.
6. Wash sections twice in 50 mL of deionized water, then in 50 mL of absolute ethanol. Let sections air-dry.

7. Stain with 1 mg/mL DAPI (for 5 to 10 min at room temperature) then wash with deionized water, ethanol, and let air-dry.
8. Microscopy is performed after embedding in low-fluorescence glycerol mountant containing an antibleaching agent. The embedding medium can also be directly amended with DAPI (final concentration, 1 $\mu\text{g/mL}$), but such a mix should be freshly prepared each month. Stained preparations can also be stored at -20°C until further processing (see **Note 6**).

3.8. Alternative: Multicolor CARD-FISH

Sometimes, it might be necessary to localize two or more target species simultaneously, for example, to clarify the distribution of microorganisms within a dense community (e.g., biofilm, symbiosis; **refs. 4, 22, and 23**). For multicolor CARD-FISH with oligonucleotide probes directly linked to HRP, the hybridization and cytochemical detection of different targets have to be performed in sequence. After the first hybridization and detection of the probe by tyramide signal amplification, the probe-delivered HRP has to be inactivated (e.g., by acid, heat, or H_2O_2). Then the next hybridization with another probe and another fluorochrome for probe detection can be performed. Choose appropriate fluorochromes for multicolor FISH. For this purpose, ALEXA dyes are the fluorochromes of choice. In our laboratory, we combine ALEXA488-, ALEXA546-, Alexa 633-, and ALEXA350-labeled tyramides with good results.

1. Inactivate the probe delivered peroxidase from the first hybridization by incubating the filter sections in 0.01 M HCl for 10 min at room temperature.
2. Wash sections once with 1X PBS, and twice with deionized water.
3. A second hybridization followed by a second CARD using another fluorescently labeled tyramide can now be performed, as described in **Subheading 3.7**.

4. Notes

1. Active ALEXA dyes are purchased from Molecular Probes Inc., Eugene, OR. Because these succinimidyl esters can hydrolyze rapidly, all reagents have to be water free, and the active dye stock as well as the tyramine HCl stock must be prepared a few minutes before use.
2. Commercially available 35 to 37% formalin solution is often stabilized with methanol, which decreases FISH signal intensity and tends to precipitate on longer storage. Use mouth protection for handling of PFA powder—it is an irritant if inhaled.
3. Some microorganisms, e.g., from anoxic sediments, may contain peroxidases or enzymes with pseudoperoxidase activity. This can be tested by incubating a filter section in amplification buffer containing H_2O_2 and fluorescently labeled tyramides. Cells with peroxidase activities will show bright fluorescence (for details, see **Subheading 3.7**). These enzymes have to be inactivated, for example by treatment with hydrochloric acid.

4. This protocol might be suitable for other samples rich in Gram-positive bacteria. As an alternative to enzymatic digestion, cell walls can also be treated with diluted acid or detergents. These strategies may be useful, for example for the permeabilization of archaeal cells.
5. Alternatively, put filter sections on glass slides coated with Parafilm; several filter sections can be placed on one slide and hybridized simultaneously with the same probe. Add hybridization mix on the samples and place the slide with the filter sections in a horizontal position into a 50-mL polyethylene tube, filled with a lint-free tissue paper and 2 mL of a formamide-water mix (use the same formamide concentration as in the hybridization buffer).
6. The tyramide signal amplification can be enhanced by the addition of salts (21). The deposition of tyramides labeled with Cy3, fluorescein, Alexa350, Alexa488, Alexa633, and Alexa546 is enhanced by presence of NaCl. Preferably, concentrations of NaCl in the amplification buffer range from 2 M to saturation. IPBA (20 mg IPBA/mg of tyramide) will also enhance the CARD-FISH signal. This works well for tyramides labeled with fluorescein, Alexa488, Alexa633, and Alexa546, but not for tyramides labeled with Cy3 and Alexa350. For other fluorescent labels, one will need to test either of the salts as well as the combination of both.
7. Possible reasons for high background fluorescence:
 - a. Too high tyramide concentration. Either decrease the tyramide concentration or increase the blocking reagent concentration.
 - b. Too high probe concentration. If the background is covered with tiny fluorescent dots, the amount of probe might be too high. Check the probe concentration; 0.2 ng/ μ L is plenty.
 - c. Too short washing after CARD. Prolonged washing in deionized water, and/or several changes with fresh water may help.
8. Possible reasons for low FISH signal intensity:
 - a. Low ribosome content of the target cells. Increase the tyramide concentration or the temperature during the tyramide signal amplification (up to 46°C). A prolonged hybridization time (up to 15 h) may also help.
 - b. Too low tyramide concentration.
 - c. The probe-delivered HRP has too low or no activity. Check the probe. The probe should be thawed only once and should not be stored in the fridge for more than 6 mo. Check the pH of the PBS, it should be approx 7.6. Check the H₂O₂ concentration and its age (it should be prepared freshly from stock before incubation). Check the reactivity of the tyramide.
 - d. The HRP is badly coupled to the probe. The amount of unlabeled oligonucleotide can be estimated spectrophotometrically.
 - e. The HRP-labeled probe cannot penetrate the cell wall. Modify the permeabilization protocol.

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IV

KINETIC (“REAL-TIME”) PCR

Introduction to Kinetic (Real-Time) PCR

John Mackay

Summary

Kinetic or real-time PCR continues to develop at a rapid rate since its development in the early 1990s. New applications are continually being found for this technique and it is replacing conventional PCR in many fields because of its speed, reduced hands-on time, and because the closed-tube format greatly reduces the chance of reaction contamination. This chapter covers the basis of kinetic PCR and also discusses some of the parameters that need to be considered even before the actual amplification—such as nucleic acid extraction and the complementary DNA synthesis step required in the instance of gene expression studies.

Key Words: cDNA synthesis; crossing point; DNA extraction; experimental design; kinetic PCR; RNA extraction.

1. Introduction

With the rapid integration of digital developments in the 1990s, it was inevitable that PCR would also join the computer revolution. Indeed, kinetic or real-time PCR may be likened to an online Southern hybridization. Similar to the PCR process itself, the digital version is still being developed for novel applications, 10 yr after first being published.

It was rapidly identified in the PCR chronology that varying amounts of template usually did not result in discernible differences in amplified DNA amounts. This was because of the plateau effect late in the PCR cycles, in which increasing cycles did not result in any further increase of amplified DNA. Only by following the reaction cycle by cycle could the informative exponential cycles be accurately determined.

Although the processes were first described in publications by Russell Higuchi and colleagues in 1992 and 1993 (1,2), it was not until the first commercial

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instruments were released in the mid- to late-1990s that the techniques started to become established in mainstream applications. The first of these instruments (Prism 7700 from Applied Biosystems) used TaqMan[®] probe chemistry, whereas the LightCycler (Idaho Technologies and now sold by Roche Applied Science) introduced the intercalating dye SYBR Green technology as a cost-effective alternative to probes. These and other fluorescent chemistries are described in Chapters 14–17. Most instruments can now use multiple chemistries, depending on the operator's preference and requirements.

Although the major purpose has been for the quantification of initial template, the speed and specificity advantages that real-time PCR offers has meant that it has replaced gel-based detection in many laboratories. This is especially the case in clinical diagnostics, in which these advantages, together with the greatly reduced contamination risks afforded by closed reaction vessels, has led to the development of real-time PCR parameters for many disease applications. These are described more fully in Chapters 14 to 16.

In addition, probes have replaced restriction endonuclease digestion and gel electrophoresis for many genotyping applications. These methods rely mostly on either the specificity of multiple probes to detect one or another allele (3) or the differential melting temperature of a single probe (4). Other strategies have used allele-specific amplification, with fluorescent detection being used solely to replace the conventional detection methods (5).

2. Principles

The most common detection technology for PCR products still remains agarose gel electrophoresis, with staining of the DNA bands using a fluorescent dye, such as ethidium bromide. However, when performing PCR and subsequently using gel electrophoresis, it is often easy to see that the amount of target DNA in the original sample does not equate to the observed band intensity of amplified DNA after gel electrophoresis (Fig. 1, inset).

The reason for this can be seen by the sigmoidal plots that represent the PCR amplification process (Fig. 1). It is only during the exponential phase of amplification that the PCR process reflects the amount of added target sequence. As conditions become unfavorable for amplification during later cycles (poor buffering conditions, enzyme inactivation, and inhibitors such as pyrophosphates), the amplification curve enters a plateau phase. It is during this phase (in which amplifications essentially catch up to each other in terms of amplified DNA amounts) that the reaction is usually measured—with technologies such as gel electrophoresis or enzyme-linked immunosorbent assay-based methods.

It is because of the detection nature of these reactions that one of its most popular terms is real-time PCR. Measuring the amount of amplified DNA at the end of each cycle via a fluorescent chemistry and linked computer allows the

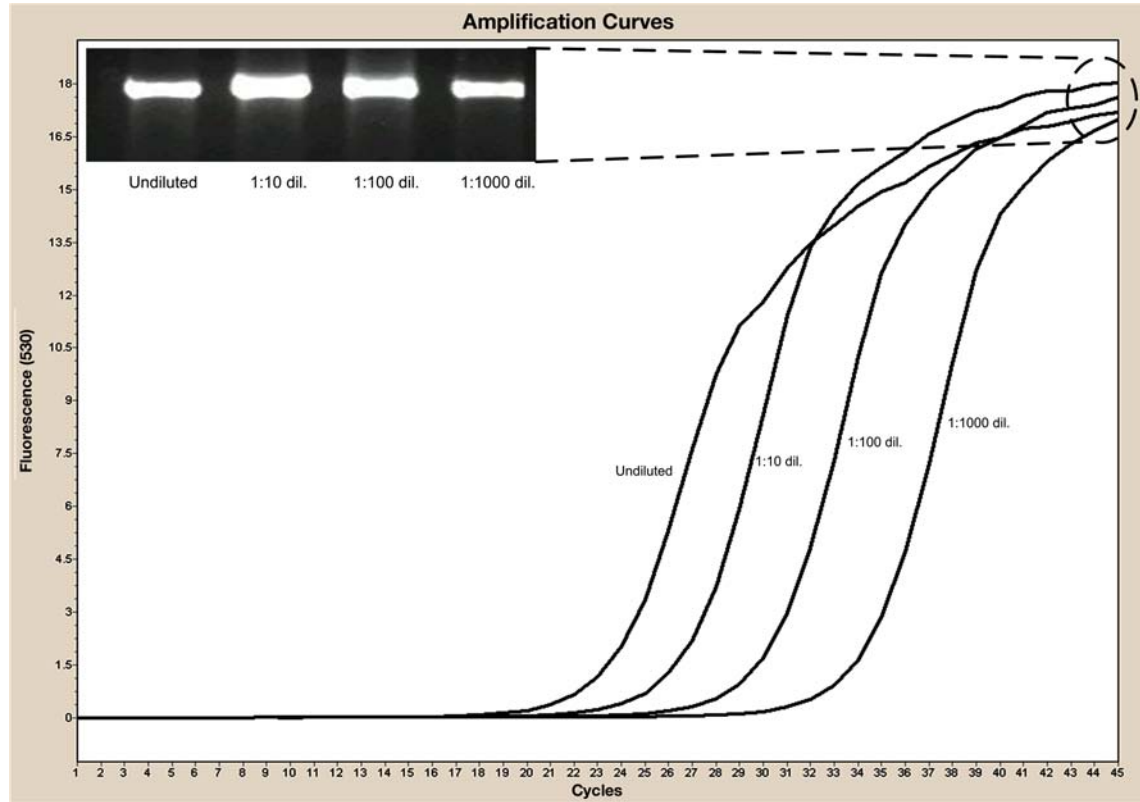


Fig. 1. A 129-bp product is amplified by real-time PCR from dilutions of bacterial DNA, ranging from neat to 1:1000. At the end of the run, the reactions were recovered and analyzed by agarose gel electrophoresis (inset). Although the dilutions could be differentiated by the cycle at which the fluorescence sharply increased (crossing point), the inset shows little difference in band intensity on an agarose gel.

operator to accurately determine the exponential cycles of the amplification process.

The point at which the amplification enters the exponential phase is directly proportional to the amount of starting template. This point is defined as the crossing point (Cp) or cycle threshold (Ct) and is either defined across all samples using a threshold band or can be determined mathematically for each amplification curve, depending on the software used.

3. Parameters Affecting Experimental Design

3.1. DNA Extraction

For quantification assays, a higher level of DNA purity is required for real-time PCR when compared with conventional amplification reactions (unpublished observations). Contaminants present in a sample may distort the fluorescence resulting in a high background level. Other substances—ranging from heme in blood through to phenolics in plant samples—can inhibit the amplification, leading to delayed detection of the signal (if at all) and artificially low copy numbers assigned as a result. In these instances, longer extraction procedures may be required, such as those detailed in Section 1 of this book, using specific buffers (e.g., CTAB) to remove contaminating substances.

To achieve the speed, purity, and robustness of DNA isolation for the number of samples often used in real-time PCR assays, column-based silica/glass fleece adsorption spin columns formats are widely used. This is often because of their easy adoption in laboratories, with only standard laboratory equipment (e.g., pipets and microcentrifuges) being required. Very high-sensitivity real-time PCR applications have been described using adapted elution protocols for these DNA extraction columns (6). An increasingly popular method—especially in higher throughput clinical detection of pathogens—is the use of instruments to automate the extraction of the sample nucleic acid. In some cases, these instruments can then prepare the amplification reactions of master mix and extracted DNA (7).

3.2. RNA Extraction

RNA is required for comparing gene expression levels. Gene quantification may be distorted by any PCR inhibitors co-purified with the RNA and, therefore, once again, a high-quality extraction is required. One of the most common methods uses an acid-phenol method based on the method of Chomczynski (8) and sold commercially as “Tri-” from a number of suppliers (e.g., Trizol™, Tripure™, or Tri-reagent™).

The other popular format is again the column-based extraction. For very small samples (e.g., laser-capture microdissected samples), mini columns are available (e.g., Arcturus) to ensure that the purified template can be eluted in very small volumes to retain a sufficient concentration for subsequent steps.

Unless an extraction protocol specifically includes a DNase digestion step, most protocols (spin-column or acid phenol, included) will result in some degree of DNA contamination (9). This may lead to artificially high target measurements caused by amplification of this contaminating DNA. Often target amplicons are designed to span across adjacent exons (allowing *distinction* of amplified DNA template), or the PCR primers themselves may span exon boundaries (preventing *any* amplification of DNA template), but this design may not be possible (e.g., in bacterial targets in which there are no intronic sequences).

There is often a reluctance to perform DNase digestion steps, especially in cases in which the target sequence may be in low abundance. This is because of the concern that the desired RNA is degraded either by the DNase itself or by the subsequent DNase inactivation steps (required to prevent degradation of subsequent amplified DNA). A number of reports have looked at optimizing the action of DNase I and also the subsequent inactivation of the enzyme while preventing the metal-catalyzed hydrolysis of the RNA template during this step (10–13).

3.3. Complementary DNA Synthesis

Most gene expression studies use two-step reverse transcriptase (RT)-PCR; that is, complementary DNA (cDNA) is prepared from the RNA and then, in subsequent reactions, an aliquot of the cDNA is used for amplification of the target gene and another aliquot is used for amplification of the reference gene. However, there are also one-step systems and kits available in which the reverse transcription and PCR may be performed in the one reaction, minimizing the opportunity for cDNA pipetting mistakes and contamination. On the downside, RNA samples are often in very limited supply, and one cDNA reaction (from 100 ng to 1 µg template RNA) can provide enough cDNA for 10 (or more, depending on the target gene concentration) amplification reactions. However, it has been described that the prepared cDNA may not be as stable as the original RNA template (14), thus, suggesting that cDNA may not be a suitable archival template.

The most popular reverse transcriptase enzymes are mutant forms engineered to be RNase H-deficient (e.g., SuperScript, Invitrogen Corp.). However, an earlier detection (lower crossing point) of many target genes has been demonstrated by performing an RNase H digest after cDNA synthesis with an RNase H minus enzyme (15), showing that the cDNA:RNA hybrid may prevent maximum primer annealing during the PCR. This improvement has also been described for conventional PCR (15). Also used are enzymes with their wild-type RNase H activity (e.g., AMV reverse transcriptase) and these have shown comparable or higher sensitivity to RNase H mutant enzymes in real-time PCR applications (T. Anderson, personal communication, 2004).

A number of enzymes are now engineered to withstand higher reverse transcription temperatures, and these higher temperatures can assist the reaction when using high G/C content RNA templates, to ensure the melting of secondary structure (ref. 16; Chapter 14).

There are a number of different priming strategies for the cDNA synthesis: oligo dT (from 15 to 20 deoxythymidine bases), random primers (from 6-base through 15-base oligonucleotides), a specific primer (e.g., the downstream PCR primer), or any combinations of these. Indeed, efficient cDNA synthesis has been shown in the absence of any of these options (17,18).

From an internet survey of real-time PCR users (<http://www.yahooogroups.com/qpcrlistserver/>), the most popular strategy was using oligo-dT (41% of respondents); exploiting the 3' poly(A) tail on eucaryotic mRNA. The next most popular was random hexamers, with 27% showing a clear preference for the oligo-dT approach, although this has typically meant that ribosomal RNAs (e.g., 18S RNA) could not be used as reference genes because of the lack of the poly(A) tail.

The optimal strategy seems to be dependent on personal preference because there is no clearly superior method described in the literature. A comparison between oligo dT, random hexamers, and a gene-specific priming strategy showed that there was no clear consensus on the optimal strategy in terms of the most sensitive (lowest crossing point) detection (18). Interestingly, the method giving the most sensitive detection was not necessarily the most reproducible, adding a further consideration to the choice of priming strategy. However, another report described the superior performance of gene-specific priming for a number of genes over oligo-dT and random hexamers methods (19).

Although most quantitative RT-PCR strategies involve taking an aliquot of the cDNA reaction (a volume typically comprising 10–50% of the total PCR volume) directly in the PCR reaction, real-time PCR allows the user to easily monitor inhibition of the PCR. For example, if amplifying a neat and 1:10 dilution of a template, it would be expected that the crossing points would be approx 3.3 cycles apart ($2^{3.3} = 10$). However, similar or even earlier crossing points can often be observed for template dilutions compared with neat concentration samples (Fig. 2). Although lower-purity nucleic acid templates can be a reason (this is also observed with DNA templates), a number of components in the cDNA synthesis reaction have been found to be inhibitory to the subsequent PCR: DTT, especially in the case of SYBR Green reactions (19), but also probe reactions (20); and the reverse transcriptase enzyme itself (20). Although purification of the cDNA may intuitively suggest limiting the sensitivity of the subsequent PCR because of loss of template during the process, in fact, purification has been shown to increase the linearity of amplification across dilutions, increase the reaction efficiency and robustness of the amplification (20,21).

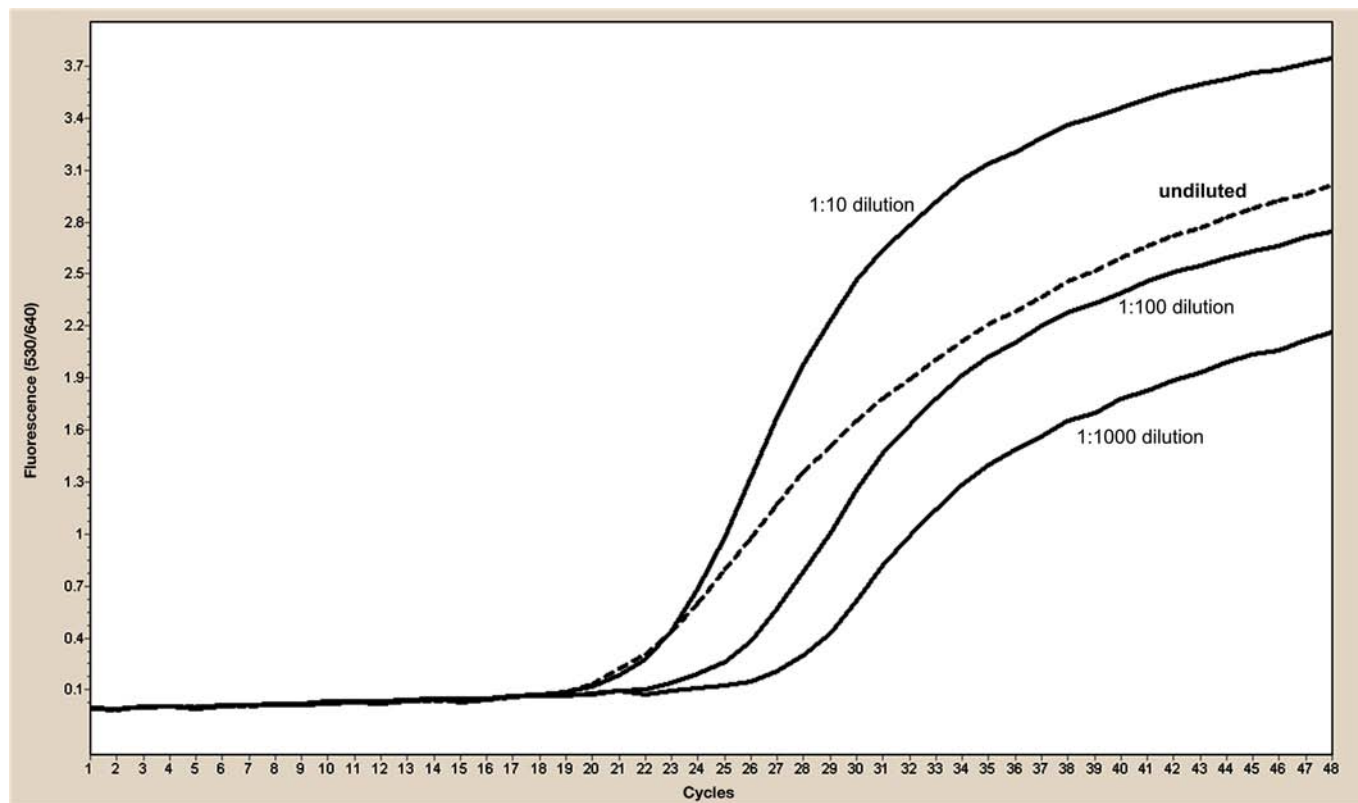


Fig. 2. Amplification of a 110-bp β -globin product from dilutions of DNA from blood using a rapid alkaline lysis method. The dotted line (---) represents the neat extract where the crossing point is delayed due to inhibition—indeed, appearing at a similar crossing point to the 1:10 dilution.

4. PCR Reaction

Kinetic PCR differs greatly from conventional PCR reactions in that there are a number of new parameters that must be considered in the case of precise and accurate quantification. Issues such as efficiency of reactions, crossing points, and standard curves are new aspects that are raised with this technology, and these are well-described in the following real-time PCR chapters in this section. As well as the primary quantification and detection uses for kinetic PCR, optimal experiment parameters can be readily identified, such as extraction methods (22) or choice of reverse transcriptase strategy (18,19).

Most operators favor the use of a master mix kit, whereby only the primers (and probe, if required) need be added, which greatly simplifies the process and decreases the opportunities for error with new users. A survey of users showed that the main reason for using kits was for the sake of reproducibility, with only 21% (for quantitative PCR) or 34% (quantitative RT-PCR) using solely individual reagents (23).

The dynamic range of an assay can be rapidly established along with the analytical sensitivity using dilutions of a known quantity sample.

By using dilutions of a sample, the efficiency of an assay can be calculated using the slope of the standard curve in the equation $10^{-1/\text{slope}}$. A well-performing assay may not have an exact efficiency of 2 (representing perfect doubling of DNA per cycle) but it should be highly reproducible between runs. Typical efficiencies range from 1.7 to just over 2—a limitation of the algorithm because 2 is obviously the maximum theoretical yield per cycle. Different assays (e.g., target and reference genes in a gene expression assay) will often have different efficiencies and allowing for these differences—should they exist—leads to improved quantification accuracy (24,25).

In summary, the use of kinetic PCR has grown exponentially, similar to the reaction itself. New applications are being published rapidly and, along with these, new methods for using and interpreting the data are generated. Although the original PCR techniques were mostly the domain of researchers for many years before being adopted for clinical diagnostic applications, this gap has now closed, with researchers and diagnostic users both exploiting the benefits of kinetic PCR. This can only further speed the widespread adoption of this exciting method.

5. Websites of Interest

There are many useful websites that users may find helpful.

1. Forums to ask questions and share information: groups.yahoo.com/group/qpcrlistserver, groups.yahoo.com/group/lightcycler, groups.yahoo.com/group/realtimemcr/.
2. Information and papers on a wide range of real-time PCR aspects. Covers upcoming meetings and seminars: www.gene-quantification.info/.

3. Technical notes (of interest to all real-time PCR) and information relating to the LightCycler: www.lightcycler-online.com.

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Validation of Short Interfering RNA Knockdowns by Quantitative Real-Time PCR

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Summary

RNA interference (RNAi) is a natural mechanism, that is triggered by the introduction of double-stranded RNA into a cell. The long double-stranded RNA is then processed into short interfering RNA (siRNA) that mediates sequence-specific degradation of homologous transcripts. This phenomenon can be exploited to experimentally trigger RNAi and downregulate gene expression by transfecting mammalian cells with synthetic siRNA. Thus, siRNAs can be designed to specifically silence the expression of genes bearing a particular target sequence. In this chapter, we present methods and procedures for validating the effects of siRNA-based gene silencing on target gene expression. To illustrate our approach, we use examples from our analysis of a Cancer Gene Library of 278 siRNAs targeting 139 classic oncogenes and tumor suppressor genes (Qiagen Inc., Germantown, MD). Specifically, this library was used for high-throughput RNAi phenotype analysis followed by gene expression analysis to validate gene silencing for siRNA that produced a phenotype. Methods and protocols are presented that illustrate how sequence-specific gene silencing of effective siRNAs are analyzed and validated by quantitative real-time PCR assays to measure the extent of target gene silencing, as well as effects on various gene expression end points.

Key Words: Dicer; gene expression; gene knockdown; gene quantification; gene silencing; housekeeping genes; nonradioactive analysis; quantitative real-time PCR; reference genes; relative quantification; RISC; RNA; RNAi; siRNA; transcription.

1. Introduction

Posttranscriptional gene silencing mediates resistance to both endogenous and exogenous pathogenic nucleic acids, such as double-stranded RNA, and manipulates the expression of functional genes by way of a mechanism known as RNA interference (RNAi; **refs. 1 and 2**). RNAi has facilitated the identification

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of gene function in a number of species (3). This natural phenomenon can be experimentally exploited to manipulate gene expression in a number of ways. These include transfecting mammalian cells with synthetically made, double-stranded, short interfering RNA (siRNA) molecules of approx 20 to 25 nucleotides, in vitro transcribing RNAs, using RNase III-derived products, and making use of in vivo expressed single-stranded transcripts that form a hairpin corresponding to a siRNA, short hairpin RNA. RNAi has been rapidly adopted as a functional genomics tool in a broad range of species and has been adapted to allow for the transient or stable knockdown of gene expression generation in cell lines and animals. Applications for RNAi in mammalian cells include determination of the function of uncharacterized genes, study of the interaction of proteins within pathways, and the development of transgenic cell lines and animals (4–6). Additionally, the RNAi mechanism has enormous potential as a therapeutic agent (7–11). RNAi has rapidly been adapted for the high-throughput analysis of gene function (12–14). These large-scale RNAi screens have facilitated the functional assignment of a number of genes with previously undefined function, and have identified a new role for certain genes (both characterized and uncharacterized) in critical pathways.

The mechanism of RNAi is mediated by RNA-induced silencing complex (RISC), which binds to complementary messenger RNA (mRNA) sequence on the target RNA molecule by aligning with the antisense strand of the siRNA and destroying the mRNAs homologous to the double-stranded siRNAs (15,16). The sequence-specific mRNA degradation is mediated by a member of the RNase III family of nucleases, commonly known as Dicer, which enzymatically cleaves long double-stranded RNAs into short siRNAs (Fig. 1; refs. 17 and 18). Consequently, siRNAs can be designed to specifically knockdown the expression of genes bearing a particular target sequence, although not all target sequences are amenable to knock-down. Specific bioinformatics strategies to design siRNAs, which increase the potency of silencing and minimize the chance of nonspecific or off target effects, have been developed by a number of commercial and academic laboratories. We have used such a strategy to design a Cancer Gene Library of 278 synthetic siRNAs against 139 known oncogenes and tumor suppressor genes (Qiagen Inc., Germantown, MD). This library has been synthesized by using TOM-amidite chemistry to yield 21-nucleotide RNA oligonucleotides, which were purified by high-performance liquid chromatography and annealed to produce high-purity and high-quality siRNA with 2-base 3' overhangs with dTdT on both strands (Qiagen Inc.).

High-throughput transfection of HeLa cells with the Cancer Gene Library of 278 synthetic siRNAs, as well as with negative- and positive-control siRNAs, on a 96-well plate, was used to screen for RNAi phenotype of decreased cell proliferation. Quantitative real-time PCR was used to validate the siRNA-mediated decrease in the level of the targeted transcript. In this chapter, we present specific

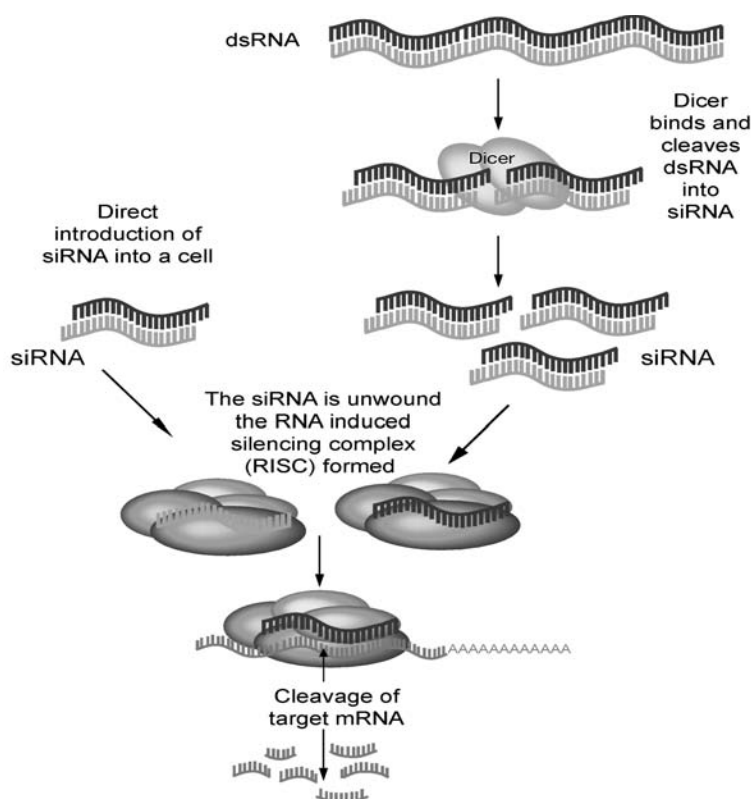


Fig. 1. RNAi mechanism. (Adapted from [ref. 18](#).)

methods and procedures for quantitative real-time PCR-based validation of the siRNA-mediated decrease in the target gene mRNA expression.

To date, many methodologies have been described as tools to investigate gene expression analysis. Common techniques for studying transcriptional analysis include: Northern blotting, *in situ* hybridization, semiquantitative quantitative real-time PCR, RNase protection assay, competitive quantitative real-time PCR, microarray analysis, and quantitative real-time PCR ([19](#)). There are significant advantages of quantitative real-time PCR compared with the rest of the gene expression quantification methods. These include enhanced sensitivity and accuracy, a large dynamic range, high-throughput capability, the ability to perform multiplex reactions, faster and more reliable amplification, and lack of post-PCR manipulations. This powerful technique has become the method of choice for rapid and quantitative monitoring of specific gene expression levels. Currently, the commercial availability of a wide range of gene expression assays for quantitative real-time PCR validation of siRNA hits and high-throughput application of these assays in 96- or 384-well formats also make this technology attractive for end point analysis.

2. Materials

2.1. Cell Culture

1. siRNA cancer gene library (Qiagen Inc.; *see Note 1*).
2. HeLa Cells (human cervical carcinoma cell line; ATCC, Manassas, VA).
3. MCF-7 Cells (human breast carcinoma cell line; ATCC).
4. Dulbecco's Modified Eagle's Medium: high glucose, with pyridoxine hydrochloride, without L-glutamine, without sodium pyruvate.
5. 5% fetal bovine serum.
6. 1% penicillin/streptomycin.
7. L-Glutamine.
8. Lipofectamine 2000 (Gibco, Invitrogen Corporation, Frederick, MD).
9. Opti-MEM I: Reduced serum medium, modification of MEM (Eagle's) 1X, with HEPES buffer, with 2400 mg/L sodium bicarbonate, with L-glutamine (Gibco).
10. 96-well plates.
11. CellTiter-Blue Cell Viability Assay (Promega, Madison, WI).
12. Fluorescent plate reader.

2.2. Quantitative Real-Time PCR

1. RNA extraction system (e.g., RNeasy Mini total RNA extraction kit, Qiagen Inc.).
2. Spectrophotometer.
3. Agarose gels and gel equipment.
4. Reverse transcriptase (e.g., ThermoScript RT-PCR System, Invitrogen).
5. TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA; *see Note 2*).
6. TaqMan® Universal PCR Master Mix (Applied Biosystems; *see Note 2*).
7. Hard-shell, thin-wall, 96-well microplates, and optical 96-well fast thermal cycling plates.
8. Real-time PCR machine (e.g., DNA Engine, Opticon 2, MJ Research Inc., ABI 7900HT Fast Real-Time PCR System).

3. Methods

Description of the methods outline the reverse transfection of siRNAs into HeLa cells, the measurement of cell proliferation (the cell viability assay) and phenotype assessment of siRNA hits, the extraction of total RNA, the synthesis of complementary DNA (cDNA), the quantitative real-time PCR assay, and the analysis of gene expression data using quantitative real-time PCR.

3.1. Reverse Transfection

1. Spot individual siRNAs (Qiagen Inc.) at a 13 nM concentration into black clear-bottom 96-well plates.
2. Add 15 μ L of freshly prepared dilution of Lipofectamine 2000/serum-free DMEM at a 1:150 ratio (13.2 μ L of Lipofectamine to 1966.8 μ L of serum-free DMEM per 96-well plate) to allow for lipid/siRNA complex formation for 30 min (*see Note 3*).

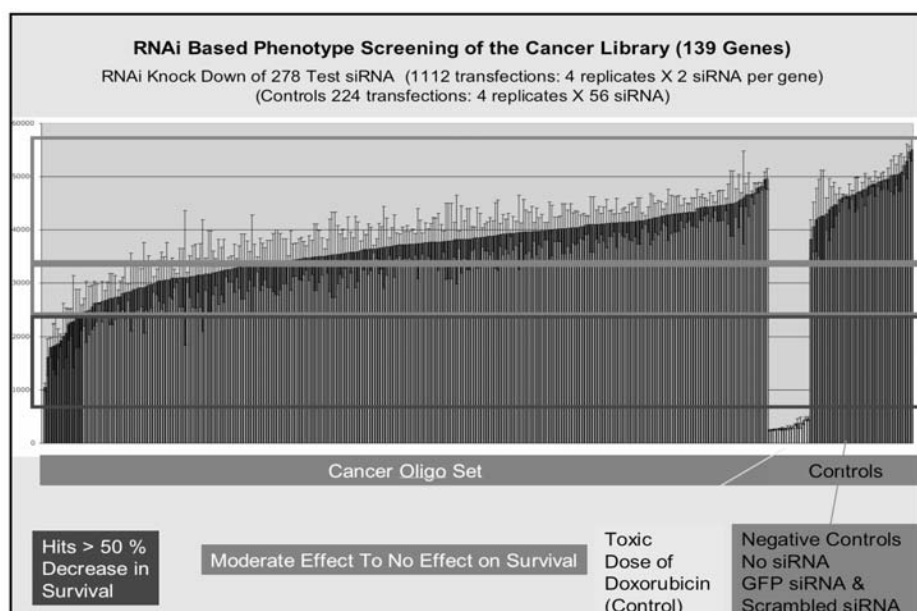


Fig. 2. RNAi-based phenotype screening.

3. Add HeLa cells at a concentration of 4000 cells/well on top of the prepared complex of 278 cancer-associated siRNAs (Qiagen Inc.).
4. Incubate the HeLa cells with lipid/siRNA complex for 72 h at 37°C under 5% CO₂. **Figure 2** describes the RNAi-based phenotype screening of the siRNA cancer library in HeLa cells (see **Note 3**).

3.2. Cell Viability Assay

Monitor the HeLa cell viability at 72 h via a proliferation assay, using the CellTiter-Blue Cell Viability Assay (Promega; see **Note 4**).

1. Add 20 µL of CellTiter-Blue reagent (at 37°C) directly to each well.
2. Incubate the plates at 37°C for 4 h (see **Note 5**).
3. Determine the fluorescence intensity in relative fluorescent units using a fluorescence plate reader (BMG), with filters set at 544 nm excitation and 590 nm emission wavelengths (see **Note 6**).

3.3. Total RNA Extraction

Completely aspirate the cell culture media after determining the fluorescent intensity of the samples from **Subheading 3.2**. Great care should be taken to avoid introducing RNases into the RNA sample during or after the isolation of RNA procedure (see **Note 7**). At this stage, either store the samples at –80°C

for later use or proceed to the total RNA extraction in Step 3 using an RNeasy Mini Kit (Qiagen Inc.; see **Note 8**).

1. Disrupt and lyse the cells in 96-well plates (growth area $\sim 0.32\text{--}0.6\text{ cm}^2$; $\sim 4\text{--}5 \times 10^4$ cells) by adding 100 μL of guanidine isothiocyanate (GITC)-containing buffer (Buffer RLT, Qiagen Inc.) into each well (see **Note 8**).
2. Pipet up and down to disrupt and lyse the cells in each well and collect the total volume in individual eppendorf tubes per siRNA treatment.
3. Pipet the pooled samples from **step 2** with the same siRNA treatment directly onto individual QIAshredder spin columns (Qiagen Inc.) placed in 2-mL collection tubes, and centrifuge for 2 min at maximum speed to homogenize the lysate (see **Note 8**).
4. Add an equal volume of 70% ethanol to the homogenized lysate and mix well by pipetting to adjust binding conditions (see **Note 9**). Do not centrifuge. If some lysate is lost during homogenization, adjust the volume of ethanol accordingly. Visible precipitates may form after the addition of ethanol when preparing RNA, but the RNeasy procedure is not affected by this (Qiagen Inc.).
5. Apply up to 700 μL of the sample, including any precipitates that may have formed, to RNeasy Spin Columns placed in 2-mL collection tubes to adsorb RNA to the membrane of the columns.
6. Close the tube gently and centrifuge for 15 s at 8000g. Discard the flow-through. Reuse the collection tubes in Step 7. At this step, on-column RNase-free DNase digestion during RNA purification can be performed according to certain RNA applications (e.g., RNase-Free DNase Set, Qiagen Inc.; see **Notes 10** and **11**).
7. Re-assemble the column and collection tube and add 700 μL Buffer RW1 to the columns (see **Note 12**).
8. Close the tube gently, and centrifuge for 15 s at greater than 8000g to wash the columns. Discard the flow-through and collection tubes (see **Note 12**).
9. Transfer the columns into new 2-mL collection tubes.
10. Pipet 500 μL of Buffer RPE onto the columns (see **Note 13**).
11. Close the tubes gently and centrifuge for 15 s at greater than 8000g to wash the columns. Discard the flow-through.
12. Add another 500 μL of Buffer RPE to the column.
13. Close the tubes gently and centrifuge for 2 min at full speed to dry the membrane (see **Note 14**).
14. To elute the ready-to-use total RNA, transfer the columns to new 1.5-mL collection tubes.
15. Pipet 30 to 50 μL of RNase-free water directly onto the membrane (see **Note 15**).
16. Close the tube gently, and centrifuge for 1 min at 8000g to elute the total RNA (see **Note 16**).

3.4. cDNA Synthesis

Synthesis of cDNA from total RNA samples is the first step in the analysis of determining the degree of silencing exerted by siRNAs on the target genes in

Table 1
cDNA Synthesis

Step 1: Reagent volumes for cDNA master mix			
Components	One reaction (100 μ L)	One reaction (20 μ L)	No. of reactions times single- reaction volume
50 μ M oligo-dT ₂₀ (50 pmol/ μ L)	5 μ L	1 μ L	
10 mM dNTP mix	10 μ L	2 μ L	
10 pg to 5 μ g RNA	— μ L	— μ L	
DEPC-treated water	— μ L	— μ L	
Final volume	60 μ L	12 μ L	
Step 2: Reagent volumes for cDNA master mix			
Components	One reaction (100 μ L)	One reaction (20 μ L)	No. of reactions times single- reaction volume
5X cDNA synthesis buffer	20 μ L	4 μ L	
0.1 M DTT	5 μ L	1 μ L	
40 U/ μ L RNaseOUT	5 μ L	1 μ L	
DEPC-treated water	5 μ L	1 μ L	
15 U/ μ L ThermoScript RT	5 μ L	1 μ L	
Final volume	40 μ L	8 μ L	

question. Using the ThermoScript RT-PCR System (Invitrogen), set up cDNA reactions as outlined (see also **Note 17**):

1. Denature the RNA and primer at 65°C for 5 min, then place on ice.
2. Prepare the master mix in **Table 1**, step 1 based on whether 20- or 100- μ L reactions are to be performed. Multiply each given volume by the number of reactions to be performed to get the total volume of each reagent.
3. Vortex the 5X cDNA synthesis buffer for 5 s just before use and keep on ice.
4. Prepare another master reaction mix on ice (**Table 1**, step 2).
5. Pipet 8 μ L (20- μ L reaction) or 40 μ L (100- μ L reaction) of master mix into each reaction tube on ice.
6. Transfer the sample to a thermal cycler, preheated to the appropriate cDNA synthesis temperature, and incubate as follows, depending on the priming method: 50 pmol/ μ L oligo-dT₂₀ primer is primed for 50 min at 50°C; 50 ng/ μ L random-hexamer is primed for 10 min at 25°C, followed by 50 min at 50°C; and 10 pmol/ μ L gene-specific primer is primed for 50 min at 50°C.
7. Terminate the reaction by incubating for 5 min at 85°C.
8. Add 1 μ L (20 μ L reaction) or 5 μ L (100 μ L reaction) of RNase H and incubate for 20 min at 37°C (see **Note 17**).

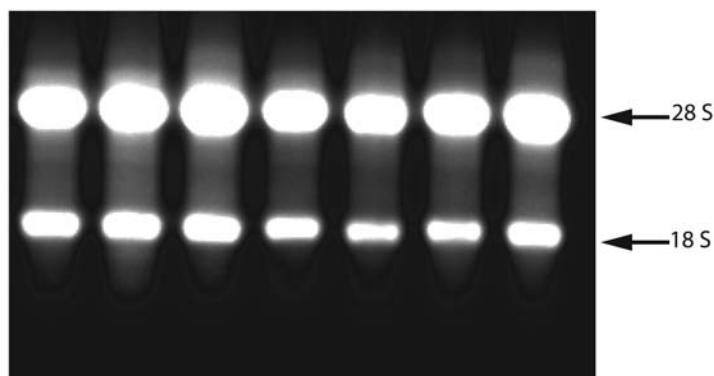


Fig. 3. Agarose gel of total RNA isolated (1–2 μ g) from HeLa cells.

9. The completed cDNA synthesis reaction can be stored at -20°C or used for quantitative real-time PCR reactions immediately.

3.5. Quantitative Real-Time PCR Assay

After the synthesis of cDNA, perform a standard quantitative real-time PCR for amplifying the target, using the cDNA as the template in a 20- μL reaction (we use TaqMan[®] Gene Expression Assays, from ABI) (see **Notes 2** and **18**, **Fig. 4**, and **Table 2**). Additionally, before starting the quantitative real-time PCR run, see **Note 19** for the amplification efficiency of quantitative real-time PCR reactions, a description of the mechanism of the TaqMan[®] probe cleavage resulting in a fluorescent signal, the quantitative real-time PCR instrument of choice, the quantitative real-time PCR reaction set-up, and quantitative real-time PCR tips.

3.6. Analysis of Gene Expression Data Using Quantitative Real-Time PCR

The two most routinely used methods of data analyses from quantitative real-time PCR are absolute quantification and relative quantification (**20–22**). Absolute quantification assesses the exact (insofar as the defined standards) copy number of the target gene, using a standard curve quantification (**23–28**), whereas relative quantification presents the relative change in gene expression compared with an untreated control/scrambled siRNA control sample (**ref. 21**, see **Note 20**). The $2^{-\Delta\Delta\text{Ct}}$ method (**21**) is an example of a relative quantification method. Use this formula to calculate the relative gene expression changes from quantitative real-time PCR experiments (see **Note 21**). The article by Livak and Schmittgen (**21**) describes the derivation, assumptions, and examples of the $2^{-\Delta\Delta\text{Ct}}$ method of relative gene quantification. The schematic presentation of the quantitative real-time PCR and the relative quantification data of gene expression after siRNA treatment is illustrated in **Fig. 4** and **Table 4**, respectively.

Table 2
Quantitative Real-Time PCR Reaction Preparation

Components	Single reaction volume (μL)
20X Target assay mix or 20X endogenous control assay mix	1
2X TaqMan® universal master mix (with or without Amp Erase UNG)	10
Template DNA + RNase/DNase-free water	9
Final volume	20
PCR conditions for DNA Engine Opticon 2 (MJ Research)	Time
1. 50°C	2 min
2. 95°C	10 min
3. 95°C	15 s
4. 60°C	1 min
5. Plate read	
6. Go to line 3 for 44 more cycles	
7. Incubate at 72°C	7 min
8. Incubate at 10°C	Forever
9. End	

4. Notes

1. A Cancer Gene Library of 278 siRNAs against 139 known oncogenes and tumor suppressor genes was designed in collaboration with Qiagen Inc., applying extensive bioinformatics strategies to ensure that siRNAs target only the transcripts under study and minimize the chance of nonspecific, off-target effects (<http://www1.qiagen.com/Products/GeneSilencing/LibrarySiRna/SiRnaSets/CancerSiRnaSet.aspx?ShowInfo=1>).
2. The information for a comprehensive collection of predesigned primer and probe sets of TaqMan® Gene Expression Assays is available on the Applied Biosystems Web site:http://myscience.appliedbiosystems.com/cdsEntry/Form/gene_expression_keyword.jsp. TaqMan® Gene Expression Assays are built on Applied Biosystems 5' nuclease chemistry. Each assay consists of two unlabeled PCR primers for amplifying the gene of interest (used at a final concentration of 900 nM each) and a TaqMan® probe with a FAM dye-labeled reporter and a Minor Groove Binder attached to a nonfluorescent quencher probe (see Fig. 4). The Minor Groove Binder increases the probe melting temperature by binding in the minor groove of a DNA duplex. This increase enables the use of probes as short as 13 bases. These shorter probes offer superior primer/probe design for improved specificity. They also provide more flexibility when designing assays for closely related sequences, such as gene family members or species-specific assays. The incorporation of a

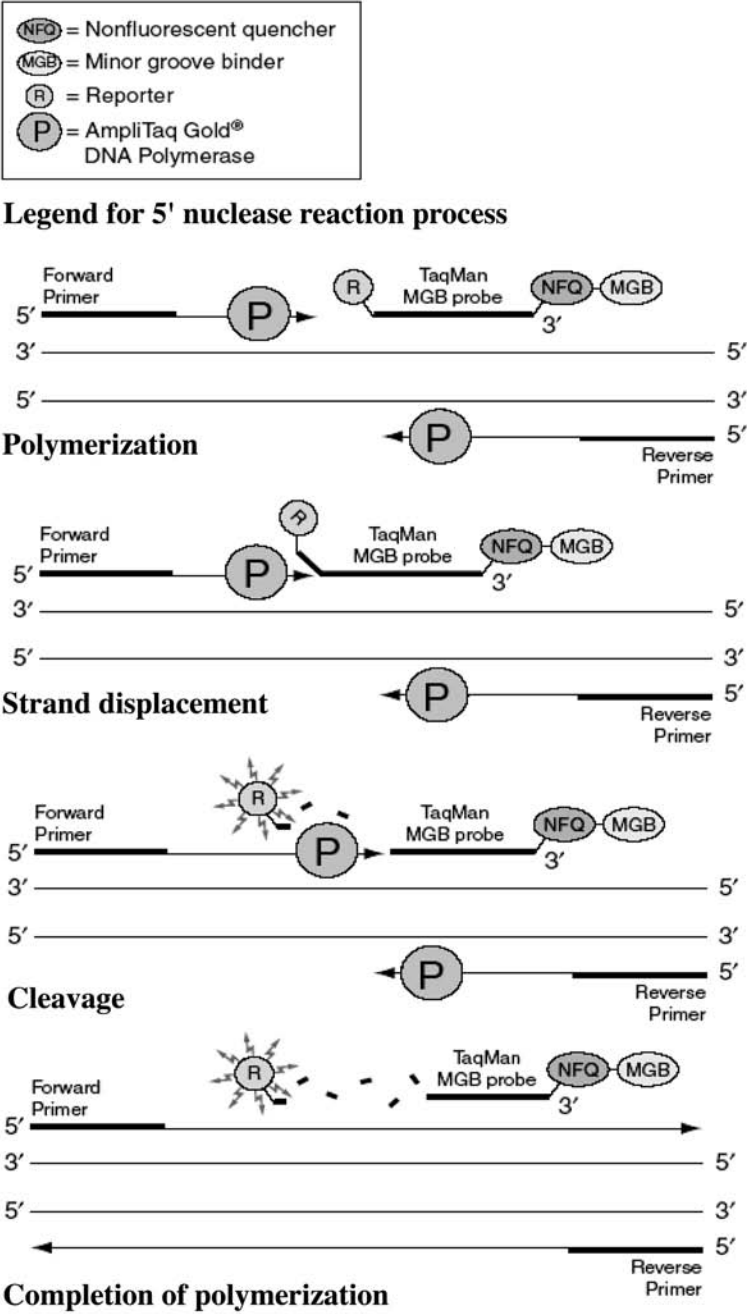


Fig. 4. 5' nuclease assay illustrating the mechanism of probe cleavage resulting in a fluorescent signal.

nonfluorescent quencher virtually eliminates the background fluorescence associated with traditional quenchers (e.g., TAMRA), providing better sensitivity and quantification precision. All components are formulated as a single ready-to-use 20X mix. TaqMan® Gene Expression Assays are designed to:

- a. Run under universal conditions for two-step quantitative real-time PCR.
 - b. Work with TaqMan® Universal Master Mix (with or without AmpErase Uracil N-glycosylase [UNG]). UNG uses an enzymatic reaction analogous to the restriction-modification and excision-repair systems of cells to specifically degrade contaminating PCR products from previous PCR amplifications in which dUTP has been incorporated, without degrading native nucleic acid templates (29–32).
 - c. Amplify target cDNA without amplifying genomic DNA, if possible. This is achieved by designing probes that cross exon–exon junctions. Further information on specific product uses is available on the ABI website (<http://www.allgenes.com>).
3. Spotting of the individual siRNAs and the lipid reagent into black clear-bottom 96-well plates can either be performed manually with a multichannel pipet or robotically. Perform duplicate transfections at two different positions to overcome position effects. The reverse transfection enhances the delivery of the siRNAs into HeLa cells compared with the conventional transfection (personal observation), in which the cells are first grown at the bottom of the 96-well plates and the lipid/siRNA complex is prepared in a separate tube and then added to the already plated cells. Additionally, this procedure accelerates the experimental process by eliminating the extra 24 h of incubation involved in the conventional transfection protocol. The experiments performed on HeLa cells have also been repeated for MCF-7 cells. The percent cell survival data of the cancer set siRNA treatments on HeLa and MCF-7 cells are graphically depicted in the multidimensional phenotype analysis (see Fig. 5). Three of the genes, A, B, and C, have been chosen as examples for subsequent validation of siRNA-mediated decrease in the targeted mRNA. Figure 6 illustrates the relative quantification analysis for genes A, B, and C.
4. The CellTiter-Blue Cell Viability Assay (Promega) provides a fluorometric method for estimating the number of viable cells present in a multiwell format. The indicator dye, resazurin, present in the buffered solution of CellTiter-Blue, is used to measure the metabolic capacity of cells (cell viability). Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and, thus, do not generate a fluorescent signal. The spectral properties of CellTiter-Blue Reagent change after reduction of resazurin to resorufin. Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin. The absorbance maximum of resazurin is 605 nm, and that of resorufin is 573 nm. Either fluorescence or absorbance may be used to record results; however, fluorescence is the preferred method because it is more sensitive.
5. Incubation of the cells at 37°C allows the cells to convert resazurin to resorufin, after which, the fluorescent signal is measured. Different cell types may take different times from reduction of resazurin to resorufin. For example, the MCF-7

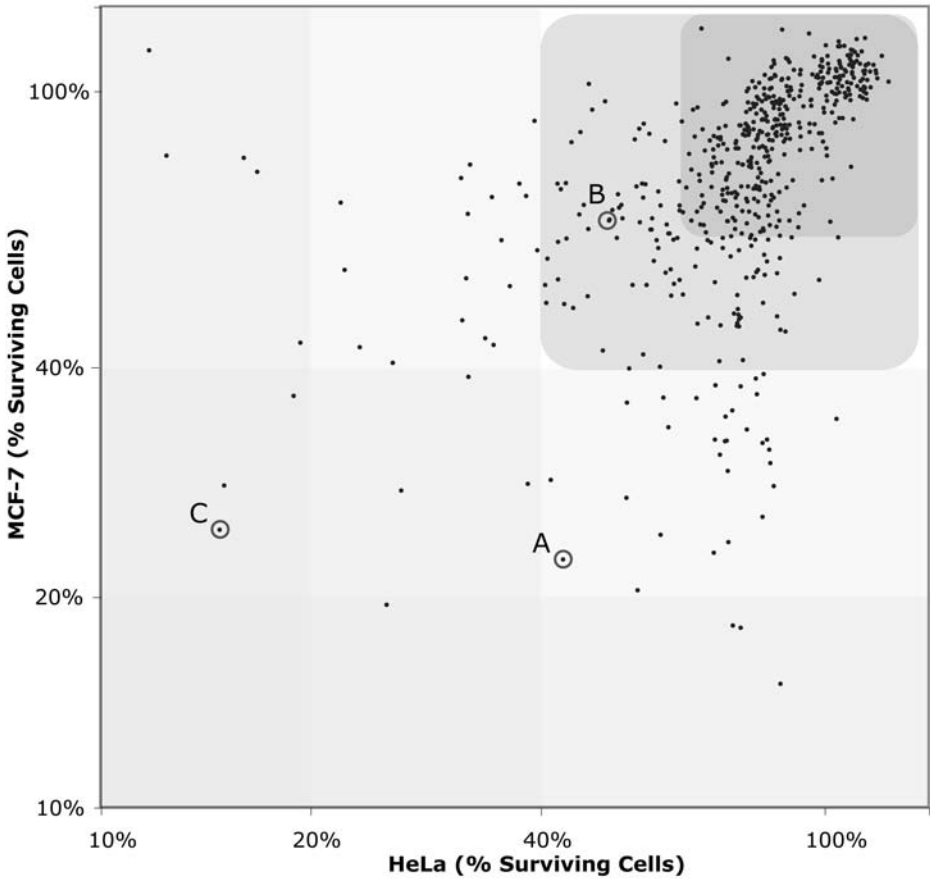


Fig. 5. Multidimensional phenotype analysis.

cells have a longer reduction time than HeLa cells; therefore, the user will have to empirically determine the amount of time for each cell line.

6. Relative fluorescent units are measured at 544 nm excitation and 590 nm emission wavelengths. All siRNA treatments, including controls (see **Subheading 3.1.**), were repeated in four different wells, and the average of the four replicates were later used to plot the effect for each siRNA; the standard deviation of the replicates were used to plot the error bars. Controls include “no siRNA,” “no cell,” and a universal nonspecific “scrambled siRNA sequence” control (Qiagen Inc.). Data are normalized to the scrambled siRNA sequence control and presented as a percentage decrease in cell viability. The efficacy of the siRNAs on phenotypic end points in cancer cells reveals that approx 5% of the siRNAs have a significant effect on HeLa cell survival (**Fig. 2**). siRNAs that had a significant effect on HeLa cell proliferation/survival were determined, and the specific sample wells were pooled for further total RNA extraction.

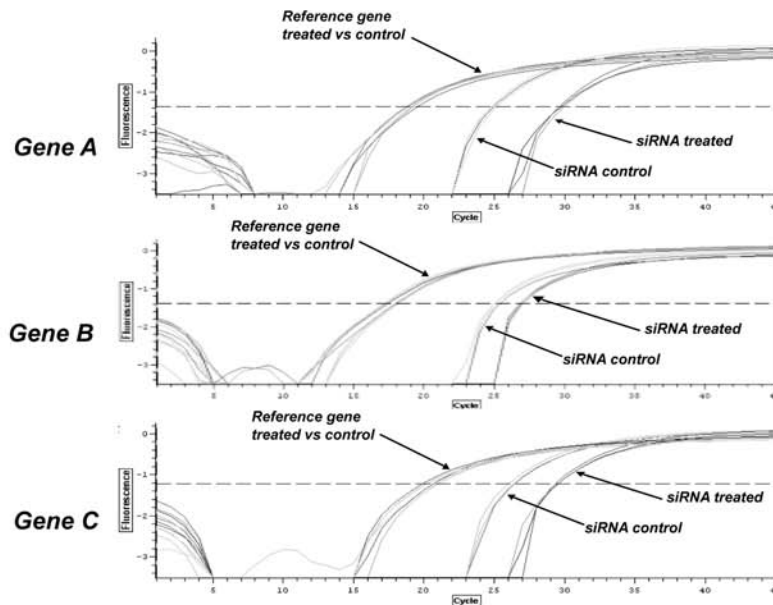
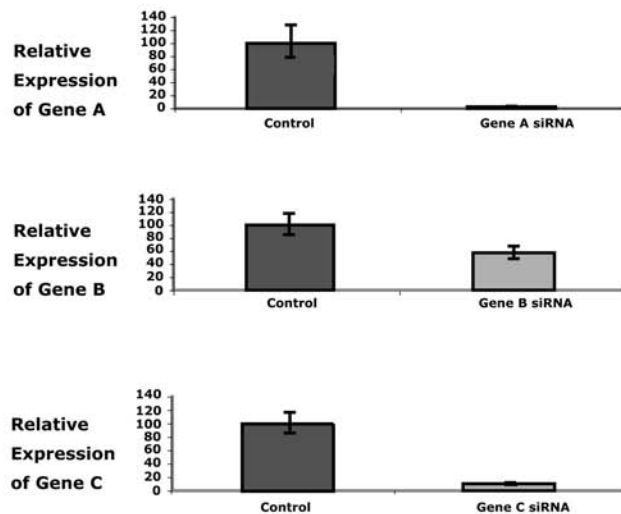


Fig. 6. Relative quantification of gene expression after siRNA treatment.

7. Handling of RNA: RNases are very stable and active enzymes that generally do not require cofactors to function. Because RNases are difficult to inactivate, and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA. Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice while aliquots are pipetted for downstream applications. The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases. Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH and 1 mM EDTA followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for 4 h or longer (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC (DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using DEPC). Fill glassware with 0.1% DEPC (0.1% v/v in water), allow to stand overnight (12 h) at 37°C, and autoclave or heat to 100°C for 15 min to eliminate residual DEPC. Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% sodium dodecyl sulfate), thoroughly rinsed with RNase-free water, and then rinsed with ethanol (plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions) and allowed to dry. Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 mL DEPC to 100 mL of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO₂. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency

in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 min. Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are, therefore, free of any DEPC contamination (Qiagen Inc.). Purified RNA may be stored at -20 or -80°C in water. Under these conditions, we have observed no degradation of RNA after 1 yr. The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA/mL. This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be performed in water. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvetts are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing the cuvetts with 0.1M NaOH and 1 mM EDTA followed by washing with RNase-free water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is:

Volume of RNA sample = 100 µL.

1/50 Dilution = 10 µL RNA sample + 490 µL distilled water.

Measure absorbance of diluted sample in an RNase-free, 1-mL cuvet;

$A_{260} = 0.23$.

Concentration of RNA sample = $40 \times A_{260} \times \text{dilution factor} = 40 \times 0.23 \times 50 = 460 \mu\text{g/mL}$.

Total yield = concentration $\times$ volume of sample in milliliters = $460 \mu\text{g/mL} \times 0.1 \text{ mL} = 46 \mu\text{g}$.

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV range, such as proteins. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Because water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination (33). For accurate values, measuring absorbance in 10 mM Tris-HCl, pH 7.5, is recommended. Pure RNA has an A_{260}/A_{280} ratio of 1.9 to 2.1. Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris-HCl, pH 7.5), with some spectrophotometers. Always be sure to calibrate the spectrophotometer with the same solution. For determination of RNA concentration, however, dilution of the sample in water is recommended (Qiagen Inc.) because the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/mL RNA) is based on an extinction coefficient calculated for RNA in water.

8. Incomplete removal of the cell culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy silica-gel membrane. Both effects may reduce RNA yield. The RNeasy Mini procedure combines the selective binding properties of a silica-gel-based membrane with the speed of

microspin technology. A specialized high-salt buffer system allows up to 600 to 750 ng (per well of a 96-well plate) of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. First, lyse the HeLa cells in each well of the 96-well plates by adding GITC-containing lysis buffer (Buffer RLT). Buffer RLT may form a precipitate after storage. If necessary, redissolve by warming, and then place at room temperature. Buffer RLT is added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane and to inactivate RNases. Buffer RLT contains GITC and is, therefore, not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling. β -mercaptoethanol (β -ME; commercially available solutions are usually 14.3 M) must be added to Buffer RLT before use. β -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 μ L of β -ME/1 mL of buffer RLT. Buffer RLT is stable for 1 mo after addition of β -ME. Pipet up and down several times and pool the quadruplicate samples of similar siRNA treatment together in a single 1.5-mL Eppendorf tube after adding Buffer RLT. Homogenize the pooled lysate using QIAshredders (Qiagen Inc.) in the presence of the highly denaturing buffer RLT, which immediately inactivates RNases, to ensure isolation of intact RNA. Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Disruption and homogenization are considered two distinct steps. Complete disruption of cells walls and plasma membranes of cells and organelles is absolutely required to release all of the RNA contained in the samples. Different samples require different methods to achieve complete disruption. Here, the HeLa cells are disrupted by the addition of the lysis buffer RLT and repeated pipetting. Incomplete disruption results in significantly reduced yield. On the other hand, homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. The QIA shredders (Qiagen Inc.) are used to homogenize the HeLa cells. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy membrane and, therefore, significantly reduced yield.

9. Add ethanol to provide appropriate binding conditions and then apply the sample to an RNeasy mini column, in which the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in at least 30 μ L of water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA because most RNAs with fewer than 200 nucleotides (such as 5.8S ribosomal RNA, 5S ribosomal RNA, and transfer RNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.
10. DNA contamination: No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, designing primers

that anneal at intron splice junctions, so that genomic DNA will not be amplified, is recommended. Alternatively, DNA contamination can be detected on agarose gels after RT-PCR by performing control experiments in which no reverse transcriptase is added before the PCR step or by using intron-spanning primers. For sensitive applications, such as differential display, or if it is not practical to use splice-junction primers, DNase digestion of the RNA on the column with RNase-free DNase is recommended. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the RNeasy procedure, the eluate containing the RNA can be treated with DNase. The RNA can then be repurified with the RNeasy cleanup protocol (Qiagen Inc.), or, after heat inactivation of the DNase, the RNA can be used directly in downstream applications. The RNeasy Mini Protocol for Isolation of Cytoplasmic RNA from Animal Cells is particularly advantageous in applications in which the absence of DNA contamination is critical, because the intact nuclei are removed. Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and the RNeasy silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., quantitative real-time PCR analysis with a low-abundance target). Use of the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive quantitative real-time PCR analyses. The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel (formaldehyde agarose gel), electrophoresis, and ethidium bromide staining. The respective ribosomal bands (**Fig. 3**) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with intensity approximately twice that of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller-sized RNAs, it is likely that the RNA sample suffered major degradation during preparation. The smears just above 28S and in between 28S and 18S ribosomal bands represent the mRNA population.

11. Cell pellets can be stored at -80°C for later use or can be used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that cell pellets can be dislodged by flicking. Homogenized cell lysates (in Buffer RLT) can be stored at -80°C for several months. To process frozen lysates, thaw samples for 15 to 20 min at 37°C in a water bath to dissolve salts. If any insoluble material is visible, centrifuge for 5 min at 3000g to 5000g. Transfer the supernatant to a new RNase-free glass or polypropylene tube and continue with the next step.

Generally, DNase digestion is not required (see **Note 10**). However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., quantitative real-time PCR analysis with a low-abundance target). In these cases, the small residual amounts of DNA remaining can be removed using RNase-free DNase I (e.g., the RNase-Free DNase, Qiagen Inc.) for the optional on-column DNase digestion, or by a DNase digestion after RNA isolation.

12. Buffer RLT and Buffer RW1 contain a guanidine salt (guanidinium thiocyanate, 25–50% and 2.5–10%, respectively); in addition, Buffer RW1 contains 2.5 to 10% ethanol. They are, therefore, not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling guanidine.
13. Buffer RPE is used at the washing step and is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
14. Residual ethanol may interfere with the downstream reactions, therefore, centrifugation at **Subheading 3.3, step 13** is very important.
15. To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate from **Subheading 3.3., step 15**. The yield will be approx 15 to 30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.
16. All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
17. mRNA can also be used depending on the purpose of the experiment. Depending on the yield of the total RNA from the cell lines (HeLa and MCF-7) grown and the siRNA treated in 96-well plates, a concentration between 100 ng and 1 μ g of total RNA is used in the 20 to 100 μ L cDNA reactions. The cDNA synthesis experiments described throughout this chapter are performed using oligo-dT₂₀ primers. This allows the detection of multiple transcripts from a single first-strand reaction. The denaturation step (**Subheading 3.4., step 1**) is optional, but the users are encouraged to include this step as part of their cDNA synthesis reaction. Heating the RNA in the absence of reaction buffer and RT enzyme before cDNA synthesis at temperatures as high as 65°C can enhance the unwinding of the secondary structures in the RNA molecule, and full-length cDNA synthesis may be achieved as a result. We use Thermoscript RT at 50°C for oligo-dT₂₀ to reduce secondary structure or to improve specificity. For convenience, the components of the cDNA synthesis reaction have been calculated not only for 20- μ L but also for 100- μ L reaction volumes. The cDNA synthesis reagents are recommended to be prepared as two separate master mixes before combination (**Steps 1 and 2**), and to be kept on ice at all times. Preparation of the master mixes allows homogenous distribution of the reaction contents to keep the conditions of the cDNA synthesis the same for all samples. Treatment of cDNA with RNase H to remove the complementary RNA before quantitative real-time PCR is optional (**Subheading 3.4., step 8**), but recommended, because digestion with RNase H will improve the quantitative real-time PCR signal of many targets and, in some cases, is required for efficient and consistent amplification of long quantitative real-time PCR templates.
18. Fluorescent reporters are the common entities for all of the quantitative real-time PCR assays. The analysis of amplification during quantitative real-time PCR is achieved by detecting the direct or indirect accumulation of the fluorescence of the newly amplified cDNA. Among the most common dyes and fluorescent detection chemistries used in quantitative real-time PCR are SYBR Green I (Molecular

Probes), TaqMan® Probes (Roche Molecular Systems), and Molecular Beacons (Research Genetics). Choosing a detection system can be a major consideration in developing a quantitative real-time PCR assay and these are described more fully in Chapter 17. Each system may have its advantages and disadvantages. Specificity, assay optimization time, and cost may be some of the concerns involved before making a decision regarding an assay of choice. The TaqMan® chemistry, an indirect assay system, is the method of choice for our study (ref. 34; Note 2 and Fig. 4). The TaqMan® Gene Expression Assays (Applied Biosystems) are ready-to-use, prevalidated, and are available for more than 300,000 quantitative real-time PCR assays for measuring the expression of genes in human, mouse, and rat. In our experience, these assays have been consistent between batches as well as in sample replicates. They eliminate the labor, expense, and bioinformatics expertise previously needed for generating high-quality quantitative gene expression data, especially important in the process of validation of large number of siRNA knockdowns.

19. One of the major concerns of the researchers involved with quantitative real-time PCR is the amplification efficiency of the PCR reaction. Efficiency estimates can vary significantly if rigorous measurement procedures are not met. Measurements must be acquired over a broad range of dilutions (5–6 logs) and should include replicates (e.g., triplicates) to reduce the effects of handling errors (e.g., pipetting accuracy). Efficiency of PCR reactions can be affected by substances such as heme, and detergents that are known to inhibit PCR. Researchers must make sure that cDNA templates are free of inhibitors. One other important issue regarding the efficiency validation of the quantitative real-time PCR is ensuring that the two amplicons have the same efficiency, if the $2^{-\Delta\Delta Ct}$ method is used (21). For example, the efficiency of an internal control (e.g., reference gene) should be the same as the target gene, in which case, the normalization factor comes into question, especially if multiplexing in the same reaction tube. If the efficiencies of the two particular genes in question are not equal, then new primers can be designed and/or optimized to achieve a similar efficiency for both the target and the reference control (see Subheading 3.6.; ref. 21). A sensitive method of measuring whether two amplification products have the same efficiency is to check how the cycle threshold (Ct) changes (ΔCt) with template dilution (21). For this, cDNA dilutions over a 100-fold range are prepared for both the target and the reference gene. For each dilution sample, amplifications are performed using TaqMan® gene expression assays or other available fluorogenic probes. The average Ct is calculated for both the template and the reference gene, and ΔCt is determined. A plot of the log cDNA dilution vs ΔCt is made. If the absolute value of the slope is close to zero, the efficiencies of the target and the reference genes are considered similar, and the $\Delta\Delta Ct$ calculation for the relative quantification of target may be used to analyze the data (21).

TaqMan® assays use a fluorescence resonance energy transfer probe as a reporter system with a 6-FAM dye-labeled reporter (see Note 2). During PCR, the TaqMan® probe anneals specifically to a complementary sequence between the sense and antisense primer sites (polymerization; see Fig. 4). If the probe is intact (at the start

of the polymerization step), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, mainly by fluorescent resonance energy transfer (35). The DNA polymerase cleaves only probes that bind to the target (strand displacement). Cleavage separates the reporter dye from the quencher dye, resulting in increased fluorescent signal by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to the probe and is amplified during the PCR process. Because of these stringent requirements, any nonspecific amplification is not detected. Polymerization of the strand continues, but the 3'-end of the probe is blocked to prevent extension of the probe during PCR (cleavage and completion of polymerization).

The two quantitative real-time PCR instruments used in our laboratory are MJ Research's Opticon 2 and ABI's 7900HT Fast Real-Time PCR System. Other qRT-PCR instruments can be used according to the researchers' needs or availability.

Table 2 describes a typical quantitative real-time PCR reaction setup in our laboratory. Each siRNA-treated sample, the suitable reference gene for the particular siRNA treatment and cell line, the nontemplate control, and the necessary group of internal and external controls are prepared in triplicate. Special care should be taken when handling the light-sensitive TaqMan® probes. It is recommended that after light-protected thawing, i.e., by holding in the hand with gloves until thawed, the probes are immediately placed on ice and covered with aluminum foil. Care should be taken to avoid light exposure to preserve the life of the fluorescent probes. The calculation for components of a 20- μ L TaqMan® reaction is given in **Table 2**. The amount of total RNA in each TaqMan® reaction is typically 10 to 50 ng. The number of reactions to be prepared can be calculated accordingly. Preparation of a master mix per siRNA-treated sample to be validated (per particular gene assay) and the corresponding reference genes are recommended. Sample distribution into each well of the 96-well plate is aided considerably by the guidance of a Pipetter Guide (e.g., Diversified Biotech). Attention should be paid to cover over the wells of 96-well plates with aluminum foil, during the process of pipetting other components of the quantitative real-time PCR reaction. Use of an automatic pipet is highly recommended for sample distribution in the wells, for well-to-well accuracy and consistency. With our MJ Research's Opticon 2 instrument, hard-shell, thin-wall, 96-well microplates with white shell and white wells are used, together with eight-strip ultraclear caps for the amplification process. Dilution of the cDNA with DNase/RNase-free water to make up for 9 μ L of volume per sample would ease the quantitative real-time PCR preparation step, especially when handling large amount of samples. Depending on the range of total RNA amount needed (10–50 ng) per quantitative real-time PCR reaction of 20 μ L, use the total volume of cDNA reaction (i.e., 20 μ L) to make up a working cDNA dilution per sample. For example, if 10 ng of total RNA/20 μ L of quantitative real-time PCR reaction is needed from a 20- μ L cDNA reaction with 1 μ g of initial total RNA input, take 0.2 μ L of cDNA and add 8.8 μ L of DNase/RNase-free water to prepare 9 μ L of volume per quantitative real-time PCR reaction. For ease of pipetting, to a total of 20 μ L cDNA reaction, add 880 μ L of DNase/RNase-free water for the final working

dilution (9 μ L of diluted cDNA per quantitative real-time PCR reaction). This, in turn, would allow for more than 100 quantitative real-time PCR reactions-worth of cDNA dilutions per siRNA-treated sample.

After the preparation of all the components of the quantitative real-time PCR reaction, pipet the contents, as listed in [Table 2](#), and cap the 96-well plates that are ready for the quantitative real-time PCR reaction at the set thermal cycler conditions for the MJ Research DNA Engine Opticon 2 (see [Table 2](#)). The thermal cycler temperature conditions for other quantitative real-time PCR instruments can be set according to the needs of researchers and the manufacturer's recommendations. Before placing the 96-well plate in the thermal cycler, it is strongly recommended that the 96-well plate be briefly centrifuged to mix the components of the quantitative real-time PCR reaction. Pipetting up and down is not recommended while distributing the reagents. This may cause pipetting errors and loss of the already pipetted small volume of reaction components. Centrifuges that accommodate rotors with 96-well plates can be used, but we find that a quicker and easier way of briefly centrifuging the 96-well plates is to use a DNA Speed Vac with a 96-well rotor (DNA 110, Savant), without turning the vacuum on. This allows a shorter time of handling the quantitative real-time PCR reactions outside just before placing the 96-well plates in the thermal cycler.

20. Unless it is necessary to report the absolute transcript copy number, determining the relative change in gene expression profiles of the transcripts should be sufficient. There are drawbacks in using standard curve quantification if a lot of sample screening is required, as in the case of siRNA validation via quantitative real-time PCR. If using absolute quantification, each target requires a standard curve quantification at least with triplicates of each dilution series over a range of 5 to 6 logs of dilutions. This would take much space on a 96-well plate, not leaving enough room for a large number of siRNA target sample analysis. This problem may be somewhat eased on a 384-well system, but, again, the more target genes to be analyzed, the larger will be the number of dilution series to be considered. The necessity for using cDNA, RNA, plasmid, oligonucleotide, or other sources for standard curve analysis introduces extra steps and further variation, causing difficulty in comparison of experiments run at different times ([22](#)). Variation can be reduced with additional controls, but this further decreases the number of target samples per plate. Moreover, in different laboratory settings, the data analysis may be difficult because of the variations in the samples used to generate standard curves, unless the data are normalized against a standard cell line. An additional source of error can also be introduced at the cDNA synthesis step, because different RNA samples may have varying efficiencies of reverse transcription. For studies with small number of samples, absolute quantification using the standard curve method may be considered. A large degree of assay optimization would not be required. Any concerns regarding the changes introduced on the internal control genes because of a specific treatment on the cells will be eliminated, but PCR efficiency should always be calculated and optimized as often as possible ([22](#)). The choice of gene expression data analysis method in our laboratory

is relative quantification. This allows us to screen and validate a large number of siRNA knockdowns in a 96-well format. By using quantitative real-time PCR in our RNAi validation experiments, we have faced some challenges, which we were able to address by testing an array of endogenous controls or reference genes that are not affected transcriptionally by the treatments with different siRNAs. These transcriptional changes and challenges are usually observed when the siRNA has an effect on the cell growth or survival. For the data obtained from the siRNA knockdowns to reflect the truth regarding the extent of silencing by the siRNAs and to be devoid of erroneous results, it is required that the expression levels of the chosen reference gene be kept unchanged between the siRNA-treated and siRNA control samples (36). Table 3 lists the different flavors of 11 reference genes that have been used in our high-throughput RNAi validation experiments for quantitative real-time PCR normalization purposes. In the event that all of these in-house reference genes are affected by our treatment of siRNAs, we will start looking for additional reference genes that are least or not at all affected. An alternative solution to the problem of variable gene expression levels between the reference genes and the target gene of interest caused by a particular treatment would be to use a software that would correct for the changes in the levels of reference genes after treatment of the cells (37). Additionally, websites, such as www.gene-quantification.info, are a valuable resource in obtaining information regarding quantitative real-time PCR in general.

21. Data analysis varies depending on the quantitative real-time PCR instruments used. Therefore, refer to the appropriate user's guide for instructions on how to analyze your data. As in the case of the conventional PCR (38), the more copies of the nucleic acid that are present at a start of a given quantitative real-time PCR reaction, the fewer cycles of the template amplification it will take to synthesize a specific number of amplicons. The Ct value, which is the number of cycles necessary for an amplification-related fluorescence to reach a specific threshold, is inversely proportional to the amount of template that was present at the start of a quantitative real-time PCR reaction. Because the amplification process in a given quantitative real-time PCR is monitored in real-time, during the exponential phase of a quantitative real-time PCR reaction—when the amplification efficiency is at its peak and least affected by reaction-limiting conditions—determination of the Ct values can be achieved (39). It is possible to roughly quantify a Ct value without having to plot a standard curve. During the exponential phase of a quantitative real-time PCR, the number of amplicons theoretically doubles during each cycle, assuming that the efficiency of the amplification is 100%. Hence, a sample with twice the number of starting copies compared with another sample will need one less cycle of amplification to build up an equal number of products. Therefore, using the difference between the Ct values of two samples, the relative difference of different samples can be determined (40). When using $2^{-\Delta\Delta C_t}$ method (relative quantification method), the amplification efficiencies of the target and reference genes must be approximately equal. To assess whether the amplification efficiencies of the target and control genes are the same, look at how ΔC_t varies with the

Table 3
Reference Genes Used in Quantitative Real-Time PCR Assays for the Validation of High-Throughput RNAi^a

Gene symbol	RefSeq	Gene name	Gene function	Assay ID (ABI)
<i>B2M</i>	NM_004048	β 2-Microglobulin	β -Chain of major histocompatibility complex class I molecules	Hs00187842_m1
<i>SDHA</i>	NM_004168	Succinate dehydrogenase complex, subunit A	Electron transporter in the TCA cycle and respiratory chain	Hs00417200_m1
<i>HMBS</i>	NM_000190	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	Hs00609297_m1
<i>YWHAZ</i>	NM_003406	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ -polypeptide	Signal transduction via binding to phosphorylated serine residues on various signaling molecules	Hs00237047_m1
<i>UBC</i>	NM_021009	Ubiquitin C	Protein degradation	Hs00824723_m1
<i>GAPDH</i>	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	Hs99999905_m1
<i>ACTB</i>	NM_001101	β -actin	Cytoskeletal structural protein	Hs99999903_m1
<i>TBP</i>	NM_003194	TATA box-binding protein	General RNA polymerase II transcription factor	Hs99999910_m1
<i>HPRT1</i>	NM_000194	Hypoxanthine phosphoribosyl-transferase I	Purine synthesis in salvage pathway	Hs99999909_m1
<i>POLR 2A</i>	NM_000937	Polymerase RNA II (DNA directed) polypeptide A, 220 kD	DNA-directed RNA polymerase II activity	Hs00172187_m1
<i>PPIA</i>	NM_021130	Peptidylprolyl isomerase A (cyclophilin A)	Accelerate the folding of proteins and may play a role in cyclosporin A-mediated immunosuppression	Hs99999904_m1

^aAssay IDs are quotations from TaqMan[®] Gene Expression Assays (ABI).

template dilutions (21). Calculate the average Ct value for both the target gene and the reference gene. Then calculate ΔCt ($\Delta\text{Ct} = \text{Ct of target} - \text{Ct of reference}$). Finally, plot the log cDNA dilution vs ΔCt . If the absolute value of the slope is close to zero, then the efficiencies of the target and the reference genes are similar. Consequently, the $\Delta\Delta\text{Ct}$ method for calculating relative quantifications may be used (21). In situations in which the efficiencies of the target and the reference genes are not equal, either design new primers or try to find another reference gene to meet the $\Delta\Delta\text{Ct}$ criteria.

To perform an accurate validation of the siRNA knockdowns, it is absolutely essential that the selection of the internal controls/calibrators for the relative quantification method ($\Delta\Delta\text{Ct}$ method) be fulfilled cautiously. The reference genes must be properly validated for each experiment to determine that experimental treatments (e.g., siRNA treatments) will not affect the gene expression of the reference gene for the normalization purpose. Untreated control samples or, in our case, scrambled siRNA-treated control samples can be considered to be the calibrator (a control sample against which the treated samples are normalized) for the relative quantification method using the $2^{-\Delta\Delta\text{Ct}}$ formula.

A typical quantitative real-time PCR run of siRNA-treated samples is prepared using triplicates of each siRNA treatment together with the appropriate controls (see Fig. 6). The Ct values obtained from quantitative real-time PCR instrumentation (e.g., Opticon 2, MJ Research) can be easily imported into a Microsoft Excel spreadsheet program. See Table 4 for a demonstration of a data analysis of siRNA treatment using relative expression method ($2^{-\Delta\Delta\text{Ct}}$). Quadruplicate samples of cells were pooled and quantitative real-time PCR was performed in triplicate on cDNA synthesized from total RNA extracted from HeLa and MCF-7 cells treated with gene A-, gene B-, and gene C-specific siRNAs. Controls included scrambled siRNA controls and β -actin as the reference control gene for normalization purposes. The data are analyzed as the fold change in gene expression normalized to an endogenous reference gene (β -actin) and relative to the scrambled siRNA-treated control using the following equations:

$$\Delta\text{Ct of treated} = \text{average target gene Ct} - \text{average } \beta\text{-actin Ct}$$

$$\Delta\text{Ct of control} = \text{average target gene Ct} - \text{average } \beta\text{-actin Ct}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct of treated} - \Delta\text{Ct of control}$$

The amount of target normalized to β -actin and relative to the calibrator is equal to $2^{-\Delta\Delta\text{Ct}}$ where “treated” refers to the target gene, and “control” refers to the scrambled siRNA control. The scrambled siRNA control is chosen as the calibrator because no change is expected to be seen as a result of scrambled siRNA treatment. In other words, this should behave as the untreated control. The choice of calibrator for the relative quantification of gene expression using $2^{-\Delta\Delta\text{Ct}}$ method depends on the type of experiment that is planned (21). Ct is exponentially correlated to copy number. Hence, as part of the final calculation, the standard error is estimated by calculating the $2^{-(\Delta\Delta\text{Ct} + \text{SD})}$ and $2^{-(\Delta\Delta\text{Ct} - \text{SD})}$ terms, where SD is the standard deviation. Thus, a range of values that are asymmetrically distributed relative

Table 4
Data Analysis Using $2^{-\Delta\Delta Ct}$ Method^a

ΔCt of treated = average target gene Ct – average β -actin Ct = 29.75 – 18.84 = 10.91	
ΔCt of control = average target gene Ct – average β -actin Ct = 24.96 – 19.29 = 5.67	
$\Delta\Delta Ct$ of treated = ΔCt of treated – ΔCt of control = 10.91 – 5.67 = 5.24	
$\Delta\Delta Ct$ of control = ΔCt of control – ΔCt of control = 10.91 – 10.91 = 0.00	
(the calibrator)	
Relative quantification of gene $A = 2^{\Delta\Delta Ct} = 2^{-5.24} = 0.03$	Relative quantification of scrambled control = $2^{\Delta\Delta Ct} = 2^{-0.00} = 1.00$
$SD^b (\Delta\Delta Ct)_{treated} = [(SD\Delta Ct_{treated})^2 + (SD\Delta Ct_{control})^2]^{0.5} = (0.19^2 + 0.30^2)^{0.5} = 0.36$	
$SD^c (\Delta\Delta Ct)_{control} = [(SD\Delta Ct_{treated})^2 + (SD\Delta Ct_{control})^2]^{0.5} = (0.19^2 + 0.30^2)^{0.5} = 0.36$	

^aRelative quantification of gene expression analysis in HeLa cells using gene A siRNAs relative to scrambled siRNA controls and normalized to β -actin (an endogenous reference gene; see **Note 20**; ref. **21**).

^bStandard deviation (see **Note 21**): SD of gene A siRNA-treated sample: $2^{-(\Delta\Delta Ct + SD)} = 0.02$; and $2^{-(\Delta\Delta Ct - SD)} = 0.03$. Gene A: (+) SD = $2^{-(\Delta\Delta Ct - SD)} - 2^{-\Delta\Delta Ct} = 0.01$; and (–) SD = $2^{-\Delta\Delta Ct} - 2^{-(\Delta\Delta Ct + SD)} = 0.01$.

^cSD of scrambled siRNA control: $2^{-(\Delta\Delta Ct + SD)} = 0.78$; and $2^{-(\Delta\Delta Ct - SD)} = 1.28$. Scrambled siRNA control, (+) SD = $2^{-(\Delta\Delta Ct - SD)} - 2^{-\Delta\Delta Ct} = 0.28$; and (–) SD = $2^{-\Delta\Delta Ct} - 2^{-(\Delta\Delta Ct + SD)} = 0.22$.

to the average value is obtained. The asymmetric distribution arises from converting the results of an exponential outcome into a linear comparison of amounts (**21**). The above equations from an actual quantitative real-time PCR validation of siRNA experiment (see **Table 4**).

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Real-Time Quantitative PCR as an Alternative to Southern Blot or Fluorescence *In Situ* Hybridization for Detection of Gene Copy Number Changes

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Summary

Changes in copy number of genes contribute to the pathogenesis of various genetic disorders and cancer. The status of a gene has not only diagnostic value but sometimes directs treatment stratification. Although, for many years, Southern blot and fluorescence *in situ* hybridization were the standard methods for the detection of deletion, duplication, or amplification of a gene, both methods have their own important limitations. Recently, real-time quantitative PCR has proven to be a good alternative for the detection of gene copy number changes. Its main advantages are the large dynamic range of accurate quantification, the absence of post-PCR manipulations, its high-throughput screening capacity and degree of automation, and the possibility to perform the assay on minimal amounts of sample DNA in just a few hours of time. In this chapter, we outline the procedure of how to develop an assay for the detection of gene copy number changes for your gene of interest. We illustrate the approach by describing a validated assay for the detection of germline *VHL* exon deletions and for determination of *MYCN* copy numbers in tumor samples.

1. Introduction

Alterations in gene copy number are responsible for many genetic disorders and lead to activation of oncogenes and inactivation of tumor suppressor genes in human cancers. Until recently, Southern blot and fluorescence *in situ* hybridization (FISH) were used as molecular detection methods for gene copy number changes. However, both methods have their own intrinsic limitations. Southern blot is laborious, time consuming, and requires large amounts of high-quality DNA. Although the strength of FISH is the direct visualization of the DNA copy number at the single-cell level, this method is not well suited for detection of partial gene deletions. In contrast, real-time quantitative PCR (qPCR) has evolved as a valuable

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alternative in molecular diagnostics. This method has many advantages, including the absence of post-PCR manipulation (significantly reducing contamination risk and hands-on time), its speed, the requirement for only minimal amounts of input DNA, and the large dynamic range of accurate quantification.

The basic principle of real-time PCR is the monitoring of the PCR product accumulation at each PCR cycle during the reaction (in “real time”) as opposed to the end point detection by conventional (quantitative) PCR methods. PCR amplicon accumulation can be measured by a variety of fluorescent detection chemistries, such as the generic double-stranded DNA binding dye, SYBR Green I (1), or sequence-specific probes such as TaqMan[®] (2), adjacent hybridization probes (1), or molecular beacons (3). Many more detection formats are available; see the gene-quantification website section on dyes and chemistries (<http://dyes.gene-quantification.info>) and Chapter 17 for an overview.

Higuchi and colleagues (4) were the first to demonstrate that there is a relationship between the moment that the fluorescent PCR signal increases above background and the initial amount of template in the tube (Fig. 1). The sooner the signal increases above background, the more template was initially present. The difficult question of “how much?” is, therefore, transformed to a more easily addressed question of “when?” during real-time PCR analysis. In practice, by measuring fluorescence during PCR, a so-called amplification plot is generated. After background correction, an arbitrary fluorescence threshold line is set, such that it crosses the amplification plot in the exponential phase of the PCR (this is the linear phase in a semilogarithmic plot, and constitutes a range of PCR cycles in which the amplification efficiency is maximal, i.e., doubling of PCR product in each cycle under optimal conditions). The fractional PCR cycle at which the fluorescence crosses the threshold is called the threshold cycle (Ct) value. As stated, there is a linear relationship between this Ct value and the logarithm of the initial template amount. By measuring serial dilutions of a DNA sample, a standard curve can be constructed that allows intrapolation of the Ct value of an unknown sample to obtain relative or absolute quantities (depending on whether the actual copy numbers in the dilutions are known). In addition to the standard curve-based quantification strategy, another popular data analysis method (the comparative Ct method) is discussed in this chapter (see **Subheading 3.5.2.1.**).

This chapter describes a real-time qPCR approach for the detection of gene copy number changes, such as deletions, duplications, or amplifications. The different steps include:

1. DNA isolation yielding PCR-grade DNA template.
2. Primer design (paying attention to primer specificity and avoiding amplicon formation with secondary structures) and experimental validation (evaluation on agarose gel [only once], melting curve analysis, and determination of efficiency).

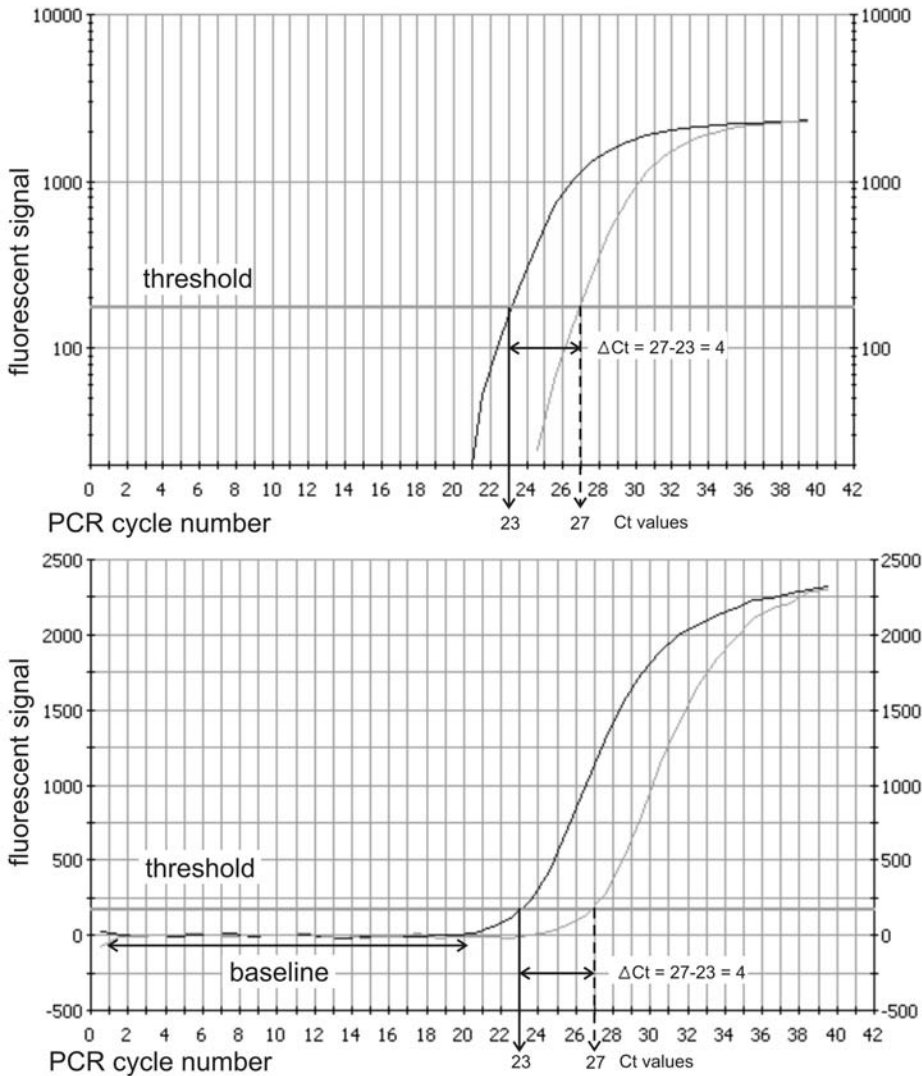


Fig. 1. Linear (bottom) and semi-logarithmic (top) amplification plot illustrating the nomenclature typically used in real-time PCR data analysis.

3. The actual PCR assay.
4. Data analysis.

In two different assays, we illustrate the use of real-time qPCR for gene copy number quantification, strongly demonstrating that qPCR is the method of choice for gene copy number assessment in routine DNA diagnosis. A first assay involves screening of germline *VHL* exon deletions in von Hippel-Lindau

(VHL) syndrome patients. The VHL syndrome is an autosomal-dominant inherited cancer syndrome caused by germline mutations or deletions of the *VHL* gene. In approximately one-fifth of all VHL syndrome families, small exon or larger gene deletions are detected. In a blind *VHL* exon deletion screening study by qPCR, we demonstrated that all deletions (exon or entire gene deletion in 10 of 24 samples) were detected. These data were in perfect agreement with previously determined Southern blot results, pointing at the high specificity and sensitivity of qPCR (5). A second assay was developed for measuring the *MYCN* gene copy number in neuroblastoma tumor samples. Amplification of the proto-oncogene, *MYCN*, is the hallmark of a genetic subgroup of aggressive neuroblastoma tumors and, as such, the status of the *MYCN* gene has become an important factor in therapy stratification. qPCR data for 175 neuroblastoma samples were highly concordant with previously determined FISH and Southern blot data (6).

2. Materials

1. PCR-grade DNA (fresh or frozen material; we have no experience yet with fixed paraffin-embedded material).
2. Primer design software (see **Subheading 3.3.3.**).
3. Oligonucleotide primers: standard desalted, 250 μ M stock solution in nuclease-free water and 5 μ M working solution in nuclease-free water; store at -20°C (try to avoid more than 20 thaw–freeze cycles).
4. Real-time PCR master mix (see **Note 1**).
5. Real-time PCR instrument (see **Note 2**).
6. Optical 96-well reaction plate and optical adhesive sealing, or individual tubes with optical caps.
7. Filter tips for pipetting (aerosol barrier; see **Note 3**).
8. Microsoft Excel template for data analysis and visualization (available from the authors on request; see **Subheading 3.5.5.**).

3. Methods

3.1. Template Preparation

Any protocol for DNA extraction that yields purified PCR-grade nucleic acids (removal of PCR inhibitors) will generate an adequate template for real-time PCR. We routinely use DNA from fresh or frozen cells (and have no experience with DNA from fixed paraffin-embedded tissue).

3.2. Reference Genes

Appropriate normalization of qPCR data is required to account for non-specific variation, such as variation in DNA input amounts or presence of PCR inhibitors, especially important in clinical samples. In the study of cancer, reference genes should be located in chromosomal regions that rarely show

genetic abnormalities in the cancer under investigation, i.e., *TNFRSF17* and *SDC4* in neuroblastoma, located on chromosome 16p13.1 and 20q12, respectively, which are not frequently altered in neuroblastoma (7). For other (constitutional) genetic disorders, in principle, every gene can be used as reference gene. In practice, reference genes are often selected in the vicinity or on the same chromosome on which the disease gene is located.

To validate candidate reference genes as proper reference genes, determine their copy number in both normal control DNA samples and affected patient or diseased tissue samples. The observed copy number ratio between the two reference genes in normal and patient DNA should be similar and both significantly equal to 1 (see **ref. 6** for more details).

3.3. Primer Design and Evaluation

The reliability and accuracy of real-time PCR lies, in part, in the specificity and reaction conditions of the primers and probe(s). Although many software tools and internet sites exist to design real-time primers and probes (summarized in **Subheading 3.3.3.**), validation experiments are required to ensure that the designed oligonucleotides perform well. It might prove difficult to design specific working primers and probes, particularly if working with highly homologous sequences (e.g., gene families).

As a golden rule, never use your old primer sequences (that you have been using successfully for many years in conventional PCR). You will save a lot of time and reagents by designing new primers, compatible with real-time PCR. Reaction condition optimization is almost never needed; follow the primer design (see **Subheading 3.3.2.**) and universal reaction setup guidelines (see **Subheading 3.4.**) and you have a high chance of a successful assay. Before designing your own set of primers, it might be interesting to take a look at the RTPrimerDB, a real-time PCR primer and probe database (8) (<http://medgen.ugent.be/rtprimerdb/>; limit search to “DNA copy number quantification/detection” applications) to see whether primers have been designed and validated for your specific gene of interest (GOI; see **Note 4**). We highly recommend submitting your validated primers to this database so that other people can benefit from your expertise. Furthermore, the parallel design of different primers for the same sequence in different laboratories is often a waste of resources and excludes a certain level of standardization and uniformity.

3.3.1. Sequence Retrieval and Preparation

Go to the Ensembl Genome Browser (<http://www.ensembl.org/>) and select your organism of interest. Search for the gene you are interested in and view (predicted) transcript information. In this frame, select “Exon information.”

Select and copy the sequence fragment of interest (see **Note 5**). Mask repeat sequences using RepeatMasker (<http://www.repeatmasker.org/>).

3.3.2. Primer Design Guidelines

Requirements for primer design:

1. Primer length: 9 bp/20 bp/30 bp (minimum/optimal/maximum).
2. Melting temperature (T_m) primers: 58°C/59°C/60°C (minimum/optimal/maximum).
3. GC content primers: 30%/50%/80% (minimum/optimal/maximum).
4. The five nucleotides at the 3'-end should have no more than two G and/or C bases, e.g., 5'-...ACTGT-3' (accept) vs 5'-...ACCGT-3' (reject). This above requirement can be set in the PrimerQuest software (see **Subheading 3.3.3.**) by adjusting the max 3' stability parameter to three.
5. Maximum T_m difference between primer pairs (°C): two.
6. Avoid runs of four or more identical nucleotides (especially G bases).
7. Amplicon length: 80 to 150 bp; avoid secondary structures by using the MFOLD software (see **Subheading 3.3.5.**).
8. If you use SYBR Green I as the detection chemistry, a probe is not needed.

3.3.3. Software

1. Primer3 (Whitehead Institute; free, Web-based [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi]).
2. PrimerQuest (Integrated DNA Technologies; free, Web-based [<http://biotools.idtdna.com/primerquest/>]).

Many other dedicated software packages (free as well as commercial) are available (see the “primer design” in the “links” section of the RTPimerDB, <http://medgen.ugent.be/rtpimerdb/>).

3.3.4. BLAST Primer Specificity Analysis

Search for short nearly exact matches using a modified BLAST search at <http://www.ncbi.nlm.nih.gov/blast/> by pasting both primer sequences separated by 2 N bases (for further details, see **Fig. 2**).

3.3.5. MFOLD Secondary Structure Evaluation

For accurate quantification during real-time qPCR, a high PCR amplification efficiency is required. One of the main factors that may influence the efficiency of a PCR reaction, and, hence, the accurate detection of Ct values, is the formation of secondary structures in the amplified fragments in the region in which the primers (and probe, in case of probe-based detection chemistries) bind at the annealing temperature. Formation of a secondary structure is not favored and, hence, does not influence the PCR reaction if the hybridization of primer:target is thermodynamically stronger than the formation of a secondary structure. The

Identities = 28/28 (100%)

③ Strand = Plus / Minus

```
Query: 26      gtcctatcctgtacttaaccacaacaacct 53
              |||
Sbjct: 175800  gtcctatcctgtacttaaccacaacaacct 175773
```

Score = 46.1 bits (23), Expect = 0.002

2 Identities = 23/23 (100%)

Strand1 = Plus / Plus

```
Query: 1      caatgttgacggacagcctattt 23
             |||
Subject: 175700 caatgttgacggacagcctattt 175722
```

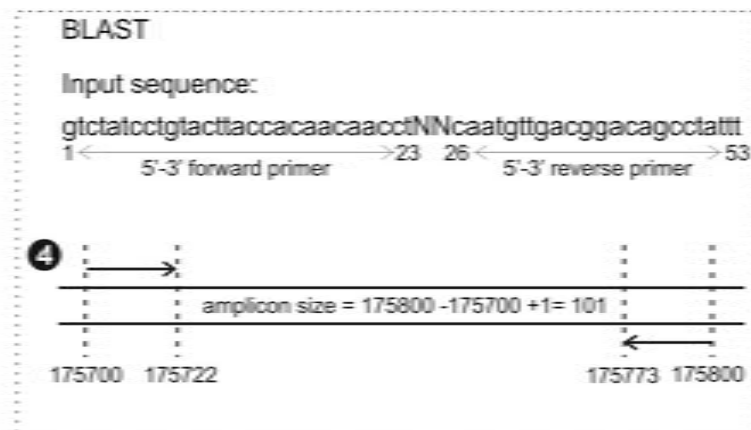


Fig. 2. Interpretation of BLAST output. The BLAST input sequence for a search of short, nearly exact matches against the chromosome or nonredundant database consists of the two primer sequences (5'–3'), separated by 2N bases (shown at the right side). Important BLAST result parameters are indicated in this figure: ❶ “Expect” value describes the number of hits one can expect by chance if searching a database of a particular size. ❷ “Identities” indicates the percentage of bases from the primer that correspond with the aligned sequence, the denominator of the fraction represents the primer length. ❸ “Strand localization” the strand in the 5' to 3' direction of a DNA helix is called the “plus” strand, whereas the strand from 3' to 5' is the “minus” strand. ❹ The PCR amplification length can be indirectly calculated based on the information provided by the BLAST output (see insert). Good primers should meet the following requirements (for both forward and reverse primers):

1. An expect value close to zero; a cut-off cannot be proposed because this value depends on the primer length.
2. An identities value of 100% for both the forward and reverse primer.
3. Primers should be located on complementary strands.
4. An amplicon length between 80 to 150 bp (see **Subheading 3.3.2**).
5. Primers should only match via BLAST analysis at the sequence of interest. Primer pairs that result in one or more hits on the same or another chromosome are not specific.

Additional information can be found on the BLAST education site (<http://www.ncbi.nlm.nih.gov/Education/index.html>).

MFOLD v. 3.1 software (free, web-based), based on minimal free energy (<http://www.bioinfo.rpi.edu/applications/mfold/>; **ref. 9**) can be used for predicting the secondary structure of single-stranded DNA; applying default settings and a temperature that is the annealing temperature of the primers, 50 mM Na⁺, and adjusting concentration of Mg²⁺ to the actual concentration in the PCR reaction. Choose amplicons with minimal secondary structures. To determine which structures are favored, look at the ΔG (free energy; kcal/mole) value. The more negative the ΔG value, the stronger the formation of the structure is favored. **Figure 3** demonstrates that the presence of secondary structures in the region in which the primers anneal significantly influences PCR amplification efficiency.

3.3.6. Experimental Primer Evaluation

Always test a new primer pair by running a standard curve (see **Heading 1.** and **Subheading 3.5.2.2.**) and calculating the PCR reaction efficiency. Efficiency should be higher than 90% (slope > -3.6; **Fig. 3**). Run PCR samples once (and never again) on an agarose gel, to verify specificity and correct amplicon length (see **Note 6**). If using SYBR Green I, ensure the presence of a single sharp peak in the melting curve (see **Note 7**).

3.4. Reaction Setup

Provided that the same primer design guidelines are used, run all quantitative assays using the same universal thermal cycling parameters (10 min at 95°C [polymerase activation], 40 cycles at 95°C for 15 s [denaturation], and 60°C for 60 s [annealing and extension]; see **Note 8**). When working with SYBR Green I detection, start a melting curve run after the 40 amplification cycles. The described “universal” approach obviates any optimization of the thermal cycling parameters and means that multiple assays can be run on the same plate without sacrificing performance. The thermal cycling parameters constitute a two-step PCR (denaturation followed by combined annealing/extension), with an extra step for melting curve analysis if SYBR Green I is used as the detection format. Although initially recommended by some companies, real-time PCR reaction optimization (especially concentration of primers and MgCl₂) is now considered not to be required. Rigorous testing in our laboratory indicated that a final concentration of 250 nM for each primer is optimal for SYBR Green I assays. Many commercial ready-to-use master mixes or core kits for real-time PCR do not need any optimization, and provide accurate and reliable results (we have good experiences with the Bio-Rad and Eurogentec kits, working at 3 mM MgCl₂).

Table 1 shows a typical real-time PCR reagent mix with the individual reagents (see **Note 9**).

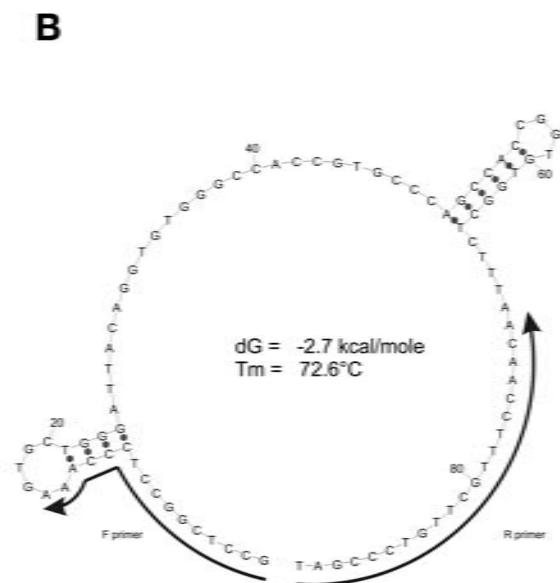
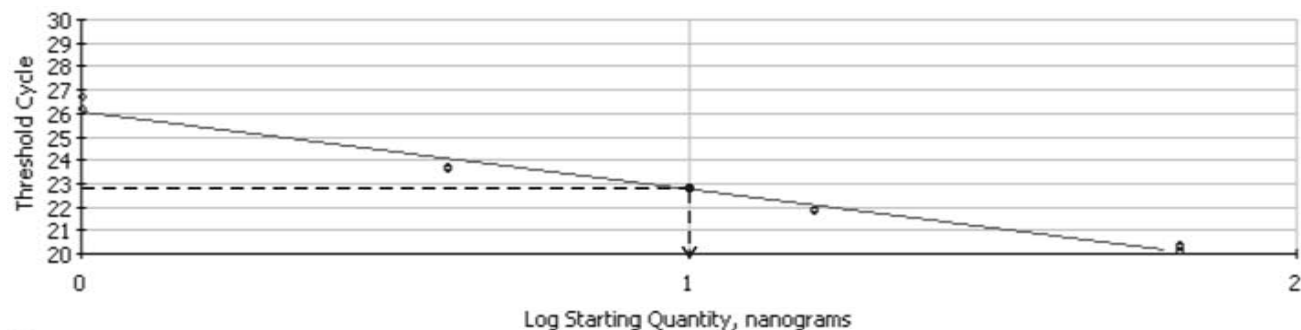


Fig. 3. (Continued)

C

Correlation Coefficient: 0.988 Slope: -3.296 Intercept: 26.042 $Y = -3.296 X + 26.042$
 PCR Efficiency: 101.1 %

• Unknowns
 ○ Standards

**D**

Correlation Coefficient: 0.909 Slope: -0.991 Intercept: 22.110 $Y = -0.991 X + 22.110$
 PCR Efficiency: 920.5 %

○ Standards

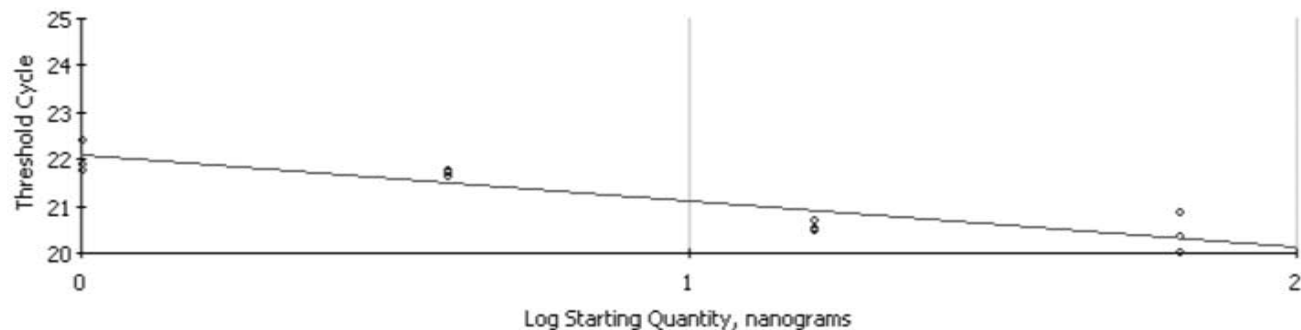


Table 1
Typical Real-Time PCR Reagent Mix

SYBR dilution (1:2000) ^a	0.75 μ L
SYBR Green I PCR mix ^b	12.5 μ L
5 μ M Forward primer	1.25 μ L
5 μ M Reverse primer	1.25 μ L
2.5 ng/ μ L DNA template	4 μ L
Nuclease-free H ₂ O	to a 25- μ L final volume

^aDepending on the reagent kit, SYBR Green I might also be present in the PCR mix.

^bBuffer containing (hot-start) *Taq* polymerase, dNTPs (200 μ M final concentration), stabilizers, and MgCl₂ (typically yielding 3 to 5 mM final concentration).

Refer to the instrument manual regarding whether a passive reference dye is required for calibration purposes (e.g., fluorescein for the Bio-Rad cyclers or ROX for the Applied Biosystems machines).

Always run duplicate reactions for gene amplification assays (example, *MYCN*) and quadruplicate reactions for deletion screening (example, *VHL*) for each sample, including the no-template control. Some practical hints for preparing a reaction plate are presented in **Notes 10–12**.

3.5. Data Analysis

3.5.1. Nomenclature

An amplification plot illustrating the nomenclature typically used in real-time PCR experiments is shown in **Fig. 1**. The amplification plot displays the fluorescence intensity vs the PCR cycle number. The baseline is defined as the PCR cycle range in which a signal is accumulating but lies beneath the detection limit of the instrument. The threshold line is used to define the Ct for each sample. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal reaches the threshold value. Different methods are available to determine the threshold value. In one method, the threshold is calculated as the average baseline value plus 10 times the standard deviation (SD) of all baseline fluorescent signals. A fluorescent

Fig. 3. Secondary structure analysis and standard curves for calculation of PCR efficiency. **(A)** No secondary structures are present in the region where the primers anneal, the secondary structure between the primer annealing sites has a very small negative ΔG value, and, hence, does not influence the amplification efficiency (see **C**). **(B)** Secondary structure is more stable than primer:target hybridization and, hence, hampers efficient annealing of the primers (see **D**). **(C)** Using the primer set from **(A)**, an almost perfect amplification efficiency (100%) is achieved. **(D)** The primer set from **(B)** results in an aberrant slope of -0.991 (920% efficiency), demonstrating the influence of secondary structures on PCR efficiency.

signal that is detected above the threshold is considered a real signal that can be used to define the Ct for a sample. Other methods arbitrarily position the threshold line in (the middle of) the linear part of the log-linear amplification plot (this is the first 5–10 cycles after the fluorescent signal increases above background, and indicates exponential amplification, in contrast to the later plateau phase). In practice, it does not matter very much where the threshold is set, as long as it is the same for all of the samples that you are comparing for your GOI.

3.5.2. Transformation of Ct Value to Quantity

3.5.2.1. COMPARATIVE CT METHOD

The Ct values of the different samples can be used to calculate the relative abundance of template for each sample. In **Fig. 1**, the solid line crosses the threshold at PCR cycle number 23, whereas the dotted line crosses at 27. By subtracting 23 from 27, there is a four-cycle difference between these two samples or a change in Ct (ΔCt) of 4. Because of the exponential nature of PCR, the ΔCt is converted to a relative abundance by $2^{\Delta\text{Ct}}$, or a 16-fold difference, in this case (see **Note 13**). This calculation forms the basis of the comparative Ct method for calculating DNA copy numbers. The ΔCt method generates raw (not normalized) quantities, which need to be normalized by dividing by a proper normalization factor (see **Subheading 3.5.4.**).

Another method that can be used to transform Ct values into normalized relative quantities is the $\Delta\Delta\text{Ct}$ method. This method relates the Ct value of your GOI in your sample to a calibrator/control sample and to the Ct value of a reference gene in both samples. Note that in the original publication of the $\Delta\Delta\text{Ct}$ method (**10**), there is no correction for a difference in amplification efficiency between the GOI and the reference gene (only the underlying requirement that the efficiency of both genes should be similar). Calculating $2 \times \Delta\Delta\text{Ct}$ between the GOI and the two reference genes, and taking the geometric mean of the two relative quantities is the same as first transforming the Ct values of the three genes into quantities using the ΔCt method, and dividing the GOI by the geometric mean of the two reference genes. Although both approaches yield the same result, we favor the ΔCt method, because:

1. It is much easier to perform in Excel.
2. It is very easy to take different amplification efficiencies for the different genes into account (just replace value 2 with the actual efficiency of the gene (e.g., 1.95 for 95%) in the formula of ΔCt).
3. It allows easy inclusion of multiple reference genes, which is a prerequisite for accurate normalization.

3.5.2.2. STANDARD CURVE METHOD

Besides the comparative Ct methods, a standard curve-based quantification method is also frequently applied for calculation of PCR efficiency and for

interpolating unknown sample quantities (**Fig. 3C**). Typically, a serial dilution of a positive control template is used to generate the standard curve. The resulting Ct values for each input amount of template are plotted as a function of the log concentration/quantities of input amounts (black circles), and a linear trend line is fit to the data. The resulting slope of the line fit to the data is used to determine the PCR efficiency, as shown in the formula. An ideal slope should be -3.32 for 100% PCR efficiency (i.e., a doubling of PCR product each cycle); in this example, it is 101.1%. The function that defines this slope is also used to calculate the amount of unknown samples by interpolation (black dot). Most real-time PCR instruments have software that can automatically compute the amount of template of an unknown sample from a standard curve. However, it can be calculated manually by putting the observed Ct value for an unknown sample into the formula: $(\text{observed Ct} - y \text{ intercept})/\text{slope}$.

3.5.2.3. CAVEATS IN DATA ANALYSIS WITH RESPECT TO REACTION EFFICIENCY

The main disadvantage of external standards (as described in **Subheading 3.5.2.2.**) is the lack of internal control for PCR inhibitors or other efficiency modulators in the DNA samples. All qPCR methods assume that the standards and the actual samples amplify with similar efficiency. Although this is true in most cases, this cannot be guaranteed in advance. The reaction efficiency calculated from the standard curve slope is, therefore, only valid for the standards, and is sometimes not transferable to the samples. The risk with external standards is that the unknown samples are amplified with varying efficiency. It is possible to test for PCR inhibitors directly to ascertain whether there is a consistent problem with unknown samples. Unrelated PCR product can be spiked into the sample, and the Ct value of the amplification can be compared with a control. A dilution series of the unknown sample can be tested, whereby PCR inhibitors are often diluted out, causing a nonlinear standard curve.

Although the standard curve is a valid quantification method in most cases, care should be taken to blindly extrapolate standard reaction efficiency to the unknown samples. Recently, several methods have been reported to determine the reaction efficiency for each individual tube based on the amplification plot. However, it is beyond the scope of this chapter to explain this in more detail. More info regarding this issue can be found on the GeneQuantification website (<http://efficiency.gene-quantification.info/>), where several approaches are listed.

3.5.3. DNA Melting Curve Analysis

An important issue related to the use of the cost-effective SYBR Green I dye is that it binds to any double-stranded DNA; the specific product, nonspecific products, and primer dimers are detected equally well. There are a number of ways to handle this problem. Careful primer design (including BLAST specificity

search, see **Subheadings 3.3.2. and 3.3.4.**), keeping primer concentrations relatively low (<300 nM), and increasing template concentration, can usually reduce primer dimers and nonspecific amplification. Hot-start techniques (in which the *Taq* polymerase is activated after an incubation at high temperature, usually several minutes at 95°C) is also helpful in reducing primer dimer and nonspecific amplification. The specificity of a SYBR Green I amplification can reliably and elegantly be monitored by generating a so-called melting curve of the PCR product (first described in **ref. 11**). Fluorescence is continuously monitored while heating the final PCR product from 60 to 95°C . Around the T_m of the amplicon (the temperature at which 50% of the products are denatured and single stranded), a sharp decrease in fluorescence is observed (because the SYBR Green I molecules can no longer bind the double-stranded DNA). With the instrument's software calculating the negative first derivative of the fluorescence over temperature, a typical melting peak is generated. A specific PCR product is characterized by a single sharp peak, whereas primer dimers generally display a lower and broader peak, at a lower temperature (**Fig. 4**; see **Note 14**). The resulting amplicon of a new primer pair is also analyzed using agarose gel electrophoresis (see **Subheading 3.3.6.**).

3.5.4. Accurate Normalization

Appropriate normalization is required to eliminate nonspecific variation, such as variation in DNA input amounts or presence of PCR inhibitors that are especially important in clinical samples. Using reference genes to normalize the data, it is not very important to use the same amount of DNA from each sample. However, for the different genes tested on the same sample, it is extremely important to use the same amount of the same concentration of DNA (and preferably originating from the same dilution). For screening copy number changes, two reference genes are needed. The use of two reference loci instead of one single locus for normalization of the data not only allows internal quality control, but also has proven to result in more robust, reliable, and accurate quantification (**5**).

3.5.5. Data Analysis and Error Propagation: Calculation of SD on Normalized Gene Copy Numbers

To calculate the SD on the final copy number of the normalized GOI (GOI_{norm}) copy number values, the error propagation rules for independent variables have to be applied.

3.5.5.1. SD OF RELATIVE COPY NUMBER VALUES

The ΔCt formula for transforming Ct values to copy numbers with the calibrator (normal control DNA sample) haploid copy number set to 1:

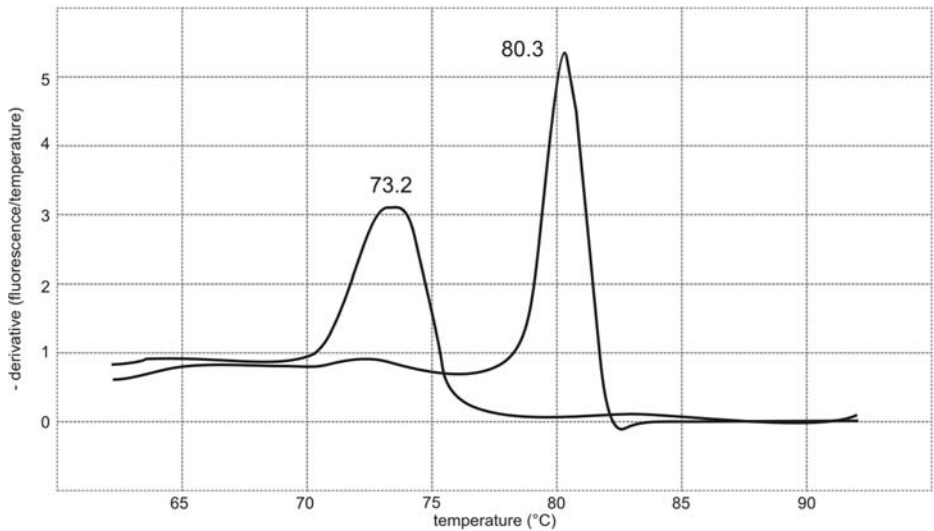


Fig. 4. Representative melting peaks of primer dimers and a specific amplicon, melting at 73.2 and 80.3°C, respectively. In general, primer dimer peaks are broader and smaller.

$$Q = E^{\Delta Ct} \tag{1}$$

$$Q = E^{(calibratorCt - sampleCt)} \tag{2}$$

where Q is the sample quantity relative to the calibrator sample, E is the amplification efficiency (2 = 100%; see also **Note 13**), and calibratorCt is the Ct value of calibrator sample.

The SD for this relative quantity Q is (see geNorm manual for derivation of the formula, <http://medgen.ugent.be/genorm/>):

$$SD\ Q = E^{\Delta Ct} \times \ln E \times SD\ sampleCt \tag{3}$$

where lnE is the natural logarithm of the amplification efficiency and SD sampleCt is the SD Ct values of the sample replicates.

3.5.5.2. SD FOR NORMALIZED HAPLOID COPY NUMBERS

Two reference genes (REFG) and one GOI—each with their own SD values (calculated as outlined in **Subheading 3.5.5.1.**)—are measured, and the geometric mean of the two REFG is calculated as a reliable normalization factor (NF₂).

The normalization factor based on two reference genes is:

$$NF_2 = \sqrt{REFG_1 \cdot REFG_2} \text{ (geometric mean)} \tag{4}$$

The SD for this NF₂ is:

Table 2
Relative Quantities Calculated for the GOI and REFG

GOI	GOI ± SD
Reference gene 1	REFG1 ± SD
Reference gene 2	REFG2 ± SD

GOI, gene of interest; SD, standard deviation; REFG, reference gene.

$$SD\ NF_2 = NF_2 \times \sqrt{\left(\frac{SD\ REFG_1}{2 \cdot REFG_1}\right)^2 + \left(\frac{SD\ REFG_2}{2 \cdot REFG_2}\right)^2} \tag{5}$$

The SD of the normalized GOI is:

$$SD\ GOI_{norm} = GOI_{norm} \times \sqrt{\left(\frac{SD\ NF_2}{NF_2}\right)^2 + \left(\frac{SD\ GOI}{GOI}\right)^2} \tag{6}$$

Alternatively, standard error (SE) values can be used instead of SD; the latter is the error on a single measured value and the former is the error on the mean (of repeated measurements).

$$SE = \frac{SD}{\sqrt{m}} \tag{7}$$

where *m* is the number of measurements, i.e., three for triplicates in a PCR. The error propagation rules are identical if using SD or SE values, just replace the SD values with SE values in **Eqs. 5** and **6**.

Note that this procedure only provides the error for the normalized gene copy number of a GOI for a single sample (mainly reflecting technical and pipetting variation, and variation among the different reference genes used for normalization).

However, if multiple samples are averaged (e.g., biological replicates [same cells independently grown or harvested], technical replicates [testing the same sample in different runs], or grouping samples with similar properties [e.g., diseased vs healthy tissue samples]), different rules apply (see the geNorm manual).

3.6. Example 1: Germline Exon Deletion Screening of the VHL Gene in Patients With VHL Syndrome

3.6.1. Template Preparation

Generate adequate template for real-time qPCR (see **Subheading 3.1.**). High-molecular-weight DNA was isolated from peripheral blood samples using the QIAamp DNA Blood mini kit according to the manufacturer’s instructions (Qiagen).

3.6.2. Reference Genes

Select reference genes (see **Subheading 3.2.**). Two reference genes were selected on the same chromosome that the disease gene is located, i.e., *ZNF80* (3q13.2) and *GPR15* (3q12.1).

3.6.3. Primer Design and Evaluation

1. Examine the RTPrimerDB to check whether primers for your genes of interest are present. Primers for the *VHL*, *ZNF80*, and *GPR15* gene were not present in the RTPrimerDB (**8**).
2. If no suitable primer pairs are present in the database, design new primers following the designing guidelines (see **Subheading 3.3.2.**). We designed multiple primer pairs for each of the three *VHL* exons and for the two reference genes. Because of the small size of the *VHL* exons, we also developed primers spanning an exon–intron boundary (see **Note 5**).
3. Test primer specificity *in silico* (see **Subheading 3.3.4.**). Primer specificity was tested by a search for short nearly exact matches against the nonredundant and chromosome database, using the BLAST program.
4. Select primers with minimal secondary structures (see **Subheading 3.3.5.**). For the amplicons generated by the primers that passed the BLAST search, secondary structures of single-stranded DNA were predicted using the MFOLD program, applying default settings and 50 mM Na⁺, 3 mM Mg²⁺, and a temperature of 60°C (which is the annealing temperature of the primers). All primers that generate amplicons with secondary structures at the primer binding sites with a significant negative ΔG (free energy in kcal/mole) value were excluded from further analysis.
5. Validate your primers experimentally (see **Subheading 3.3.6.**). Amplification efficiency was evaluated by generating a standard curve. PCR products were run on an agarose gel to ensure correct amplicon length and specificity.
6. Primer sequences are available in the public RTPrimerDB database (<http://med-gen.ugent.be/rtpriimerdb/>; gene [RTPrimerDB-ID]: *VHL* [1023, 1024, and 1025], *GPR15* [1022], and *ZNF80* [1021]; **ref. 8**).

3.6.4. Reaction Setup (see **Subheading 3.4.**)

In addition to test samples, each assay must include:

1. A no-template control.
2. Calibrator DNA (a normal control or a mixture of genomic DNA from the organism of interest).
3. A positive control for deletion, duplication, or amplification (depending on the kind of assay to be developed).

We used the SYBR Green I detection method for quantification of the three test amplicons (*VHL* exon 1, 2, and 3) with unknown copy number and of the two reference genes *ZNF80* and *GPR15*. A 15 μ L reaction was performed following the cycling program: 10 minutes at 95°C, 40 cycling at 95°C for 15 s

and 60°C for 60 s. After PCR amplification, a melting curve is run to guarantee the specificity in every well of the reaction plate.

Each assay included:

- No-template control (in duplicate).
- 10 ng of calibrator DNA (in quadruplicate; Roche human genomic DNA).
- 10 ng of a positive control for deletion (in quadruplicate).
- 10 ng of test DNA (in quadruplicate).

3.6.5. Data Analysis and Error Propagation

1. First transform the Ct value quantities (either by using standard curves or the comparative Ct method). Here, calculation of the gene copy number was performed using the comparative Ct method (see **Subheading 3.6.2.1.**), according to the geNorm manual (<http://medgen.ugent.be/genorm/>; **ref. 12**).
2. Exclude reaction wells with obvious PCR reaction failure or clear outlier values (difference between Ct and mean Ct > 0.3) from further data analysis.
3. Calculate the arithmetic means of replicated Ct values.
4. Transform these arithmetic means to relative quantities with the copy number of the calibrator set to 1, using the Δ Ct formula, and an amplification efficiency of 97% (determined on the basis of a standard curve consisting of a DNA dilution series).
5. Normalize these relative quantities by dividing the *VHL* copy numbers by the geometric mean of two reference gene copy numbers (*ZNF80* and *GPR15*). Using this method, a haploid copy number of 1 is expected for a normal sample and a value of 0.5 for a sample with a *VHL* deletion.

We developed an Excel template for automated calculations, error propagation, and graphical representation of the results (available from the authors on request, **Fig. 5**).

3.7. Example 2: Detection of MYCN Oncogene Amplification in Neuroblastoma Tumors

Details regarding assay design and data analysis are described by De Preter and colleagues (**6**). Only substantial differences with the previous example are indicated in this section.

Two reference genes (*TNFRSF17* and *SDC4*) were selected in chromosomal regions that are rarely affected in neuroblastoma (based on comparative genomic hybridization results of more than 200 primary neuroblastomas; **ref. 7**).

Primer sequences are available in the public RTPrimerDB database (<http://medgen.ugent.be/rtpriimerdb/>; gene [RTPrimerDB-ID]: *MYCN* [11], *TNFRSF17* [14], and *SDC4* [15]; **ref. 8**).

Each assay included duplicate reactions instead of quadruplicate reactions because the difference in gene copy number caused by amplification is much larger compared with a single-copy loss in the case of a deletion.

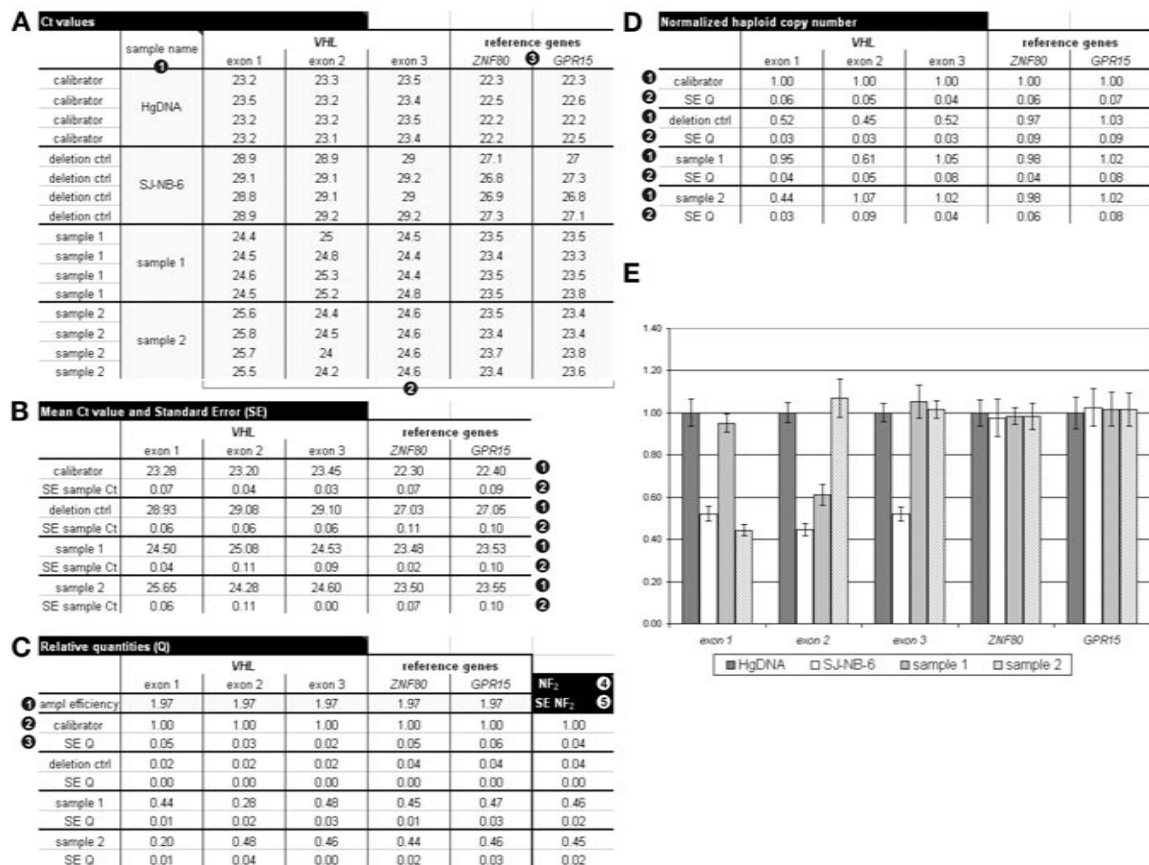


Fig. 5.

If using the standard curve method for data analysis in the case of gene amplification assays, use a dilution series of a sample that is amplified for the gene under investigation. In this way, the copy number values from both amplified and single-copy samples can be reliably interpolated from the standard curve.

3.8. Overview of Relevant Internet Links

1. Gene quantification: general website regarding all aspects of real-time PCR (quantification strategies, fluorescent detection chemistries, determination of amplification efficiency, and so on): <http://www.gene-quantification.info/>.
2. RTPrimerDB: public database for real-time PCR primer and probe sequences for all popular detection chemistries (with links to primer design software, real-time PCR machine vendors, and so on): <http://medgen.ugent.be/rtpimerdb/>.
3. geNorm: normalization using multiple reference genes: <http://medgen.ugent.be/geNorm/>.
4. qpcrlistserver: qPCR discussion group: <http://groups.yahoo.com/group/qpcrlistserver/>.

4. Notes

1. We have had good experiences with iQ SYBR Green Supermix (Bio-Rad) or the SYBR Green I qPCR core reagents (Eurogentec).
2. Any real-time PCR machine is acceptable. Currently, we are using a GeneAmp 5700 thermal cycler (Applied Biosystems) and an iCycler iQ real-time PCR detection system (Bio-Rad).
3. A repetition pipet to distribute the master mix in the reaction tubes is recommended, especially if dealing with many samples, to increase reproducibility and to reduce hands-on time.
4. If you are new to real-time PCR, order a pair of primers (e.g., for a reference gene) that have been shown to work and convince yourself that you can perform the PCR and/or obtain good standard curves.

Fig. 5. (Continued) Microsoft Excel template for data analysis and visualization. **(A)** Importing data: ❶ Type sample name in this column. ❷ Paste Ct values for the three *VHL* exons and two reference genes for each sample (up to four replicates). ❸ Fill in reference gene names. **(B)** Calculation of mean Ct values and SE: ❶ In these rows: arithmetic mean of replicates. ❷ In these rows: $SE = SD/\sqrt{4}$ (in case of four replicates) (see **Subheading 3.6.5, Eq. 7**). **(C)** From Ct values to relative quantities: ❶ Paste amplification efficiency (see **Subheading 3.5.2.2**). ❷ Relative quantities (Q) are calculated using the comparative Ct method, using provided amplification efficiencies (C❶), and setting calibrator Q to 1. ❸ Error propagation using formula $SE\ Q = E\Delta Ct \times \ln E \times SE\ sample\ Ct$. ❹ NF2, normalization factor based on two reference genes **Eq. 4**. ❺ Standard error NF2 (based on **Eqs. 5 and 7**). **(D)** ❶ Normalization: Divide all relative quantities (Q) by the normalization factor NF₂. ❷ Error propagation: $SE\ GOI_{norm}$ (based on **Eqs. 6 and 7**). **(E)** Graphical representation of the data.

5. If the exon you are interested in is too small or if no suitable primer pairs can be designed, you can add adjacent intron sequence and develop primers spanning an intron–exon boundary.
6. Some researchers advise sequencing the amplicon; however, this is not always straightforward, because of the small size of the fragment.
7. Sometimes a specific PCR product can generate a (atypical) melting curve with two or more peaks caused by regions in the amplicon that are characterized by different melting temperatures (for instance, a sequence with a GC-poor and GC-rich region).
8. Both annealing and extension can be performed at the same temperature (60°C); there is no need for a separate extension at 72°C during 1 min. Although the extension rate of the enzyme is slower at 60°C, it will certainly be sufficient to extend a 250-bp amplicon at 60°C for 1 min.
9. Having performed 25-μL reactions for many years, we now routinely use a 15-μL reaction. All components of the reaction mix are downscaled in the same proportion, but the amount of template DNA remains unchanged. In this way, we obtain a slight increase in sensitivity (lower Ct values, because of the higher initial template concentration) and a 40% decrease in reagent cost.
10. For the PCR master mix, make an excess of one reaction (for <20 reactions) or an excess of 5% (when dealing with >20 reactions). Always run duplicate reactions for each sample, including the no-template control. Work with filter tips in a dedicated PCR workstation (no flow) equipped with UV decontamination bulbs.
11. Prepare the reaction mix (reagents for quantification and primers) in a pre-PCR room to avoid carry-over contamination; this is a room different from the lab in which the DNA is prepared or in which post-PCR manipulations are performed. It might also help to use uracyl-*N*-glycosylase and dUTP nucleotides in the PCR. During an initial step at 50°C, the uracyl-*N*-glycosylase enzyme cleaves contaminating PCR products (carry-over from previous runs).
12. After preparing a 96-well plate for qPCR analysis (reaction mix and added DNA samples), shake the plate on a plate shaker (or vortex) to mix the DNA with the reaction mix, and centrifuge the plate shortly to spin down the reaction mixture and remove air bubbles. Always check the wells for air bubbles, because air bubbles can cause unusual reaction plots and, hence, inaccurate quantification of the gene copy number.
13. In the comparative Ct method, the value of 2 is used as the base in the formula $2^{\Delta C_t}$. A value of 2 means that the reaction efficiency of the PCR was 100%, which is almost never the case. The base value should be adjusted to the actual PCR efficiency (e.g., a value of 1.90 should be used if the efficiency is 90%). Most people determine the reaction efficiency once using a standard curve, and, later on, use this value in their comparative Ct analytical procedure.
14. Although it is possible to collect fluorescence data at a higher temperature (between the T_m of the nonspecific/primer dimer signal and the true signal; thus, removing the contribution of nonspecific signal to the measurement), this is not recommended because the simultaneous amplification of the nonspecific product(s) can adversely affect the amplification of your sequence of interest.

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Design and Work-Up of a New Molecular Diagnostic Assay Based on Real-Time PCR

Harald H. Kessler

Summary

In routine molecular diagnostics, real-time PCR has made a major impact because of the faster time to the result; decreased hands-on time; and virtual elimination of the major issue in the early days of PCR, sample contamination from previously amplified DNA. The shorter time to the result for a real-time PCR assay means that a new diagnostic application may be developed relatively quickly. This chapter discusses the fluorescent chemistries typically used in molecular diagnostic applications and the additional features, such as internal controls, which are highly desirable in these assays. After an assay has been developed, the assay's performance must also be evaluated before the assay can be implemented in the testing laboratory.

1. Introduction

PCR has been used for almost every aspect of biotechnology. Today, PCR-based assays are recognized as reference standard assays for the sensitive and specific diagnosis of a number of pathogens. Moreover, in the routine diagnostic laboratory, PCR-based assays have been used for genetic analysis, including gene expression studies, mutation analysis, and pharmacogenomics. Confirmation of PCR amplification products, however, can involve laborious methods. Longer than 10 yr ago, kinetic PCR analysis by real-time monitoring of DNA amplification reactions was described ([1](#)). This technology, also called real-time PCR, has significantly simplified routine molecular diagnostics. Today, the number of commercially available assays based on real-time PCR is continuously increasing, but the majority of tests still involve in-house developed ("home-brewed") assays. When establishing a home-brewed assay, several issues must be addressed. Issues include detection formats, introduction of an internal control (IC), quantitation, and evaluation of the assay.

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2. General Considerations

PCR-based assays generally involve three major steps. In the first step, target nucleic acid is extracted, also called sample preparation. In the second step, the PCR, which includes a denaturation, an annealing, and an elongation step, is performed. Both of the primers must have a similar annealing temperature and a similar GC content. 3'-end GC clamps should be avoided by allowing a maximum of two G or C bases within the last five nucleotides at the 3'-end of the oligonucleotide. The third step includes hybridization and detection of amplification products. The introduction of probes prevents false-positive results caused by nonspecific amplification products and guarantees specificity of results. Therefore, hybridization must be included in molecular assays, to be applicable in the routine diagnostic laboratory.

Real-time PCR uses fluorescence to detect PCR amplification. The linear correlation between PCR product generation and fluorescence intensity can be monitored. For real-time PCR, instruments that combine a thermal cycler and fluorometer are used. These instruments include optics for fluorescence excitation and emission collection, as well as software that can process and analyze data. Rapid thermal cycling in real-time PCR instruments is made possible by rapid air exchange or rapid thermal conductivity through solid-phase material surrounding the reaction vessel and by a high surface-to-volume ratio of the PCR mix. The latter property is facilitated by narrow elongated reaction cuvetts.

Compared with conventional PCR-based assays, real-time PCR offers several important advantages. Real-time PCR combines amplification of target DNA with detection of amplification products in the same closed vessel. Therefore, the potential for contamination is significantly reduced. With real-time PCR assays, the analytical turnaround time is significantly shorter than that required for conventional PCR assays. In contrast to conventional PCR, real-time PCR allows for log-phase analysis. Therefore, the quantitation range for real PCR assays is significantly greater (5–6 logs) than for conventional PCR assays (2–3 logs).

When establishing a home-brewed assay, it is advisable to use a primer pair that has already been published in a highly recognized journal. This helps to avoid extended specificity testing. The published sequences, however, should always be subjected to BLAST analysis to ensure that the correct sequence has been written.

3. Detection Formats

Detection formats include detection of double-stranded DNA and probe detection formats. The SYBR Green I dye intercalates into double-stranded DNA. A fluorescence signal is not only generated by amplification products, but also by primer dimers and other PCR artifacts. This technology is, thus,

similar to detection of amplification products by gel electrophoresis, and is less common than probe formats in the routine diagnostic laboratory in the assays of sensitive and specific pathogen detection. It does, however, allow melting curve analysis (see **Subheading 3.5.**), and may be useful for a first evaluation during early developmental steps.

For use in the routine diagnostic laboratory, introduction of probes is usually required for maximum specificity. To ensure strong binding of the probe(s) during annealing, the melting temperature (T_m) of the probe(s) should be 5 to 10°C higher than that of the primers. Consequently, probes are usually between 25 and 30 nucleotides long and their design may be sometimes difficult to optimize because of formation of secondary structure or because of sequence variability. To overcome these problems, locked nucleic acid (LNA) probes may be used. LNA is a new class of DNA analog whose incorporation into oligonucleotides results in a significant increase in the thermal stability of duplexes with complementary DNA (2). A general increase of 3 to 8°C per modified base may be expected. Consequently, shorter probes can be used for hybridization-based assays (3–7).

Probe detection formats, which have been most frequently adapted to real-time instruments, include hybridization probes, TaqMan® probes, molecular beacons, and single probes.

3.1. Hybridization Probes

The hybridization probes format uses two different fluorescence-labeled oligonucleotides. The donor probe carries a fluorescein label at its 3'-end, whereas the acceptor probe is labeled with a different fluorescein label at its 5'-end. When the fluorescein at the donor probe is excited, it emits fluorescent light at a certain wavelength. The sequences of the two probes are selected so that they hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes into close proximity. When both of the dyes are in close proximity, the energy emitted excites the dye attached to the acceptor probe that subsequently emits fluorescent light at a longer wavelength. This energy transfer, referred to as fluorescent resonance energy transfer (FRET), is highly dependent on close proximity (between 1 and 5 nucleotides) of the oligonucleotides. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. Because the signal is only emitted when both oligonucleotides are hybridized, fluorescence is measured just after the annealing step. After annealing, the temperature is raised, and the hybridization probes are displaced by the polymerase. At the end of the elongation step, the amplification product is double-stranded and the probes are too far apart to allow FRET.

When using the hybridization probes format, it is essential that both of the probes cannot be elongated during PCR. For the donor probe, the fluorescein at the 3'-end blocks elongation, whereas, for the acceptor probe, a nonfluorescent blocking agent, typically, a phosphate group, is linked to the 3'-end. Sudden loss of probe fluorescence has been observed. Photobleaching and/or repeated freeze-thaw cycles have been correlated with the phenomenon, but an additional mechanism for FRET probe failure has been described: loss of the phosphate blocker from the 3'-end of the acceptor probe (8,9). If this occurs, then the acceptor probe can act as a PCR primer. To prevent this, the phosphate blocker may be replaced by a carbon spacer as a blocker of potentially enhanced stability (9).

Amplification products are designed not to be too long, because shorter amplification products amplify more efficiently than longer ones and are more tolerant to reaction conditions. For gene expression studies, the amplification product should be designed across an intron-exon boundary, so that complementary regions in any contaminating genomic sequence are not amplified and products represent PCR of the complementary DNA template. If using FRET hybridization probes, use of LNA may be of special importance. Introduction of LNA residues offers the advantage of modulating the required T_m of probes without any modification of the sequences, and has been shown to be very useful for single nucleotide polymorphism detection (10,12).

3.2. *TaqMan Probes*

The TaqMan probe format uses an oligonucleotide with a fluorescein label (reporter dye) at its 3'-end and a different fluorescein label (quencher dye) at its 5'-end. The TaqMan probe anneals to the target DNA. During elongation, the 5' exonuclease activity of the polymerase excises the reporter dye. Because of this separation of the reporter dye from the quencher dye, the reporter dye emits fluorescent light at a certain wavelength. In contrast to all other detection formats, complete hydrolysis of the probes by the DNA polymerase is essential to yield precise results. In addition to choice of the adequate polymerase, factors that additionally influence hydrolysis include concentration of probes, the primer-probe distance, and avoidance of regions that would produce either primer:primer dimers or primer:probe dimers (13).

3.3. *Molecular Beacons*

The hairpin-shaped molecular beacon is nonfluorescent because of its stem hybrid that keeps the reporter dye close to the quencher dye. When the probe sequence in the loop hybridizes to its target, the quencher dye is separated from the reporter dye and the fluorescence is restored. Assays that use molecular beacons are difficult to optimize. The stem structure forms by an intramolecular

hybridization event and the signal yield is very sensitive to hybridization conditions. Calculation of the assay kinetics may, thus, be rather complicated.

3.4. Single Probes

The single probe format is similar to the hybridization probes format, but it uses only a single probe. During the annealing step of PCR, the probe hybridizes specifically to the target sequence of interest. When hybridized, the probe emits a greater fluorescent signal than it does when it is not hybridized to its target. In addition to DNA probes, peptide nucleic acids may be used. With this format, changes in fluorescent signal depend solely on the hybridization status of the probe. The single-probe format may especially serve as tool for mutation detection, but is limited by unspecific background fluorescence (14,15).

3.5. Melting Curve Analysis

The temperature at which double-stranded DNA separates can vary greatly depending on the length, but mainly depends on the sequence (GC content). After completion of the amplification protocol, the temperature is steadily increased while the fluorescence is monitored. Fluorescence decreases as the temperature increases. At a certain temperature, an abrupt decrease of fluorescence can be observed because of the melting of a product. The T_m of a product is defined as the temperature at which half of the DNA is single stranded, and represents the steepest decrease of the fluorescent signal. This can be identified conveniently as the peak value in the negative derivative of the melting curve. Melting curve analysis can be performed for all detection formats, except for the TaqMan probe format, because signal generation depends on the hydrolysis of the probe.

The T_m profile can provide additional information, e.g., the genotype of the DNA product (16). Even single-base differences in heterozygous DNA can change its T_m . A heterozygous sample contains two DNA sequences, each of which melts at a different temperature, resulting in a two-peak curve. Unexpected melting peaks may indicate primer:primer or primer:probe dimers or sequence variants (Fig. 1).

4. Internal Control

Amplification may fail in a reaction because of interference from PCR inhibitors. Consequently, an IC should be incorporated in every molecular assay to exclude false-negative results. To ensure an accurate control of the entire molecular assay, the IC should be added to the specimen before the start of the nucleic acid extraction procedure. This guarantees validation of the entire analytical testing process. No matter what IC is chosen, it must be ensured that the IC is added at a suitable concentration to prevent extreme competition with the target template for reagents (17).

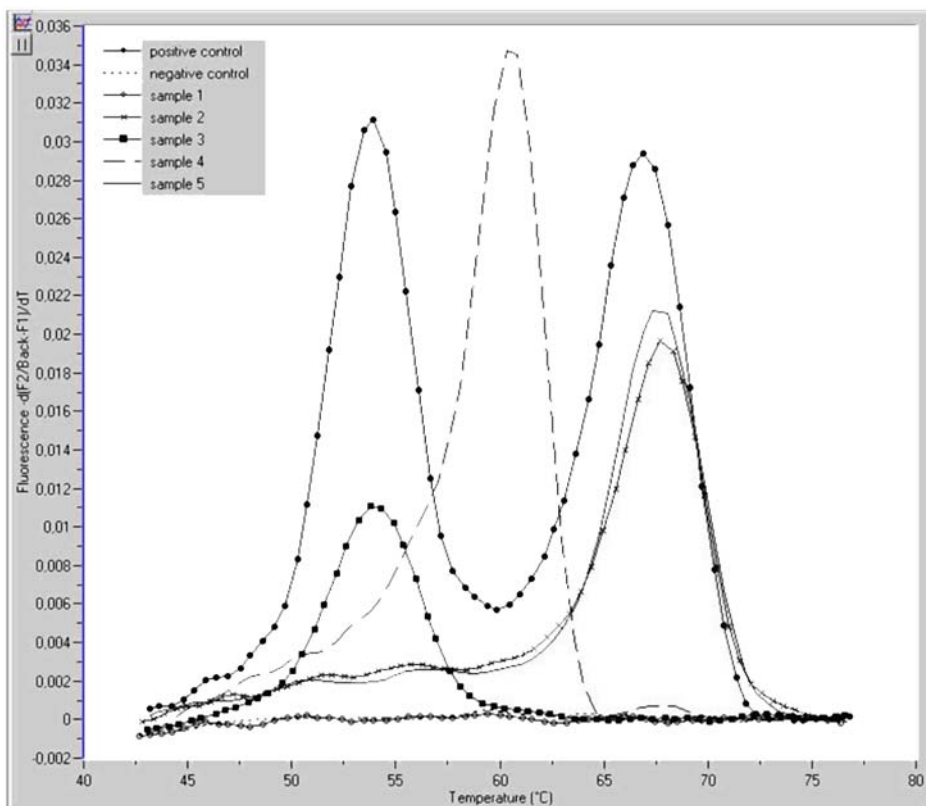


Fig. 1. Detection of herpes simplex virus (HSV) type 1 and type 2 DNA by real-time PCR. Melting curves of clinical samples (genital swabs). In sample 3, HSV-1 was detected; in samples 2 and 5, HSV-2 was found. The positive control contained both HSV-1 and HSV-2. Sample 4 shows an unexpected melting peak at 60.5°C. Sequence variation in the HSV DNA polymerase gene may produce melting peak values that differ from expected values for HSV type 1 or type 2. (Modified from [ref. 16.](#))

4.1. Homologous IC

The homologous IC is a DNA sequence (for DNA amplification targets) or an in vitro transcript (for RNA targets) consisting of primer binding regions identical to those of the target sequence, a randomized internal sequence with a length and base composition similar to those of the target sequence, and a unique probe-binding region that differentiates the IC amplification product from the target amplification product. If using the hybridization probe format, the IC amplification product is detected by IC-specific hybridization probes labeled with a fluorescence dye different from those used for the detection of the target amplification product. It has recently been shown that IC DNA can

conveniently be generated by applying composite primer technology (18,19). Because ICs consisting of bare, unprotected DNA may become degraded during storage or within the clinical specimen before nucleic acid extraction, introduction into λ -phage particles may be useful (20).

4.2. Heterologous IC

In contrast to a homologous IC, the heterologous IC presents a second amplification system within the same reaction vessel. The control must have the same or similar amplification efficiency (see **Heading 5.**) as the target. Plasmids or housekeeping genes (e.g., β -microglobulin) can be used as heterologous ICs. IC-specific probes must be labeled with a fluorescence dye different from those used for the detection of the target amplification product.

5. Quantitation

In theory, PCR amplification results in an exponential increase of amplification products according to the formula $N = N_0 - 2^n$, where N is the number of amplified molecules; N_0 is the initial number of molecules; and n is the number of amplification cycles. In reality, however, PCR amplification consists of three distinct phases. In the initial lag phase, no product formation can be detected. In the second phase, a more or less exponential increase of amplification products can be observed, whereas, in the third phase, the plateau effect occurs. The plateau effect may be caused by several factors, for example, product inhibition, decrease of enzyme stability and reagent concentration, and reassociation of amplification product followed by competition with primers. The result of PCR amplification is, thus, better expressed with the formula $N = N_0 - (E_{\text{var}})^n$, where E is the amplification efficiency.

In contrast to conventional PCR, which only allows quantitation by end-point analysis, real-time PCR permits analysis of the complete amplification course. This, however, requires knowledge of two values. First, the crossing point (C_p), also called the threshold value, must be determined. The C_p is defined as the point at which the fluorescence curve correlated with accumulation of the amplification product intersects the background fluorescence line. This point may be between two successive cycles (i.e., it may be a fractional number). The C_p value depends on the background fluorescence, the signal noise, and the C_p calculation method (21). In optimized assays, standard errors of less than ± 0.2 cycles can be achieved. If the amplification efficiency would be 2.0, the minimum relative error for quantitation would be 10 to 20%. Second, the amplification efficiency must be known. The maximum value for efficiency is 2.0, corresponding to an exponential increase of amplification products. In reality, efficiency values usually vary between 1.5 and 1.9. Because low efficiency may limit the sensitivity, assays must be optimized for maximum efficiency.

Table 1
Suggested Steps for Evaluation of a Newly Established Molecular Assay

What to evaluate	How to evaluate	Which kind of assay
Precision	International standard; reference material	Qualitative; quantitative
Detection limit	Dilution series	Qualitative
Linearity	Dilution series	Quantitative
Interassay variation	Samples aliquoted and analyzed one time on each of several days	Quantitative
Intra-assay variation	Samples aliquoted and analyzed several times in one run	Quantitative
Performance in the routine diagnostic laboratory	Testing of clinical specimens; comparison to reference assay ("gold standard")	Qualitative; quantitative

In routine real-time PCR assays, quantitation is usually performed with the use of external standards (22,23). From the data obtained by a dilution series of an external standard, the standard curve is generated. The concentration of the target, which is amplified in the same run but in a separate vessel, can be derived from the standard curve. A prerequisite for this quantitation approach is the assumption of identical amplification efficiencies in standards and in samples. This quantitation approach may, however, be impaired by inhibitors, which may be present in different concentrations in standards and in samples. To overcome this problem, an internal standard must be introduced. As described in **Subheading 4.1.**, an analytically distinguishable standard template is added to the sample and co-amplified in the same reaction (24,25). However, even this approach poses problems because of different fluorescence dyes used to distinguish the sequences of the standard template, and those of the sample that may be overcome by introduction of an algorithm for normalization of possible differences in amplification efficiencies (26). Together with internal standardization, quantitative results may be obtained by melting curve analysis. The area under the curve is proportional to the amount of the amplification product. Melting curve analysis, however, may be used only if the T_m s of standard template and sample are significantly different (27).

6. Evaluation

Before use in the routine molecular laboratory, the newly established assay must be checked regarding its analytical and diagnostic accuracy. Analytical accuracy comprises analytical characteristics of the assay, whereas diagnostic accuracy refers to the ability of the assay to identify a condition of interest. In studies of diagnostic accuracy, the results of the assay are compared with those obtained by

a reference assay (“gold standard”) in a group of patients suspected of having the condition of interest. The term “accuracy” in this context, thus, refers to the amount of agreement between the studied assay and the reference assay. To obtain such data, guidelines toward diagnostic accuracy of medical tests have recently been published (28,29). Suggested steps for evaluation of a newly established molecular assay for use in the routine diagnostic laboratory are shown in Table 1.

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Real-Time PCR Fluorescent Chemistries

John Mackay and Olfert Landt

Summary

There are more than a dozen formats available for the fluorescent detection of amplified DNA in kinetic (real-time) PCR. These chemistries are adaptable to most real-time PCR instruments and may offer benefits over the usual manufacturer-recommended chemistries for the instrument. The most popular chemistries are the generic dye, SYBR Green I, TaqMan®, and hybridization probes. However, there are now new dyes being reported with superior fluorescent detection and product resolution; as well as new probe formats that may offer advanced multiplexing opportunities for quantification and genotyping.

Key Words: Fluorescent chemistries; hybridization probes; hydrolysis probes; melting curves; molecular beacon probes; quenched dye primers; scorpion primers; SYBR Green I.

1. Introduction

Real-time (kinetic) PCR is based on fluorescence changes during the generation of the PCR product. Once a scientist has undertaken the decision to perform real-time PCR experiments, there can often be considerable concern regarding the appropriate fluorescent chemistry to use. Conversely, users may use a particular chemistry only because that is the chemistry used by others in the laboratory. The purpose of this chapter is to describe many of the chemistries available, with their applications. Many real-time PCR instruments, despite often being linked to one or two chemistries, can use a number of different chemistries that the operator may choose. It may only be a matter of using a more suitable dye for the instrument of choice than was used in the original description of the chemistry.

It should be pointed out that real-time PCR is typically no more sensitive than conventional PCR, in that a product that is not visible in an agarose gel will also

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usually not generate a fluorescent signal. Furthermore, the generation of byproducts such as primer dimers affect not only SYBR Green-based assays by these spurious signals; but also any probe-based assay, by lowering the fluorescent signal, and, thus, shifting the cycle threshold or crossing point values and yielding unexpected quantification signals. Therefore, the quality of real-time PCR data is very much dependent on an adequate primer selection. Simply adding a probe or probes to a poor reaction will not increase the sensitivity; it will merely hide the nonspecific amplification products that are limiting the reaction sensitivity.

There are three basic groups of fluorescent chemistries for real-time PCR. The first group uses a double-stranded DNA binding dye to detect the amplified DNA in the reaction, in an analogous manner to staining DNA on an agarose gel. The second group is based on primer consumption methods. The third group uses one or more fluorescent probes, generally consisting of DNA, but also modified nucleotides, such as locked nucleic acids (LNAs) or analogs, such as peptide nucleic acids (PNAs) that only change fluorescence as the target DNA is amplified.

2. DNA Binding Dyes

DNA binding dyes offer the simplest introduction to new users because only the primer sequences and template are needed; a requirement familiar to all PCR users. It also offers a rapid and economical method for users performing reactions with a wide variety of genes (e.g., microarray validation).

2.1. SYBR Green I

Real-time PCR was originally described in the early 1990s using ethidium bromide as the detection chemistry (1,2). The most commonly used intercalating dye in today's instruments is SYBR[®] Green I (Molecular Probes), first introduced on the LightCycler instrument (Fig. 1; ref. 3). SYBR Green I is approx 10 to 25 times more sensitive and exhibits a much greater specificity for double-stranded DNA than ethidium bromide (4). SYBR Green I also demonstrates high stability during thermal cycling, remaining more than 90% active during the repeated heating to PCR denaturation temperatures (5).

SYBR Green I is an asymmetrical cyanine dye that likely binds to the minor groove of DNA, as deduced from its structure and similarity to other minor groove dyes (6). After binding to double-stranded DNA, its fluorescence is enhanced 800- to 1000-fold and, thus, it represents a sensitive method for the detection of amplified DNA (7). Although claims have been made regarding limited sensitivity of detection with SYBR Green I compared with probe chemistries; as with conventional PCR, this sensitivity is largely dependent on the primers used in the PCR. With appropriate design, SYBR Green I offers similar sensitivity to probe applications (8), and, indeed, has demonstrated superior performance in some cases (9).

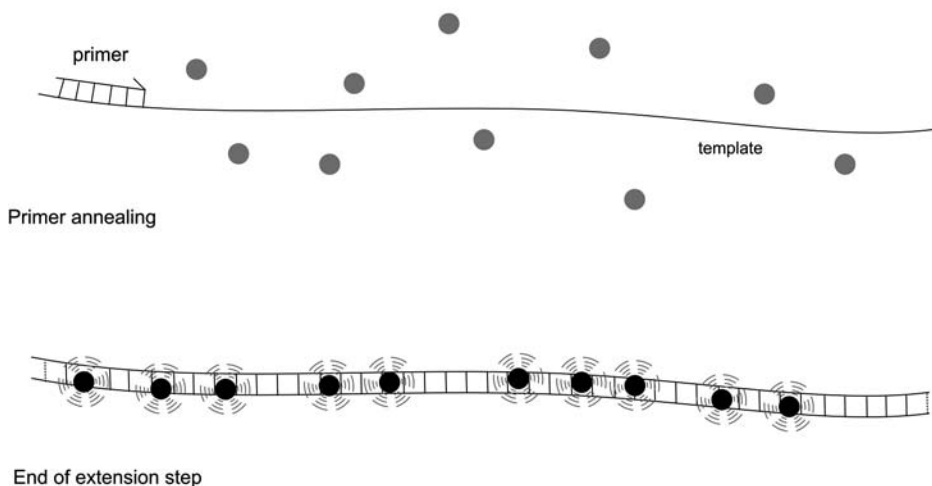


Fig. 1. SYBR Green I dye binds to the double-stranded DNA as the primer is extended, with up to a 1000-fold increase in fluorescence. Maximum fluorescence is at the end of the extension step, at which point, there is the highest amount of double-stranded DNA.

2.2. Melting Curves and Peaks

The major issue with SYBR Green I detection is that all products amplified will bind SYBR Green and contribute to the signal generated. Thus, if primer dimers or other nonspecific products are amplified, these will lower the crossing point (cp)/cycle threshold (ct) and artificially “increase” the resulting target concentration. Alternatively, an amplification curve might be observed despite the absence of the desired target sequence.

These nonspecific products can be distinguished from desired products with an elegant technique, performed at the end of the amplification cycles (10,11). Melting curves (also called dissociation curves) are used to monitor the melting temperature (T_m) of the amplicons. If the instrument is programmed to continuously monitor the fluorescence during the gradual heating of the amplified reactions, then the temperature at which the amplicons denature—visualized as a sharp decrease in fluorescence because of loss of SYBR Green binding—may be readily identified. For easier identification of the temperature at which half the amplicons are denatured (T_m), the fluorescence is plotted as a negative first derivative to turn the maximal rate of fluorescence decrease into a turning point: the melting peak (Fig. 2). The expected T_m for a given amplicon can be calculated using the formula:

$$T_m = 81.5 - 16.6 \times \log_{10} \left\{ \frac{[\text{salt}]}{(1 + 0.7 \times [\text{salt}])} \right\} + 0.41 \times \%GC - (500/L) \\ + \{2.09 \times e^{(-1.18 + \text{SYBR dilution})}\}$$

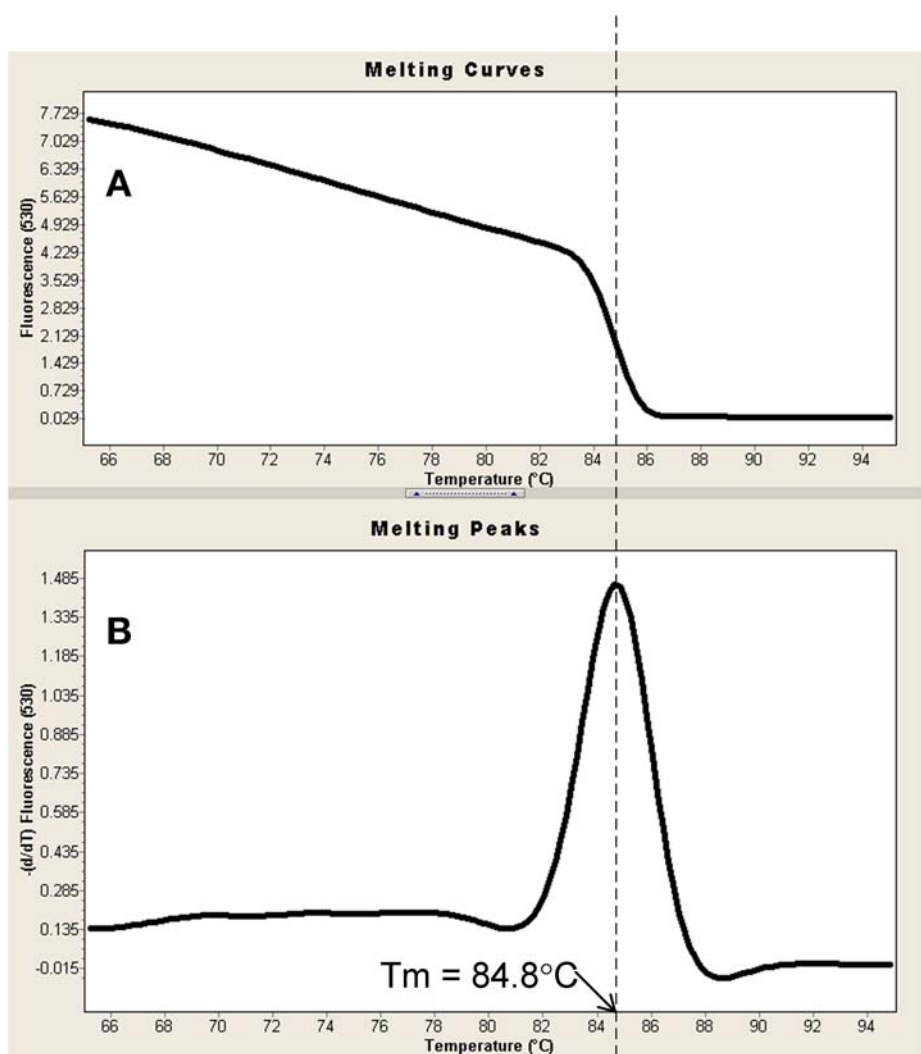


Fig. 2. The sharp decrease in fluorescence (A) as the PCR amplicon denatures and the SYBR Green dissociates is most conveniently displayed as a first negative derivative melting peak (B).

where [salt] is the salt concentration; %GC is the percentage GC content of the amplicon, L is the length in base pairs and SYBR dilution is the fold dilution of stock concentration.

Although different-length products may have melting peaks of different temperature (i.e., allowing primer dimers to be distinguished from target amplicons), it is the GC content of the product that has the largest impact, as shown

in the formula, allowing similar length products that may result in one band if resolved on an agarose gel to be differentiated on the basis of their GC content.

2.3. Elevated Acquisition

Although SYBR Green binds to all amplified double-stranded DNA, it is possible to use the knowledge gained from melting curves to exploit the differences in T_m between the desired and nonspecific products (should they be amplified), to greatly reduce any SYBR fluorescence contributed by primer dimers and other nonspecific products.

With a standard reaction, the fluorescence is acquired at the stage at which there is the highest amount of double-stranded DNA (and, thus, the most SYBR Green fluorescence), i.e., at the end of the extension step. This step is typically performed at 72°C. At this temperature, both the desired and any nonspecific products will be double-stranded and, thus, the SYBR Green signal generated at each cycle may have a contribution from both of these DNA types. By adding an additional step per cycle (if permitted by the instrument software) and acquiring the fluorescence at a higher temperature (below that of the specific product but above that of the nonspecific products) any signal generated by nonspecific products is not measured. In this way, the dynamic range and sensitivity of a SYBR assay may be extended (12–14).

However, this method should not be a substitute for good primer design—a design that should be aimed to generate the most specific amplification. In our experience, using this technique to attempt to overcome large amounts of primer dimer results in lower fluorescence for many samples. This tends to counter any possible sensitivity increase.

2.4. Other Dyes

2.4.1. BEBO

BEBO—a name certainly easier to remember than 4-[(3-methyl-6-(benzothiazol-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methylpyridinium iodide—is another asymmetric cyanine dye that is also a minor groove binder (MGB; **ref. 15**). This dye and its derivative BOXTO (both from TATAA Biocenter) have shown similar performances in quantitative PCR and melting curve applications to SYBR Green I; albeit displaying a lower analytical sensitivity, with a crossing point for a given template concentration approximately four cycles higher. These dyes are not yet widely used.

2.4.2. LC Green I

LC Green I is used in high-resolution melting curve analysis for mutation analyses (16,17). LC Green I can be used at a saturating concentration, unlike

the high dilution used with SYBR Green, because of the inhibitory effects of SYBR Green on PCR (18); therefore, mutation detection is possible by whole-amplicon melting (19) or by using unlabeled probes covering a known mutation (20). Slight DNA changes are enough to affect the LC Green binding at heteroduplexes vs homoduplex molecules, and this is reported via high-resolution melting curve analysis.

2.4.3. SYTO9

SYTO9 (Molecular Probes) is a dye originally used in cell viability assays and has been demonstrated as another potential alternative to SYBR Green I (21). In a multiplex application in which SYBR Green melting curves were previously unable to differentiate two amplicons (22), SYTO9 allowed the resolution of sharp melting peaks for each product.

2.5. Applications

With a good assay designed to generate highly specific amplicons, SYBR Green has been used in all applications. Although it is commonly suggested that only probe-based assays be used in diagnostic applications to guarantee specificity of the amplified product (see Chapter 16), many diagnostic assays have been published using SYBR Green I (see Chapter 15; refs. 23 and 24). Indeed, with the rapid mutation rates of some viruses potentially reducing or preventing probe binding, dye-binding detection may be the only option for some targets and more so in the future (25), especially in the case of rapidly diverging RNA viruses (26).

SYBR Green is a favorite technology of many researchers because of the availability of their primers and, usually, a wide variety of genes to be quantified. It is especially useful in applications such as microarray validation—an application with relatively few samples using a particular primer set but many different genes to quantify (27,28). SYBR Green assays are relatively quick to set up for new targets of interest and primers can be rapidly designed using a number of publicly available software programs, such as Primer3 (see websites of interest in **Heading 6.**).

3. Quenched Dye Primers

These technologies rely on fluorescently labeled primers that only provide a fluorescence increase after primer extension during amplicon formations. They monitor the consumption of primers and not necessarily the formation of the correct PCR product and, as such, they require the same careful validation as with SYBR Green I if used for any diagnostic procedure. In contrast to the SYBR Green I method, these methods allow multiplex applications.

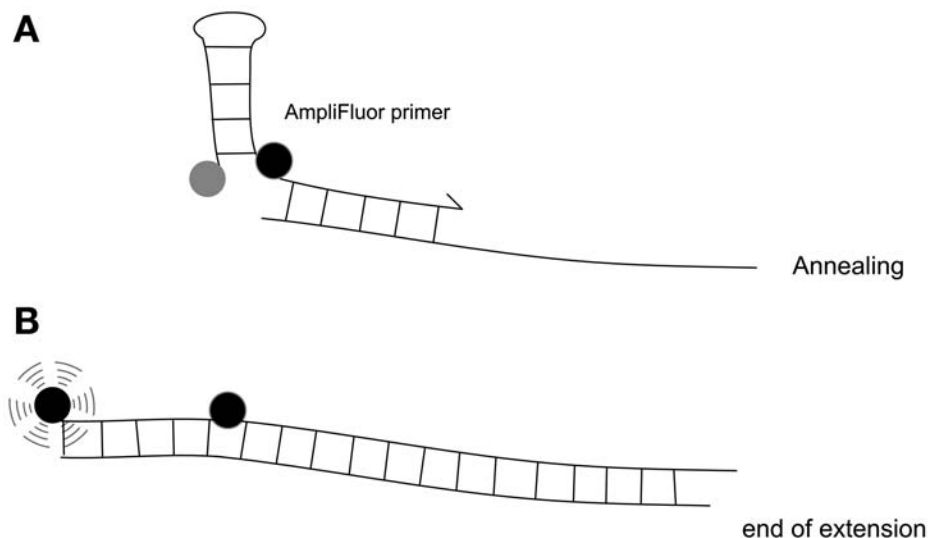


Fig. 3. As the AmpliFluor primer remains in a hairpin structure (A), it stays in a quenched state due to the close proximity of the quencher dye to the reporter dye. After primer extension (B), the polymerase copies the hairpin structure, spatially separating the reporter from the quencher, and allowing fluorescent signal to be measured.

3.1. AmpliFluor

AmpliFluor primers (Chemicon) are PCR primers with a specific intramolecular “hairpin” sequence at the 5′-end. Although this hairpin sequence is bound (Fig. 3), the fluorescein signal is quenched by a nearby dye. As the primer is unfolded during the double-stranded DNA synthesis in the PCR, the hairpin unfolds, and the fluorescein signal is free from the quenching effect. This hairpin method is similar to the molecular beacon probe format described in **Subheading 4.3.**, and is also used as part of the Scorpion probe format in **Subheading 4.4.**

AmpliFluor also relies on the specificity of the primers because any amplified product contains unfolded primers and will thus contribute to fluorescence. It is perhaps for this reason that AmpliFluor primers have been reported to be less sensitive than a TaqMan assay (29).

3.2. LUX Primers

LUX (Light Upon eXtension) primers (30) rely on only a single label. Rather than use a quenching dye, LUX primers are designed in a hairpin structure (Fig. 4), so that the fluorescent dye is quenched by a nearby guanosine in the sequence (31). This hairpin structure of the primer is also reported to minimize nonspecific amplification (32)—an important consideration because, once again,

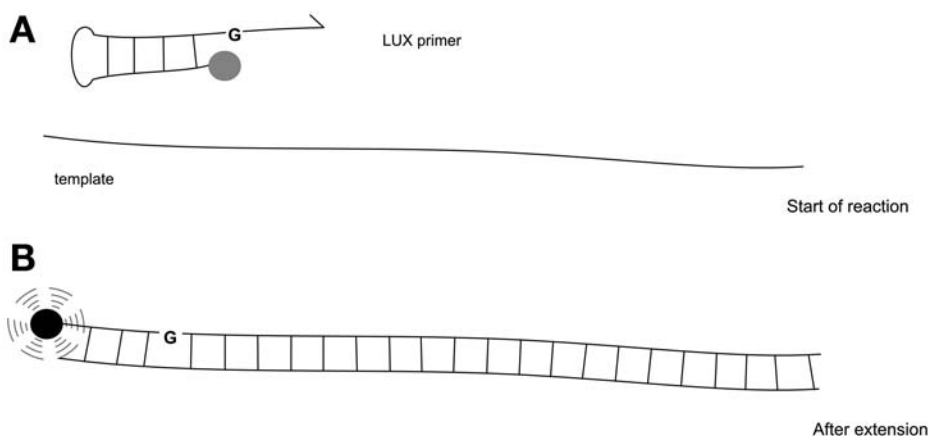


Fig. 4. The LUX primer fluorescence group is quenched by a nearby guanosine (A). The reporter is separated from the quenching “G” as the template is copied and the hairpin LUX primer unfolds (B).

all amplified products will contain fluorescing structures. LUX primers, with the added amplification specificity of the hairpin structure, may offer an advantage in the amplification of consensus sequences, in which sufficient sequence may not be available to use probes (N. Walker, personal communication, 2003). LUX primers may be readily designed on a website (www.invitrogen.com), and have been used in applications such as viral detection (33) and bacterial quantification (N. Walker, personal communication, 2003).

3.3. Self-Quenched Primers

In this method, one of the PCR primers is labeled at the 5′-end with a reporter dye, such as FAM. A complementary oligonucleotide, preferably a PNA labeled with a quencher dye, such as DABCYL, binds to the 5′-end of the primer, thus, quenching the fluorophore. During the PCR extension, the PNA is displaced, allowing the reporter fluorescence to be detected (34). As with all of these formats, the detection of primer dimers remains an issue.

4. Probe-Based Chemistries

Nearly all probe-based chemistries (and indeed, the AmpliFluor primers in **Subheading 3.1.**) rely on a process known as fluorescent/Förster resonance energy transfer (FRET). Although this name is often given to the hybridization probe format in **Subheading 4.2.**, it represents a more general principle. For kinetic PCR, this typically uses two dyes bound to the probe(s). The emission spectrum of one dye overlaps the excitation of the other dye, such that when the two dyes are spatially close to one another (<100 Å; **ref. 35**), one dye acts either

as a quencher of the first dye's reporter signal (hydrolysis probes, molecular beacon probes, AmpliFluor, and Scorpion primers) or as an acceptor/reporter dye (hybridization probes). Only during the specific amplification of the target sequence, when the probe binds to an internal amplicon sequence, is the reporter either separated from the quencher or brought into contact with the donor dye. Both of these options result in a fluorescent signal increase.

These probes are typically DNA-based oligomers, with various conjugated dyes, but newer adaptations include the use of modified nucleotides, such as LNA bases or adding of the MGB group, as well as DNA analogs, such as PNA. All of these modifications allow shorter probe sequences to be used, while maintaining a sufficiently high T_m for specific annealing.

4.1. Hydrolysis (TaqMan) Probes

TaqMan probes are the original and most well-known probe system used in kinetic PCR—indeed the name is often used as the name of the first commercial instrument: the Prism 7700 (Applied Biosystems). In truth, it was developed as a PCR reporter system before kinetic PCR was published (36) and, in its early stages, used thin-layer chromatography as the detection format. It was only after the introduction of the FRET concept (labeling the probe with a reporter and quencher dye [37]) that it became of use in the closed-tube format of kinetic PCR (38).

The original hydrolysis probes used FAM as the reporter dye and TAMRA as the quencher dye, and this is still the most popular format in use today. Hydrolysis probes are a single linear oligonucleotide labeled with a reporter dye at one end (preferentially the 5'-end) and a quencher dye at the other end. With a T_m of 8 to 10°C higher than that of the primers, the probe will anneal to the target sequence before the primers to ensure detection as the nearby primer is extended.

As well as a polymerase activity for DNA synthesis, *Taq* polymerase also contains a 5' nuclease activity. This activity is used when the *Taq* polymerase encounters the bound probe during the primer extension; hydrolyzing the probe (hence the name), and releasing the FAM (reporter) dye from the quenching effect. Once the probe is hydrolyzed sufficiently to be displaced, the polymerase continues the synthesis of the amplicon DNA (Fig. 5).

As mentioned, the most popular dye combination is the reporter FAM and the quenching dye TAMRA, which, itself, is a fluorescent dye. In addition to FAM, there is a wide variety of different dyes spanning the visible spectrum. This allows multiplex assays to be run. Quencher dyes must exhibit a spectral overlap with the fluorophore and, thus, must be selected accordingly. Newer quencher dyes are now available, such as the Black Hole Quencher (BHQ 1, 2, and 3) dyes (Biosearch Technologies Inc.), QSY7 Eclipse Dark Quencher

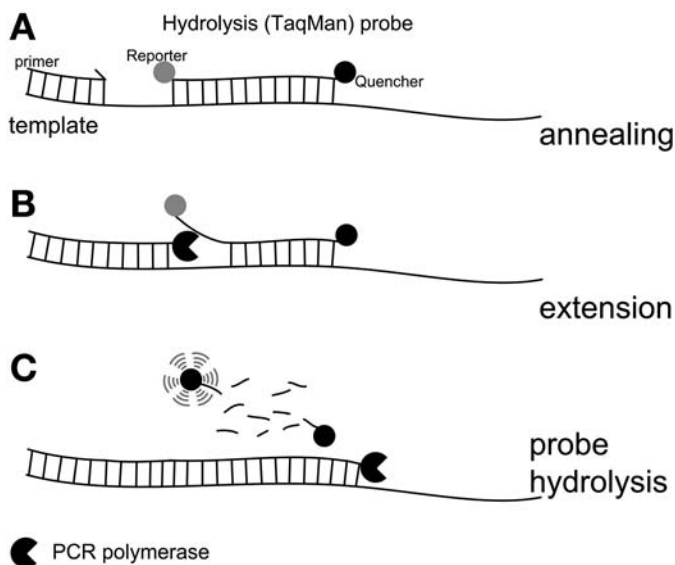


Fig. 5. The hydrolysis probe reporter dye is quenched while the probe remains intact (A). During the primer annealing and extension, the probe is cleaved (B) by the 5' nuclease activity of the polymerase, separating the fluorophore, and allowing fluorescence (C).

(Epoch), and Blackberry (Berry and Associates Inc.) that are nonfluorescent and are reported to result in better signal-to-noise ratios with less background fluorescence (for review, see [ref. 39](#)). In the case of replacing TAMRA, this allows further multiplexing opportunities for additional reporter fluorophores, as a result of removing the fluorescence in the range of 580 nm.

Another recent modification is the use of conjugated MGB to the end of the hydrolysis probe ([40](#)). By stabilizing the probe on the DNA, the T_m of the probe is increased, allowing shorter sequences to be used. This is particularly useful for specificity in genotyping applications based on this probe format.

4.1.1. Design

A number of design features are to be considered in designing hydrolysis probes. Most of these are automatically taken into account with specific hydrolysis probe design software. (e.g., Primer Express[®], Applied Biosystems) or adapting free online software to take into account the primer and probe T_m requirements (e.g., Primer3). Some of these design features include:

- The 5' nucleotide being other than a guanosine (G), because of the quenching of the reporter dye.
- Fewer Gs than Cs in the probe to generate maximum fluorescence.

- A probe T_m of 8 to 10°C degrees higher than the primers to ensure annealing before the primers.
- The PCR primer annealing to the same strand should be close to the probe, without overlapping.

4.1.2. Applications

As the only chemistry originally available on the first kinetic PCR instrument, hydrolysis probes were used in researchers' initial quantitative experiments. Therefore, many primer and hydrolysis probe sequences are available either on databases (e.g., <http://medgen.ugent.be/rtrprimerdb/>), probe suppliers (e.g., TIB MOLBIOL, Germany; or Fluorescentric, Inc.) or commercially, as ready-to-use assays (www.allgenes.com). Another commercial source is the Probe Library (Exiqon), which uses a set of 90 shortened (because of the use of modified LNA nucleotides) dual-labeled hydrolysis probes to cover the transcriptome of a number of species.

Literature searches will also turn up numerous primer and probe combinations. Assays have been published for many infectious diseases, such as West Nile Virus (41), enterovirus (42), and a number of bacterial infections (43–45). There is also a wide variety of research applications published for genes of interest, although, as mentioned in **Subheading 2.5.**, SYBR Green is often a preferred detection format in many research laboratories.

Another major application for hydrolysis probe assays is single-nucleotide polymorphism (SNP) typing, whereby two probes are used; one matching each allele perfectly. They are differentiated by different reporter fluorophores and rely on the specific hydrolysis of only the probe(s) representing the alleles present in the sample. As an example, if the FAM-labeled probe represents allele A and the VIC-labeled probe represents allele B, a sample containing allele A will have a high FAM signal and no VIC signal, whereas a heterozygote sample will have hydrolysis of both probes (46). This is best represented as a scatter plot.

4.2. Hybridization Probes

Hybridization probes (HybProbe[®] probes) generally use two single-dye labeled oligonucleotides binding adjacently on the target and bringing the dyes into proximity, to communicate through FRET. Using fluorescein as the donor dye, and an acceptor dye (usually emitting in the 600–720 nm range), the general format is for one of the oligonucleotides to be labeled at the 3'-end, typically with fluorescein, and the other oligonucleotide labeled at the 5'-end, with the acceptor dye (although this labeling order may be reversed). This 5' dye-labeled probe is also modified with, typically, a 3' phosphate group, to prevent the extension of the probe by *Taq* polymerase, although one report has suggested

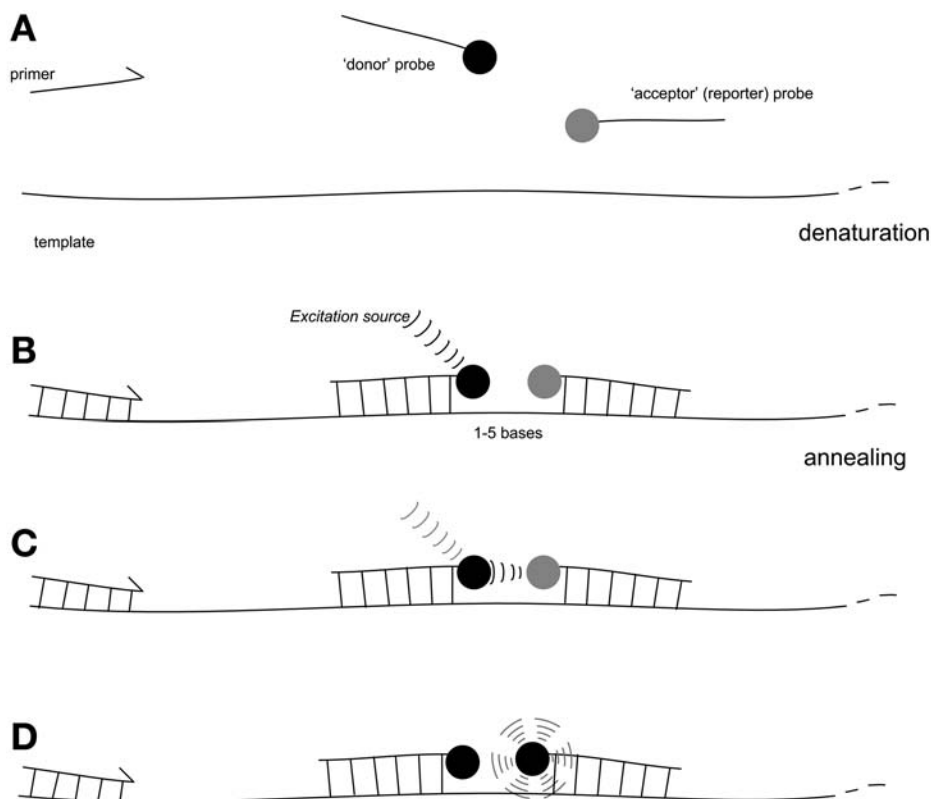


Fig. 6. The reporter (acceptor) and donor fluorophores are on separate oligonucleotides (A) in the hybridization probe format. After annealing with the fluorophore ends 1 to 5 bases apart, the donor fluorophore is excited (B), and its emission (C) excites the nearby reporter fluorophore, which emits at its characteristic wavelength (D).

an alternative blocker to prevent extension (47). Indeed, early hybridization probes used biotin as the 3' blocking group (48,49).

These probes are designed so that they anneal with the dye labeled ends 1 to 5 bases apart (Fig. 6) during the annealing phase of the PCR. With this format, it is usually the acceptor dye that is being measured, although there is also a variation in which the PCR is monitored using the decrease in fluorescein signal as the reaction progresses.

The signal from hybridization probes is measured at the end of the annealing phase before the probes are displaced by the PCR polymerase. When the two probes are annealed, the emission from the fluorescein dye is transferred to the acceptor dye, which emits at its characteristic longer wavelength. The latest

chemistry developments allow four acceptor dyes to be spectrally separated and used as acceptor dyes with fluorescein.

Hybridization probes use typical three-step PCR and, during the extension phase of the PCR (after the signal has been measured), the probes are displaced from the DNA rather than cleaved, as with hydrolysis probes. However, a comparison of standard *Taq* with an exonuclease deficient strain (Stoffel fragment) still showed that there was some degree of probe hydrolysis, as evidenced by the higher fluorescence levels and capillary electrophoresis results resulting with the Stoffel enzyme (50). A similarly modified *Taq* polymerase also gives superior results in multiplex applications (51). As well as quantification, hybridization probes also provide a powerful means of mutation detection through the use of melting curves (for a review, see ref. 52). After the PCR and a moderate temperature hold to allow the probes to bind to the amplified sequences, the temperature is slowly increased, while monitoring the fluorescence, as described in **Subheading 2.2**. The presence of a mutation(s) underneath the probe will cause the probe to dissociate at a lower temperature, leading to a melting peak of a lower T_m , compared with the wild-type peak. Hybridization probes can detect multiple mutations with one probe, unlike other methodologies (53), evidenced by unexpected changes in the melting curves.

4.2.1. Variations

Displacement probes (54) consist of a specific hybridizing sequence labeled with a reporter dye and a complementary quenching oligonucleotide. In the absence of complementary amplified DNA, the two oligonucleotides are bound, bringing the quenching dye into close proximity with the reporter dye. The reporter probe will bind preferentially to the desired amplicon over its binding to the quencher probe.

Another variation of the hybridization probe format is the “primer:probe” approach, in which one of the primers is labeled internally (on an appropriate thymidine near the 3′-end of the primer). The probe is labeled at the 3′-end, and is designed so that the two dyes are within approx 5 nucleotides, to allow the FRET process to occur. This approach is of similar sensitivity to the conventional method (55), and is particularly useful for genotyping (56,57) or for consensus sequences that do not permit an additional hybridization probe (unpublished data).

4.2.2. Design

As with hydrolysis probes, a number of aspects are taken into account when designing hybridization probes, although many researchers choose to use design services of commercial custom oligonucleotide synthesis companies (examples being TIB MOLBIOL, Germany; Metabion, Germany; or Fluorescentric,

Inc.). Specialized software may also be used, such as LightCycler Primer Probe Design Software (Roche Applied Science) or MeltCalc (www.meltcalc.de; **ref. 58**).

As already described, hybridization probes are two (or more in special designs) oligonucleotides that are designed to anneal with a 1- to 5-bp gap. Similar to hydrolysis probes, they should also have a T_m higher than the primers (5–10°C) to ensure binding during the PCR annealing. Other design considerations include:

- Placing the probes away from the primer annealing on the same strand (*cf.* hydrolysis probes) to prevent displacement by polymerase activity at the annealing temperature before the signal can be measured at the end of this step.
- Avoiding complementarity to the 3'-end of the primers, to prevent primer extension off the probes.
- Ensuring the 5'-labeled probe is blocked at the 3'-end with the addition of a phosphate group (most commonly).

To increase T_m differences between wild-type and mutant peaks in SNP designs, shortened probes can be designed with the inclusion of higher T_m nucleotide analogs, such as LNA (**59,60**).

4.2.3. Applications

The majority of hybridization probe applications have been published for the LightCycler instrument and cover similar applications to hydrolysis probes. Many clinical applications for viruses (**61–63**), bacteria (**64–67**), and fungi (**68,69**) have been published. A large number of research applications, such as cytokine quantification (**70,71**), have also been described. Genetic SNP assays have been reported with hybridization probes (**52**), and a number of multiplex applications have also been described (**72,73**).

4.3. Molecular Beacon Probes

Molecular beacons are oligonucleotide sequences that are designed to form hairpin structures, which keep them in a quenched state (**Fig. 7**). The hairpin has two complementary arms and a loop structure designed to anneal to the desired amplified sequence (**74**). At the end of one arm is a reporter dye and at the other, a nonfluorescent quencher (typically DABCYL). In the absence of target DNA, the probe remains bound in the self-complementary form, thus, keeping the two dyes very close to one another and resulting in a very efficiently quenched and nonfluorescent probe. The binding of the loop structure to amplified DNA (designed to be favored over the stem binding) forces the arms of the probe (and dyes) apart and, thus, fluorescent signal can be measured.

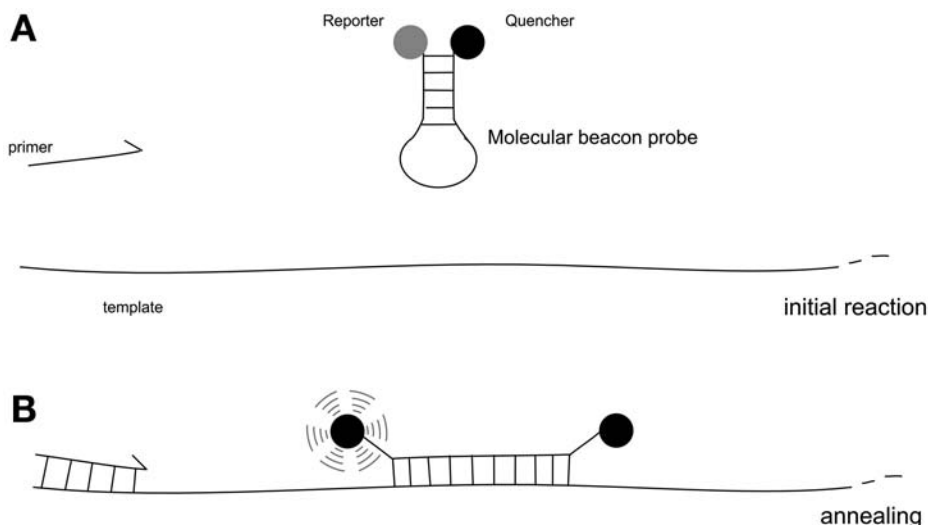


Fig. 7. The molecular beacon probe exists in a quenched hairpin form when free in solution (A). The stem loop contains sequence complementary to the amplified target that forms a more stable complex than the self-complementary “arms” of the beacon. This favors the annealing of the probe (B) to the amplified DNA—and separation of the fluorophore from the quencher—over the probe remaining in a hairpin structure.

Because DABCYL may be used as a quencher for a number of dyes (74), it is possible to use multiple molecular beacons, e.g., for SNP analysis, with a probe perfectly matching each allele (similar to hydrolysis probes). Perfectly matched beacons exhibit a very high annealing specificity over those with a single-base mismatch, in the case of a SNP present, and the signal generation is again similar to hydrolysis probes. The perfectly matched probe generates signal for the respective allele, whereas both beacons generate signal in the case of heterozygotes (75).

Another option for multiplexing molecular beacons uses a third dye and a FRET process in addition to the reporter/quenching process within the probe. Wavelength-shifting molecular beacons use the usual reporter dye (placed internally on one of the arms of the probe) as a FRET partner to a reporter fluorophore on the end of the same probe arm, which fluoresces at its characteristic wavelength (76).

4.3.1. Design

Initially, a drawback of molecular beacons was the inability to rapidly design suitable structures. Many beacons were initially designed by trial and error, choosing a sequence with the appropriate complementary arms and checking

secondary structures with a program, such as mfold (77), to ensure that the beacon favored binding to the amplified sequence at the annealing temperature over the arms self-annealing. Thankfully, there are now software programs to aid the design of these forms, such as BeaconDesigner (www.premierbiosoft.com).

4.3.2. Applications

Molecular beacons have been used in several forms for intracellular detection in vivo (78,79). Examples of SNP applications include drug-resistance mutations in *Mycobacterium tuberculosis* (80) and p53 mutations (81). They have been used in the detection of a number of pathogens, such as hepatitis A, salmonella, and Group B streptococci (82–84).

4.4. Scorpion Primers

Scorpion primers (DxS Genotyping Ltd., United Kingdom) use elements of two chemistries already described in this chapter, in **Subheadings 3.1.** and **4.3.:** AmpliFluor primers and molecular beacons—and yet represent a unique methodology.

As initially described, a Scorpion “uses a primer with an integral tail that is used to probe an extension product of the primer” (85). Perhaps this is best understood diagrammatically (Fig. 8). As with AmpliFluor primers, there is a hairpin structure at the 5′-end of the primer. However, unlike AmpliFluor, this hairpin structure cannot be fully copied by the polymerase during the PCR, because of the presence of a blocking molecule (hexethylene glycol) immediately 3′ to the hairpin. The loop of the hairpin is complementary to an internal amplicon sequence and allows the 5′-end of the primer to bend over (similar to a scorpion’s tail) and bind to the matching sequence separating the complementary arms of the hairpin and, thus, separating the reporter and quencher dyes to allow fluorescence.

Because the probe is essentially attached to the primer, this unimolecular event happens very quickly, allowing good results with rapid cycling times, in comparison with molecular beacons (86). With standard cycling times on other real-time PCR instruments, although the crossing points between samples with these different chemistries is similar (indicating similar analytical sensitivities), the fluorescence levels of amplification curves with Scorpion primers are much higher.

4.4.1. Design

Design of Scorpion primers seems to not be straightforward, with the multiple elements to be considered of the molecule, i.e., the T_m of the 3′ PCR primer aspect, the T_m of the hairpin loop acting as the probe, and the complementary

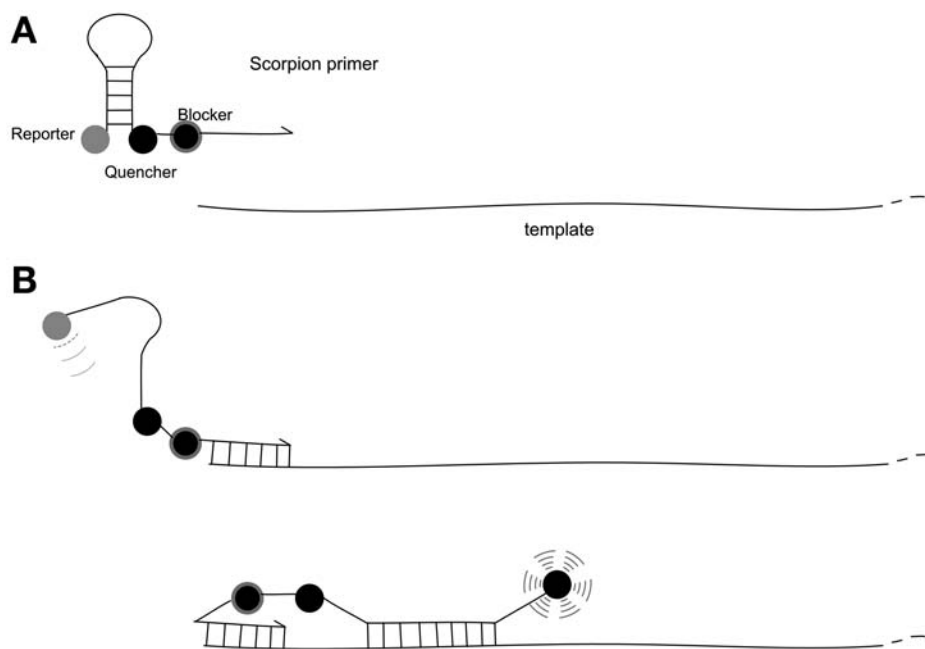


Fig. 8. The Scorpion primer contains a “self-probing” tail that is quenched in its free state (A). After the 3'-end binding as the PCR primer, the hairpin structure unfolds (B) and the stem-loop structure of the Scorpion primer binds to a complementary sequence downstream from the primer sequence, in the preferred manner of the molecular beacon probe (Fig. 7). This again separates the reporter fluorophore from the quencher.

sequences of the hairpin arms. A second form of the Scorpion primer is possible with the quencher on a second “duplex” oligonucleotide (87). As with original molecular beacon design, secondary structure prediction programs, such as mfold, will prove useful; however, design software is available at www.DNA-software.com.

4.4.2. Applications

As a newer technology, there are relatively few applications, although the technology is being licensed and developed by DxS Genotyping. Scorpions have been used in the detection and quantification of human papillomavirus (88) and also for a number of genotyping applications. Using allele-specific PCR, Scorpions may be used to detect specific amplification of one or more alleles (86). To perform both allele-specific PCR reactions in the same vessel, a different dye combination can be used for one of the probes. The use of a format similar to the wavelength-shifting molecular beacons described in Subheading 4.3.: using a second reporter dye near a FAM “harvester” dye and

allowing the FRET process to result in fluorescence of the ROX dye, in the case of the specific-allele amplification (87). However, the separate reactions may be easier to initially develop because only the allele-specific base (plus any other mismatches required for highly specific binding of the Scorpion primer) requires modification for the appropriate allele.

4.5. Other Probe Designs

4.5.1. Light-Up Probes

Light-up probes combine the fluorescent specificity of SYBR Green-like dyes for double-stranded DNA with the high hybridization specificity of PNA oligomers (89,90), and do not rely on the FRET process. Light-up probes use a different asymmetric cyanine dye (thiazole orange) conjugated to the amino terminus of the PNA. As with modified oligonucleotide probes (e.g., those with MGB or LNA moieties), the uncharged nature of PNA and its higher DNA binding activity means that shorter probe sequences may be used, while still maintaining a higher T_m than with the primers for real-time PCR monitoring.

4.5.2. Simple Probe

Simple Probe oligonucleotides are single hybridization probes used solely for genotyping (because of the relatively low fluorescent change upon binding to their target sequence). The probe is labeled with a single fluorescein group and a nonfluorescent quenching group, and is designed over the mutation(s) of interest. In an unbound state, the Simple Probe is quenched, but its fluorescence increases after binding to the complementary sequence. At the end of the PCR, the Simple Probe may be melted from the amplified sequence and the genotype determined, as with hybridization probes (91,92).

5. Summary

There is a wide choice of chemistries available, most of which are adaptable to the majority of real-time PCR platforms. Factors influencing the chemistry choice are availability of an application protocol and support for the particular chemistry in case troubleshooting is necessary. Hydrolysis probes, SYBR Green, and hybridization probes remain the most popular options (89), but new chemistries will continue to be developed as the increase in real-time PCR continues unabated.

6. Useful Websites

Websites useful for information on SYBR Green properties are: www.gene-quantification.com; www.lightcycler-online.com; www.molecularbeacons.org; and www.probes.com. For Scorpion primers: www.dxsgenotyping.com. For

light-up probes: www.lightup.se; www.tataa.com also discusses light-up probes. For predicting secondary structure: www.bioinfo.rpi.edu/applications/mfold/. For the Primer3 primer design program: frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

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V_____

MICROARRAYS

Microarrays

An Overview

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Summary

Gene expression microarrays are being used widely to address a myriad of complex biological questions. To gather meaningful expression data, it is crucial to have a firm understanding of the steps involved in the application of microarrays. The available microarray platforms are discussed along with their advantages and disadvantages. Additional considerations include study design, quality control and systematic assessment of microarray performance, RNA-labeling strategies, sample allocation, signal amplification schemes, defining the number of appropriate biological replicates, data normalization, statistical approaches to identify differentially regulated genes, and clustering algorithms for data visualization. In this chapter, the underlying principles regarding microarrays are reviewed, to serve as a guide when navigating through this powerful technology.

Key Words: Amplification; ANOVA; cDNA; clustering; dye-swap; expression matrix; expression vector; fluorescence; gene expression; hierarchical; hybridization; *k*-means; long oligomers; microarrays; mRNA; oligonucleotides; probe; RNA; target; *t*-test.

1. Introduction

In the past several years, we have witnessed remarkable progress in the completion of draft genome sequences for human, mouse, rat, *Drosophila*, *Arabidopsis*, and hundreds of microbial species. Couple this sequence information with complementary methodologies (e.g., expressed sequence tags sequencing projects for eukaryotic organisms) to catalog the expressed gene repertoire of an organism, and it becomes clear that we are arriving at an increasingly impressive “parts list” (1). However, interpreting these gene lists in terms of an organism’s underlying biology remains a challenge. This is compounded by the fact that more than half of the identified genes of most organisms have no

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obvious biological function. Moreover, predicted gene function based on sequence homology to genes with known cellular roles remains to be validated in the laboratory. Hence, DNA microarrays have become a universal tool to link gene expression with biological consequence (2). The power of microarrays is in their ability to simultaneously assess the expression of thousands of genes in a massively parallel fashion and across numerous conditions (e.g., comparing across time, treatment groups, genetic strains, and so on) to uncover higher-order organization of gene transcriptional behavior and, ultimately, to understand biology. The advantage of microarray analysis is not in viewing the expression of genes as individual components, but rather in visualizing the data as a “composite image” to understand biological processes (2).

2. Microarray Platforms

2.1. Overview

The fundamental steps behind a DNA microarray experiment are as follows: obtain RNA from two or more experimental groups that are being compared, convert the RNA to antisense RNA (aRNA) or complementary DNA (cDNA; herein referred to as the target), label the target with a fluorophore, hybridize the labeled target against thousands of DNA probes/elements (representing genes) immobilized on a solid support surface, and measure the relative expression (i.e., fluorescence) of each gene in each of the groups. However, how these steps are accomplished can be quite varied. There is no single “microarray platform” and new technologies are being introduced in an attempt to improve throughput and sensitivity, such as the Universal Hexamer Array from Agilix (3) and Illumina Beadarray (4). That being said, the most established microarray platforms in use are the commercially available Affymetrix GeneChips (Santa Clara, CA, <http://www.affymetrix.com/index.affx>), in-house manufactured PCR amplicon-based cDNA arrays, and, more recently, long oligomer arrays (e.g., 70-mer oligonucleotides) that are manufactured in-house or commercially available (see Agilent, Palo Alto, CA, <http://we.home.agilent.com>; Sigma-Genosys, Jamesburg, NJ, <http://www.sigma-genosys.com/oligonucleotide.asp>; Illumina, San Diego, CA, <http://www.oligator.com>; Operon, Huntsville, AL, <http://www.operon.com>; and MWG Biotech, High Point, NC, <http://www.mwg-biotech.com>).

The 25-mer short oligonucleotide probes contained in the Affymetrix GeneChips are synthesized directly onto a solid matrix using a proprietary photolithographic technology (5,6). The probes are “freely” moving, being tethered at one end to the solid support surface. A key advantage of this platform over nonsynthetic methods (mechanically spotted arrays; see **Subheading 2.1.**) is that the burdensome aspects of probe handling and tracking are eliminated. Another advantage of this high-precision photolithographic approach is that the use of synthetic reagents minimizes chip-to-chip variation. However, one

drawback is the expense incurred by these arrays, which can be 5- to 10-fold higher than in-house manufactured arrays.

For PCR amplicon and long oligomer arrays, the probes are either mechanically “spotted” onto modified glass slides by direct contact printing or deposited onto the slide surface by ink jet printing (7,8). After spotting, the probes are subjected to UV irradiation to covalently attach the probes onto the surface of the slide. Typically, the spotted PCR amplicons are 500 to 1000 bases in length, whereas the long oligomers are 55 to 70 nucleotides long. Alternatively, long oligomers can be synthesized *in situ* onto the glass slide surface (see Agilent). In the case of the Agilent long 70-mer arrays, similar to the Affymetrix GeneChips, one end of the probe is tethered to the solid support surface. It is thought that this design strategy, in contrast to UV-crosslinked probes that lie flat on the slide surface, increases the available probe surface area for probe–target hybridization and, hence, provides greater signal-to-noise benefits. To mimic this design of freely moving probes, both PCR amplicons and long oligomers can be modified at one end by the addition of a 5′ amino group for covalent attachment onto preactivated slides (9,10). Long oligomer arrays are gaining widespread popularity among the spotted array platforms. Problems associated with PCR amplicon-based arrays, such as clone tracking, handling of glycerol stocks, and failed PCR amplifications, are avoided when using long oligomers. With the completion of numerous microbial, plant, and eukaryotic genomes, as well as extensive expressed sequence tags data, there is sufficient sequence information to design unique long oligonucleotide probes capable of distinguishing homologous genes, alternative splice variants, and partially overlapping genes found on opposite DNA strands of compact genomes. As such, long oligomer probes have an added flexibility over PCR amplicons.

2.2. Comparative Analysis of Different Platforms

Inherent across all of these platforms is a multitude of different methodologies for generating labeled target from starting RNA, various hybridization and wash conditions, different microarray scanners (which are not necessarily interchangeable across platforms), a host of image segmentation and quantification techniques, and a multitude of approaches for background noise estimation and normalization. For a more thorough discussion of these differences and their impact on expression measurements, we refer the reader to more specialized reviews (11–13). Comparative analysis of the various platforms has been reported in the literature, with somewhat varying outcomes. Although there seems to be good concordance of gene expression measurements between long oligomer and PCR amplicon-based cDNA arrays (14), there have been conflicting reports regarding the correlation between Affymetrix and amplicon-based measurements (15–17). A careful analysis of the probe sets from each platform

contributing to the discordant data is warranted in the very near future. For example, are the discrepancies caused, in part, by unwanted cross-hybridization properties exhibited by probes in one platform but not by the analogous probes in another platform, or are the conflicting results a reflection of an inability of the longer PCR amplicon-based probes to differentiate alternatively spliced transcripts? Ultimately, it will be up to the user to identify the best platform for their particular application and to validate the microarray results using an alternative RNA quantification method (e.g., Northern blot, real-time reverse transcriptase PCR, and so on; **ref. 18**).

3. RNA-Labeling Strategies

3.1. Affymetrix Arrays: One-Color Scheme

The RNA-labeling protocol in use for the Affymetrix GeneChip is vastly different from the approach favored by microarrays comprising the longer probes (i.e., 70-mer oligonucleotides and PCR amplicons). Affymetrix arrays use a one-color scheme by using a single fluorescent label (i.e., phycoerythrin). Expression profiles of each sample are generated on separate chips, and the different fluorescent images are compared against one another for determination of differential gene expression (**5**). Using the Eberwine method (**19**), total RNA (5 to 10 μ g) from an individual sample is primed with an oligo-dT primer with a T7 promoter sequence at the 5'-end (**Fig. 1**). Reverse transcriptase is added to generate single-stranded cDNA, followed by addition of RNase H, DNA polymerase, and DNA ligase to synthesize double-stranded cDNA containing the T7 promoter. In the presence of biotin-labeled rNTPs, T7 RNA polymerase uses the double-stranded cDNA as a template to synthesize multiple copies of biotinylated aRNA. The biotinylated aRNA is fragmented, hybridized onto the GeneChip, and stained with a streptavidin-phycoerythrin conjugate before scanning to generate a fluorescent image.

3.2. PCR Amplicon and Long Oligonucleotide Arrays: Two-Color Scheme

In the two-color format, specifically developed for long oligomer and PCR amplicon-based arrays, the two RNA samples being compared are reverse transcribed into cDNA (in separate reaction tubes), and different fluorescent tags (typically Cy3 and Cy5) are incorporated into the two cDNA molecules. Next, the Cy3- and Cy5-labeled cDNA targets are co-hybridized overnight onto a single array, and scanned the following day to generate a two-channel fluorescent image (**7**). The relative Cy3 and Cy5 fluorescent intensities associated with each probe on the array provide comparative gene expression information in the two samples.

There are two main protocols for target labeling, and both require 10 to 15 μ g total RNA as the starting material. In the direct-labeling approach (**7**), a Cy3

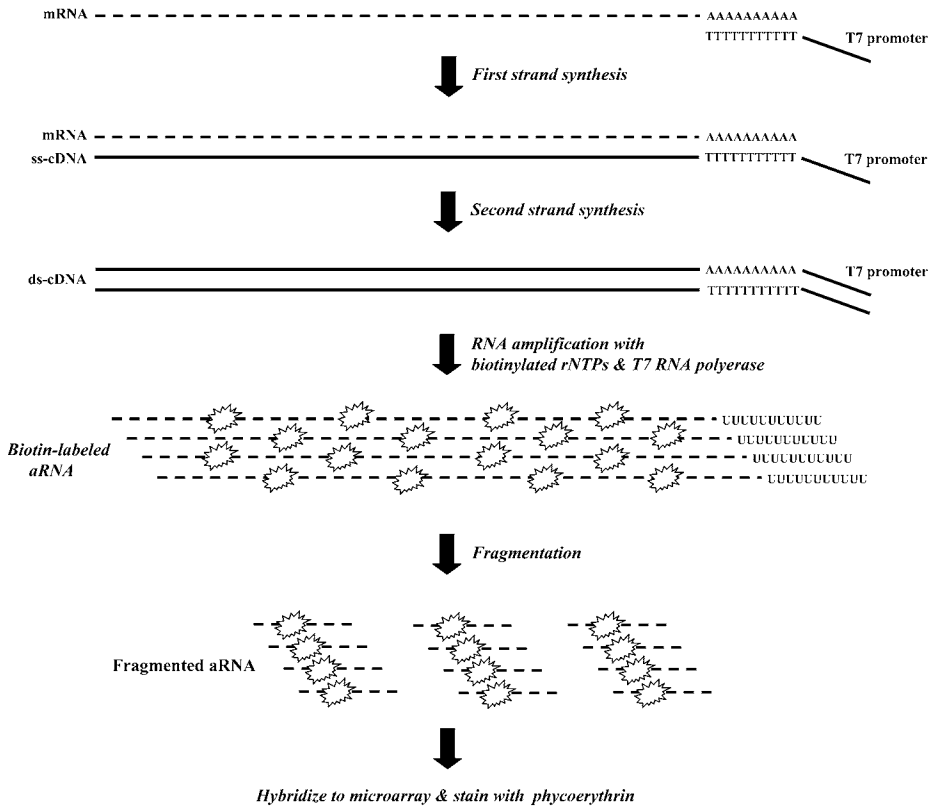


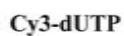
Fig. 1. T7 RNA polymerase amplification method to generate biotinylated aRNA for hybridization onto Affymetrix GeneChips.

or Cy5 fluorescently conjugated nucleotide (e.g., Cy3-dUTP or Cy5-dUTP) is directly incorporated into the first-strand cDNA synthesis (**Fig. 2A**). This particular labeling strategy was used in many of the early microarray publications. However, the large bulk of the fluorescent dye presents significant steric hindrance problems for the reverse transcriptase, resulting in low incorporation of Cy dye molecules into the cDNA target. Moreover, differences in the sizes of the Cy3 and Cy5 fluorophores resulted in unevenly labeled cDNA target (i.e., the cDNA labeled with the larger Cy5 molecule typically exhibits poorer dye incorporation compared with the cDNA labeled with the smaller Cy3 molecule). Hence, the indirect-labeling approach was developed to eliminate these problems (**Fig. 2B**; **refs. 20** and **21**). Instead of using Cy dye-conjugated dUTP, an aminoallyl dUTP is used in the reverse transcription reaction, followed by chemical coupling of the aminoallyl-labeled cDNA with an NHS ester of the Cy dye. There is less global dye bias between the two labeled targets because both

A

mRNA ----- AAAAAAAAAA
TTTTTTTTT T oligo(dT) primer

mRNA ----- AAAAAA
 TTTTTT
 1st strand cDNA



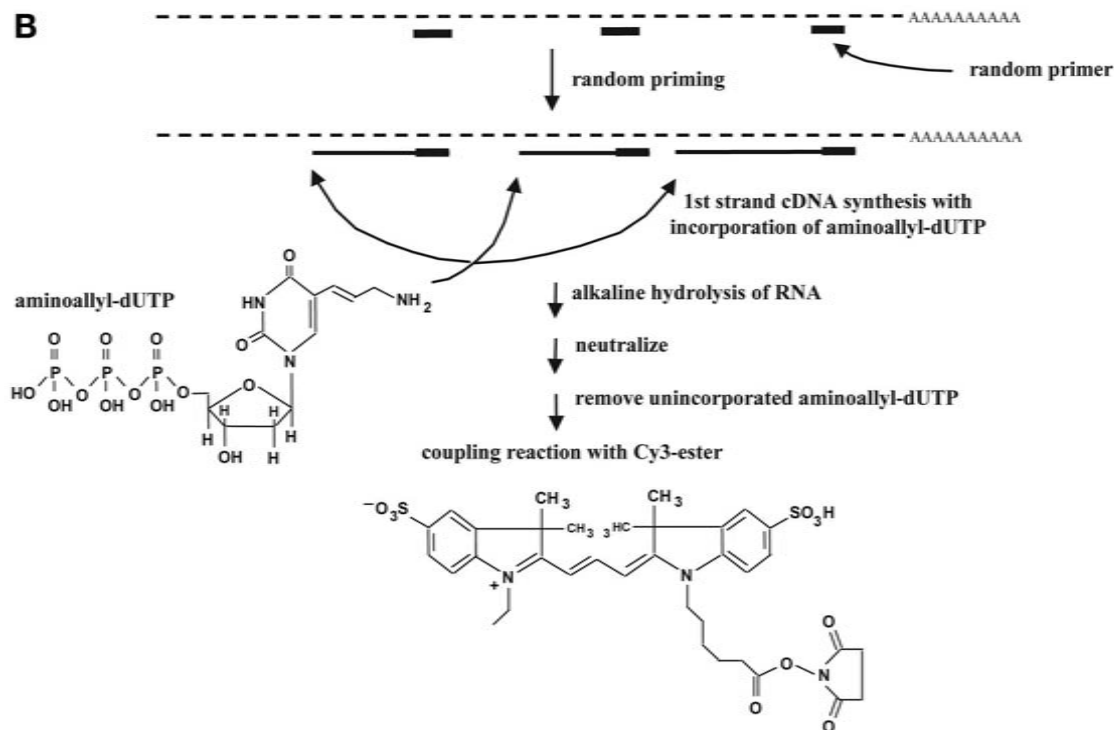


Fig. 2. (A) Direct labeling of cDNA target for hybridization onto two-color arrays. In separate reaction tubes, RNA samples A and B are reversed transcribed into cDNA that has been labeled with Cy3 and Cy5, respectively. Shown is the reaction for sample A. Cy3- and Cy5-labeled targets are co-hybridized onto the microarray. Bulky Cy dye coupled dUTP is inefficiently incorporated into first-strand cDNA during the reverse transcriptase reaction, leading to chain termination and low Cy dye specific activity. **(B)** Indirect labeling of cDNA target for hybridization onto two-color arrays. Aminoallyl-coupled dUTP is more efficiently incorporated into first-strand cDNA by reverse transcriptase. Random hexamer priming of mRNA for first-strand cDNA synthesis is depicted, but oligo-dT priming works as well.

RNA samples are reverse transcribed into cDNA using aminoallyl dUTP, and the labeling of one cDNA target with the Cy3 ester and the second cDNA target with the Cy5 ester involves a chemical and not an enzymatic reaction. The density of labeling with the indirect method can be as high as approx 1 fluorophore per 6 nucleotides, compared with 1 fluorophore for every 20 to 50 nucleotides in the direct method.

3.3. Signal Amplification for Two-Color Arrays

In the labeling strategies described thus far, the amount of total RNA required typically ranges between 10 to 15 μg . However, what happens if there is less RNA available? One possibility is to pool RNA samples from multiple biological replicates, and the relative merits and statistical implications of such an approach have been described (22). If pooling is not a viable option, there are a number of alternative labeling strategies specifically designed to handle total RNA yields ranging from 0.5 to 2 μg . In the case of the Affymetrix GeneChip, the Eberwine T7 RNA polymerase method can readily handle this amount of starting RNA because it is, in essence, a linear RNA amplification scheme (19). For long oligomer and PCR amplicon-based cDNA arrays, the tyramide signal amplification and dendrimer schemes are available. In the tyramide signal amplification method MICROMAX TSA Labeling and Detection Kit (Perkin Elmer Life Sciences, Boston, MA), biotin-conjugated dNTPs (used as a hapten for subsequent Cy5 labeling) and fluorescein-conjugated dNTPs (used as a hapten for subsequent Cy3 labeling) are incorporated in the first-strand cDNA synthesis reaction of samples A and B, respectively (Fig. 3A). The two hapten-coupled cDNA targets are co-hybridized onto the microarray overnight. After stringent washing, the microarray is incubated with an antibody against fluorescein, conjugated to horseradish peroxidase (HRP), which binds specifically to fluorescein-labeled cDNA target and enzymatically catalyzes the deposition of Cy3-labeled tyramide. After HRP inactivation, streptavidin-HRP binds to biotin-labeled cDNA and catalyzes the deposition of Cy5-labeled tyramide. The resulting 100-fold signal amplification relative to the direct-labeling protocol allows for the use of much less RNA starting material, although the major disadvantage is the high labor cost.

In the signal amplification method using the dendrimer technology (also referred to as the three-dimensional [3D] multilabeled structure protocol; ref. 23), first-strand cDNA synthesis is performed using an oligo-dT primer with a short “dendrimer capture sequence” at the 5′-end (Fig. 3A). After cDNA synthesis, a dendrimer (oligonucleotides crosslinked into a 3D structure) containing approx 300 Cy3 fluorophores is annealed to the cDNA via a complimentary sequence to the dendrimer capture sequence found on the 5′-end of each cDNA molecule. cDNA from a second sample is coupled with Cy5-dendrimers and

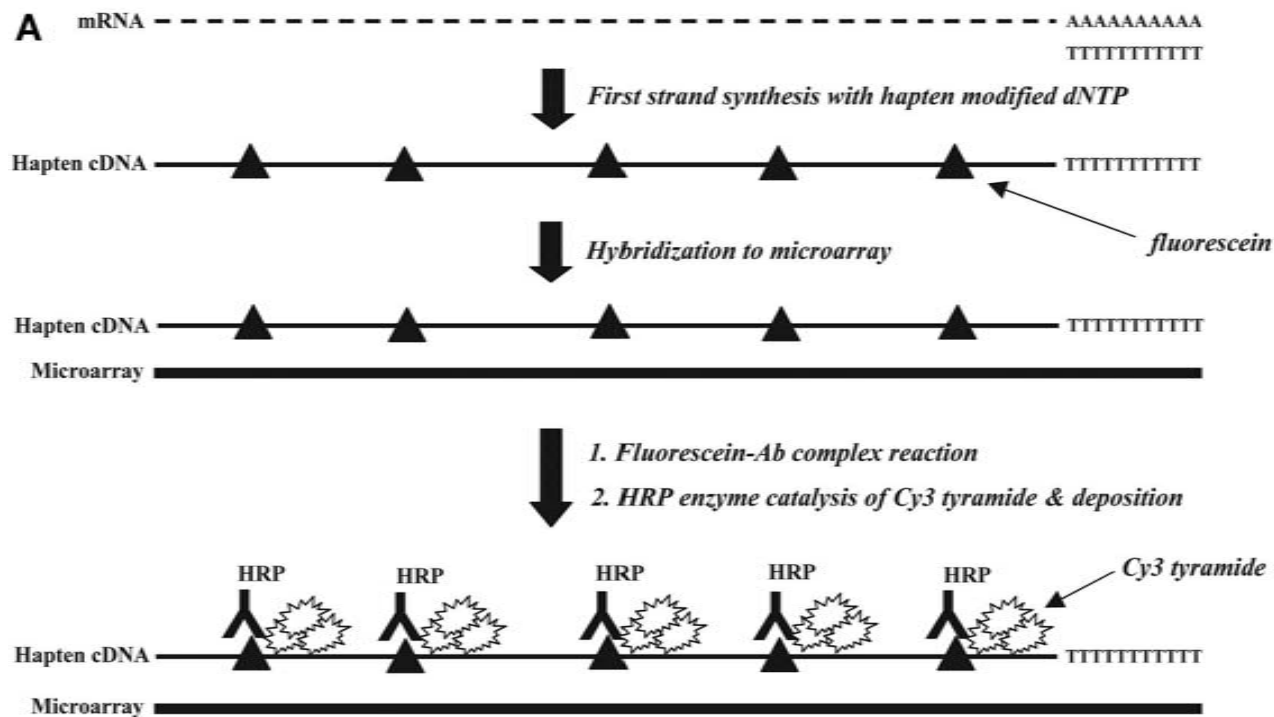


Fig. 3. (Continued)

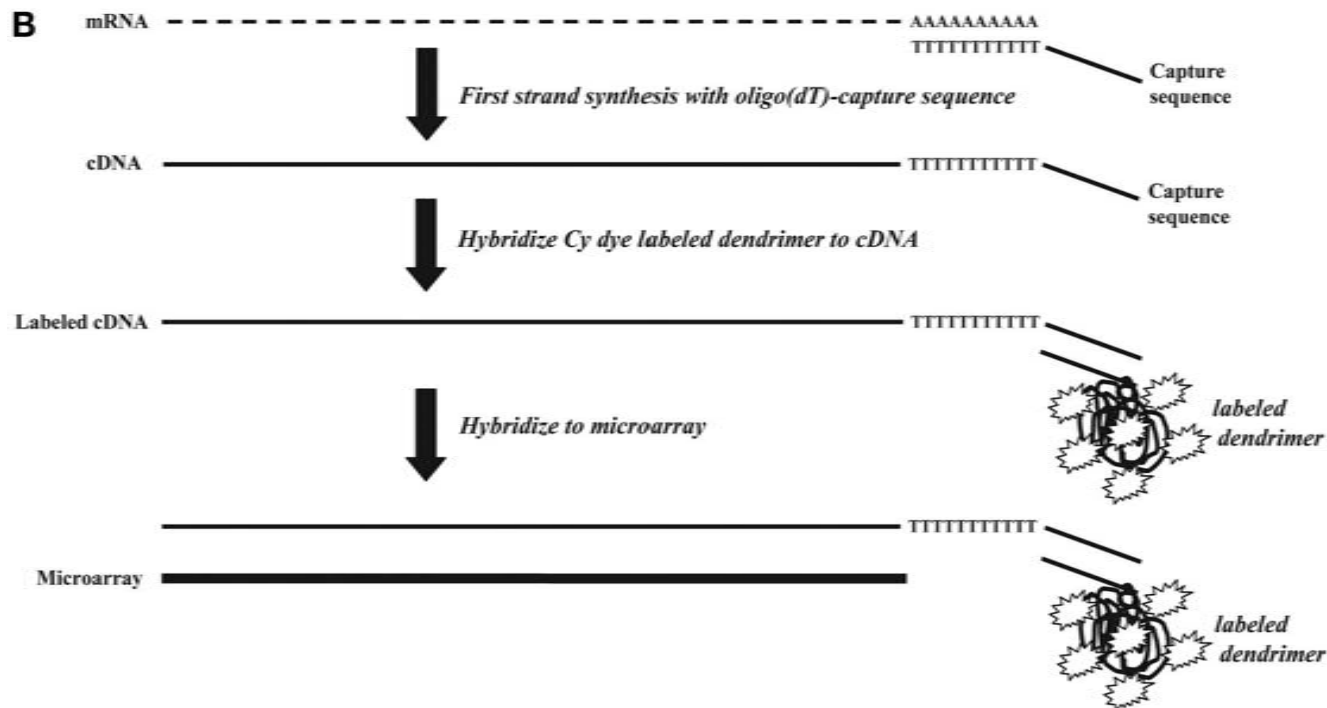


Fig. 3. (A) Tyramide signal amplification scheme for two-color arrays. In separate reaction tubes, RNA samples A and B are reversed transcribed into cDNA that has been labeled with fluorescein and biotin, respectively. Shown is the reaction for sample A. Fluorescein- and biotin-labeled cDNA targets are co-hybridized onto the microarray. The array is treated sequentially with an anti-body (Ab) to fluorescein, conjugated with HRP and Cy3 tyramide, followed by streptavidin–HRP and Cy5 tyramide. **(B)** Dendrimer signal amplification scheme for two-color arrays. In separate reaction tubes, RNA samples A and B are primed with an oligo-dT primer with capture sequence, and reversed transcribed into the cDNA target. Cy3 and Cy5 dendrimers are annealed to the sample A and B cDNAs, respectively, and co-hybridized onto a microarray.

co-hybridized with the cDNA coupled to Cy3-dendrimers. By using fluorescent dendrimers, a 10- to 100-fold signal enhancement relative to the direct-labeling method is achieved.

3.4. RNA Amplification for Affymetrix and Two-Color Arrays

But what happens when only a very limited amount of RNA (10–100 ng and less) is available, as in the case of a laser capture microdissected sample? In this scenario, many laboratories have relied on the Eberwine T7 RNA polymerase amplification approach or modifications thereof. The first round of amplification yields an approx 1000-fold increase of the original amount of starting messenger RNA (mRNA), whereas two rounds will yield an approx 10,000-fold enrichment (24). For PCR amplicon-based microarrays, the aRNA resulting from the first round of amplification is not used for direct hybridization onto the array, but, instead, is used as a template in the indirect-labeling protocol for the generation of fluorophore-containing cDNA target (Fig. 4). If a second round of amplification is necessary, the aRNA from the first round is converted to first-strand cDNA using random hexamer primers (Fig. 4). Next, the first-strand cDNA is primed with the oligo-dT–T7 primer for generation of the second-strand cDNA. The resulting double-stranded cDNA template with T7 promoter is used to run-off a second round of aRNA, which serves as the template for the generation of fluorophore-labeled cDNA via the indirect-labeling approach. For the Affymetrix GeneChip, the user bypasses the direct/indirect-labeling step.

It is important to note that this particular RNA amplification scheme can only be used for Affymetrix GeneChip and PCR amplicon-based arrays and is not applicable to the long oligomer arrays. The orientation of the probes in the long oligomer microarray is in the sense direction, as is the case of the labeled cDNA target derived from standard Eberwine RNA amplification coupled with the indirect-labeling approach. Hence, a modification of the Eberwine method is necessary if first-strand cDNA synthesis is initiated with an oligo-dT primer (no T7 RNA promoter sequence); second-strand cDNA is primed with random nonamers containing a T7 RNA promoter sequence appended to the 5'-end. The resulting double-stranded cDNA is used in a one-round RNA amplification reaction to synthesize sense-strand RNA, which serves as the template for generation of fluorophore-labeled (–)cDNA target. If two or more rounds of amplification are required, the T3 RNA polymerase amplification scheme described by Xiang et al. (25) is a viable option (Fig. 5). Lastly, these so-called “linear” RNA amplification schemes can still introduce biases in the amplified product, especially as the number of rounds increases; hence, important safeguards and quality control assurances must be in place to ensure fidelity of the gene expression measurements (24).

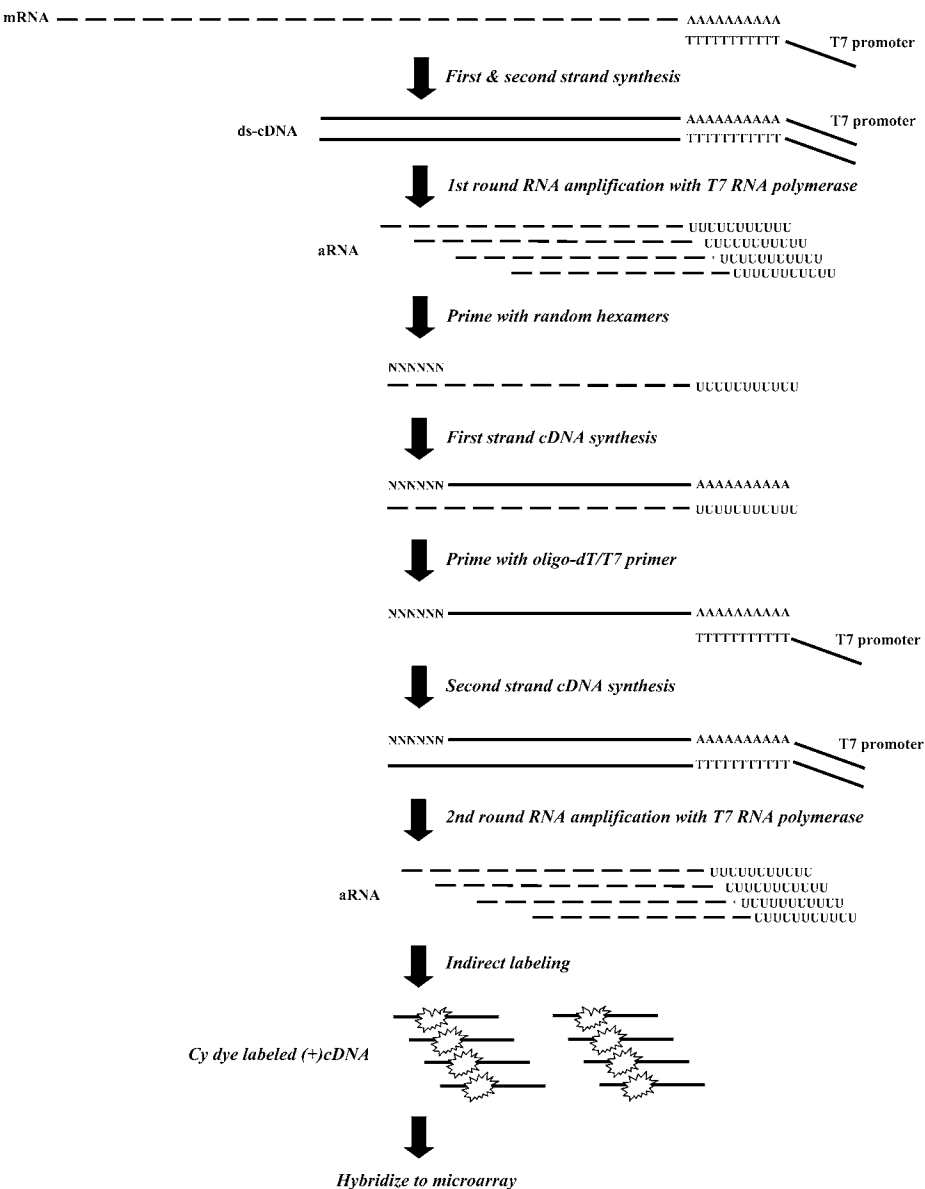


Fig. 4. Two rounds of T7 RNA polymerase amplification for extremely low amounts of starting RNA. Biotinylated aRNA generated from the second round of amplification can be hybridized onto Affymetrix GeneChips, or nonbiotinylated aRNA from the second round can be converted into Cy dye-labeled cDNA target via the indirect-labeling method and hybridized onto two-color arrays. Note that the orientation of the cDNA is sense (+) and, hence, can only be hybridized onto PCR amplicon-based arrays and not long oligomer arrays.

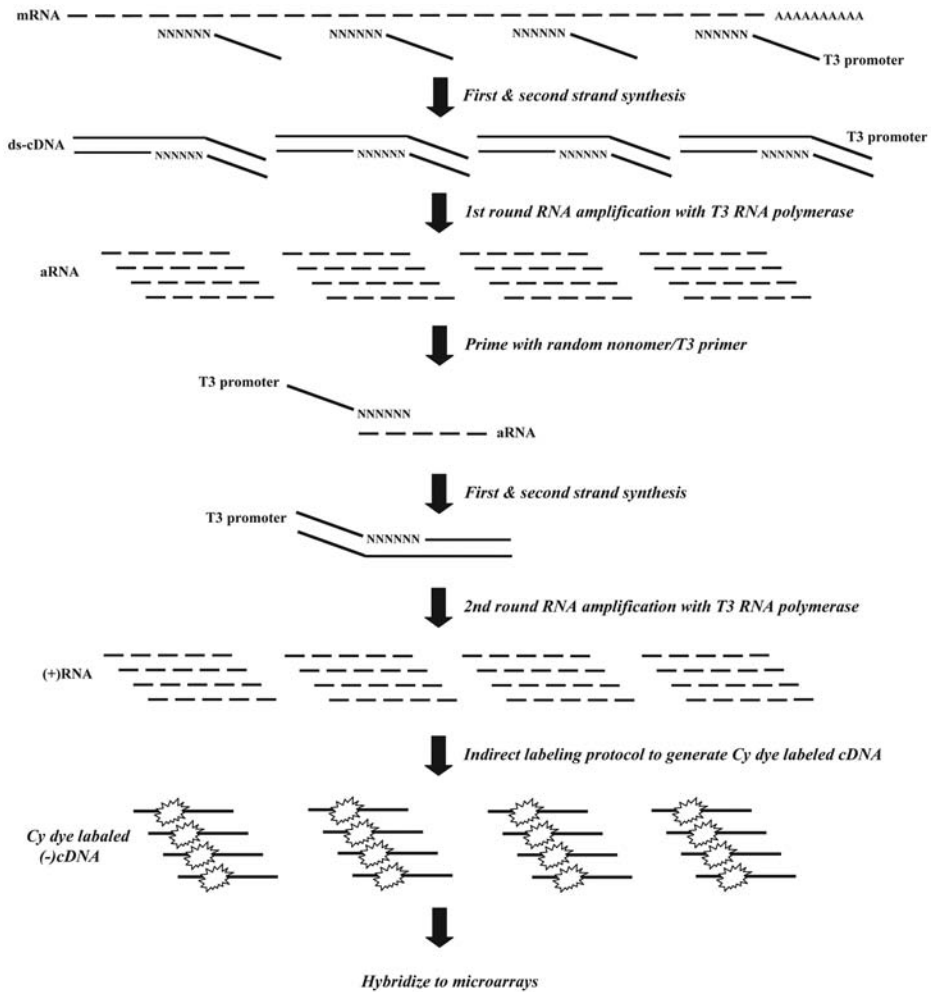


Fig. 5. RNA amplification strategy to generate Cy dye-labeled (-)cDNA target for hybridization onto long oligomer arrays.

4. Study Design and Objectives

4.1. Sample Allocation Overview

The abundance of gene expression data in the literature has sometimes fostered a false sense that microarray data can be collected in a relatively spontaneous or unplanned manner. This could not be further from the truth. Because PCR amplicon and long oligomer microarrays both use a two-color system, an investigator has to decide how to allocate samples to labels and to microarrays. With the Affymetrix GeneChip, RNA samples are individually labeled and

hybridized to individual chips, thereby obviating the need to contend with sample allocation issues. There have been a number of recent reviews discussing sample allocation strategies for microarrays using the two-color system (26–29). The most commonly used of the design strategies can be broken down into four major categories: direct comparison with dye-swap, balanced block design, reference sample design, and loop design. Although more extravagant and complicated schemes have been put forward, these four design strategies are conceptually straightforward and easier to implement, and, in the majority of instances, have sufficient statistical power to identify differentially regulated genes.

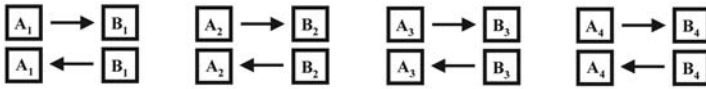
4.2. Direct Comparison Design

The direct comparison is favored if an investigator wishes only to compare the relative gene expression of two classes of samples or experimental groups (Fig. 6). The groups being compared can be untreated and drug-treated cell lines, gene-knockout mouse strain and wild-type mouse strain, chromosome-substituted rat strain and parental rat strain, and so on. On each microarray, RNA samples from the two experimental groups are labeled and co-hybridized onto the same gene chip. The major advantage of the direct comparison design can be found in its name, that is to say, “differential expression of genes in samples A and B is more efficiently measured (and hence more accurate) when comparisons are made on the same array.” This is in contrast to indirect approaches, such as the reference and loop design (see their description in Subheadings 4.4. and 4.5.), in which differential gene expression in the two experimental groups must be inferred because interrogations are performed on separate microarrays, leading to greater variance in the determinations (30). If using the direct-labeling approach (and to a lesser extent with the indirect-labeling approach) we, as well as others, have noted that a small proportion of mRNA species will label with one dye preferentially over the other, leading to a gene-specific dye bias effect. This phenomenon persists even after data normalization to account for global dye biases (i.e., less efficient incorporation of Cy5 vs Cy3 during direct labeling); the reasons for this gene-specific bias are not known, but may be related to distinct physiochemical properties of individual mRNA species. To account for gene-specific dye bias effects, a dye-swap (also referred to as flip-dye or dye reversal) hybridization is performed (Fig. 7). This replicate hybridization, although requiring more starting material RNA, serves two important functions:

1. Identifies gene-specific dye bias artifacts and allows the investigator to exclude such genes from further downstream analysis.
2. Increases the precision of the gene expression measurement because dye-swap hybridizations are akin to performing a technical replicate.

The direct comparison design with dye-swap hybridization uses n arrays for n samples in which each sample is divided into two aliquots.

Direct Comparison with Dye Swap:



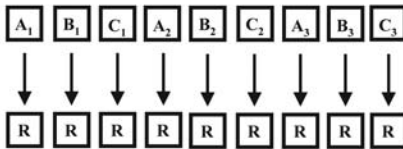
- RNA sample is not limiting
- Flip dyes account for any gene-specific dye bias effects

Balanced Block Design:



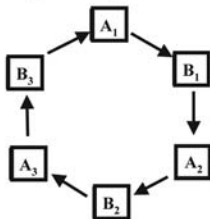
- RNA sample is limiting
- Balanced blocking accounts for any gene-specific dye bias effects

Reference Design (Indirect Comparison):



- More than two samples are compared (e.g. time course; tumor classification)
- Flip dyes are not necessary but can be done to increase precision
- Ratio values are inferred (indirect)
$$(A_1/R_1)/B_1/R_1 = A_1/B_1$$
- Requires common RNA reference for all hybs

Loop Design:



- Suitable for 2 or more experimental groups
- Flip dyes are not necessary but can be done to increase precision
- Loop design ‘unravels’ with a single poor hybridization
- ANOVA analysis is performed to estimate $\log_2$ ratios

Fig. 6. RNA sample allocation strategies for two-color microarrays. RNA samples are represented as boxes. Each arrow represents a single hybridization assay (hyb), with the “head” of each arrow representing Cy5 labeling and the “tail” representing Cy3 labeling. Experimental groups are indicated by letters in boxes and biological replicates are indicated by numbered subscripts. For example, there are two experimental groups A and B and each experimental group is comprised of four biological replicates.

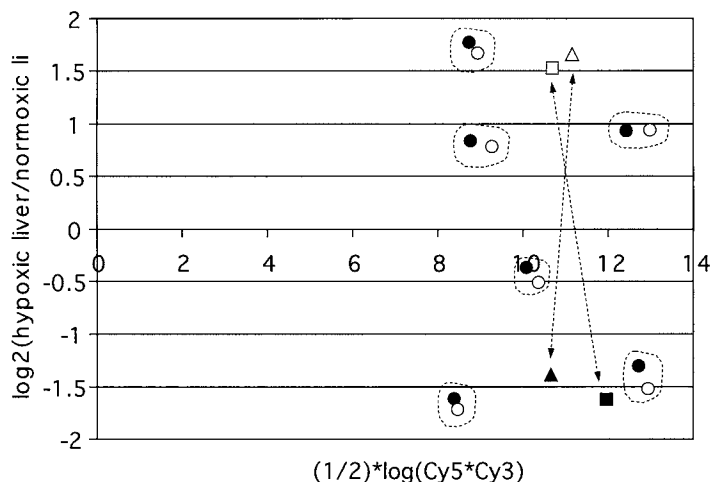


Fig. 7. R-I plot revealing gene-specific dye labeling bias. Three hybridizations (biological replicates) were performed comparing the livers from hypoxic and normoxic Dahl Salt-Sensitive rats. A direct comparison (hypoxic vs normoxic) with dye-swap hybridization strategy was implemented, and the averaged results for eight genes are plotted. For six genes, plotted as open circles representing “forward” hybridizations and closed circles representing dye-swap hybridizations (labeling in the opposite direction), there was no evidence for gene-specific dye labeling bias. This was the case for the majority of genes surveyed. However, the plots of two genes are shown (triangle and square), exhibiting gene-specific dye labeling bias. Forward hybridizations indicated that these two genes were upregulated in hypoxic liver, but dye-swap hybridizations showed the opposite trend.

4.3. Balanced Block Design

If a direct comparison-type strategy is warranted, but the RNA samples are limiting (and the investigator wishes to avoid RNA amplification), and there are concerns of gene-specific dye bias, then a balanced block design is a good alternative (Fig. 6). Here, the direction of the labeling is switched back and forth among a prescribed number of hybridizations; each hybridization represents a biological replicate. In this scheme, mRNAs suffering from labeling bias with a particular fluorophore can be accounted for without resorting to technical replicates. This design uses $n/2$ arrays for n samples.

4.4. Reference Sample Design

If more than two classes of samples or experimental groups are being compared, such as a time-course experiment or classifying multiple tumor types, it is usually more efficient to design array experiments in which each experimental

group is compared back to a common reference RNA sample (**Fig. 6**). The efficiency of using a reference design becomes readily apparent as more and more experimental groups are added, resulting, if one chooses a direct comparison design, in a bewildering number of hybridizations (e.g., experimental group A vs experimental group B, B vs C, C vs D, D vs E, A vs C, A vs D, A vs E, B vs D, and so on), which becomes too cumbersome, cost prohibitive, and/or RNA sample limiting. There is an additional advantage to the application of the reference design, the presence of any gene-specific dye bias affects all arrays similarly and, hence, does not confound experimental group comparisons (**29**). As a result, dye-swap experiments are not necessary, but can be included to increase the precision of the gene expression measurements. In the absence of dye-swap experiments, the reference design uses n arrays for n nonreference samples; or if dye-swap hybridizations are included, $2n$ arrays for each nonreference sample (two aliquots are required for each nonreference group). The reference design is particular well-suited for class discovery using cluster analysis, but any relative gene expression comparisons between groups must be inferred, leading to greater variance in expression measurements. For any gene, the relative expression measurement $\log_2(A/B)$ is estimated by taking the difference $\log_2(A/R) - \log_2(B/R)$, where A, B, and R are experimental group A, experimental group B, and reference, respectively. The reference sample RNA may be biologically relevant, such as a time-zero sample from a time-course experiment, but the reference need not have any biological relevance whatsoever. In this case, the reference RNA can be derived from a mixture of RNA from multiple cell lines or tissues. It is only important that the reference RNA sample is able to hybridize to the majority of probes on the microarray (typically >90% of the probes have a positive signal), and is available in a sufficient quantity to cover all microarray experiments, because even different batches of reference RNA may have quite different expression profiles. Hence, care must be taken in planning experiments and estimating the amount of reference RNA required. Interestingly, genomic DNA has recently been advanced as a better alternative, because this nucleic acid is not subject to biological variance associated with different batches of RNA isolations, and, as a result, represents an “inexhaustible reference source” (**31**).

4.5. Loop Design

In the loop design approach, which may serve as an alternative strategy to the reference design, samples representing two or more experimental groups are compared with one another in a “head-to-tail” fashion, resulting in the formation of a loop (**Fig. 6**; **ref. 26**). This design uses n arrays for n samples, using two aliquots of each sample. By using this configuration, gene-specific dye bias effects are accounted for because each RNA sample in the loop is used once as

the head (i.e., labeled with Cy3) in one hybridization, and as the tail (i.e., labeled with Cy5) in a second hybridization. Analysis of variance (ANOVA) techniques have been developed that allow $\log_2$ ratio values (relative expression values between two samples) to be estimated for each sample comparison (26). A drawback to this approach can be observed if samples number four or more in a loop, namely, gene expression comparisons between samples not directly connected to each other must be inferred (27). Moreover, samples at opposite ends of the loop require the greatest inferences, resulting in the least accurate gene expression measurements. This becomes a very distinct disadvantage as loops become larger and larger. Lastly, the loop design is less robust against the presence of poor-quality hybridizations in which a single bad array can unravel the loop.

4.6. Defining the Number of Biological Replicates Needed

A common question raised by investigators, regardless of the microarray platform used, is “How many biological replicates are needed?” The number of independent biological replicates needed depends on such factors as the objectives of the experiment and the inherent noise of the biological system. Because gene expression measurements from microarrays can be rather variable, it is important to have some type of assurance that our determinations are not false positives. It is important to distinguish biological replication from technical replication. The dye-swap hybridizations represent a form of technical replication, whereby the precision of our measurements is increased by repeated hybridizations with the same RNA samples. By comparison, biological replication is essential to draw conclusions that are valid beyond the scope of the tested samples (e.g., is there a statistically significant difference between treatments?). To estimate the sample size required to achieve the aims of our study, a power calculation is applied. It takes into account the variance of individual measurements, the acceptable false-positive rate, and the desired discriminatory power of the microarray. Simon and Dobbin (29) described a relatively simple power calculation that can be applied to both two-color microarrays using a reference design and single-color Affymetrix GeneChips if comparing two experimental groups/classes. This approach assumes that the gene-specific expression measurements (e.g., $\log_2$ values) are approximately normally distributed for each class. We let σ denote the standard deviation of the log expression level among samples within each class, and suppose that the means of the two classes differ by δ for a particular gene. For $\log_2$ values, a $\delta = 1$ would correspond to a twofold difference in gene expression between classes. We assume that the two classes are compared at the level of expression of each gene, and that a statistically significant difference occurs on rejection of the null hypothesis at a significance level α . Because thousands of genes are analyzed simultaneously on

an array, the significance level α can be set stringently to limit the number of false positives. The statistical power of our calculation is defined by $1 - \beta$, where β is the false-positive rate. Under these parameters, the approximate number of independent biological samples is n , where

$$n = 4(z_{\alpha/2} + z_{\beta})^2 / (\delta/\sigma)^2 \quad (1)$$

and $z_{\alpha/2}$ and z_{β} denote the corresponding percentiles of the standard normal distribution (32). It has been suggested that a good general guideline is to choose $\alpha = 0.001$ and $\beta = 0.05$ (29). For a 10,000-element array, $\alpha = 0.001$ results in an average of 10 false-positive genes and $\beta = 0.05$ provides a 95% probability of detecting a significant change in gene expression. Using $\alpha = 0.001$ ($z_{\alpha/2} = 3.29$), $\beta = 0.05$ ($z_{\beta} = 1.645$), $\delta = 1$, and $\sigma = 0.35$ in **Eq. 1**, we find that a total of 12 samples, 6 for each of the two classes, are required for comparing the two classes and identifying genes exhibiting a significant twofold change. For one-color Affymetrix GeneChips, n = number of arrays; and for the two-color format, $n/2$ = number of arrays.

A second simple approach to estimate the adequacy of the number of biological replicates in a microarray experiment is based on determining the degrees of freedom (27). This can be determined by counting the number of independent biological replicates (e.g., independent animals, independent cell line cultures, or independent pools of microdissected tissues) and subtracting the number of distinct treatments from the number of independent biological replicates. If $df = 0$, there may be no information available to estimate the biological variance, and, hence, the scope of one's conclusions will be limited to the samples themselves. A good guide at the experimental design stage is to have $df = 5$ or greater (27).

5. Systematic Assessment of Microarray Performance

How do we assess the performance of our microarrays? This is an especially relevant question to the novice beginning their first microarray hybridization, and to the experienced user interested in testing a new labeling protocol, RNA extraction method, or RNA amplification scheme. An approach to monitor microarray performance that is gaining widespread popularity is the adoption of external RNA controls, also referred to as spike-in controls or exogenous controls (5,14,33–36). External controls help to identify systematic problems associated with target labeling, array hybridization, and scanning. Typically, external controls are RNA molecules that are synthetically manufactured by *in vitro* transcription. The essential feature of external RNA controls is that the user can introduce predefined amounts to the biological RNA sample. Several external controls are recommended to cover a broad range of expression levels (1–5 copies per cell for rare transcripts, and ~100–300 copies per cell

for moderately expressed transcripts in mammalian organisms). If they are spiked differentially (e.g., two- and threefold differences) into the two RNA samples that are being compared, the external controls mimic differentially expressed genes. Hence, the external controls provide an important benchmark for quality control assessment, and, in many laboratories, the external controls are routinely used in all microarray experiments.

The critical requirement for external RNA controls is that they are representative of the endogenous biological mRNAs in terms of length and sequence characteristics (e.g., GC content, and secondary structure). In addition, cross-hybridization toward the endogenous transcripts should be avoided. Hence, for example, plant-specific RNA external controls can be used when interrogating mammalian RNA samples (14). In terms of the microarrays, the probe sets that recognize the fluorophore-labeled external targets are frequently printed across different sectors of the microarray glass slide, thereby allowing assessment of intraslide variability, whereas interslide variability is assessed across multiple independent hybridizations and target labeling. The external probe elements can also serve as negative controls if the external RNA is not added to the labeling reaction. In the absence of external RNA spiking, nonspecific hybridization of fluorophore-labeled sample target should be negligible, otherwise, it may be an indication that wash conditions are not sufficiently stringent.

6. Identifying Differentially Regulated Genes

The study of gene expression with microarrays has evolved from a qualitative endeavor during its early years to a more quantitative pursuit in more recent years. Statistical procedures for determining differentially regulated genes are just one aspect of this evolution. Even if data mining analysis is going to be performed using one or more of the widely used visualization tools (e.g., cluster analysis; see **Heading 7.**), it is frequently useful to reduce the data set to those genes that can best distinguish between the experimental groups. The earliest microarray papers typically used an *ad hoc* approach to define differentially regulated genes. For example, all genes exhibiting a twofold difference in expression (up or down) between experimental groups were deemed interesting, thus, ignoring biologically relevant genes exhibiting smaller changes. Furthermore, with this approach, there is no associated value that indicates the level of confidence in the designation of genes as differentially regulated.

The *t*-test is a simple, statistically based method for detecting differentially regulated genes (37). This statistical approach can be used for both Affymetrix GeneChips and two-color arrays using a reference or balanced block design. However, a drawback to using the *t*-test on microarray data is the resulting phenomenon known as the *multiple testing problem* (38). Consider a cut-off for differential expression of $p < 0.05$. We would expect 5% of the nondifferentially regulated genes on the array to reach “statistical significance” (false-positives).

Because large numbers of tests are being conducted on a single array, this is equivalent to saying that we expect 500 genes to be identified as significant on a 10,000-element array, when, in fact, they are not differentially regulated. To control for these false-positives resulting from the multiple testing problem, a Bonferroni correction is commonly implemented. The nominal false-positive rate is divided by the number of tests (in this case 10,000) to yield the effective rate. For a 10,000-element array, the Bonferroni-corrected p value is reduced to $p < \alpha/N$ array elements or, in our example, $p < 0.000005$. In practice, this correction is too severe, and typically leads to very few identified differentially regulated genes. There are, however, less conservative corrections that can be applied, including the *adjusted Bonferroni correction*, which ranks gene by their t statistic and then applies increasingly less stringent criteria to subsequent genes in the list until an appropriate threshold p value is reached. Alternatively, the Westfall and Young stepdown p values rely on permutation testing to select appropriate significance cutoffs. Both of these approaches, along with the more conservative Bonferroni technique, correct for multiple testing by controlling the familywise error rate, which is the probability of accumulating one or more false-positive errors over a number of statistical tests (37).

For two or more experimental groups assayed on Affymetrix GeneChips or two-color arrays, significance analysis of microarrays (SAM) is a popular approach to identify differentially regulated genes (39). SAM uses an adjusted t statistic along with permutation testing to estimate the false-discovery rate in any user-defined set of significant genes. Alternatively, ANOVA techniques have been described for microarray experiments assaying three or more experimental groups (37). Finally, a one-sample t -test with a multiple testing correction, or a variant, such as one-sample SAM, can be implemented for two-color arrays using a direct comparison or balanced block design. Designs of this type involve the co-hybridization of two experimental groups on the same array, and the primary question is whether the $\log_2$ expression ratio values are consistently significantly different from zero.

It is important to note that a good foundation in statistics is increasingly critical in microarray applications, but it is not a substitute for good experimental design. For example, in the absence of dye-reversal hybridizations to account for gene-specific dye bias effects, no amount of statistical gymnastics will rescue an investigator from potentially pursuing these false-positive genes in downstream functional analysis.

7. Visualizing Expression Data

7.1. Getting Started

The starting point in the analysis of expression data is the collection of raw expression measurements. For two-color arrays, these measurements are typically performed by image analysis software, such as TIGR Spotfinder (<http://www.tm4.org/spotfinder.html>) or ScanAlyze (<http://rana.lbl.gov/EisenSoftware.htm>),

that detects the fluorescence intensity of each fluorophore (Cy3 and Cy5) on each array spot. After taking into account and correcting for factors such as local background estimates and spot morphology, the software will output a pair of intensity values for each spot—an estimate of the expression level for both conditions in the hybridization.

Before any biologically relevant expression analysis can take place, these raw intensity values must first be normalized. Normalization algorithms can help to reduce the effects of systemic biases, such as differences in labeling efficiencies and spatial variation across the array, and to facilitate comparisons between data sets. Data filtering techniques are often applied near the normalization steps to reduce the complexity of the data set by removing data that are of questionable or poor quality. There are many normalization algorithms available, ranging from simple scaling techniques, such as total intensity normalization (12), to the advanced lowess normalization (40). Other algorithms exist to deal with the rationalization of dye-swap (41) and replicate data. Examples of available normalization packages include MIDAS (<http://www.tm4.org/midas.html>) and ArrayNorm (<http://genome.tugraz.at/Software/>).

7.2. Working With Expression Data

The relationship between the expression measurements for a particular array element in a two-color array can be summarized by the ratio of its intensity values. This expression ratio is calculated by dividing one intensity value (associated with one dye) for a given element by the other intensity value for that same element (associated with the second dye): intensity1 = 20,000 and intensity2 = 10,000.

$$\text{expression ratio} = \frac{\text{intensity1}}{\text{intensity2}} = \frac{20,000}{10,000} = 2.0$$

The community standard has been to use $\log_2$ ratios instead of basic (nonlog) ratios. Using $\log_2$ ratios to represent relative expression levels offers several advantages over basic expression ratios. Consider a fivefold change in expression: intensity1 = 50,000 and intensity2 = 10,000.

$$\text{basic ratio} = \frac{\text{intensity1}}{\text{intensity2}} = \frac{50,000}{10,000} = 5.0$$

$$\log \text{ ratio} = \log_2 (\text{basic ratio}) = \log_2 (5.0) = 2.32$$

In the opposite case, intensity2 is five times larger than intensity1: intensity1 = 10,000 and intensity2 = 50,000.

$$\text{basic ratio} = \frac{\text{intensity1}}{\text{intensity2}} = \frac{10,000}{50,000} = 0.2$$

$$\log \text{ ratio} = \log_2 (\text{basic ratio}) = \log_2 (0.2) = -2.32$$

A comparison of the basic ratios from these examples reveals two arithmetically accurate results that are reciprocals of each other. The basic ratios, 5.0 and 0.2, are asymmetrically distant from the basic ratio that represents a lack of expression change, 1.0. On the other hand, the corresponding $\log_2$ ratios, 2.32 and -2.32 , are equally distant from the $\log_2$ ratio that represents a lack of expression change, 0.0. The nature of the logarithm is such that an n -fold change in expression will result in a log ratio that is equal in magnitude to another n -fold change in the opposite “direction.” This trait makes comparisons involving log ratios more intuitive than their basic counterparts, because overexpressed and underexpressed elements are treated symmetrically. From this point on, expression values will be represented by log ratios.

The expression level of an element in a specific hybridization experiment can be summarized by its log expression ratio. One technique to compare the expression levels of an element across experiments is to examine the corresponding series of log ratios. For example, the expression of element A across four experiments (numbered 1–4) can be represented by the four ratios shown in **Table 1**.

This sequence of log ratios is known as an *expression vector*. It represents the expression of an element across multiple experiments. The expression vector can serve as a profile of the specified array element; such profiles are necessary if determining the similarity of expression levels between multiple elements. It is also possible to generate expression vectors to represent the profiles of experiments instead of array elements. Many clustering and classification techniques will operate on expression vectors of experiments as well as vectors of elements.

A natural extension in working with expression vectors is to evaluate, in tandem, those vectors that cover the same series of experiments. “Stacking” such expression vectors produces a structure known as an *expression matrix*. In the example in **Table 2**, note the addition of the expression vectors representing the expression levels of elements B, C, and D across the same set of experiments as the expression vector corresponding to element A. Each intersection of an element and an experiment is a matrix cell that contains a log ratio; this value represents the expression of the specified element in the specified experiment.

One way to think about an expression vector is as a series of Cartesian coordinates that define an element’s location in n -dimensional expression space, where n is equal to the number of experiments in the vector. In the example in **Table 3**, there are four expression vectors, each with three data points (i.e., $\log_2$ ratios). Each of these vectors can be represented as a triad of Cartesian coordinates: element A = (3.0, 4.0, 5.0); element B = (2.0, 3.0, 4.5); and element C = (−3.0, −2.0, 3.0).

Table 1
Expression of Element A Across Four Experiments

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Element A	Log ratio A1	Log ratio A2	Log ratio A3	Log ratio A4

Table 2
Expression Matrix

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Element A	Log ratio A1	Log ratio A2	Log ratio A3	Log ratio A4
Element B	Log ratio B1	Log ratio B2	Log ratio B3	Log ratio B4
Element C	Log ratio C1	Log ratio C2	Log ratio C3	Log ratio C4
Element D	Log ratio D1	Log ratio D2	Log ratio D3	Log ratio D4

Table 3
Expression Vectors

	Experiment 1	Experiment 2	Experiment 3
Element A	3.0	4.0	5.0
Element B	2.0	3.0	4.5
Element C	−3.0	−2.0	3.0

With these sets of coordinates, each vector can now be plotted on a 3D graph (Fig. 8). Note that elements A and B have similar log ratios in each of the three experiments, both in terms of magnitude and sign. These two elements appear near each other on the graph, but substantially further from element C, whose log ratios are less similar to those of elements A and B. Mathematical formulas called *distance metrics* will be used (see Subheading 7.3.) to quantify these observations and to facilitate the analysis of expression vector relationships (i.e., clustering).

7.3. Clustering: An Overview

One branch of microarray data analysis is the exploration of the expression patterns that arise for array elements within a series of experiments. Identifying array elements with similar expression patterns may provide evidence of a biological relationship between the represented genes. The use of clustering algorithms is a common method of evaluating these patterns of expression and organizing related elements.

Clustering algorithms can be divided into a few functional categories. Agglomerative methods, such as hierarchical clustering (42,43), start with

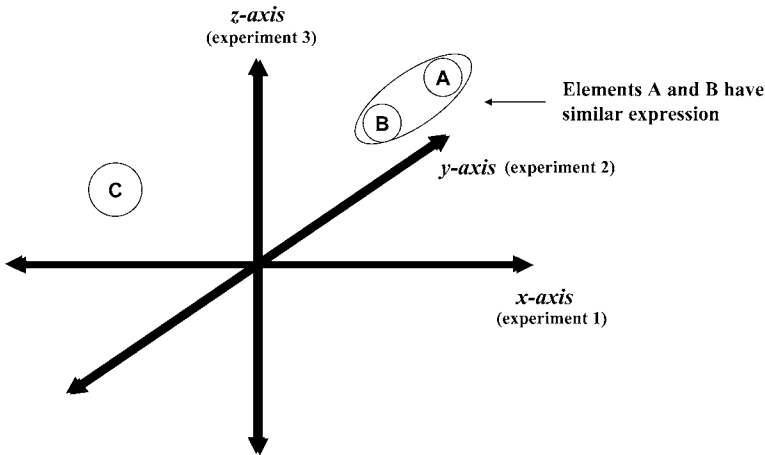


Fig. 8. Expression vectors as points in a three-dimensional (3D) expression space. Microarray data mining involves looking for genes with “similar” patterns of expression. If three hybridization experiments are considered, the expression vector for each gene is represented by a point in 3D space, where the expression measure ($\log_2$ ratio value) for gene i in experiment 1 is its x coordinate, the expression measure for gene i in experiment 2 is its y coordinate, and the expression measure for gene i in experiment 3 is its z coordinate. In such a geometric representation, expression vectors for gene elements A and B have similar expression patterns.

individual elements and iteratively build up larger structures by associating similar elements with each other. Divisive methods, such as k -means clustering (44,45), seek to take a large collection of elements and segregate them into groups containing elements with similar expression patterns. Other algorithms transform the input expression matrix to facilitate user-defined element groupings or may incorporate approaches from multiple algorithm categories. The hierarchical clustering and k -means clustering algorithms are described in **Subheadings 7.4** and **7.5**.

Clustering algorithms rely on mathematical formulations to determine how similar elements are to each other. The terms similarity and distance are inversely related; two elements are considered similar if the distance between their expression vectors is low. Conversely, a larger distance between a pair of expression vectors indicates a lower level of similarity between the associated elements. It is this measured distance between expression vectors that is used when decisions are made to cluster elements.

There are many methods available to measure the distance between expression vectors; these are collectively known as *distance metrics*. Each distance metric uses a formula that can take two expression vectors and compute a numeric distance measurement. Some clustering algorithms were designed with

a particular distance metric in mind, whereas others are compatible with several metrics. The selection of the distance metric to use is an important decision, because each metric is capable of uncovering different features of the data set.

Two common distance metrics are Euclidean distance and centered Pearson correlation coefficient. Euclidean distance is based on the two-dimensional Pythagorean theorem:

$$d_{A:B} = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2} \quad (2)$$

where $d_{A:B}$ is the distance between points A and B, point A is defined by the coordinates (x_1, y_1) and point B is defined by the coordinates (x_2, y_2) . In the Euclidean distance metric, each experiment in the expression vector is treated as a dimension. If the expression vectors each contain two ratios (i.e., there are two experiments) then the distance formula could be written in a form similar to **Eq. 2**. The distance between two expression vectors, each containing n log ratios, can be calculated using the general equation:

$$d_{A:B} = \sqrt{\sum_{i=1}^n (x_i - y_i)^2}$$

where $d_{A:B}$ is the distance between expression vectors A and B, x_i is the log ratio from expression vector A and y_i is the log ratio from expression vector B, both from the experiment at position i . The Euclidean distance metric exhibits a commutative behavior; the distance between expression vectors A and B is equal to the distance between expression vectors B and A. The smallest distance possible is the distance between an expression vector and itself, 0. There is not a defined upper limit for distance.

To measure the similarity of the shapes of two expression vectors, a centered Pearson correlation coefficient distance metric is used. The shape of an expression vector is most apparent by graphing experiments on the x-axis, and component log ratio values on the y-axis (**Fig. 9A**). The value of the centered Pearson correlation coefficient, r , for two expression vectors each containing n log ratios, is calculated as:

$$r = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 \sum_{i=1}^n (y_i - \bar{y})^2}}$$

where x_i is the log ratio from expression vector A, and y_i is the log ratio from expression vector B, both for the experiment at position i , $\bar{x}$ is the mean log ratio from expression vector A, and $\bar{y}$ is the mean log ratio from expression vector B.

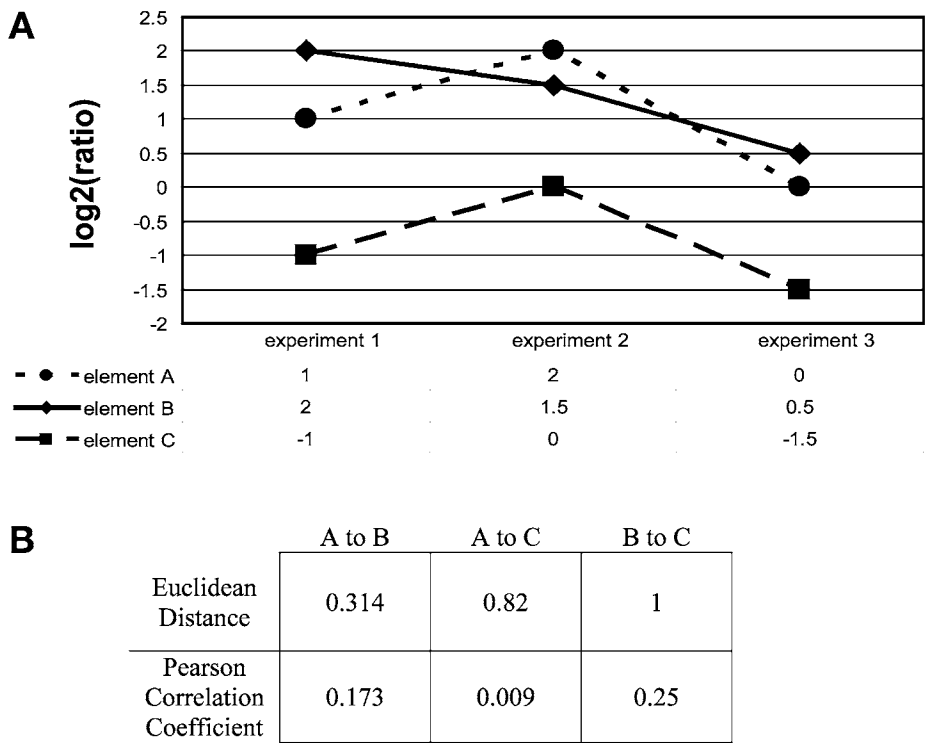


Fig. 9. (A) The expression vectors A, B, and C are shown in both tabular and graphical form. **(B)** Distances between each of the vectors were calculated using Euclidean distance and Pearson Correlation Coefficient. The distances have been scaled such that the minimum distance is 0 and the maximum distance is 1. Note that the most similar vectors are A and B if using the Euclidean Distance metric, whereas the Pearson Correlation Coefficient metric shows A and C to be most similar.

Values for r range from -1 to 1 . The magnitude of the r -value indicates the strength of the correlation, and the sign indicates whether the correlation is direct or inverse. Expression vectors with strong direct correlation (i.e., similar shapes) will have an r -value close to 1 . In the case of vectors with a strong inverse correlation (i.e., opposite shapes), the r -value will be close to -1 . A pair of expression vectors with weak correlation (i.e., neither directly nor inversely correlated) and independent shapes will have an r -value close to 0 . Two other forms of the Pearson correlation coefficient are also widely used. An uncentered version takes into account the magnitude of expression changes within each vector when calculating r . The Pearson squared form treats pairs of correlated vectors in the same manner as anticorrelated vectors.

Similar to the Euclidean distance metric, the centered Pearson correlation coefficient also exhibits commutative behavior. A comparison of these two metrics is illustrated in [Fig. 9B](#).

7.4. Hierarchical Clustering

Hierarchical clustering is an agglomerative clustering method that offers an intuitive visual result in the form of a tree diagram and provides insight into the degree of relationship that elements have with each other. The algorithm takes a collection of independent elements and progressively joins them into increasingly larger clusters.

The preliminary step in creating a hierarchical tree is the calculation of the pairwise distances between every element and every other element to determine which elements are most closely related. A *distance matrix* can be constructed to store all of the calculated distance values as an $n \times n$ grid, where n is the number of elements involved in the analysis. Each row and column represent an element and the matrix cells contain the pairwise distance between the row element and the column element, each calculated using the same distance metric ([Table 4](#)).

If the distance metric used has a commutative behavior (i.e., the distance from element A to element B is equal to the distance between element B and element A) then the distance matrix will be symmetrical about the diagonal (upper left to lower right). From a computational perspective, this reduces the total number of distance calculations by approximately half.

Once the distance matrix has been constructed, the algorithm will enter an iterative stage in which the following steps will be performed a number of times equal to $n - 1$. At the start, each element in the distance matrix is treated as a “cluster.” As the algorithm progresses, these single-element clusters will be combined to form progressively larger nested clusters.

The steps in the algorithm are:

1. Determine which two clusters are the most similar by finding the smallest distance value from the distance matrix.
2. Combine these two clusters together to form a larger cluster.
3. Recalculate only the distances between this cluster and all other clusters. A predetermined *linkage method* will dictate the procedure to use when calculating the distance between clusters that contain more than one element.
4. Continue with the next iteration at **step 1**. Look for the next smallest distance value from the distance matrix.

There are several linkage methods to choose from in deciding how to measure distances between clusters. Consider two clusters, A and B, each with five member elements. The *single linkage* method sets the distance between clusters

Table 4
Distance Matrix

	Element A	Element B	Element C	Element D
Element A	Distance AA	Distance AB	Distance AC	Distance AD
Element B	Distance BA	Distance BB	Distance BC	Distance BD
Element C	Distance CA	Distance CB	Distance CC	Distance CD
Element D	Distance DA	Distance DB	Distance DC	Distance DD

A and B to equal the smallest distance between any element contained in cluster A and any element contained in cluster B. The opposite approach, *complete linkage*, uses the largest distance between any element contained in cluster A and any element contained in cluster B as the intercluster distance. *Average linkage* calculates the average distance between elements in both clusters and sets this value as the intercluster distance.

The result of this algorithm is a series of progressively larger nested clusters, and a table of relevant intercluster distances. It is a relatively straightforward task to use this data to create a graphical depiction, known as a dendrogram (Fig. 10). The clusters that were joined together in the earliest iterations are connected by short branches (i.e., elements with the most similar patterns of expression), whereas clusters that were joined later are connected by increasingly longer branches (i.e., less similar). A color scheme is applied to the dendrogram to provide an intuitive representation of overexpressed and under-expressed genes. A color gradient running from black to red represents log ratios from zero to a positive end-point value, respectively, along with a color gradient running from black to green to represent log ratios from zero to a negative end-point value, respectively.

7.5. k-Means Clustering

If there is an *a priori* hypothesis regarding the number of clusters into which the elements in the data set should be partitioned, the divisive *k*-means clustering method can be used to perform the partitioning. The goal of the algorithm is to divide the elements into *k* distinct clusters; each cluster should end up containing elements that are more similar to each other than to elements in other clusters. The value for *k* must be set by the user before the start of the algorithm.

The *k*-means algorithm consists of the following steps:

1. Each element is assigned randomly to one of the *k* clusters.
2. An expression vector is used to represent each cluster by computing the mean expression vector of all elements in that cluster. If the median expression vector is used instead, this method is called *k*-medians clustering.

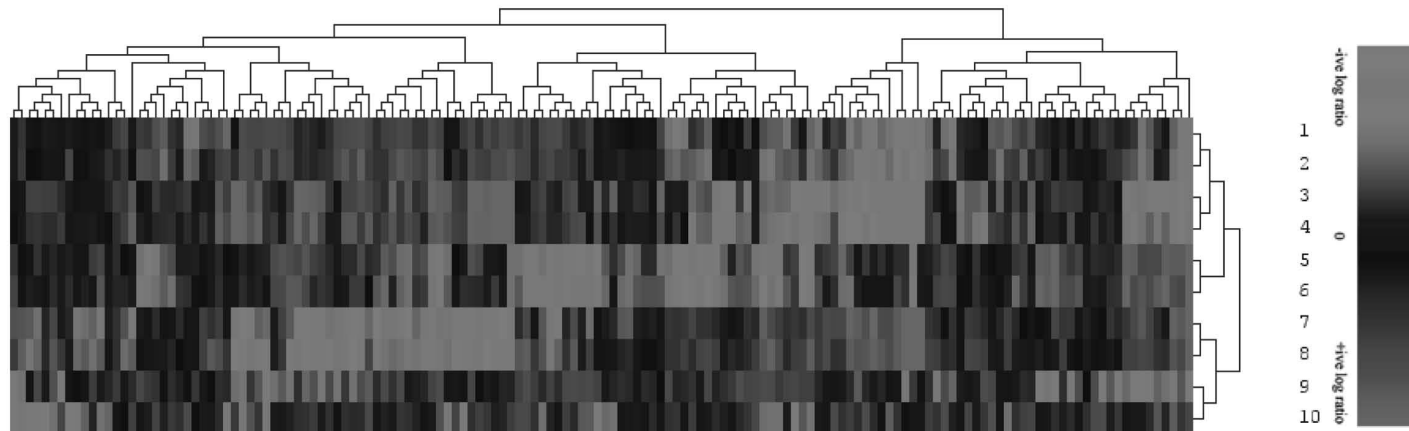


Fig. 10. A dendrogram derived from hierarchical clustering. Hierarchical trees have been constructed for both gene elements (rows) and experiments (columns). Shorter branches indicate smaller distances between the expression vectors, and a closer relationship.

1 3 3 4 5 5 7 8 9 10



Element 82
Element 115
Element 129
Element 141
Element 59
Element 83
Element 99
Element 113
Element 123
Element 128
Element 149
Element 2
Element 29
Element 33
Element 41
Element 60
Element 45
Element 109
Element 118
Element 71
Element 78
Element 102
Element 103
Element 89

1 3 3 4 5 5 7 8 9 10



Element 5
Element 13
Element 17
Element 19
Element 32
Element 36
Element 40
Element 66
Element 130
Element 10
Element 16
Element 18
Element 31
Element 35
Element 43
Element 53
Element 54
Element 58
Element 61
Element 63
Element 64
Element 67
Element 68
Element 81
Element 85
Element 94
Element 111
Element 112
Element 125
Element 132
Element 137
Element 138
Element 144

1 3 3 4 5 5 7 8 9 10



Element 12
Element 90
Element 104
Element 126
Element 25
Element 72
Element 88
Element 119
Element 124
Element 127
Element 143
Element 3
Element 20
Element 46
Element 97
Element 98
Element 101
Element 110
Element 122
Element 134
Element 135
Element 37
Element 47
Element 57
Element 77
Element 79
Element 86
Element 96
Element 147
Element 23
Element 80
Element 148
Element 121

1 3 3 4 5 5 7 8 9 10



Element 28
Element 76
Element 114
Element 131
Element 133
Element 136
Element 1
Element 4
Element 26
Element 38
Element 42
Element 50
Element 52
Element 62
Element 69
Element 70
Element 73
Element 75
Element 120
Element 145
Element 146

Fig. 11. (Continued)

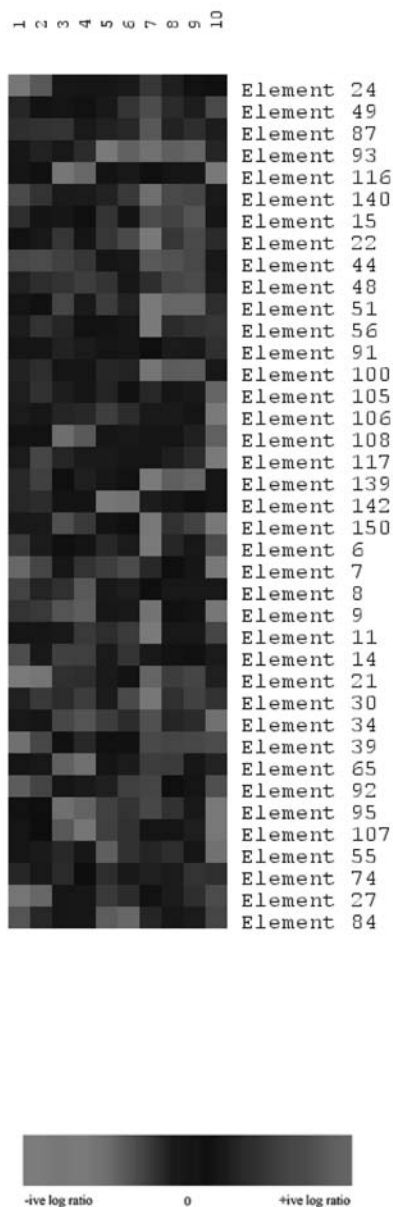


Fig. 11. *k*-Means clustering. A *k* of five was chosen, and five clusters were produced. Two elements from within the same cluster have a similar appearance, whereas two elements from different clusters will look less alike.

3. Perform **steps 3a,b** once for every element, in turn. A single iteration of this step involves the evaluation all elements.
 - a. Select an element and find the cluster with which it has the most similarity (i.e., into the cluster with a mean expression vector least distant from the element's own). If the element is not already a member of this cluster, move it there.
 - b. If the element was moved into a different cluster, recalculate the mean expression vector for the cluster it was moved from and for the cluster to which it was moved. Continue to **step 3a**.
4. If no elements were moved during the most recent iteration of **step 3**, then all elements are currently in their most ideal clusters and the algorithm is finished. Otherwise, begin the next iteration of **step 3**.

The result of this algorithm is a collection of k clusters (**Fig. 11**), each containing the elements that most closely matched the cluster's mean expression vector at the time each element was assigned.

There are many software packages available that give the user the ability to perform analyses similar to those described in **Subheadings 7.4** and **7.5**. Recommended open-source systems (**46**) include TM4 (<http://www.tm4.org>; **ref. 47**), BioConductor (<http://www.bioconductor.org>; **ref. 48**), and BASE (<http://base.thep.lu.se>; **ref. 49**). Each of these systems are available free of charge and source code is provided (additional visualization and analysis schemes are available, and we refer the reader to the pertinent reviews in **refs. 50** and **51**).

It is important to note that the results of clustering algorithms are merely mathematical interpretations of the data and may not necessarily correlate with biological organizations. The algorithms that are chosen in the course of the analysis of a data set, as well as the specific parameter settings used, will have a significant effect on the conclusions that can be drawn from the analysis. Such conclusions should not be taken as absolute facts but rather as hypotheses that can be further examined.

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Oligonucleotide Microarrays for the Study of Coastal Microbial Communities

Gaspar Taroncher-Oldenburg and Bess B. Ward

Summary

DNA microarrays are well suited as a tool for analyzing functional gene diversity as well as community composition in aquatic environments. Microarrays allow for the semi-quantitative characterization of target genes by means of specific hybridization of labeled target gene sequences, amplified from the environment, to the corresponding oligonucleotide probes on the slide. Specificity and sensitivity are determined by the probe design. In their current implementation, environmental DNA microarrays are useful for analyzing microbial communities as well as for analyzing the presence of functional genes involved in larger biogeochemical processes, such as nitrogen cycling. Here, we lay out a basic protocol to analyze genes in the environment, which can be applied to most target genes of interest.

Key Words: 70-mer; Chesapeake Bay; Choptank River; functional gene; hybridization; *nirS*; nitrite reductase; oligonucleotide microarray.

1. Introduction

The analysis of functional diversity and its dynamics in the environment is essential for understanding the microbial ecology and biogeochemistry of aquatic systems. Specific enzymes, encoded by their corresponding genes, mediate the different steps involved in elemental cycling processes. Determining the presence and/or expression of these genes provides a first look at the tip of the regulatory hierarchy and permits the correlation of DNA sequence patterns with biogeochemical dynamics (1,2). Such analyses have been traditionally performed at the single gene level. Given their ability to interrogate the environment by analyzing many different genes at once, DNA microarrays now afford an ideal tool for identifying and quantifying multiple microbial genes simultaneously, and for

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evaluating distribution of the genes in the environment (3–6). Oligonucleotide microarrays are the format of choice for such applications because they provide the highest versatility in terms of probe design (optimization of probe binding characteristics) and microarray design flexibility (the capability of adding/removing probes from the array in successive rounds of analysis). The probes, the oligonucleotides bound to the array's surface, hybridize to the fluorescently labeled target sequences amplified from DNA extracted from an environmental sample. After scanning of the hybridized microarray and image analysis, the identity of each of the targets is determined. The results are rendered semiquantitative by performing competitive hybridizations in which the sample is co-hybridized with a reference sample, labeled with a different fluorophore, and the fluorescence intensity ratios are evaluated (Fig. 1). An approach that is more quantitative involves the use of internal standards. To illustrate the implementation of DNA microarrays for the detection and quantification of functional genes in the environment, we describe the development and application of a 70-mer oligonucleotide microarray containing 64 probes representing as many different sequences of the nitrate reductase gene, *nirS*, for analyzing the nitrogen cycle diversity in the Choptank River–Chesapeake Bay system (7,8).

2. Materials

2.1. Equipment

1. Thermocycler.
2. Microarrayer.
3. Centrifuge (with 96-well plate and 50-mL tube adaptors).
4. Hybridization oven (50–80°C).
5. Shaker.
6. Microarray scanner (e.g., GenePix 4000A, Axon Instruments Inc.).

2.2. Environmental DNA Isolation

1. Sterivex filter capsules (0.2- μ m pore size filter, Millipore Inc.).
2. FastDNA SPIN kit for soil (Qbiogene, Inc.).
3. GentraPureGene DNA isolation kit (Gentra Systems).

2.3. Microarray Fabrication

1. Amino-saline-coated glass slides (CMT_GAPS, Corning Inc.).
2. Oligonucleotide probes (70-mers or 90-mers [stock solution: 1 μ g/ μ L in 50% DMSO] adjusted to a concentration of 0.05 μ g/ μ L in 50% DMSO).

2.4. Target Labeling

1. Random hexamers or gene specific primers.
2. dNTPs and Cy3 and Cy5 dCTP (Amersham Biosciences).
3. DyeEx spin columns (Qiagen).

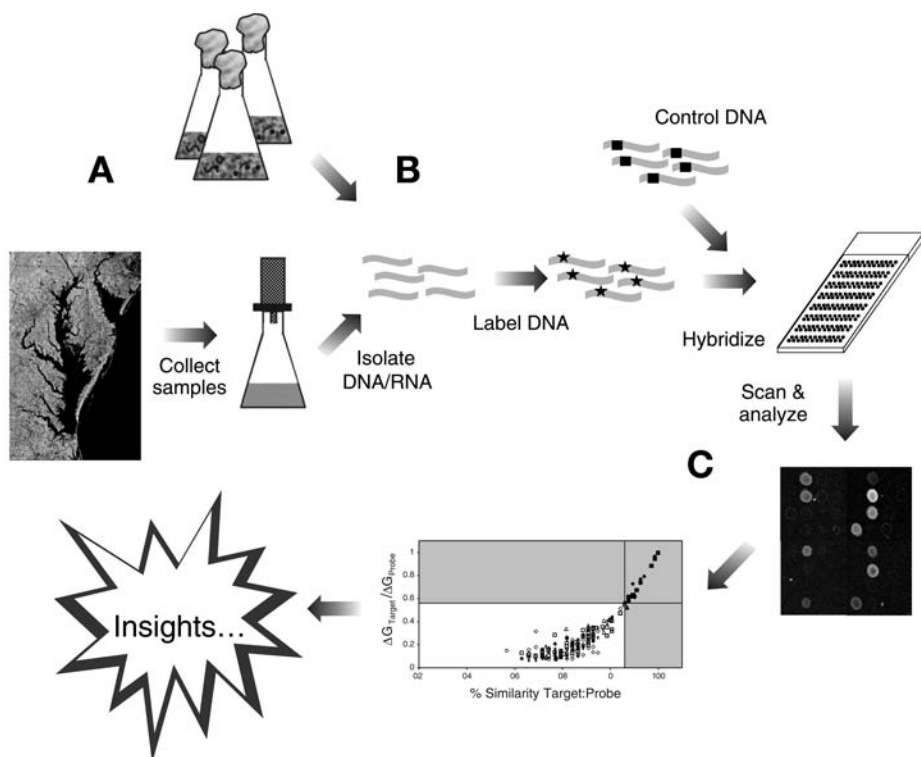


Fig. 1. Flow-chart representing the different steps involved in applying microarrays to microbial community and functional studies in aquatic ecosystems. **(A)** Samples are collected from a range of sites to be compared and prepared using a variety of methods (including filtration, centrifugation, and so on). The approaches described here for analyzing ecosystems can also be adapted to studying cultures, mesocosms, and other artificial population setups. **(B)** Samples are labeled and hybridized, either in a competitive approach with a reference sample (a base-line population or mix of populations against which all of the samples are compared), or in a noncompetitive approach, in which the reference or standard is built into the probes spotted on the microarray. **(C)** After hybridization, the fluorescence readings are analyzed using a variety of filters and adjustments to extract semiquantitative abundance values (see **Subheadings 3.2–3.4.** for detailed descriptions of these three steps).

2.5. Hybridization and Data Acquisition

1. Hybridization chambers (e.g., Corning, Inc.) and glass slide covers (22 × 60 mm).
2. Prehybridization buffer: 0.75 M NaCl, 0.075 M Na citrate, 1% blocking reagent (bovine serum albumin), and 0.1% sodium dodecyl sulfate (SDS).
3. Poly(A) DNA (Amersham Biosciences) solution: 1 µg/µL (in H₂O).
4. Hybridization buffer: GlassHyb (Clontech Laboratories, Inc.).

5. 1X standard sodium citrate (SSC): 0.15 M NaCl and 0.015 M sodium citrate.
6. Low-stringency washing buffer: 1X SSC and 0.1% SDS.
7. Medium-stringency washing buffer: 0.1X SSC and 0.1% SDS.
8. High-stringency washing buffer: 0.1X SSC.

3. Methods

3.1. Probe Design and Microarray Construction

This section describes the process leading from target sequence alignment to probe sequence optimization and microarray fabrication. Four parameters are important for optimal hybridization and for minimizing the potential for cross-hybridization among probes:

1. Sequence identities less than 87%.
2. Random distribution of mismatches.
3. Target-to-probe perfect match to mismatch binding free-energy ratios higher than 0.56.
4. A GC content in the probe region of approx 50%.

These guidelines can be modified to adapt to the particular needs and characteristics of the functional gene of choice.

3.1.1. Sequence Alignment and Clustering

The probes are best designed based on sequence information derived from clone libraries obtained from the ecosystem under study (1,9–12). Alternatively, comprehensive sequence information regarding the functional gene of interest compiled from existing databases can also be used as a starting point. Next, the optimal sequence segment of the target gene is identified by determining the 70-bp stretch with the best compromise between gene specificity (minimal cross-hybridization with other target gene sequences in GenBank) and high sequence variability among all of the sequences aligned for the genes of interest. The preceding determination is best performed manually using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) searches to determine sequence similarity of the candidate 70-bp stretches with the rest of GenBank. Subsequently, the oligonucleotide sequences (70-mers) are aligned, using a sequence analysis software package (e.g., Sequencher from GeneCodes Corp.). Individual distance matrices (percent identity) for all of the probe sequences are generated with the PAUP software package (v. 4.0b8a; ref. 13; see Note 1). A tree is constructed to identify deep-branching, representative sequences for their use as cluster-specific probes (Fig. 2).

3.1.2. Free-Binding Energy Calculations

To optimize the specificity of the probes, free-binding energy (ΔG^0) for each potential hybridization pair on the microarray is calculated to choose those probes

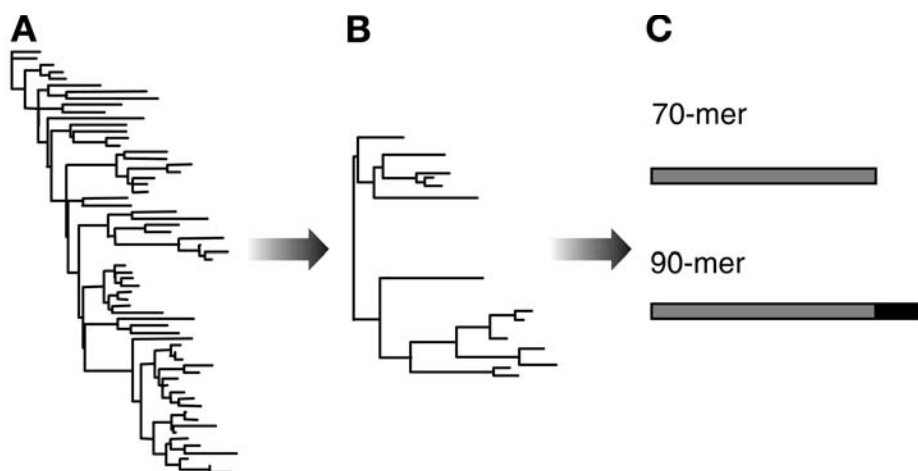


Fig. 2. Probe design. **(A)** A comprehensive catalog of each target gene is generated and represented as a phylogenetic tree. **(B)** Representative probes for the different branches are chosen based on sequence characteristics, such as GC content and free binding energies, and a reduced representation of the sequence space to be analyzed is generated. **(C)** Two types of probes are described in the text: 70-mers for competitive hybridization setups and 90-mers incorporating a reference sequence for noncompetitive hybridizations (gray lines indicate gene-specific sequences; black lines corresponds to the reference oligonucleotide).

with minimal theoretical cross-hybridization (14,15). These calculations are temperature dependent—in most cases, hybridizations can be performed in the range of 55 to 65°C. For the application described here, all calculations were performed for 65°C, the temperature that was found to yield optimal fluorescence and hybridization specificity for the *nirS* array. Using the web-based mfold software (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna>; refs. 16 and 17), the free-binding energy of a folded nucleic acid strand can be determined. For the purpose of our calculations, an artificial AATT bridge must be introduced between the forward sequence of a probe and the reverse complemented sequence of each possible target (all of the other probe sequences). This generates a loop with a ΔG_{65}^0 of 3.4 kcal/mol that allows the mfold algorithm to functionally align the sequences properly. This bridge-specific free-energy value is eventually subtracted from the total ΔG_{65}^0 value of every sequence pair analyzed.

3.1.3. DNA Microarray Printing

1. Determine number of probes to be spotted and number of arrays to be printed.
2. Following the instructions for the arrayer, determine the physical distribution of the slides on the arrayer and spotting patterns. Spotting indices can generally be created using common spreadsheets, such as Excel.

3. Adjust the concentration of the oligonucleotides probes (70-mers) to 0.05 $\mu\text{g}/\mu\text{L}$ in 50% DMSO (see **Note 2**).
4. Spot in triplicate on CMT-GAPS amino-silane-coated glass slides (see **Note 3**).
5. After spotting, carefully remove the slides from the arrayer and transfer them to a metal slide holder, or to their original packaging for further processing. The time lapse between removing the slides from the arrayer and crosslinking of the probes, the next step in the microarray fabrication process, should not exceed 2 to 3 h.
6. Crosslink the probes to the arrays by transferring the slides to a metal slide holder and placing them in an oven to bake at 80°C for 3 h.
7. After crosslinking, keep the microarrays in their original packaging, protected from light and at room temperature, in a desiccator under vacuum or with N_2 .

3.2. Environmental DNA Isolation and Labeling

1. Collect environmental samples using conventional sediment and water sampling devices: use cut-off plastic syringes to obtain sediment samples from sediment cores; for water samples, filter 2 to 8 L of water, depending on the biomass, onto a Sterivex capsule.
2. Flash-freeze the samples with liquid N_2 to preserve the integrity of the nucleic acids.
3. Store on dry ice or at -80°C until processing.

3.2.1. DNA Extraction From Sediment Samples

A small amount of sediment (~ 0.5 g) is solubilized in 2 mL of the resuspension buffer included in the FastDNA SPIN kit for soil, and then processed following the directions provided by the manufacturer with the kit (see **Note 4**).

3.2.2. DNA Extraction From Water Column Samples

Particles captured on Sterivex filter capsules are stored at -80°C dry, without buffer, and extracted with kits designed to yield total nucleic acids or DNA or RNA alone. For DNA, the Gentra Puregene tissue extraction protocol is satisfactory, with minor modifications to the lysis volume requirements. For RNA, the Ambion RNAqueous 4PCR kit yields good-quality RNA from the capsules. Alternative kits are probably acceptable, but have not been exhaustively tested in our laboratory.

3.2.3. Target DNA Labeling

Using 10 to 20 ng of DNA from the environmental samples obtained in **Subheadings 3.2.1–3.2.2.**, set up two separate PCR reactions, with Cy3 and Cy5 dCTP, respectively. In a final volume of 20 mL, these reaction mixes should contain 2.5 mM dATP, dGTP, and dTTP; 1.875 mM dCTP; 0.625 mM Cy3 or Cy5 dCTP; and 0.1 μM primers (depending on your goals or the complexity of the sample, you may use random hexamers or primers specific to your target genes).

Parameters for the 30-cycle PCR are as follows: denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. At the end, an additional extension at 72°C for 10 min should be performed.

Remove unincorporated Cy dCTPs with a DyeEx spin column following the manufacturer's protocol.

DNA concentrations in the resulting solutions are determined from the absorbance values at 260 nm (DNA) and 550 nm (Cy3; $E_{\text{Cy3}} = 150,000 \text{ /M/cm}$) or 649 nm (Cy5; $E_{\text{Cy5}} = 250,000 \text{ /M/cm}$).

3.3. Competitive Two-Color Microarray Hybridization and Data Acquisition

All hybridizations are performed in duplicate on two identical DNA microarrays (18). Microarray A is used to hybridize two samples to be compared, or a sample and a reference mix, that have been labeled with two different fluorophores (in this case Cy3 and Cy5 [Amersham Biosciences])—microarray B is used to hybridize a label-inverted set of samples, or a sample and reference, i.e., the samples labeled with Cy3 or Cy5 are now Cy5 or Cy3 labeled, respectively. This experimental design results in duplicate data sets of replicate spots per slide for each gene—a total of six to eight values per probe, depending on the array layout (19,20).

3.3.1. Hybridization

1. Prehybridize microarrays in freshly made prehybridization buffer. The original microarray plastic containers designed to hold five slides are well-suited for prehybridizing two to three slides with a volume of 20 to 40 mL of prehybridization buffer in a hybridization oven, at the hybridization temperature (55–65°C, depending on the melting temperature of the probes) and for 45 min.
2. After prehybridization, place the slides in a glass slide holder and dip them five times in MilliQ water (in a staining jar) at room temperature to remove excess buffer.
3. Wash once in isopropanol, followed by a quick centrifugation at 1700g for 5 min (see **Note 5**).
4. Place prehybridized slides immediately in hybridization chambers and place cover slips on top of the spotted segment of the microarray (see **Note 6**).
5. Prepare hybridization mix as follows: mix desired quantities of labeled target (usually 2 μL of each of the differentially labeled samples, or sample and reference) with 4 μg of poly(A) DNA—alternatively, other nonspecific DNA can be used to block background hybridization.
6. Denature the mix at 96°C for 3 min and place it on ice until ready for hybridization.
7. Add preheated (65°C) hybridization buffer (72 μL of GlassHyb) to the denatured mix and apply the 80 μL hybridization mixture by capillarity, with a 100 μL pipet tip between the cover slip and the slide.

8. Close the hybridization chamber, quickly wrap it in aluminum foil, and place it in a hybridization oven at 65°C for 16 to 18 h.

3.3.2. Posthybridization Processing

1. After hybridization, remove the slides from the hybridization chambers and place them individually in 50-mL, aluminum foil-covered tubes containing 45 mL of low-stringency washing buffer to start a washing sequence in three buffers of increasing stringency (low → medium → high). All washes should be performed while gently shaking the tubes on a shaker.
2. After approx 30 s of gentle shaking, the cover slips should fall off the slides.
3. Remove the cover slip from the tube with tweezers to avoid scratching the hybridized microarray surface.
4. After 5 min, transfer the slide to the medium stringency buffer and shake for another 5 min.
5. Transfer the slide to the final high stringency wash and shake for 5 min.
6. After the high-stringency wash, transfer the slides to tubes containing 45 mL of MilliQ water and gently shake for 5 min.
7. Finally, dip the slide in 100% ethanol and quickly dry it by centrifugation (see **Note 5**).

3.3.3. Scanning

After the posthybridization washes, the dry microarrays should be kept in the dark and at room temperature. The slide to be scanned is placed in the scanner's microarray holder and prescanned. Most commercially available scanners have a prescan setting, which provides a low-resolution, quick-pass scan of the microarray. The purpose of the prescan is to check the overall success of the hybridization and to optimize the intensity levels of the two preset fluorescence channels (Cy3 [550 nm] and Cy5 [649 nm]); avoiding potential photobleaching of the fluorophores as a result of long exposure to excitatory wavelengths. This is achieved by changing the power settings of the laser. After scanning, the grid representing the locations of the probes is placed on the image, and the analysis is performed (see **Note 7**). The results are summarized in an Excel format spreadsheet.

3.4. Data Processing

All of the values used during processing are derived from the median feature fluorescence or background fluorescence data reported on the GenePix software-derived spreadsheet. For the first two processing steps, the raw fluorescence data reported for the Cy3 and Cy5 channels are used. Subsequently, the $\log_2$ of the fluorescence ratios at each spot (Cy5/Cy3) is applied (see **Note 8**). Likewise, after the first two steps, the average of the ratios of the filtered spots and their standard deviations are calculated for their application in all further

analysis steps. The calculations are all conveniently set up in an Excel spreadsheet format.

3.4.1. Spot and Feature Evenness

This step is designed to remove features of high internal variability (e.g., doughnut-shaped spots). Only those features for which more than 90% of the signal pixels exceed the local background for either or both channels (Cy3 or Cy5) by at least two standard deviations of the local background fluorescence are accepted for further analysis.

3.4.2. Background Filter

To eliminate features whose quality may be compromised by unusually high background fluorescence levels caused by local slide inconsistencies or hybridization-related artifacts (e.g., “comets” caused by probe smearing or non-specific dye spots), only those spots for which the local background signal is within two standard deviations of the global background level of the slide are accepted. Next, filter all of the remaining “good” values (e.g., at least two of three or five of eight replicates per probe) are averaged, and their standard deviations determined—these values are used for the remainder of the analysis steps.

3.4.3. Consistency and Reproducibility Check

In this step, all of those features for which only one of the label-reverse microarrays (see **Subheading 3.4.1.**) shows a valid signal, as determined from **Subheadings 3.4.1.** and **3.4.2.** are removed from further analysis.

3.4.4. Dye Normalization Filter

To account for the difference in fluorescence intensity between Cy3 and Cy5, the fluorescence ratios obtained from the label-reverse microarrays must be normalized. For every probe, the ratio of the $\log_2$ fluorescence ratios (see **Note 8**) from a pair of label-reverse microarrays is calculated. This ratio must exceed the median value of Cy5/Cy3 ratios determined for the entire slide to be considered a significant ratio, and its contributing values accepted for further analysis. This step is analogous to the background filter applied earlier for the single fluorescence channels.

3.4.5. Labeling Efficiency Normalization

All corresponding pairs from a pair of label-reverse microarrays are plotted to obtain a linear regression through the points (i.e., relative fluorescence intensity of slide A vs slide B). Differences in labeling efficiency and quantum efficiency (QE) of each fluorophore ($QE_{\text{Cy3}} = 0.38$; and $QE_{\text{Cy5}} = 0.28$) result in linear regressions with slopes close to one, but ordinate intercepts significantly

different from zero. To normalize the values, all of the fluorescence ratio values must be adjusted to one-half the Euclidian distance between them and their respective inverse values. This step mathematically removes any fluorescence bias introduced by the labeling reactions as well as by the differences in fluorescence intensity between the two dyes, and adjusts the intercepts to zero.

3.4.6. Outlier Determination

Relative differences among samples in the fluorescence of particular targets can be small. A good criterion to define the significance of a change in fluorescence ratio in the context of a particular experiment is to set a threshold for the fluorescence ratio at which a spot will be considered to be significantly different from a 1:1 ratio. We have empirically determined that fluorescence ratios higher than the average of the standard deviation of all of the positive features on a pair of slides sets a realistic limit for considering a value to be different from the 1:1 ratio.

3.4.7. Data Representation

We have found that one of the most intuitive ways to represent hybridization results is by means of overlaying the hybridization data over a distance tree representing the similarity among the probes spotted on the microarray (**Fig. 3**). This provides an immediate assessment of target distribution and possible cross-hybridization issues.

3.5. Alternative Internal Standard Microarray Hybridization and Data Acquisition Approach

In this approach, each probe consists of two parts: the 70-mer gene-specific probe oligonucleotide (the same 70-mers as used in the competitive approach) and a 20-mer reference oligonucleotide (**21**). Thus, each spot contains an internal standard, and it is not necessary to perform inverse-labeling experiments.

3.5.1. Hybridization Scanning

The protocols are essentially the same in **Subheadings 3.3.1. to 3.3.3.**, except that 200 pmol of Cy5-labeled antisense 20-mer is added to each hybridization mixture. Poly(A) is not necessary if only prokaryotic sequences are used (i.e., there has been no amplification with oligo-dT primers).

3.5.2. Data Processing

Either median or mean fluorescence values can be used for analysis. The user should investigate the data scatter resulting from both kinds of analyses and choose the approach that provides the most robust replication. The procedures for elimination of bad features (excess background, local inconsistencies,

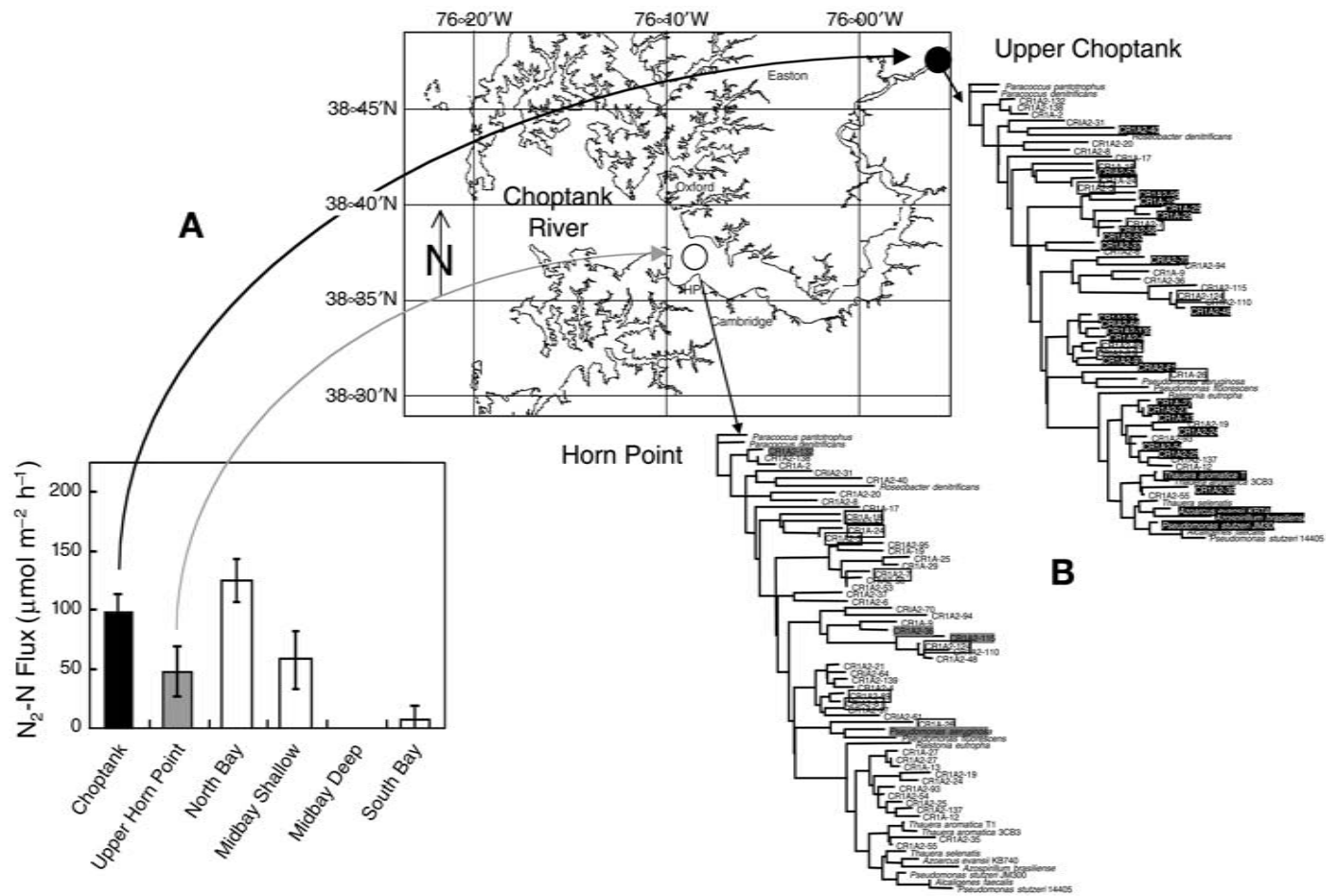


Fig. 3. (Continued)

and so on) are similar to those used for the competitive approach, except that there is no longer a need for a label-inverse check and to normalize for dye intensity, because the standards are built into the 90-mer probes and red and green fluorescence from different slides are not being compared, respectively. Instead, after the appropriate elimination of spots with low-quality signal or nonsignificant fluorescence, an average green-to-red fluorescence ratio is computed for each set of replicate spots. This ratio is a quantitative representation of signal intensity for each probe, because the same amount of reference 20-mer was spotted in each dot. This ratio can be normalized to the Cy5 signal strength of a designated standard probe to normalize for possible systematic variability in hybridization strength in the standard in different locations on the array.

4. Notes

1. Several software packages are available for aligning sequences and generating distance matrices. Different distance trees based on neighbor joining, maximum likelihood, or parsimony distance matrices can be generated. The topology of each of these trees will vary slightly, but to group sequences within the 87% identity threshold, any of the algorithms will suffice, and multiple different analysis will ensure robustness of the resulting trees.
2. The probes are best aliquoted at a concentration of 1 $\mu\text{g}/\mu\text{L}$ in 50% DMSO in 96- or 384-well plates and kept sealed at -80°C . Probes can be diluted to a working concentration of 0.05 $\mu\text{g}/\mu\text{L}$ in 50% DMSO and kept at -20°C . When ready for spotting, plates are thawed, and briefly vortexed and centrifuged to ensure homogeneity of the probe solutions.
3. In our hands, the CMT-GAPS amino silane-coated glass slides from Corning have had the most consistent and reliable performance if used as a platform for oligonucleotide (70-mer) spotting and hybridization. New slides are constantly being developed and commercialized, therefore some side-by-side testing and comparing would be advisable when getting started with your own project.
4. A range of kits is available for extracting nucleic acids from sediment samples. It is advisable to perform side-by-side comparisons for the specific kind of sediment to be analyzed because differences in the physicochemical characteristics of a sediment affect the performance of the extraction kits. It is further advisable to optimize the extraction conditions for one's particular samples, and to pool extractions or samples, especially if PCR is used to generate the target fragments.
5. Processing (washing and drying) of individual slides can be easily performed in 50-mL plastic Corning tubes. The slides are transferred with tweezers from tube

Fig. 3. (Continued) Example of a semiquantitative study along a nutrient gradient in the Choptank river. **(A)** Sampling sites and their nitrate levels. **(B)** "Community trees" showing different distributions of nitrite reductase genes at the two sampling points.

- to tube containing successive buffers (45 mL each), thereby minimizing the risk of the microarrays drying out. After the last rinse, the slides are inserted in dry 50-mL tubes fitted with a lint-free tissue (e.g., KimWipe, Kimbely Clark Corp.) at the conical bottom to absorb moisture during centrifugation.
6. The cover slips can be placed directly on the slide, but a distribution of the hybridization solution that is more uniform is generally achieved by slightly lifting the cover slip from the microarray. We have found that the easiest way to do this is by cutting two to three 25-mm strips of Parafilm and wrapping one around either end of the cover slip or the slide—this provides a minimal separation between the cover slip and the slide that ensures better capillary distribution of the hybridization solution.
 7. Automatic identification and quantification of the spots is usually very reliable, provided the grid has been properly placed over the spots and the background is low. Manual adjustments can be made if necessary.
 8. Following standard procedure for evaluating microarray data, $\log_2$ values are used to represent both relative increases and decreases in relative fluorescence on the same scale. On a linear scale, a twofold increase translates into a value of 2, whereas a twofold reduction is equivalent to a value of 0.5. This results in an asymmetrical graphical representation of analogous relative changes. To circumvent this issue, the ratios are represented on a $\log_2$ scale, such that the values for the above ratios are 1 and -1 , respectively.

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