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Large-Scale Generation of Plasmids that Express Type I Interferon

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1. Introduction

Large-scale preparations of plasmid DNA encoding cytokine cassettes are continuously gaining in importance in many areas of gene-therapy ***(1)*** including DNA vaccinations with plasmid cytokine adjuvants ***(2–4)***, tumor research ***(5)***, and treatment of infectious diseases ***(6,7)***. Small-scale plasmid preparations (approx 10–100 µg DNA) usually result in adequate yields, whereas large-scale (>1 mg DNA) preparations of plasmid DNA can result in disproportionally small yields of DNA. We have found that using Terrific broth (TB) rather than Luria-Bertani (LB) broth in available kits significantly (approx fivefold) increases plasmid DNA yields (*see* **Note 1**). This suggestion is especially true for low or very low copy plasmids. In this chapter, we give an overview of different plasmid purification techniques commonly used, and describe advantages and disadvantages for each method.

If plasmid DNA is used for transfection purposes, it is advantageous to use a method yielding low lipopolysaccharide (LPS) contamination. It is well established that LPS contamination negatively influences transfection efficiency ***(8)***. In addition, LPS alone is a strong inducer of chemokine and cytokine synthesis ***(9)***. LPS contamination of DNA obtained from commercially available kits is reportedly much lower compared to DNA using a single CsCl gradient purification scheme ***(8)***. Another advantage of commercially available kits is the reduced time needed, and the use of less health-hazardous chemicals. Ready-to-use DNA can be obtained with kits in less than 1 d compared to approx 5–7 working days needed for the CsCl preparation. In our laboratory, we transfect murine eyes/vaginas with 100 µg type I interferon (IFN) plasmid DNA per eye and per vagina to protect animals from otherwise lethal herpes simplex virus type 1 (HSV-1) and HSV-2 infections ***(6,7)*** in the

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hopes of utilizing this technique for the human condition. We have also initiated the generation of human IFN-β plasmid DNA for use in higher vertebrates. In these experiments between 2–4 mg of high-purity (260:280 ratio = 1.7–2.0), supercoiled plasmid DNA is needed.

Figure 1 gives an overview of the process from cloning the type I IFN gene into the desired plasmid vector, isolating the plasmid, and finally using the plasmid constructs in in vitro and in vivo studies.

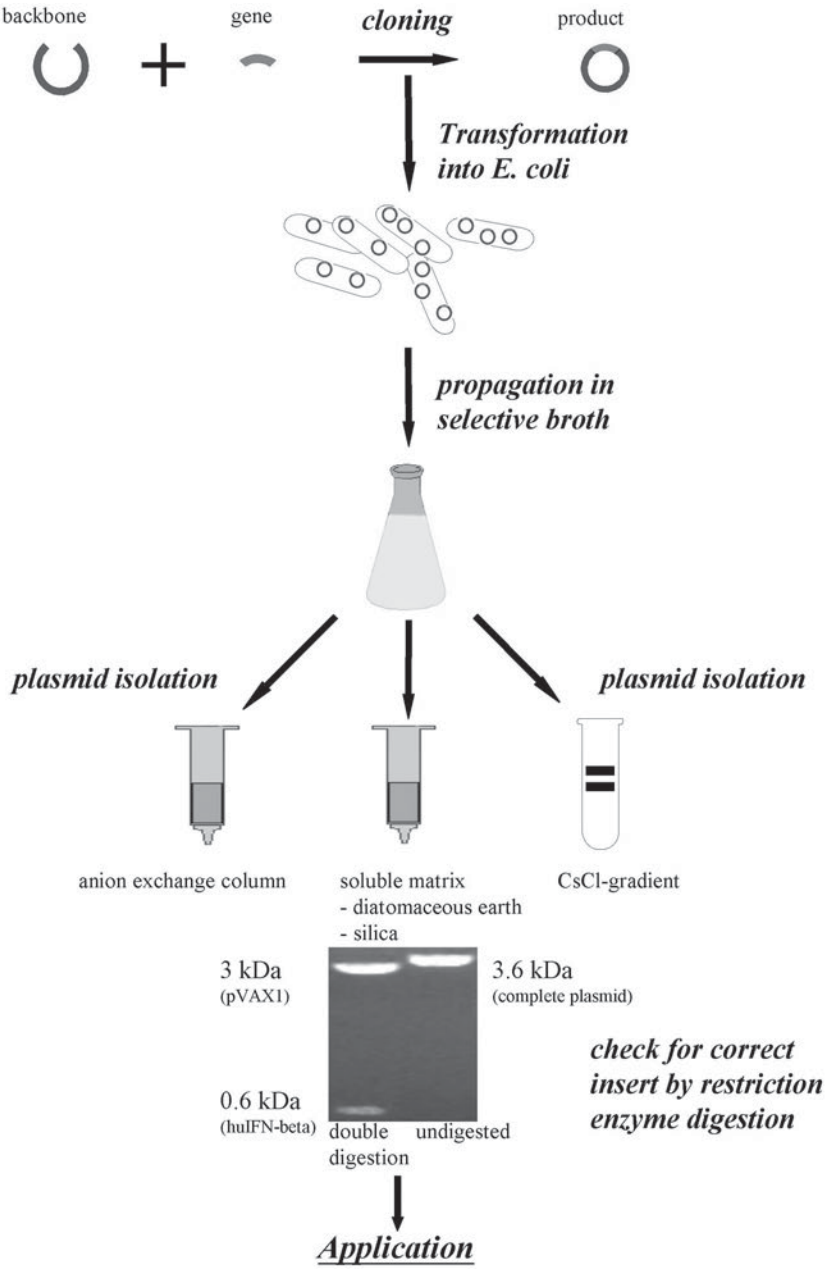
2. Materials

1. Luria-Bertani broth: 10 g/L tryptone peptone and 5 g/L yeast extract (both from Becton Dickinson, Sparks, MD), and 10 g/L NaCl (Fischer, NJ). After all ingredients are dissolved, autoclave broth immediately and let it cool before inoculating with bacteria. We use 1 mL of frozen bacterial stock (1 mL bacterial culture gently mixed with 480 µL of 50% sterile glycerol, stocks frozen at –80°C) per L TB (*see* **Note 2**).
2. TB: Prepare a stock solution of 0.17 *M* KH_2PO_4 (11.5 g/500 mL), 0.72 *M* K_2HPO_4 (62.7 g/500 mL) (J.T. Backer Inc., Phillipsburg, NJ) and autoclave. Mix 12 g/L trytone peptone and 24 g/L yeast extract (both from Becton Dickinson), and 4 mL glycerol (Fischer), adjust with 900 mL deionized water, and autoclave. When the broth has cooled, add 100 mL of your sterile stock potassium phosphate solution and inoculate broth (for bacterial inoculum, *see* **item 1**).
3. Agar plates: To prepare the plates, use the same broth as described above and add 15 g/L agar (DIFCO Laboratories, Detroit, MI) and pour into sterile dishes after adding the selective antibiotic.
4. Miniprep kits: Qiaprep® Miniprep (Qiagen, Valencia, CA) or Wizard® Minipreps (Promega, Madison, WI).
5. TE buffer, LPS free (GIBCO, NY).

3. Methods

1. Take three agar plates containing the recommended concentration of selective antibiotics. Transfer 25 µL, 50 µL, and 100 µL of the transformed bacteria suspension onto the agar plates, spread the liquid evenly, and incubate plates at 37°C for 24 h.
2. Using a sterile wire loop, select five typical, single colonies (2–3 mm diameter) and inoculate them separately into 6 mL of TB. Incubate in a shaking incubator at 37°C, 250 rpm for about 8 h.
3. Isolate DNA from the 8 h cultures, using plasmid miniprep kits for each day culture (5 mL) to find the sample with the highest DNA yield (minipreps take about 1 h).

Fig. 1. *(opposite page)* The process from cloning the type I IFN gene (we used human/macaque type I IFN) into the FDA-approved pVAX1 plasmid vector (Invitrogen®, Carlsbad, CA) (3000 bp size) to the application. The plasmid is transformed into the *Escherichia coli* strain INVαF' One shot® (Invitrogen®) and grown up



in TB followed by purification using different methods. The insert, in this case human IFN-β (600 bp), was verified using restriction enzyme digestion (*Kpn*I and *Xho*I, New England Biolabs, Beverly, MA), yielding a 600 bp and 3000 bp band on a 1% agarose gel, containing 0.5 µg/mL ethidium bromide. Freshly isolated high-grade DNA is then used for in vitro and in vivo studies.

Table 1
Advantages and Disadvantages of Different Methods to Purify Plasmid DNA

Considerations	Anion exchange column (Quiagen)	Diatomaceous basis (Bio-Rad)	Silica based (Promega)	CsCl gradient
Time needed from lysate step to ready to use DNA	4 h up to 2 d, depending on the preparation size	~2 h	2 h up to 1 d	approx 5–7 d
Yields	max DNA binding capacity per column 10 mg	max DNA binding capacity 3 mg	max DNA binding capacity 3 mg	no limit
Others	Column can be recalibrated Special kits for LPS removal available Long waiting times for during binding step and washing steps (time consuming steps)	Cannot be recalibrated All steps can be done with a large bench-top centrifuge	Cannot be recalibrated Highest LPS residues of all preparartion choices Special kits for LPS removal available	Higher chance of RNA/ chromosomal DNA contamination Use of toxic substances like CsCl and ethidium bromide

[a]In all methods the purity of the DNA, as determined by the absorption ratio measured at 260/280 nm, is in the range 1.7–1.9.

4. Inoculate 1 mL of the day culture in 1 L of TB and incubate for 16–18 h in a shaker incubator (250 rpm) at 37°C.
5. Select the appropriate kit according to your desired DNA yield (*see* **Table 1** and **Notes 3** and **4**) and follow the kit protocol. If you scale up the culture volumes to obtain higher yields, do not forget to scale up the recommended lysate and neutralization volumes appropriately. (For example, in the standard protocol 500 mL broth is used, if you desire 1000 mL broth, then you also have to double the volume of the resuspension, lysate, and neutralization buffers.)
6. Dry down the DNA pellet and dissolve it in LPS-free TE buffer (GIBCO) (*see* **Note 5**).
7. The concentration of the purified plasmid DNA can be measured by spectrophotometry at 260 nm. The ratio between 260 nm (nucleic acids) and 280 nm (protein) should be between 1.7–2.0 for high purity.
8. Agarose gel analysis may reveal ribosomal RNA contamination. If ribosomal RNA is suspected, digest RNA with RNase followed by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction (*see* **Note 6**).

9. To verify the correct gene insert and orientation, restriction enzyme digestion should be carried out using unique restriction sites. Restriction enzyme maps are included for each commercially available vector. A restriction enzyme map can be obtained using the World Wide Web (*see* **Note 7**).

4. Notes

1. Another reason for reduced yields can also be a gradual loss of plasmid copies/bacterium. This is especially true when bacterial broth aliquots are frozen back intermittently instead of freezing back a large number of vials from a freshly transformed and high-yield bacterial broth. Using aliquots from one broth batch will ensure similar yields each time. If reduced yields are encountered over time, we recommend transforming competent bacteria again and continuing with **Subheading 3., step 1**.
2. Another suggestion is to scrape a piece of frozen bacteria (approx 10 µL) with a small spatula out of a stock vial, let it thaw on selective agar, spread it out when thawed, incubate the plates for 18 h at 37°C, pick a well–sized colony (approx 2–3 mm diameter), grow an 8-h culture, and inoculate broth with 100–1000 µL. With this method, the culture will grow much faster to the desired density.
3. If more than 3 mg of freshly isolated DNA are needed, then Qiagen“ Plasmid Giga Kits (Qiagen) can be used and reused several times. Therefore, scale up your broth to 2–3 L volumes and follow the kit protocol. Save the flowthrough lysate and wash the column once with distilled water followed by an equilibration using 50 mL equilibration buffer. Rerun the flowthrough lysate through the column as described in the kit protocol. Be aware that keeping the used columns for more than ~10 h may result in bacterial contamination of further preparations.
4. If less than 3 mg DNA is needed, then Quantum Prep® Plasmid Maxiprep Kit (BioRad, Hercules, CA) spin kits with a 3 mg capacity are the faster and more economical alternative. These columns cannot be reused.
5. A good method to take out the ethanol/isopropranolol precipitated DNA is by using a P1000 pipet to transfer the DNA pieces into a 1.5-mL sterile tube. Quickspin at 12,000*g* for 1 min and repeat until all DNA pieces are transferred into the tube. Dry the DNA pellet in a speedvac without heat. Prewarmed sterile water or TE buffer to approx 60°C facilitates DNA solubilization. Never vortex DNA, which can cause plasmid DNA shearing; fingerflicking the tube works well.
6. RNase A can be DNase contaminated. If so, heat inactivate the DNase at 100°C for 30 min, dissolve in TE buffer.
7. The nucleotide sequence of the gene of interest can be obtained from the NIH gene bank (for example http://www.ncbi.nlm.nih.gov). To receive a complete restriction enzyme map of the gene, you can cut and paste the gene sequence into an easily accessible program (for example, http://www.firstmarket.com/cutter/cut2.html). The manufacturer of the plasmid includes a plasmid description with a restriction enzyme. With this information, it should be possible to choose two unique restriction enzymes to cut the plasmid construct such that one can easily differentiate the correct insert, backbone, and orientation of the transgene.

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Identification of *trans*-Acting Factors by Electrophoretic Mobility Shift Assay

Rebecca J. Chan, Min You, and Gen-Sheng Feng

1. Introduction

Cell stimulation with a growth factor or cytokine results in a myriad of intracellular activities, including up- and down-regulation of multiple signal transduction pathways, culminating in observable cellular functions, such as modulation of cell motility, alteration of cell proliferation rate, or induction of apoptosis. Understanding the molecular mechanisms that mediate these cellular functions is of critical importance as aberrations of these pathways lead to human maladies such as autoimmune disease, malignancy, and susceptibility to infection. The most distal components of the signal transduction pathways are the transcription factors. These *trans*-acting proteins bind to *cis*-acting DNA elements and, together with the basal transcriptional machinery, control the rate of gene transcription. One of the most useful and common techniques employed in studying transcription factors is the gel mobility shift assay. The overall principle behind this technique involves the use of a radiolabeled piece of DNA mixed with a nuclear protein extract. The protein–DNA complex has a higher molecular weight than the DNA alone resulting in a slower moving or "shifted" band on a nondenaturing polyacrylamide gel (*see* **Fig. 1**). These assays have traditionally been used to investigate the binding of novel transcription factors to undefined regions of genomic DNA, typically immediately upstream of the transcription start site of a gene of interest. Using this technique, many ubiquitous and cell type–specific transcription factors as well as their DNA consensus sequences have been described ***(1–5)***.

More recently, however, the use of these assays has been extended to measure the activity of a given signal transduction pathway in response to a cell stimulation. For example, it is known that the growth-inhibitory cytokine interferon-γ signals to the signal transducers and activators of transcription

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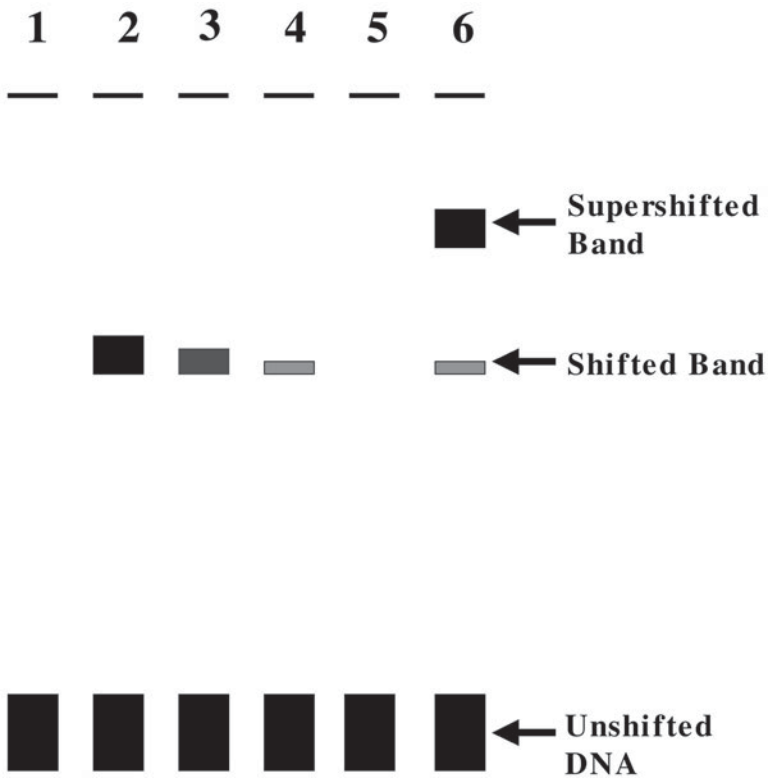

Fig. 1. Schematic diagram of gel shift, competition, and supershift assays. Lane (1) radiolabeled probe alone; (2) radiolabeled probe plus nuclear protein extract; (3–5) radiolabeled probe, nuclear protein extract, and increasing molar amounts of unlabeled DNA; (6) radiolabeled probe, nuclear protein extract, and antibody.

(STAT) transcription factors ***(6)***. When investigating the role of the cytoplasmic tyrosine phosphatase Shp-2 in mediating signals from the interferon-γ receptor to the STAT proteins in the nucleus, You et al. employed the gel shift assay using the STAT-binding consensus sequence (hSIE, 5) and nuclear protein prepared from wild-type (WT) and mutant (Shp-$2^{-/-}$) murine embryonic fibroblast cell lines. As seen in **Fig. 2**, the activity of STAT binding was much greater in the Shp-$2^{-/-}$ cells following stimulation with interferon-γ compared to that observed in the WT cell line. The use of the gel shift assay in this capacity allowed the authors to observe that Shp-2 functions to downregulate signal relay from the interferon-γ receptor to the STAT proteins ***(7)***.

This chapter compiles the methods needed to perform the gel shift assay. It has been organized into separate descriptions for each component needed to

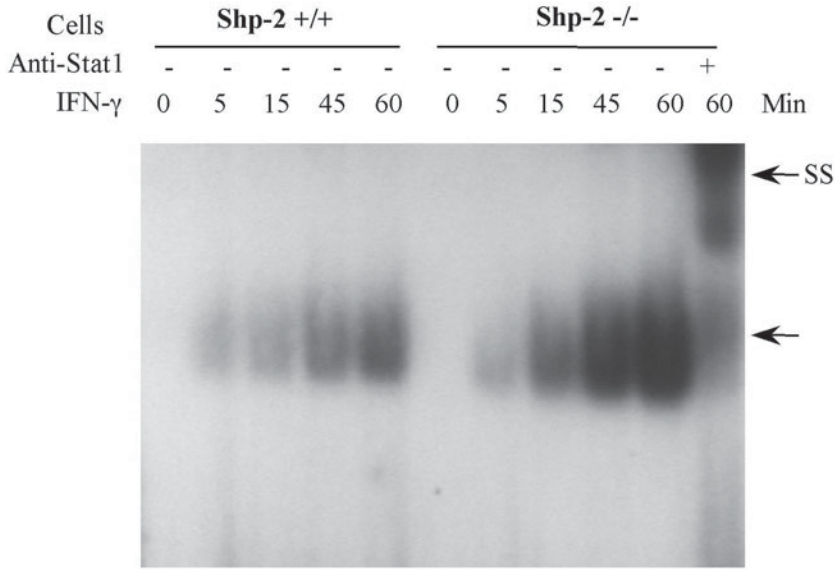

Fig. 2. Gel shift assay used to examine interferon-γ-stimulated STAT activity using radiolabeled STAT consensus sequence (hSIE) and nuclear protein extracts from WT and Shp-2 -/- murine embryonic fibroblast cells. The addition of anti-STAT1 to binding reaction results in a supershifted (SS) band.

perform a successful assay, starting with the nuclear protein extract, followed by probe preparation, the binding reaction, and, finally, gel electrophoresis and detection.

2. Materials

2.1. Equipment

1. Dounce homogenizer.
2. Motor-driven Teflon pestle.
3. Gauze or cheesecloth.
4. 25-gauge needles.
5. Ultraviolet (UV) irradiation source.
6. Polyacrylamide gel electrophoresis apparatus.
7. Gel dryer with attached vacuum.
8. Autoradiograph film and intensifying screens.

2.2. Reagents

1. Phosphate-buffered saline (PBS).
2. Enhance (New England Nuclear, Boston, MA).

3. Aprotinin: 1000X stock solution of 5 mg/mL in water.
4. Leupeptin: 1000X stock solution of 30 mg/mL in water.
5. Pepstatin A: 1000X stock solution of 5 mg/mL in water.
6. Na_3VO_4: 2500X stock solution of 500 mM in water.
7. Buffer A: 10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, and add fresh, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.5 m*M* dithiothreitol (DTT), 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
8. Buffer C: 20 m*M* HEPES, pH 7.9, 25% glycerol, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* ethylenediaminetetracetic acid (EDTA), and add fresh, 2 m*M* PMSF, 0.5 m*M* DTT, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
9. Buffer D: 20 m*M* HEPES, pH 7.9, 20% glycerol, 0.1 *M* KCl, 0.2 m*M* EDTA, and add fresh, 2 m*M* PMSF, 0.5 m*M* DTT, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
10. Homogenization buffer: 0.3 *M* sucrose, 10 m*M* HEPES, pH 7.6, 0.74 m*M* spermidine, 0.15 m*M* spermine, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 10 m*M* KCl, and add fresh, 0.5 m*M* PMSF, 1 m*M* DTT, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
11. Cushion buffer: homogenization buffer with 2.2 *M* (rather than 0.3 *M*) sucrose.
12. Nuclear lysis buffer: 10% glycerol, 10 m*M* HEPES, pH 7.6, 100 m*M* KCl, 3 m*M* $MgCl_2$, 0.1 m*M* EDTA, and add fresh, 0.1 µ*M* PMSF, 1 m*M* DTT, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
13. Nuclear dialysis buffer: 20% glycerol, 20 m*M* HEPES, pH 7.6, 100 m*M* KCl, 0.2 m*M* EDTA, and add fresh, 0.1 m*M* PMSF, 2 m*M* DTT, 1 m*M* $NaMoO_4$, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
14. Hypotonic buffer: 20 m*M* HEPES, pH 7.9, 20 m*M* NaF, 1 m*M* EDTA, 1 m*M* EGTA, and add fresh, 0.1 m*M* PMSF, 1 m*M* DTT, 0.2 m*M* Na_3VO_4, 5 µg/mL aprotinin, 30 µg/mL leupeptin, 5 µg/mL pepstatin A.
15. Acrylamide (acrylamide:*bis*-acrylamide ratio 80:1).
16. Low-ionic strength electrophoresis buffer: 6.7 m*M* Tris-HCl, pH 7.9, 3.3 m*M* sodium acetate, 1 m*M* EDTA.

3. Methods

3.1. Preparation of Nuclear Protein Extract

3.1.1. Tissue Culture Cells

The following protocol is based on that of Dignam et al. ***(8)*** and is the most commonly used method for the preparation of nuclear protein extracts from tissue culture cells. The general principle of the method involves swelling the cells in a hypotonic salt solution followed by mechanical disruption of the cytoplasmic membrane, collection of the nuclei by centrifugation, and mechanical disruption of the nuclear membrane to release the nuclear contents.

1. Grow tissue culture cells of interest to approx 80% confluence on tissue culture plates.

2. Aspirate media and rinse plates of tissue culture cells with 5 mL ice-cold PBS two times (*see* **Note 1**).
3. Collect cells in 5 mL PBS per 15 cm tissue culture plate or in 2 mL per 10 cm tissue culture plate using a rubber policeman. (DO NOT TRYPSINIZE TO REMOVE CELLS.) Pool cells together into a graduated conical tube. Remove 10 µL and quantitate using trypan blue exclusion dye and hemicytometer (*see* **Note 2**).
4. Centrifuge cells at 228*g* for 10 min using a standard swinging bucket rotor in a tabletop centrifuge.
5. Aspirate the supernatant and determine the packed cell volume (PCV) of the pelleted cells. Resuspend the cells in 5 PCV of Buffer A and incubate on ice for 10 min.
6. Collect the swollen cells by centrifugation at 228*g* for 10 min (the PCV should have increased by approx twofold).
7. Resuspend the cellular pellet in 2 PCV Buffer A and place the cellular suspension in a prechilled glass Dounce homogenizer.
8. Using a B-type pestle, dounce the cell suspension with 10 up and down strokes. Replace the homogenized cell suspension into a centrifugation tube.
9. Centrifuge 514*g* for 10 min to pellet the nuclei. The pellet will be loosely adherent. Carefully decant the supernatant and resuspend the loose pellet in 2 mL Buffer A (*see* **Note 3**).
10. Centrifuge at 25,000*g* for 20 min, i.e., approx 18,000 rpm using a JA20 or JA21 rotor or 16,000 rpm using a JA20.1 rotor (Beckman-Coulter) to remove residual cytoplasmic material and to obtain the crude nuclear pellet (*see* **Note 4**).
11. Resuspend crude nuclear pellet in 3 mL Buffer C per 10^9 cells as determined in **step 3**.
12. Using a B-type pestle, Dounce the nuclear suspension 10 up and down strokes.
13. Stir nuclear suspension on ice in cold room using a magnetic stirring bar or by rocking on a nutator for 30 min.
14. Collect the nuclear suspension and place in centrifuge tubes. Centrifuge 25,000*g* for 30 min, i.e., approx 18,000 rpm using a JA-20 or JA-21 or 16,000 rpm using a JA-20.1 rotor (Beckman-Coulter).
15. Collect the supernatant and place in dialysis tubing. Dialyze against 75 volumes Buffer D in cold room for 5 h to overnight (*see* **Note 5**).
16. Centrifuge dialysate 25,000*g* for 20 min. Save the supernatant and discard the pellet.
17. Aliquot the supernatant into microfuge tubes and quick-freeze in ethanol–dry ice bath. Store at –70°C.

3.1.2. Tissue

Nuclear proteins obtained from tissue culture cells provide a useful reagent for the study of transcription factors and gene transcription rates; however, prolonged time in culture may result in significant deviations from the original tissue from which the tissue culture cell line was derived. For this reason, the preparation of transcriptionally functional nuclear proteins from primary tis-

sue is of use. The following is based on a protocol developed by Hattori et al. ***(9)*** for the extraction of hepatic nuclear proteins; however, it can be easily applied to any solid organ.

1. Dissect tissue of interest from euthanized animal. Mince tissue to 3-mm cubes using scissors in appropriate volume homogenization buffer (*see* **Notes 6** and **7**).
2. Dilute minced tissue in approx 5X volume of homogenization buffer.
3. Homogenize with two to three strokes with a motor-driven Teflon pestle.
4. Filter resulting homogenate through gauze or cheesecloth to remove large particulate matter.
5. Mix the homogenate with 2X volume cushion buffer.
6. Layer the homogenate–cushion buffer mixture onto 0.4X volume of cushion buffer in polyallomer ultracentrifuge tubes (for example, layer 25 mL of homogenate onto 10 mL of cushion buffer).
7. Centrifuge at 76,000*g* using a swinging ultracentrifuge rotor (for example, 24,000 rpm using a SW28 rotor or 26,000 rpm using a SW30 rotor, Beckman-Coulter) for 50 min.
8. Decant the supernatant and invert tube to allow remaining buffer to drain from nuclear pellet.
9. Resuspend the nuclear pellet in 1/7X volume nuclear lysis buffer (for example, if total volume of homogenate and cushion buffer was 35 mL, resuspend nuclear pellet in 5 mL) and move to prechilled Dounce homogenizer.
10. Using a Dounce homogenizer, subject nuclei to one stroke using a B pestle. Bring volume to 8X current volume using nuclear lysis buffer.
11. Add 10% volume of 4 *M* ammonium sulfate, pH 7.9, mix well, and gently rock 30–60 min at 0–4°C on a nutator.
12. Centrifuge at 100,000*g* (35,000 rpm) using a 55.2 Ti ultracentrifuge rotor (Beckman-Coulter) for 60 min or at 150,000*g* (40,000 rpm) using a SW 50.1 ultracentrifuge rotor (Beckman-Coulter) for 90 min to pellet the chromosomal DNA.
13. Remove the supernatant, taking great care to avoid the chromosomal DNA pellet, and measure the supernatant volume.
14. To precipitate the nuclear proteins, add 0.33 mg/mL ammonium sulfate to supernatant in five increments over a 30-min period with constant agitation at 0–4°C.
15. Centrifuge at 100,000*g* (35,000 rpm) using a 55.2 Ti ultracentrifuge rotor (Beckman-Coulter) for 20 min or at 84,000*g* (30,000 rpm) using a SW 50.1 rotor (Beckman-Coulter) for 30 min to pellet nuclear proteins.
16. Discard supernatant and resuspend the nuclear proteins in 0.5–2 mL nuclear dialysis buffer.
17. Transfer to dialysis tubing and dialyze against 250–500 mL dialysis buffer for 1 h and then against another 250–500 mL dialysis buffer overnight.
18. Lysate may be clarified by centrifuging at 15,996*g* in a tabletop microfuge for 10 min at 4°C.
19. Aliquot and quick-freeze in ethanol-dry ice bath. Store at –70°C.

3.1.3. "Mini" Nuclear Extract Preparations

When characterizing a novel transcription factor, it is typically necessary to prepare large quantities of nuclear proteins using the protocols described above. However, when the gel shift assay is used as an end point to define the strength of a given signal from the cell surface into the nucleus of the cell, it is expedient to utilize a shortened version of the protein extract preparation that is much easier and less time consuming. Two very similar protocols, with slight variations, of the "mini" nuclear protein extract are described below (*see* **Note 8**).

3.1.3.1. Protocol #1

1. Grow tissue culture cells to approx 80% confluence. Begin with approx 5×10^6 cells for each "mini" nuclear extract prep.
2. Prior to factor or cytokine stimulation, "starve" cells in a serum-free media, such as Dulbecco's modified Eagle's medium (DMEM) with 0.5% bovine serum albumin (BSA), for 12–36 h.
3. Stimulate cells with cytokine or factor of interest for appropriate amount of time.
4. Quench stimulation by placing the plates of tissue culture cells on ice and washing with ice-cold PBS.
5. Collect the cells in ice-cold PBS and pellet cells using a tabletop microfuge at 1306*g* for 5 min.
6. Resuspend the cells in 3 PCV hypotonic buffer.
7. Allow cells to swell on ice for 10 min.
8. Lyse cells by passaging through a 25-gauge needle five times.
9. Collect nuclei using a tabletop microfuge at 15,996*g* for 10 min.
10. Extract the nuclear proteins by resuspending the nuclear pellet in 2.5 PCV hypotonic buffer supplemented with 0.42 *M* NaCl and 20% glycerol.
11. Centrifuge for 20 min using a tabletop microfuge at 15,996*g*.
12. Transfer the supernatant to a new tube.
13. Store extracts at –70°C.

3.1.3.2. Protocol #2

1. As described in the previous protocol, grow tissue culture cells of interest to about 80% confluency, "starve" cells for appropriate time, and stimulate with cytokine.
2. Wash and collect cells in ice-cold PBS.
3. Pellet cells using a tabletop microfuge at 1306*g*.
4. Resuspend cells in 3 PCV Buffer A.
5. Vortex for 10 s and allow cells to swell for 10 min.
6. Subject cellular suspension to three cycles of quick freezing and thawing by placing tubes in an ethanol–dry ice bath for 1 min followed by a 37°C water bath for 1 min and 30 s three times.
7. Pellet nuclei using a tabletop microfuge at 15,996*g* for 10 min.

8. Resuspend nuclei in 100 µL Buffer C.
9. Hold on ice for 10 min.
10. Centrifuge 20 min using a tabletop microfuge at 15,996*g*.
11. Transfer supernatant to new tube.
12. Store extracts at –70°C.

3.2. Preparation of Nucleic Acid Probe

By far, the most commonly utilized and reliable method of labeling nucleic acids for gel shift assays is the use of the ^{32}P-labeled deoxyribonucleoside triphosphates (dNTPs) or ribonucleoside triphosphates (NTPs). As there are many well-developed commercially available enzymes supplied with reaction buffers and protocols that allow dependable incorporation of these isotopes into DNA fragments, this section will focus on the principles of preparing a radiolabeled probe. Specific enzyme reaction conditions, in addition to those supplied by the manufacturer of the DNA-modifying enzyme, can be found in detail in *Current Protocols in Molecular Biology* ***(10)***.

End-labeling of the DNA is recommended to ensure a uniform length of the DNA probe for use in gel shift assays. The chosen method of end-labeling depends primarily on the nature of the DNA fragment ends to be labeled. For DNA fragments excised from a plasmid using restriction endonucleases, the kinase reaction, which transfers the terminal (γ) phosphate group from [γ^{32}P]ATP to the 5'-hydroxyl end of DNA, should be used for blunt-ended DNA; however, it is critical to note that a dephosphorylation of the "cold" phosphate from the 5'-hydroxyl group is mandatory prior to the labeling reaction. Alternatively, T4 DNA polymerase can also be used to end-label blunt-ended DNA fragments; however, a preliminary empirically determined, limited digestion by the 3' to 5' exonuclease activity of this enzyme is necessary. The kinase reaction may also be utilized for DNA fragments with 3'-recessed ends; however, if one wishes to forego the dephosphorylation step, the Klenow reaction can be used to "fill-in" the 3'-recessed end using the appropriate [α–^{32}P]dNTPs. For annealed oligonucleotides, the kinase reaction is the easiest as most oligonucleotides are available without the 5'-hydroxyl phosphate.

Following the labeling reaction, it is necessary to separate the unincorporated radiolabeled ribonucleoside triphosphates or deoxyribonucleoside triphosphates from the radiolabeled DNA fragment. This can be accomplished by centrifuging through a Sephadex G25 (for oligonucleotides) or Sephadex G50 (for DNA fragments) column. To ensure adequate labeling, it is prudent to estimate the specific activity by dividing the total number of counts incorporated into the probe by the total amount of DNA. For the best results, use probes with a specific activity of at least 10^8 cpm/µg.

3.3. Detection of DNA-Nuclear Protein Interactions

3.3.1. The Binding Reaction

Conditions for the binding reaction are fairly standard; however, depending on the nature of the protein extract and of the DNA consensus sequence, some variations may need to be investigated. For example, more or less poly dI·dC may need to be used to inhibit nonspecific interactions and optimize the specific nuclear protein–DNA interaction of interest. The actual amount of protein extract may also need to be varied. Below is a general starting point for the binding reaction ***(11)***; however, it is important to realize that these conditions are not absolute and may need to be varied based on the considerations described above.

1. Mix 2–10 fmol of radiolabeled DNA with 10–20 µg nuclear protein extract and 2 µg poly dI·dC in 10 m*M* HEPES, pH 7.9, 60 m*M* KCl, 1 m*M* EDTA, 1 m*M* DTT, and 7% glycerol in a final volume of 20 mL.
2. Hold on ice for 20–40 min.
3. Electrophorese on nondenaturing polyacrylamide gel (*see* **Subheading 3.3.5.**).

3.3.2. The Competition Assay

The competition assay is a very important control reaction, as it clarifies the specificity of the DNA–nuclear protein interaction. Unlabeled or "cold" DNA included in the binding reaction competes with the radiolabeled DNA for binding to the nuclear protein of interest. If the DNA–nuclear protein interaction is a specific one, the amount of shifted radiolabeled probe should decrease in a dose-dependent manner with increasing molar amounts of unlabeled DNA (*see* **Fig. 1**). This simple variation can also be employed as a pertinent negative control as unrelated unlabeled DNA sequences included in the reaction should have no effect on the amount of shifted radiolabeled probe.

1. Mix 10–20 µg nuclear protein extract, 2 µg poly dI·dC, and a 10- to 1000-fold molar excess of unlabeled DNA in the binding buffer, without radiolabeled probe.
2. Hold on ice for 10–20 min.
3. Add radiolabeled DNA and hold on ice for an additional 20–40 min.
4. Electrophorese on nondenaturing polyacrylamide gel (*see* **Subheading 3.3.5.**).

3.3.2. The Antibody Assay

An additional assay used to characterize the immunologic properties of the bound nuclear protein is the antibody assay. The effect of the antibody in the binding reaction varies depending on whether it recognizes the nuclear protein at or away from the DNA binding domain ***(12)***. Binding of the antibody at the DNA binding domain precludes the formation of the nuclear protein–DNA

complex; therefore, the amount of shifted radiolabeled probe is actually diminished. Alternatively, binding away from the DNA binding domain causes an increased molecular weight of the complex; therefore, the complex mobility is decreased further resulting in a supershifted band (*see* **Figs. 1** and **2**). If the nuclear protein of interest has previously been well defined, this assay may be performed to verify the immunologic identity of the shifted protein. However, if one is characterizing a novel nuclear protein or attempting to determine the identity of a nuclear protein binding to a novel DNA sequence, this assay can be used to define immunologic similarities of the nuclear protein in question to known transcription factors.

1. Mix 10–20 µg nuclear protein extract, 2 µg poly dI·dC, and antibody of interest (1:1000 to 1:100 dilution) in the binding buffer, without radiolabeled probe.
2. Hold on ice for 10–20 min.
3. Add radiolabeled DNA and hold on ice for an addtional 20–40 min.
4. Important negative controls include a reaction containing the antisera of interest without nuclear protein extract and a reaction containing "irrelevant" antisera.
5. Electrophorese on nondenaturing polyacrylamide gel (*see* **Subheading 3.3.5.**).

3.3.4. The UV Crosslinking Assay

In order to estimate the molecular weight of an unknown DNA–binding protein, one can subject the radiolabeled DNA–protein complex to UV irradiation. The UV irradiation causes the formation of covalent bonds between the pyrimidines in the DNA probe and amino acid residues on the bound protein (*see* **Note 9**). The overall result is the covalent attachment of the radioactive signal to the DNA-binding protein in question, allowing for electrophoresis under denaturing conditions in order to determine the molecular weight of the unknown protein (*see* **Note 10**). One protocol will be described that utilizes an end-labeled oligonucleotide as the probe ***(12)*** and is basically an extension of the gel shift assay.

1. Prepare binding reaction as described under **Subheading 3.3.1**.
2. Cover the tube containing the binding reaction with a UV-transparent plastic wrap.
3. Place the tube 5 cm from the UV irradiation source.
4. Subject the binding reaction to UV irradiation at a wavelength of 254 nm for 5 min to 3 h (*see* **Notes 11** and **12**).
5. Electrophorese the binding reaction on a nondenaturing polyacrylamide gel as described under **Subheading 3.3.5**.
6. Autoradiograph the gel for 1–3 h at 4°C.
7. Excise the shifted DNA-nuclear protein complex from the gel.
8. Place the excised gel piece on a denaturing sodium dodecyl sulfate (SDS)–polyacrylamide gel (a stacking gel layered over a 10% polyacrylamide gel).

9. Use radiolabeled molecular weight markers.
10. Electrophorese for 2– 3 h at 35 mA. Remove the area of the gel at the dye front where the majority of noncovalently attached radiolabeled DNA will run and discard.
11. Fix the remaining gel with 7.5% acetic acid and 50% methanol for 60 min.
12. Saturate the gel with a fluor (for example, Enhance, New England Nuclear) to improve sensitivity.
13. Dry the gel for 1 h at 60°C and 1 h at 80°C.
14. Autoradiograph the gel with intensifying screens at –80°C for 1–4 d.

3.3.5. Electrophoresis

The most critical point of this step is to use nondenaturing conditions to allow the DNA to remain double-stranded and the nuclear protein to remain bound to the DNA. Below is the general procedure.

1. Prepare a 3.5–5% polyacrylamide gel (acrylamide:*bis*-acrylamide ratio 80:1) containing 2.5% glycerol in 6.7 m*M* Tris-HCl, pH 7.9, 3.3 m*M* sodium acetate, and 1 m*M* EDTA. For 40 mL of gel mixture, add 100 µL 30% ammonium persulfate and 34 µL TEMED for polymerization.
2. Using low-ionic-strength electrophorsis buffer (6.7 m*M* Tris-HCl, pH 7.9, 3.3 m*M* sodium acetate, and 1 m*M* EDTA) in the upper and lower gel apparatus chambers, prerun gel for 1–2 h at 100 V (*see* **Note 13**).
3. Load samples into gel wells (*see* **Note 14**).
4. Run samples for 2–3 h, depending on the size of the probe and the concentration of the gel (*see* **Note 15**).
5. Dry the gel under vacuum.
6. Place on autoradiograph film overnight with intensifying screens.

3.4. Conclusion

The gel shift assay has been used extensively in the study of nuclear proteins and transcription factors. Additionally, its use has been extended to study the role of signaling molecules and signal transduction pathways in response to various cell stimuli. This assay continues to be a cornerstone in the armamentarium of protocols used to define the molecular mechanism controlling cellular function and cellular response to external stimuli. The authors have attempted to compile the pertinent principles and methods needed to perform this assay expediently and successfully.

4. Notes

1. All solutions and centrifuges should be maintained at 4°C. All tubes should be prechilled on ice. If the procedure is performed at room temperature, care should be taken to maintain all solutions and cell suspensions on ice.
2. For an estimation, 20 plates (15 cm) of HeLa cells at 80% confluency will usually yield approx 10^9 cells total as determined in **step 3**.

3. The supernatant obtained at **step 9** of this protocol contains the cytoplasmic proteins, which can be precipitated with a high salt buffer; however, the methodology for this procedure is beyond the scope of this chapter. For details, refer to original protocol by Dignam et al. ***(8)***.
4. At **steps 10**, **14**, and **16**, for low-volume preparations, the suspension may be placed in 1.5 mL microfuge tubes and centrifuged using a tabletop microfuge for 20 min at 15,996*g*.
5. For low-volume protein extract preparations, use as small dialysis tubing as possible. Alternatively, place the extract to be dialyzed into a 1.5-mL microfuge tube, cover the microfuge tube with one layer of dialysis tubing and secure in place with a rubber band. Invert the tube so that the extract covers the dialysis tubing and place the tube (in the inverted position) into the dialysis buffer. It is typically necessary to attach the inverted tube to the side of the beaker of dialysis buffer using a paper clip. Although this technique sounds rather cumbersome, it is worth the effort for low-volume extracts.
6. One of the difficulties in working with primary tissues is the increased amount of proteases; therefore, care should be taken to maintain all solutions and centrifuges at 0–4°C. Also, consider performing as many steps as possible in the cold room.
7. Depending on the size of the organ or the amount of tissue of interest, buffer volumes will need to be adjusted accordingly. For a reference, Hattori et al. ***(9)*** used 10 mL homogenization buffer for two rat livers.
8. The nuclear protein extract prepared by these methods is in a relatively high concentration of NaCl (0.42 *M*). This NaCl concentration is too high for many nuclear protein–DNA interactions to occur efficiently; therefore, one should dialyze the nuclear extracts prior to use in the binding reaction or should plan the binding reaction such that the final NaCl concentration is sufficiently low. As the typical final volumes using these methods are quite low, dialysis, when necessary, should be performed using the 1.5-mL microfuge method described previously.
9. In general, UV crosslinking works best with AT-rich DNA sequences, as the thymidine is the most reactive in response to UV light.
10. As the nucleic acid probe is not digested in the protocol, use the shortest oligonucleotide probe as is possible. The probe length should not exceed 50 bp.
11. The necessary time for UV irradiation will vary depending on the nature of the DNA–protein interaction and will need to be determined empirically.
12. An alternate protocol involves the use of bromodeoxyuridine-substituted probes. These probes are more sensitive to UV-induced crosslinking and in some cases are useful. However, the probe preparation is labor-intensive, as it requires subcloning the protein binding sequence of interest into M13. For more details on this protocol, refer to *Current Protocols in Molecular Biology* ***(10)***.
13. Owing to the low-buffering capacity of the electrophoresis buffer, it is necessary to recirculate the buffer during electrophoresis. This can be achieved using a pump with a flowrate of 5–30 mL per min. Alternatively, buffer from the upper and lower reservoirs can be removed, mixed, and returned to gel apparatus approximately every hour during electrophoresis.

14. It is not necessary to add loading dye, as the glycerol in the reaction mixture is sufficiently dense to ensure adequate sinking of the samples to the bottom of the well. However, it is prudent to load one lane on the gel with bromphenol blue in order to track the progression of the electrophoresis.
15. In a 3.5% gel, the bromphenol blue will run at approx 100 bp while in a 5% gel, it will run at approx 60 bp. The goal is to separate sufficiently the bound from the unbound radiolabeled DNA without running the unbound radiolabeled DNA off the gel (as this will result in excessive radioactivity in the lower chamber of the electrophoresis apparatus).

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3

Modulation of the Interferon-γ Signal by Transfection of Cells with an Antisense-RNA Expressing Vector

Leen Delrue and Marc De Ley

1. Introduction

Apart from cell–cell contacts, signal transduction mechanisms active during immune defense are mainly regulated by cytokines. They accomplish their pleiotropic, paracrine, and/or autocrine biological activities as a result of the contact with a membrane receptor followed by signal propagation through a series of signal transduction partners and eventually reaching the nucleus where activation of gene transcription leads to the expression of specific proteins. Application of highly specific inhibitors of these paths is the basis for their unraveling, including the identification of parallel paths and the occurrence of "crosstalk." The elucidation of specific inhibitors also has possible medical applications.

Indeed, although cytokines mainly have a protective function, they are increasingly being considered as harmful in case of an imbalance in their production and/or action. As a result of overproduction or hypersensitivity, the immune system may become directed against "self" structures. Therefore, research on inhibitors has become as important as that on the cytokines themselves. This inhibition is possible by interfering in a strictly specific manner with the cytokine network and will eventually throw new light on the mode of action and regulation of the signal pathway.

A variety of technologies are applicable for this purpose ***(1)***. Monoclonal antibodies directed against the cytokine or its membrane receptor are able to block the biological response. However, they cannot be used against an intracellular target. Another attractive way is the interference at the DNA/RNA level with antisense oligonucleotides. After careful selection of the target sequence, they are capable of potent and specific inhibition of gene expression,

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which can identify the role of specific genes in biological processes ***(2)***. The main drawbacks of external administration of antisense oligonucleotides are the difficult penetration through the membrane and the breakdown by extracellular nucleases. These can be circumvented by transfecting cells with an expression vector harboring the target sequence in the antisense direction. This will eventually lead to the development of stable transfectants defective to a variable degree in their response to cytokines ***(3)***.

Our attention was primarily focused on human interferon-γ (HuIFN-γ), a T-lympfocyte-derived cytokine that affects a variety of cells by its antiproliferative, antiviral, and immunomodulatory activities ***(4)***. Its receptor (IFN-γR) consists of two subunits, the α-chain exhibiting ligand-binding properties and the β-chain (also called accessory factor-1, AF-1), required primarily for signaling ***(5)***. We were able to construct stable antisense transfectants for AF-1 through transfection of cells with a pcDNA3-derived vector carrying the cDNA for AF-1 in antisense orientation. After extensive biological and biochemical characterization of these cells, we conclude that we successfully introduced antisense AF-1 expression. This technology, however, is generally applicable to almost any component in cytokine signal transduction.

2. Materials

1. 1.5- and 15-mL tubes.
2. Corex tubes.
3. Culture flasks: 25, 80, and 175 cm^2 (Nunc, Roskilde, Denmark).
4. 24- and 96-well plates (flat bottom) (Nunc).
5. Distilled, deionized, autoclaved water.
6. 100% (v/v) and 70% (v/v) ethanol.
7. Chloroform.
8. Isopropanol.
9. Ice.
10. Water bath at 37°C.
11. (Shaking) incubator.
12. Agarose.
13. "Wizard clean up" kit (Promega, Madison, WI).
14. 0.22-μm filter.
15. Gene Pulser® and Capacitance Extender (Bio-Rad, Hercules, CA).
16. Electroporation cuvets with a 4-mm electrode gap.
17. Fetal calf serum (FCS) and newborn calf serum (NCS) (Gibco-BRL, Invitrogen Corp., Carlsbad, CA) (inactivate prior to use by incubating for 30 min at 56°C).
18. 0.011% (w/v) $NaHCO_3$, pH 7.0.
19. 0.025% (w/v) glucose.
20. L-Glutamine: 200 m*M* (Gibco-BRL).
21. RPMI-1640 and minimal essential medium (MEM) medium (Gibco-BRL).

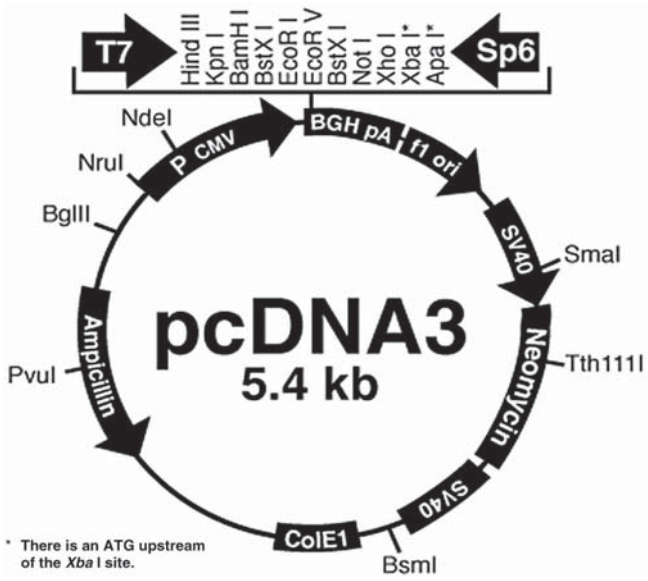

Fig. 1. "pcDNA3" is the parent vector of most of Invitrogen's different mammalian expression vectors and it is the mostly used mammalian expression vector worldwide. Important are the high level of constitutive transcription from mammalian enhancer–promoter sequences, the polyadenylation signal, and the transcription termination sequences from the bovine growth hormone gene to enhance RNA stability, the ampicillin resistance gene and ColE1 origin for selection and maintenance in *E. coli*, and the neomycin resistance gene for selection of G418 resistant stable cell lines.

22. Ampicilline: stock solution, 25 mg/mL; working concentration, 100 µg/mL.
23. Gentamycine: 50 mg/mL (Gibco-BRL).
24. Geneticin (G418) stock solution, 50 mg/mL in medium; work concentration, 360 µg/mL for THP-1 cells and 480 µg/mL for A549 cells. (*See* **Note 1**.)
25. LB medium (37°C): 25 g/L Luria Broth Base (Gibco-BRL) in distilled, deionized, autoclaved water.
26. Agar plates: 1.5% (w/v) agar and 100 µg/mL ampicillin in Luria-Bertani (LB) medium (*see* **Note 2**).
27. Competent *Escherichia coli* cells (*see* **Note 3**).
28. THP-1 cells ***(6)*** and A549 cells ***(7)***.
29. Expression vector: pcDNA3 (available from Invitrogen) (http:\\www.invitrogen.com) (**Fig. 1**).
30. Target sequence: AF-1 cDNA, cloned between the two *Sfi*I sites in pCEV15 (**Fig. 2**) (*see* **Note 4**).
31. Restriction enzymes (*Bam*HI and *Bgl*II) and their accompanying commercial buffers (REact3, Gibco-BRL).

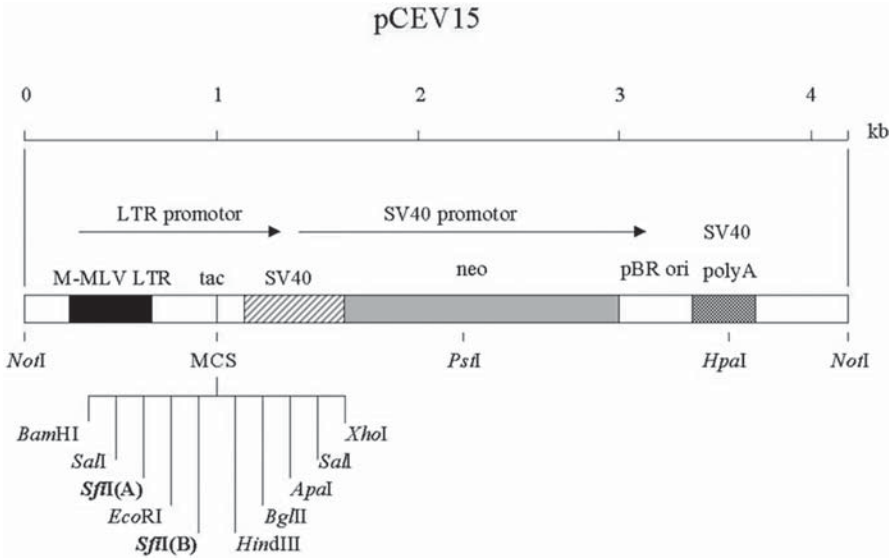

Fig. 2. The AF-1 cDNA is cloned between the two *Sfi*I-sites, A and B, of the pCEV15 vector ***(9)***, which was rescued from an M426 library ***(10)***. This vector is designed as eukaryotic expression vector, utilizing the M-MLV LTR promoter and containing the SV40 early promoter-driven *neo* gene as a selectable marker.

32. T4 DNA ligase and the accompanying 10X ligase buffer (Boehringer, Mannheim, Germany).
33. TGE buffer: 50 m*M* glucose, 10 m*M* ethylenediaminetetraacetic acid (EDTA), 25 m*M* Tris-HCl, pH 8.0.
34. Freshly prepared lysozyme solution: 10 mg lysozyme in 1 mL of TGE buffer.
35. Trypsine solution: 0.25% (w/v) in 0.04% (w/v) KCl, 0.22% (w/v) $NaHCO_3$, 0.68% (w/v) NaCl, 0.1% (w/v) glucose, 0.0005% (w/v) phenol red (Gibco-BRL).
36. Lysis buffer: 0.2 *M* NaOH, 1% (w/v) sodium dodecyl sulfate (SDS).
37. 3 *M* KOAc buffer, adjusted to pH 4.8 with HOAc.
38. 3 *M* NaOAc buffer, adjusted to pH 5 with HOAc.
39. RNase A and RNase A buffer: 10 m*M* Tris-HCl, pH 7.4.
40. Equilibrated fenol: phenol buffered with 0.1 *M* Tris-HCl, pH 8.0.
41. STE buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 M NaCl, 1 m*M* EDTA.
42. *Sol*I buffer: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA.
43. *Sol*II buffer: 0.2 *N* NaOH, 1% (w/v) SDS.
44. *Sol*III buffer: 60 mL 5 *M* KOAc, 11.5 mL HOAc, 28.5 mL H_2O.
45. RC-5B centrifuge with an SS-34 rotor (Sorvall Inc., Norwalk, CT).
46. Biofuge 13 centrifuge (Heraeus, Hanau, Germany).
47. PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 6.5 m*M* Na_2HPO_4, pH 7.4.

3. Methods

3.1. Preparation of Insert and Expression Vector

1. The insert (= AF-1 cDNA) and the expression vector (= pcDNA3) are both cut with the same (unique) restriction enzyme(s) (*see* **Note**s **5** and **6**) in such a way that the target sequence can be inserted in the expression vector in an antisense orientation downstream of the promoter. Mix DNA together with the restriction enzyme (1 U/µg DNA) and its accompanying commercial buffer and incubate at the prescribed temperature (usually 1 h at 37°C). In our case, the vector was digested with *Bam*HI, the cDNA with *Bam*HI and *Bgl*II.
2. Separate the restriction fragments in order to obtain pure insert and expression vectors by agarose gel electrophoresis or with a commercially available kit such as "Wizard clean up" (*see* **Note 7**).
3. Determine their concentration on an agarose gel by comparing the bands with a marker of known weight.

3.2. Preparation of Recombinant Vector

1. Mix the digested and purified target sequence and the expression vector in as many molar ratios as possible. For a 1:1 molar ratio, this means:

   (bp insert)/(bp vector) = (ng insert)/(ng vector)

   Always take a minimum of 100 ng vector. Add T4 DNA ligase and the accompanying ligase buffer and keep the total reaction volume as small as possible (*see* **Note 8**). If necessary, use distilled, deionized, autoclaved water in order to reach the total reaction volume.
2. Incubate 16 h at 16°C.

3.3. Transformation of Competent E. coli Cells

1. Add 50–150 ng ligation product to 100 µL of competent *E. coli* (e.g., DH5α cells) and incubate for 15 min on ice.
2. Submit the *E. coli* cells to five "heat-shocks" by alternately incubating the cells for 30 s on ice and 30 s at 37°C.
3. Incubate again for 15 min on ice.
4. Add 1 mL of prewarmed (37°C) LB medium to the cells and let them grow for 45 min in a shaking incubator.
5. Centrifuge 15 s at 14,000 rpm in a Biofuge 13 centrifuge and remove some supernatant if necessary. This will result in a higher number of colonies.
6. Apply the cells to an agar plate, supplemented with the appropriate antibiotic (e.g., 100 µg/mL ampicillin).
7. Incubate overnight at 37°C (*see* **Note 9**). Only those bacteria, transformed with the recombinant vector, will grow, due to the presence of the antibiotic resistance gene.

3.4. Analysis of the Bacterial Clones

1. Suspend several colonies each to 10-mL LB medium with appropriate antibiotic (e.g., 100 µg/mL ampicillin) and allow overnight growth by shaking at 37°C.
2. Of the full-grown culture, 1.5 mL is centrifuged (15 s, 14,000 rpm in a Biofuge 13 centrifuge).
3. Remove the supernatant and disperse the bacterial pellet completely by vortexing. Resuspend the cells in 100 µL of a freshly prepared lysozyme solution and incubate for 5 min on ice.
4. Add 200 µL of lysis buffer and mix carefully. Again, incubate for 5 min on ice.
5. Neutralize with 150 µL ice-cold KOAc buffer and incubate for 15 min on ice.
6. After centrifugation (2 min, 14,000 rpm in a Biofuge 13 centrifuge), precipitate the DNA in the supernatant with 2.5 vol of 100% (v/v) ethanol. Incubate for 30 min at –80°C and centrifugate for 15 min at 14,000 rpm. Remove the ethanol and allow the pellet to dry.
7. Submit the pellet to an RNase A treatment (1 h at 37°C) by adding 100 µg/mL RNase A in 50 µL RNase A buffer.
8. Purify the plasmid DNA with phenol–chloroform and an ethanol precipitation (*see* **Notes 10** and **11**).
9. Make a glycerol stock of the remainder of the bacterial culture [15% (v/v) glycerol].

3.5. Large-Scale Plasmid Preparation (8)

Several micrograms of plasmid DNA are necessary to transfect cells.

1. Seed a glycerol stock of bacteria containing the recombinant vector into 500 mL LB medium with 100 µg/mL ampicillin and allow growing overnight in a shaking incubator at 37°C.
2. Harvest the cells by centrifugation at 3000 rpm in a RC-SB centrifuge with an SS-34 rotor for 20 min at 4°C. Remove the supernatant completely.
3. Wash the cells with 100 mL of ice-cold STE buffer and centrifuge again.
4. After removing the supernatant, resuspend the bacterial pellet in 18 mL *Sol*I buffer.
5. Add 2 mL of a freshly prepared lysozyme solution and 40 mL of freshly prepared *Sol*II buffer. Mix gently and incubate for 5–10 min at room temperature.
6. Add 20 mL of ice-cold *Sol*III buffer and mix well. Store on ice for 10 min. A white precipitate that consists of chromosomal DNA, high-molecular-weight RNA, and potassium/SDS/protein/membrane complexes should form.
7. Centrifuge the lysate at 10,000 rpm for 20 min at 4°C in a Sorvall RC-5B rotor SS-34. Allow the rotor to stop without braking.
8. Filter the supernatant through a 0.22-µm filter.
9. Precipitate the plasmid DNA by adding 0.6 vol isopropanol. Store at room temperature for 10 min.
10. Recover the DNA by centrifugation at 10,000 rpm in a RC-5B centrifuge with an SS34 rotor for 20 min at room temperature.

11. Wash the pellet with 70% (v/v) ethanol at room temperature.
12. Remove the ethanol completely, allow drying of the DNA in the air, and dissolve in 3 mL distilled, deionized, autoclaved water.
13. Purify the plasmid by centrifugation on a CsCl gradient or with commercially available kits.
14. Determine the purity and concentration with a spectrophotometer at 260 and 280 nm.

3.6. Growing Cells

We choose to transfect a premonocytic cell line, THP-1, and a lung carcinoma cell line, A549.

THP-1 cells are grown in RPMI medium supplemented with 2 m*M* L-glutamine, 50 mg/L gentamycine, 0.025% (w/v) glucose, and 10% (v/v) FCS. Twice a week, cells are subcultured to a density of $2–5 \times 10^5$ cells/mL by adding fresh medium.

A549 cells are grown in MEM supplemented with 2 m*M* L-glutamine, 50 mg/L gentamycine, 0.025% (w/v) glucose, 0.011% (w/v) $NaHCO_3$ (pH 7.0), and 10% NCS. These adherent cells were subcultured weekly in a 1:7 ratio after treatment with trypsine.

3.7. Transfection of the Cells with Recombinant Vector by Electroporation

1. Isolate cells from a logarithmically growing culture by centrifugation (10 min, 200*g*), wash with ice-cold PBS and resuspend the pellet in growth medium at a density of 5×10^6 cells/0.5 mL. Mix the plasmid DNA (15–20 µg in 20 µL PBS) (*see* **Note 12**) and the cells in an electroporation cuvet with a 4-mm electrode gap. Mix well and store the cuvette for 10 min on ice.
2. Apply a single electrical pulse at 210 V and a capacitance of 500 µF. Chill again for 10 min.
3. Add 1 mL prewarmed culture medium (37°C) to the electroporation cuvet, mix gently, and transfer to a culture flask containing 20 mL of medium.
4. Incubate the cells 24–48 h at 37°C.

3.8. Selection and Cloning of the Transfected Cells

1. Distribute the cells over a 24-well plate.
2. Add a small amount of the appropriate antibiotic (geneticin) to every well. Increase this concentration over a period of 7–10 d to a concentration at which the nontransfected cells die and the transfected cells keep growing.
3. Select for at least 4 wk. Remove the dead cells two or three times a week by centrifugation of the plate, removing the medium, and adding fresh medium with antibiotic.
4. Clone the remaining living cells by distributing the cells of one well to one or more 96-well plates such that there is theoretically 1 cell/well. It takes almost 2 wk before clones become visible. Then transfer the cells first to a 24-well plate and then to a small culture flask (25 cm^2) before characterizing the cells.

4. Notes

1. Determine the right concentration of antibiotic before selecting the transfected cells. This can be done by incubating nontransfected cells with different amounts of antibiotic in a 24-well plate. Choose the lowest concentration at which the cells die.
2. Agar plates with 100 µg/mL ampicillin should be prepared prior to use. Plates can be stored at 4°C (2–3 wk).
3. Competent cells are prepared with $CaCl_2$ and $MgCl_2$ starting from an exponentially growing bacterial culture.
4. Here, we assume that we have already pure cDNA. If the cDNA is still inserted in a vector, first amplify the cDNA and cut the target sequence with the appropriate enzyme(s).
5. If only one restriction enzyme was used, dephosphorylate the vector. To do so, incubate 17 µL (± 10 µg) vector solution with 1 µL calf intestinal alkaline phosphatase (CIP) and 2 µL 10X dephosphorylation buffer (Boehringer) and incubate for 1 h at 37°C. Inactivate the enzyme by a subsequent incubation at 65°C for 15 min.
6. If two different restriction enzymes are used and they require a different buffer, first apply the enzyme that needs the buffer with the lowest salt concentration. If necessary, remove remaining salt between two digests by submitting the DNA to an ethanol precipitation.
7. DNA can be purified from an agarose gel with commercially available kits like the Nucleotrap Extraction Kit (Macherey-Nagel, Düren, Germany) or Qiaex II Extraction Kit (Qiagen, Chatsworth, CA).
8. Before adding T4 DNA ligase, incubate the DNA mixture for 10 min at 65°C and cool immediately on ice. This can avoid self-ligation of the vector.
9. After transformation of competent *E. coli*, do not incubate longer than 20 h at 37°C, in order to avoid the formation of satellite colonies.
10. To remove proteins from a DNA solution, add 0.5 vol of equilibrated phenol and 0.5 vol of chloroform, mix well, and centrifuge for 5 min at 14,000 rpm in a Biofuge 13 centrifuge. Mix the upper water phase with 1 vol of chloroform and centrifugate again (5 min, 14,000 rpm). DNA is recovered from the solution by an ethanol precipitation.
11. To recover DNA from a solution, precipitate the DNA with 1/10 volume of 3 *M* NaOAc, pH 5.0 and 2.5 vol ice-cold 100% (v/v) ethanol. Incubate for minimum 20 min at –80°C and centrifugate for 15 min at 14,000 rpm in a Biofuge 13 centrifuge. Remove the ethanol and rinse the DNA with 70% (v/v) ethanol. Allow the pellet to dry and dissolve in the appropriate buffer.
12. Before transfecting cells, make sure that the plasmid is very pure. This can easily be done with commercially available kits, e.g., Nucleobond Ax (Macherey-Nagel) or Qiagen-tip 100 (Qiagen).

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4

Competitive RT-PCR to Quantify Small Amounts of mRNA

Gabriela Aust

1. Introduction

Monitoring cytokine responses to various stimuli or determining the expression pattern of cytokine mRNAs requires sensitive technologies for cytokine mRNA quantitation where there is a limited quantity of material, for example, when working with biopsies. Owing to their amplifying effect, reverse transcriptase-polymerase chain reacttion (RT-PCR)–based methods permit the analysis of minimal starting quantities of nucleic acids. Because of the exponential nature of PCR, introduction of a competitive internal standard (competitor) has proven to be of great advantage for quantitation. Competitive RT-PCR is capable of ruling out tube-to-tube and sample-to-sample variation, because the competitor and the target of interest are amplified in the same reaction tube, compete for the same enzyme and nucleotides, and, thus, are subjected to identical amplification conditions *(**1**,**2**)*. This is achieved by the special construction of the competitor, which bears the same primer-binding regions as the target of interest, but the sequence in between is modified in such a way that amplification products derived from the competitor and the target can be differentiated.

It is possible to design heterologous internal competitors containing the primer-binding sites only, or homologous competitors, which are additionally identical in the sequences over a great range. Competitive RT-PCR has a very high degree of validity in relative quantification for comparing nucleic acid levels between samples. For absolute quantitation, it is most important to demonstrate that the amplification efficiency of the target and the competitor are equal. A nearly identical homologous competitor can exactly simulate the natural target during RT-PCR and, can therefore serve as a reference for absolute quantification *(**3**)*. Under several conditions, the method is also appropriate for

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quantification of low-copy targets *(4)*. The theoretical limit of template detection (one molecule/PCR) is achieved when reliable hot-start protocols are used, the formation of primer–dimer artifacts is avoided, and an appropriate detection system is linked to the amplification system.

1.1. Construction of the Competitor

Many methods for competitor construction involve the deletion or insertion of DNA sequences in the wild-type gene in order to produce a competitor that differs in size by a set number of nucleotides. Such strategies are often used on competitors in gel electrophoresis analysis. The protocol described here presents an easy way to construct a competitor that yields a smaller PCR product compared to wild-type DNA (*see* **Fig. 1**). The competitor, with its predefined size, is generated and proved in two consecutive amplifications. Standard RT-PCR equipment, the 5' and 3' primers for a chosen DNA sequence and an additional internal 3' hybrid primer are needed. In the first PCR, the normal 3' primer is replaced by the 3' hybrid primer matching *x* bp downstream to a 20-bp sequence. Because the limited resolution of common agarose gel electrophoresis, there should be a size difference of at least 10% between the target of interest and the competitor to ensure competitor and target PCR product separation.

In addition to the 20-bp matching bases, the 3' hybrid primer has a tail of 20 bp at the 5' end, which is identical to the normal 3' primer, thus serving as a linker between these two primers in the second amplification step. In both amplification steps, the same 5' primer is used. Once the competitor has been designed, it is important to validate the internal control. Validation requires demonstrating that the competitor amplifies with equal efficiency *(5)*. For quantitating samples, several competitive RT-PCRs with a constant but unknown amount of the target of interest and varying but known amounts of the competitor have to be performed.

1.2. Detection System

The amplification system has to be linked to an appropriate detection system. RT-PCR quantitation is dependent on the ability to accurately determine the concentration of PCR products after amplification. Amplification products have to be equipped with any kind of label that can be detected subsequently, in either a direct or indirect way. Generally, detection strategies for amplification products can be divided into two parts: assay systems that are capable of detecting the presence or absence of amplification products (nonsequence-specific detection systems) and assay systems that are specific for amplification products with a given sequence (sequence-specific detection systems) *(6)*. A common, easy, and cheap format for estimating the concentration of PCR prod-

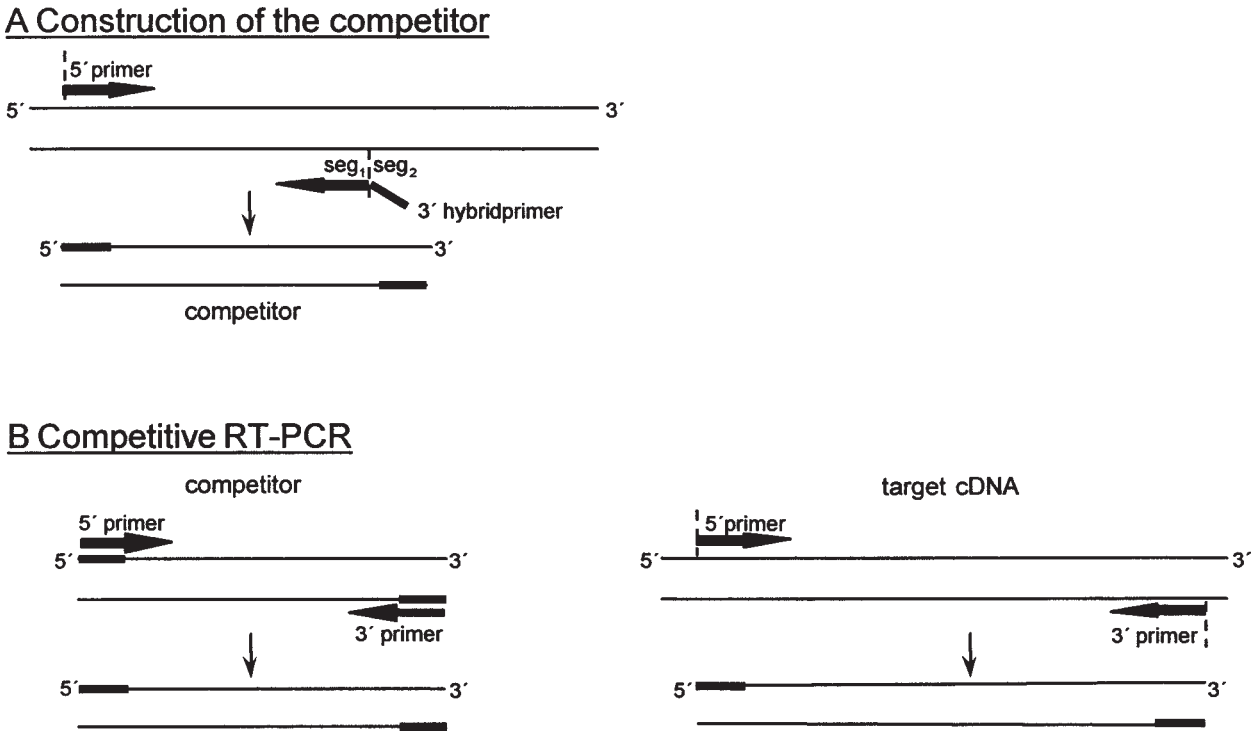

Fig. 1. Schematic view of internal competitor generation and cDNA quantitation. The same 5' primer is used in both amplification steps. In the first amplification step, the 3' hybrid primer replaces the normal 3' primer on a cDNA template serving normally as the positive control, an *x* bp fragment is generated containing the 3' hybrid primer sequence. During the second reamplification step, the original 3' primer replaces the 3' hybrid primer. The competitor produced serves as the template.

ucts is to electrophorese the samples in agarose or polyacrylamide gels, and to stain them with helix-intercalating dyes such as ethidium bromide or with higher sensitivity SYBR green. Such stained gels can be rapidly and accurately quantitated using a video documentation system and image analysis software. The method yields reproducible results for a wide range of DNA fragment sizes with a variability of generally less than 20%. However, this detection method only has a small dynamic range. Only nearly equimolar amounts of the competitor and target result in reliable quantitation compared to other, more expensive imaging systems that have dynamic ranges of 10^3–10^4-fold *(7)*.

2. Materials

2.1. RNA Isolation and cDNA Synthesis

1. RNA isolation: RNeasy Mini Kit (QIAgen, Valencia, CA).
2. DNase (RNase free; Roche Molecular Biochemicals, Basel, Switzerland) with reaction buffer (20 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1 m*M* $MnCl_2$).
3. First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Freiburg, Germany).

2.2. Construction of the Competitor, RT-PCR

1. High-quality *Taq* DNA polymerase (1 U/µL) with 10X PCR reaction buffer (100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 500 m*M* KCl, pH 8.3; Roche).
2. dNTPs, containing 100 m*M* dCTP, dATP, dGTP, and dTTP (Roche).
3. 10 m*M* 5' and 3' primers, 3' hybrid primer for the housekeeping gene and specific cDNAs.

2.3. Cloning, Purification, Stabilization, and Quantitation of the Competitor

1. Luria-Bertani (LB) medium (1 L): 10 g bacto-tryptone (Sigma, St. Louis, MO), 5 g bacto-yeast extract (Sigma), 5 g NaCl, pH 7.0.
2. LB agar: add 15 g of agar (Gibco-BRL, Invitrogen, Carlsbad, CA) to 1 L of LB medium and sterilize by autoclaving. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100 µg/mL and pouring plates. Approximately 1 h prior to transformation, spread each plate with 20 mL X-Gal [50 mg/mL in dimethyl formamide (DMF), Sigma] and 100 mL isopropylthio-β-galactoside (IPTG) (100 mM; Sigma).
3. SOC medium (100 mL): 2.0 g bacto-tryptone, 0.5 g bacto-yeast extract, 1 mL 1 *M* NaCl, 0.25 mL 1 *M* KCl, 1 mL 2 *M* Mg^{2+} stock (1 *M* $MgCl_2$; 1 *M* $MgSO_4$), 1 mL 2 *M* glucose, bring to 100 mL with distilled water, pH 7.0.
4. pGEM-T vector for TA cloning (Promega Corporation, Madison, WI); kit includes 2X rapid ligation buffer and T4 ligase.
5. Competent *Escherichia coli* (JM 109 or another strain).
6. Restriction enzymes and the recommended enzyme reaction buffers of the manufacturers.
7. QIAquick PCR Purification Kit and QIAprep Spin Miniprep Kit (QIAgen).
8. λ-DNA (Promega).

2.4. Agarose Gel Electrophoresis

1. Electrophoresis running buffer (5X stock TBE): 54 g of Tris base (Sigma), 27.5 g boric acid (Sigma), 20 mL 0.5 *M* disodium-ethylenediaminetetraacetic acid (EDTA) (pH 8.0) per liter.
2. Agarose MP (multipurpose; Roche).
3. Ethidium bromide stock solution: 10 mg/mL (Sigma), store in the dark at 4°C; use 4 µL/100 mL TBE buffer and agarose gel to stain the gel during electrophoresis.
4. Loading buffer: 30% glycerol, 0.25% xylene cyanol (Sigma).
5. 100 bp ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany).

3. Methods

3.1. RNA Isolation and cDNA Synthesis

The ability to isolate clean, intact RNA is essential in quantitating mRNA. Competitive RT-PCR does not require mRNA that is free of contaminating rRNA and tRNA. There are many commercial kits available for the purification of RNA. Those that combine the selective RNA binding properties of a silica-gel–based membrane with the speed of microspin or vacuum processing technology are quick and easy to use. The method is advisable when isolating RNA from small quantities of varying starting material.

1. Isolate RNA according to manufacturer's instructions using the RNeasy Mini Kit. Elute the RNA in 30 µL double-distilled water (ddH_2O).
2. Determine RNA concentration by OD measurements at 260 nm (RNA: $OD_{260} \times$ dilution $\times$ 40 ng/mL, when measured in 1-cm cuvets). Concentrate the RNA samples, if necessary or possible, to 0.5–1 µg/µL with an evaporation centrifuge.
3. In parallel, analyze an RNA sample (1 µL) on a 1% agarose gel to confirm that RNA had not been degraded and does not contain genomic DNA (gDNA). gDNA forms an additional band with higher molecular weight than the two rRNA bands. gDNA contamination is a real problem for competitive RT-PCR, acting as an additional competitor.
4. In the case of DNA contamination, insert a DNase digestion step. Use 1 U DNase (RNase-free) per 1 µg total RNA in reaction buffer (in most cases provided with the DNase), incubate 30 min 25°C, and stop the reaction by inactivating the enzyme 5 min, 75°C.

As for RNA isolation, a number of optimized kits make the reverse transcription easy. We have had good experiences with the First-Strand cDNA Synthesis Kit from Amersham Pharmacia Biotech using the oligo-dT primer. Up to 5 µg RNA could be inserted in one synthesis. Transcribe the same amount of RNA of each sample. Check for correct RNA isolation and cDNA synthesis by a simple PCR for a housekeeping gene (*see* **Note 1**).

3.2. Construction of the Competitor

1. Design the 5' and 3' primer and the 3' hybrid primer (*see* **Note 2**).
2. For the first amplification, use 1 µL of a cDNA, which must contain the target of interest in a 25-µL reaction mix containing 2.5 µL PCR buffer, 2 µL dNTPs, 1 µL of the 5' primer, and 1 µL of the 3' hybrid primer, and 0.5 U *Taq* polymerase. Amplify by standard PCR: template denaturation at 94°C for 10 s, primer annealing for 25 s (choose the annealing temperature according to the sequence of the 5' primer), template elongation at 72°C for 35 s; 35 cycles.
3. After the first amplification, remove a 10-µL aliquot of the reaction mixture, add 3 µL of loading buffer and analyze it on a 1% agarose gel in 1X TBE buffer. Only the band of the expected competitor size should be detected (*see* **Note 3**).
4. In the second amplification, reamplify 1 µL of a $1:10^3$ dilution of the first reaction mixture containing the competitor with the original 5' and 3' primer under the same PCR conditions. By analysis of the PCR product through agarose gel electrophoresis, only one band with the size of the competitor should be detected. This PCR product has identical 5' and 3' ends to the normal PCR fragment obtained from the target and the same DNA sequences—except for the missing *x* + 20 bp. Thus, the PCR product of the first amplification step works as the competitor.
5. Purify the PCR product from the first amplification to remove excess primers using a spin column (QIAquick PCR purification Kit) and elute the purified PCR product in 30 µL ddH_2O.

3.3. Cloning the Competitor

To get a large amount of the competitor, it is advisable to clone it into a vector. The easiest way to clone the competitor obtained after amplification with a *Taq* polymerase producing single 5' A-overhangs, is to use vectors with single 3' T-overhangs at the insertion site and permitting blue/white selection.

1. Set up a ligation reaction using 3 µL of the purified competitor, 5 µL of 2X ligation buffer, 1 µL of pGEM-T vector, and 1 µL T4 DNA ligase. Incubate it for 1 h at room temperature and then at 4°C until use.
2. Thaw one vial (50 µL) of competent *E. coli* (for example, JM 109) on ice. Add 7 µL of the ligation reaction. Allow the transformation to proceed on ice for 20 min. Heat-shock the cells at 42°C for 45–50 s and place them back on ice for 2 min. Add 950 µL of SOC medium and incubate at 37°C for 1.5 h in a shaking incubator.
3. Spread transformation onto LB agar plates containing ampicillin, IPTG, and X-Gal. Incubate inverted at 37°C overnight.
4. Using a sterile pipet, tick-pick five white colonies, and with each one, inoculate 5 µL overnight cultures in LB medium containing 50 µg/mL ampicillin. Grow overnight in a shaking incubator.
5. Isolate plasmid DNA of 3–5 mL of overnight culture using a QIA prep Spin Miniprep Kit. Elute purified plasmid DNA in 50 µL Tris-HCl, pH 7.5 or water.
6. Check 10 µL of the plasmid DNA for competitor content, first by digestion with restriction enzymes, and second by reamplification of 1 µL of a $1:10^3$ dilution of

the plasmid with the 5' and 3' primers. The orientation of the competitor in the plasmid does not matter.

3.4. Purification, Stabilization, and Quantitation of the Competitor

Normally, there is no need to cut out the competitor sequences from the recombinant plasmid DNA, and even opening of the plasmid is not necessary.

1. Determine the concentration of the competitor plus vector easily by OD measurement at 260 nm (dsDNA: $OD_{260} \times$ dilution $\times$ 50 ng/mL) in 1-cm cuvets: 1 pmol of 1000 bp DNA = 0.66 µg; 1 pmol of (competitor plus vector) bp = *x* µg; 1 fmol (10^{-15} mol) of (vector plus competitor) bp = *x* ng.
2. Adjust the concentration of the vector plus competitor (= competitor) to 1 fmol (10^{-15} mol)/10 µL. The competitor is further diluted in a log scale of standard dilutions to 10^{-23}mol/10 µL. Now, the competitor theoretically contains only one molecule.
3. Determine the sensitivity in simple 25-µL PCR reactions using 10 µL competitor under optimized conditions. In most cases, the competitor works up to 10^{-23} mol/PCR, although such sensitivity is not necessary for many applications.
4. Highly diluted competitor solutions were stable at –20°C for up to 1 yr in the presence of carrier λ-DNA (10 ng/µL), whereas progressive loss of competitor DNA with increasing storage time was observed when carrier DNA was omitted from the solution *(**8**)*. It is possible to lyophilize the highly diluted competitor plus carrier DNA in a 0.2-mL thin-walled PCR tube (10 µL/PCR tube) and to store them in a comfortable format at room temperature.

3.5. Competitive RT-PCR

1. We recommend quantitating first the cDNA of a housekeeping gene (*see* **Note 1**). If equal amounts of RNA have been transcribed and all conditions have been kept constant, the results of housekeeping gene quantitation should not vary over a large range (*see* **Notes 4** and **5**).
2. To find out the competition level of the competitor, the competitor dilutions (comfortable lyophilized in the PCR reaction tube) were each coamplified with an equal amount of cDNA. Perform PCR using standard protocols.
3. Prepare a 1.5% agarose gel in 1X TBE buffer in a gel cassette, mount it into the electrophoresis chamber, and fill the buffer tanks of the chamber with TBE puffer. Add 2 µL of 5X gel loading buffer to an 8-µL aliquot sample of each PCR. Load the samples and the DNA standard onto the gel, start electrophoresis. After electrophoresis, document the band pattern by measuring the intensity of ethidium bromide fluorescence with a very good video documentation system (for example, a cooled CCD 8-bit image sensor) and analyze the data with an image software (for example, Phoretix 1 D plus software; Phoretix International, Newcastle-upon-Tyne, UK).
4. This calibration serves to determine the competitor concentration leading to a signal strength equivalent to the PCR product (*see* **Note 6**). Use this competitor

concentration to check all samples. An increased mRNA expression can be deduced from a stronger PCR signal compared to the internal competitor after PCR. The fold increase of the mRNA relative to the calibrated sample can be determined by using higher concentration of the competitor to equivalent signal strength of the PCR product as described above (*see* **Fig. 2**). The copy number of the competitor in all dilutions used for calibration can then be calculated. If both the competitor and the PCR product to be quantified show equivalent band strength on an agarose gel, they contain approx the same copy number (*see* **Note 7**).

4. Notes

1. The mRNA of "housekeeping" genes are thought to be present in all cells and to be expressed constitutively nearly independently of the cell cycle, activation, and supply with nutrients and oxygen. In most situations, this is also the case. However, for all housekeeping genes, adaptive regulation has been found to be exerted at transcriptional and posttranscriptional levels.
2. The primers constitute the factor that is the least predictable and most difficult to troubleshoot. Several computer programs can assist in primer design. Primer–dimers are a common artifact, most frequently observed when small amounts of a template are taken through many amplification cycles. They form when the 3' end of one primer anneals to the 3' end of the other primer, and polymerase then extends each primer to the end of the other. The ensuing product can compete very effectively against the PCR product of interest, thus, preventing exact cDNA quantitation. Primer–dimers can best be avoided by using primers without complementarity, especially in their 3' ends.
3. If you obtain more than the expected band, change the primer pair. Take into consideration splicing variants of the target of interest and unspecific binding to genes with high homology. Competition must be limited to two targets to get proper results.
4. Quantitation of small amounts of RNA depends on an optimized PCR. Every element of PCR can affect the outcome. There are several PCR optimization kits and proprietary enhancers on the market. Optimization kits generally provide a panel of buffers in which the pH, buffer, nonionic detergents, and additives are varied, $MgCl_2$ may be added at several concentrations, and enhancers such as dimethylsulfoxide (DMSO), glycerol, formamide, betaine, and/or proprietary compounds may be chosen.
5. *Taq* DNA polymerase retains some activity, even at room temperature. Under such nonstringent annealing conditions, products can be generated from annealing of primers to target DNA at locations of low complementarity or having complementarity of just a few nucleotides at the 3' ends, creating new templates "tagged" with the primer sequences. Subsequent cycles amplify these tagged sequences in abundance, both generating nonspecific products and reducing amplification efficiency of specific products by competition for substrates or polymerase. Cooling all components of the reaction mixture to 0°C prior to mixing is convenient and the least expensive method, but is also the least reliable.

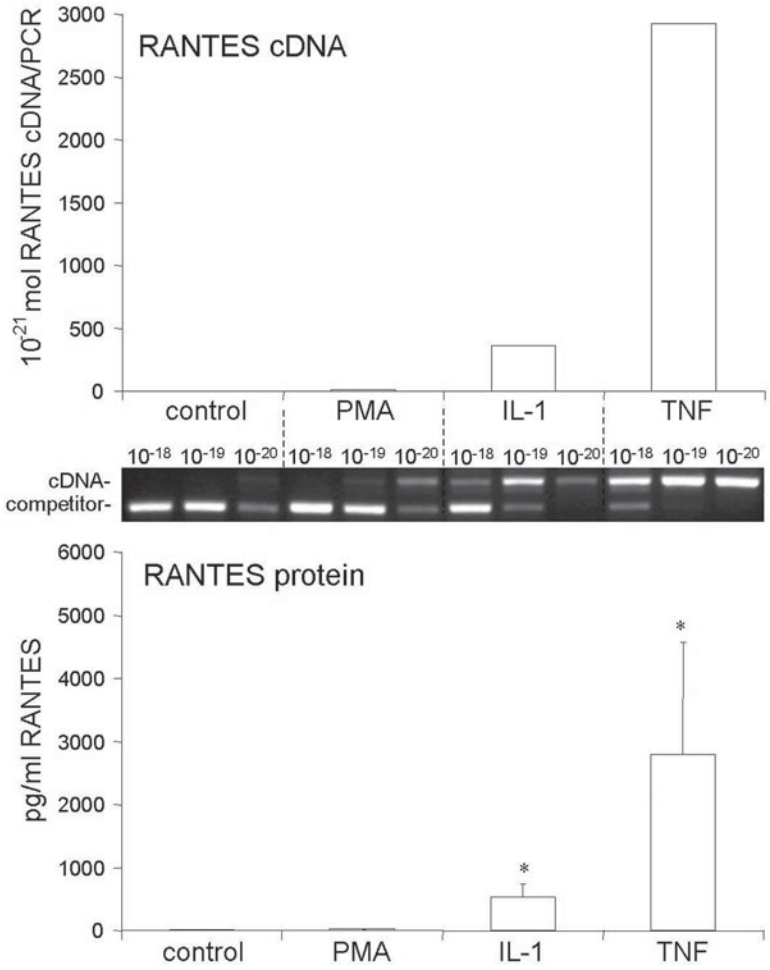

Fig. 2. Expression of the chemotactic cytokine RANTES in stimulated fibroblast cultures (PMA, phorbol myristate acetate; Il-1, interleukin-1; TNF, tumor necrosis factor-α). For competitive RANTES RT-PCR, defined concentrations of the RANTES competitor were coamplified with identical target cDNA aliquots in the same PCR tube. The target cDNA and competitor PCR products were separated by gel electrophoresis. The original gel electrophoresis results are shown in the lower part of the figure. The concentration of the competitor is indicated above the gel electrophoresis and increases 10-fold from lane to lane. RANTES protein determined in cell supernatants by a commercial ELISA; *significant differences between basal and stimulated RANTES levels, $n = 3$, $p < 0.05$.

Transferring the PCR reaction tubes from the ice slurry to a 95°C preheated thermocycler block may improve chances of success.

6. A nearly identical homologous competitor can exactly simulate the natural target during RT-PCR. However, this raises the problem of heteroduplex formation. Heteroduplexes consist of one-strand target cDNA and one-strand competitor. Its molecular weight lies exactly between that of the competitor and the target cDNA, thus forming an additional band in gel electrophoreses. This can complicate PCR product quantitation. Each half of the amount of the heteroduplex band should be added to the competitor and target cDNA.
7. Competitive RT-PCR has been successfully to quantitate DNA and RNA. Quantitation with photoimaging systems has a dynamic range limited to a target-to-competitor ratio of about 1:10 or 10:1. In fact, the best accuracy is obtained by finding the equivalence point at which the ratio of the target cDNA to the competitor is 1:1. To accomplish this, several dilutions of the competitor must be tested in order to achieve a suitable ratio of target cDNA to competitor.

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5

In Situ Hybridization for Cytokines in Human Tissue Biopsies

Emily Mathey, John Pollard, and Patricia Armati

1. Introduction

In situ hybridization (ISH) involves hybridization of labeled nucleic acid probes with the target mRNA. As cytokines are soluble mediators exported from the cells in which they are produced, ISH is a powerful technique that can be used to identify the exact identity and location of the cells that produce these soluble mediators. The technique of ISH is useful as a tool to locate mRNA species in histological samples of human tissues, in particular human nerve sections *(**1**)*. Using histological sections of such tissues has several distinct advantages, which complement studies that use extracted nucleic acids. For example, ISH can localize mRNA within single cells, can follow cell development regarding the expression of the target mRNA in embryos, and can be combined with immunohistochemistry *(**2**)* to detect the mRNA and the proteins encoded by them.

The digoxigenin (DIG) detection system is a non-radioactive labeling method designed for use in ISH with DIG-labeled oligoprobes *(**3**,**4**)* (*see* **Fig. 1**).

2. Materials

All heat-stable solutions, glassware, and apparatus used in ISH should be autoclaved at 130°C for 30 min to inactivate RNases. Wear disposable gloves throughout the ISH procedure to prevent contamination of materials with RNases. All rinse steps should be performed in troughs or jars.

1. Coplin jars.
2. Glass slides.
3. Incubator/oven set at 37°C.
4. 2% 3-aminopropyltriethoxysilane (APTS) in dry acetone (make up and use in fume hood).

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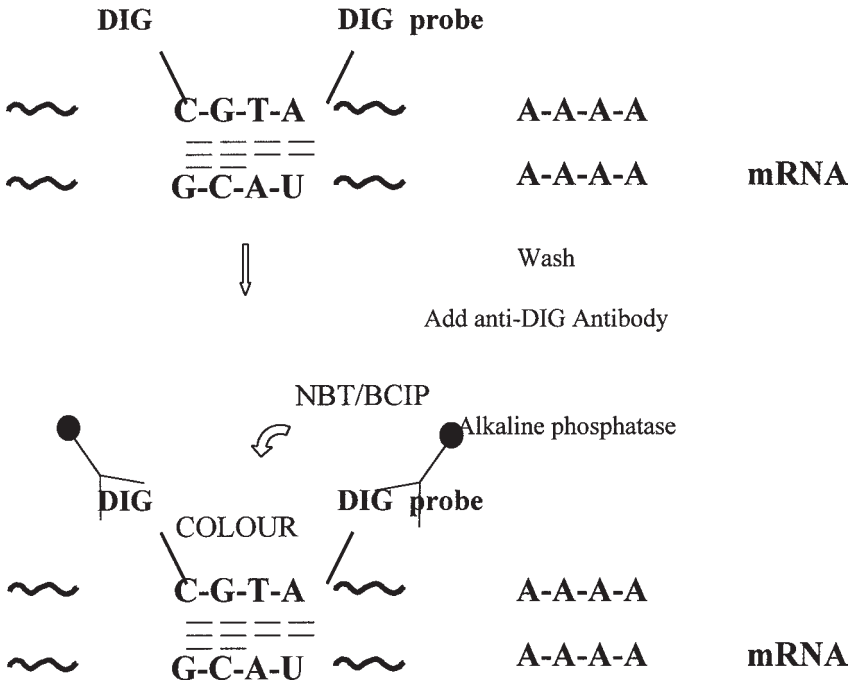

Fig. 1. Schematic of ISH technique. The DIG-labeled probe DNA binds to the target mRNA species. After the hybridization reaction, an anti-DIG alkaline phosphatase conjugated antibody is applied to the section. The alkaline phosphatase acts on the NBT/BCIP substrate to produce a color precipitate. The color precipitate is used to visualize the location of the mRNA.

5. Dry acetone.
6. Distilled water.
7. 4% paraformaldehyde in phoshate-buffered saline (PBS) (make fresh each time).
8. 20X sodium saline citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate (store at 4°C).
9. PBS (store at 4°C).
10. Buffer 1: 100 m*M* Tris-HCl and 150 m*M* NaCl, pH 7.5 (store at 4°C).
11. Buffer 2: 100 m*M* Tris-HCl, 100 m*M* NaCl, and 50 m*M* $MgCl_2$, pH 9.5 (store at 4°C).
12. Buffer 3: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0 (store at 4°C).
13. 100%, 95%, 70%, 50% ethanol.
14. 0.3% Triton X-100 in PBS.
15. 1 µg/mL Type XIL bacterial protease (Sigma, St. Louis, MO).
16. Hybridization chamber: Place a cloth on the bottom of a plastic sealable container. Moisten cloth with 6X SSC and seal container with parafilm when in the oven.
17. Hybridization solution made up in MilliQ water: 6X SSC, 1X Dendhardt's solution, 0.1 m*M* adenosine triphosphate (ATP), 200 µg/mL degraded free acid DNA from herring sperm, 2.0 m*M* sodium pyrophosphate (decahydrate) (store at 4°C).

18. Cytokine oligonucleotide probes (R & D Systems, Minneapolis, MN) (*see* **Note 1**).
19. Sheep antidigoxigenin–alkaline phospahatase conjugated antibody (Roche cat. no. 1 093 274, Basel, Switzerland) diluted 1:500 with buffer 1 containing 1% sheep serum and 0.3% Triton X-100.
20. Nitroblue tetrazolium salt (NBT, 18.75 mg/mL)/5-bromo-4-chloro-3-indolyl phosphate (BCIP, 9.4 mg/mL) stock solution in 67% DMSO (Roche cat. no. 1 681 451) diluted 1:50 in buffer 2 (Make up fresh each time in fume hood and store in the dark at 2–8°C).
21. Aquamount (BDH, Poole, England).
22. Coverslips.

3. Methods

3.1. Coating of Slides

1. Wash slides in a beaker of detergent for 10 min with periodic agitation.
2. Rinse slides for 5 min in distilled water by half filling the beaker, swirling the slides, and then decanting the water.
3. Place slides in acetone for 1 min.
4. Immerse slides in 2% APTS for 5 min.
5. Rinse slides in distilled water for 2 min and then leave slides to dry at room temperature.
6. Store slides in a slide box until needed.

3.2. Sectioning of Biopsies

1. For paraffin-embedded biopsies, cut 5 µm sections onto APTS-coated slides. Cover sections with MilliQ water and place on a 40°C hot plate to allow section to dry down onto slide (*see* **Note 2**).
2. Bake sections onto slides by overnight incubation in a 56°C oven. Store slides at 4°C until needed.
3. For frozen biopsies, cut 8-µm sections and air-dry onto APTS-coated slides. Bake sections onto slides in a 40°C oven overnight.

3.3. Pretreatment of Tissue

1. Place slides in Coplin jars. Do not let sections dry out at any point.
2. Dewax paraffin sections by immersion in dipentene for 20 min followed by rehydration in graded alcohol, 100%, 100%, 95%, 70%, and 50% for 2 min each.
3. Wash sections in PBS for 5 min. Permeabilize the sections with 0.3% Triton X-100 in PBS for 15 min and then wash twice in PBS for 5 min each time.
4. Further permeabilize with Type XIL bacterial protease at 22°C (1 µg/mL in PBS) for 5 min and then wash in PBS for 5 min (*see* **Note 3**).
5. Post-fix sections in 4% paraformaldehyde in PBS for 5 min at 22°C.
6. Acetylate tissue with an incubation in 0.25% acetic anhydride in 0.1 *M* triethanolamine for 10 min at 22°C and then wash with distilled water for 1 min.

3.4. Hybridization

1. Prepare a hybridization chamber by placing a cloth on the bottom, so that it covers the entire base, a plastic sealable container. Moisten cloth with 6X SSC.
2. Apply 50 µL of hybridization buffer (6X SSC, 1X Denhardt's solution, 0.1 *M* ATP, 2 m*M* sodium pyrophosphate, and 200 mg/mL denatured herring sperm DNA) to each section. Place slides on the bottom of the container and incubate for 30 min at 37°C inside an oven or incubator.
3. Make up a 30-n*M* solution of digoxigenin-labelled probe in hybridization buffer (*see* **Note 4**).
4. Apply probe solution to sections. Cut small squares of parafilm and place over sections as a coverslip. Place slides on the bottom of the container and seal the lid of the chamber further by wrapping parafilm around the edges of the lid. Incubate at 37°C for 18 h.

3.5. Posthybridization Washes

1. Heat all solutions to the required temperature in the oven before carrying out the washes. After hybridization wash sections in 6X SSC at 22°C for 10 min, followed by washes in 2X SSC and 0.2X SSC twice each for 5 min at 37°C and then immerse in 2X SSC for 1 min.

3.6. DIG Detection

1. Immerse sections in buffer 1 at 22°C.
2. Incubate sections in buffer 1 containing 2% normal sheep serum, 3% BSA, and 0.3% Triton X-100 for 1 h.
3. Dilute antidigoxigenin antibody (Roche) 1:500 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100 for 3 h.
4. Remove unbound conjugate by washing in buffer 1 for 15 min and then in buffer 2 for 15 min at 22°C.
5. Dilute NBT/BCIP 1:50 with buffer 2.
6. Apply NBT/BCIP solution to each section and incubate in the dark at 22°C.
7. Monitor reaction product by light microscopy over 7–12 h (*see* **Note 5**).
8. Terminate reaction when color precipitate has developed by immersing in buffer 3 (*see* **Fig. 2**).
9. Rinse slides under running tap water and drain onto blotting paper. Allow sections to dry.
10. Mount sections in Aquamount.

4. Notes

1. Oligonucleotide probes have several advantages over RNA and cDNA probes for ISH. Oligoprobes of 30 bases penetrate cells more easily than larger probes and can be designed to have similar GC contents, which permits constant hybridization conditions to be employed. The lower sensitivity of oligoprobes, compared

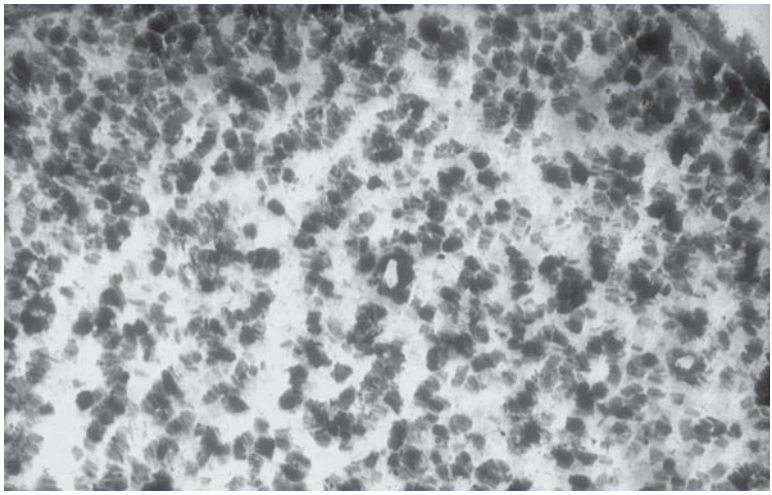

Fig. 2. TNF-α mRNA expression in endoneurial infiltrates in a human chronic inflammatory demyelinating polyneuropathy (CIDP) sural nerve biopsy.

with RNA and cDNA probes, can be overcome by using a cocktail of probes, each of which is specific for different regions of the same mRNA species. Moreover, suitable cocktails of probes for most cytokines are commercially available (R & D Systems) *(**5**)*.

2. The ability to perform retroperspective ISH analyses on archival tissue biopsies provides a large pool of patient specimens to select from. However, such tissue has often been fixed using suboptimal fixatives for ISH. Unfixed frozen biopsies are most suitable for ISH analyses as the most appropriate fixation methods can be chosen prior to the ISH.
3. Tissue pretreatments are designed to maximize the accessibility of the mRNA to the probe while at the same time maintaining tissue morphology and retention of the mRNA within the tissue. However, different tissue types often respond differently. For example, reports in the literature suggest the use of different concentrations of proteinase K from 1 to 20 μg/mL and incubation times ranging from 5 to 50 min. This difference in response necessitates extensive "trial and error" methods to determine the correct concentration and length of incubation of the enzyme.
4. Adequate controls are fundamental to the interpretation of ISH results. A negative tissue control, i.e., tissue without the target mRNA, must be used to ensure that the probe is binding to the specified target cytokine mRNA. The *negative tissue control* evaluates the stringency of the hybridization. For example, if the hybridization and posthybridization washes are of adequate stringency, the probe will not bind to non-homologous sequences. The negative control also assesses

the level of nonspecific absorption of the probe to cellular components and the amount of signal produced when DIG is introduced into the system. The negative tissue control is analogous to using a non-homologous probe in the tissue of interest. A *positive tissue control* is also necessary to ensure that the probe is binding and can be detected using the conditions in the protocol. A *technical control* on the tissue of interest using a poly-d(T) probe is necessary to evaluate probe penetration into the tissue, amount of mRNA degradation by RNases and the functioning of the immunological detection system. Poly-d(T) binds to all cellular mRNA species. Finally, a *detection control* must be used to gauge nonspecific staining. Nonspecific staining can occur as a result of the immunological detection procedure. The amount of such background staining due to the nonspecific binding of the detection antibody or precipitation of the color substrate can be appraised by a further control, the omission of the probe.

5. Formation of a precipitate in the color solution can sometimes contribute to nonspecific staining and dirty the section. If the reaction is monitored every hour by light microscopy, the sections can be washed and fresh solution applied if the precipitate starts to form.

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6

Purification, Identification, and Synthesis of Chemokines

Patricia Menten, Anja Wuyts, and Jo Van Damme

1. Introduction

Chemokines, i.e., low-molecular-weight chemotactic cytokines, are generally produced in very small amounts. Hence, liters of conditioned medium from primary cells or cell lines are necessary as start material to obtain microgram amounts of natural chemokine (*see* **Note 1**). A substantial quantity of a particular chemokine is only produced by certain cell types, providing that the cells are stimulated with an appropriate endogenous (cytokine) or exogenous (viral, bacterial, or plant products) inducer. Thus, if a specific immunological or biological assay is available, it is recommended to determine the optimal combination of cell type and inducer, as well as the timepoint of maximal production of the chemokine, prior to large-scale production. Maximal production of chemokines is often reached 48–72 h after addition of the inducer.

Owing to the large volumes (1–10 L) of conditioned medium that need to be processed, an initial concentration step of the medium is required prior to purification. In our laboratory, adsorption to controlled pore glass beads (CPG) or silicic acid and elution at acidic or neutral pH are routinely used for the concentration of the chemotactic activity present in the conditioned medium (***1***,***2***). Adsorption to CPG or silicic acid results in a 10-fold reduction of the initial volume and a 50-fold decrease of the protein content (*see* **Note 2**). Because more than 50% of the amount of each chemokine can be recovered, this procedure yields a 25-fold increase in the specific chemotactic activity, which indicates that this concentration step also results in a partial purification of chemokines.

After the initial concentration step, chemokines are isolated by means of three successive chromatographical steps, e.g., antibody or heparin-Sepharose

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affinity chromatography, cation-exchange chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC). Heparin-Sepharose affinity chromatography is based on the common high affinity of chemokines for heparin. This procedure allows for the separation of chemokines from the majority of the proteins in the CPG/silicic acid eluate. A more than 50-fold purification and 20-fold concentration can be accomplished. As a result of the diverging affinities of chemokines for heparin, a partial fractionation of the chemokines is achieved as well. Because chemokines are basic proteins with a high isoelectric point (pI), cation-exchange chromatography at low pH can further remove irrelevant proteins and separate chemokines from each other. Finally, chemokines are purified to homogeneity by RP-HPLC based on their hydrophobic interactions with the column matrix.

The column fractions obtained by affinity chromatography, cation-exchange chromatography, and RP-HPLC are checked for protein composition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*see* **refs. *3,4*** and **Note 3**), for biological activity (chemotaxis), and/or for immunoreactivity (ELISA) to decide which fractions will be retained and pooled for further processing. If a pure chemokine is obtained after purification, its identity can be determined by NH_2-terminal amino acid sequence analysis and mass spectrometry. Once the complete amino acid sequence (about 70 residues) of the chemokine is known, large amounts of chemokine, necessary for a detailed biochemical and biological characterization, can be chemically synthesized.

2. Materials

2.1. Protein Concentration

2.1.1. Adsorption to CPG

1. Spinner flask (Bellco Glass, Inc., Vineland, NJ).
2. Controlled pore glass beads (CPG, particle size 120–200 mesh, pore size 35 nm; Trisopor; REATEC GmbH; Weiterstadt, Germany).
3. Phosphate-buffered saline (PBS), pH 7.4. Storage at 4°C.
4. 10 m*M* glycine-HCl, pH 3.5. Storage at 4°C.
5. Elution buffer: 0.3 *M* glycine-HCl, pH 2.0. Storage at 4°C.
6. Sintered glass filter (porosity 2 or 3; Schott; Mainz, Germany).

2.1.2. Adsorption to Silicic Acid

1. Spinner flask or other suspended stirring device.
2. Silicic acid (Matrex Silica, particle size 35–70 µm, pore size 10 nm; Amicon, Inc., Beverly, MA). Storage at 4°C.
3. Polypropylene centrifuge bottles (1 L; Nalgene®, Nalge Nunc International; Rochester, NY).
4. PBS, pH 7.4. Storage at 4°C.

5. PBS, 1 *M* NaCl, pH 7.4. Storage at 4°C.
6. Elution buffer A: PBS, 1.4 *M* NaCl, pH 7.4, containing 50% ethylene glycol. Storage at 4°C.
7. Elution buffer B: 0.3 *M* glycine-HCl, pH 2.0. Storage at 4°C.
8. Dialysis and concentration buffer: 20 m*M* Tris-HCl, pH 7.4, containing 15% polyethylene glycol (average MW 20,000; Fluka Chemie AG, Buchs, Switzerland). Storage at 4°C.
9. Dialysis membranes with a molecular weight cutoff of 3.5 kDa (Spectra/Por®, Spectrum Medical Industries, Inc.; Laguna Hills, CA).

2.2. Chemokine Purification

2.2.1. Heparin-Sepharose Affinity Chromatography

1. Dialysis membranes with a molecular weight cut-off of 3.5 kDa.
2. Equilibration buffer: 50 m*M* Tris-HCl, 50 m*M* NaCl, pH 7.4. Storage at 4°C.
3. Elution buffer: 50 m*M* Tris-HCl, 2 *M* NaCl, pH 7.4. Storage at 4°C.
4. Heparin-Sepharose column (e.g., 1.6 cm × 40 cm; CL-6B; Amersham Pharmacia Biotech, Uppsala, Sweden).
5. Coomassie blue G-250 binding assay for the measurement of the protein concentration (Bio-Rad, Hercules, CA). Storage at 4°C.
6. Bovine serum albumin (BSA). Storage at 4°C.

2.2.2. Cation-Exchange Chromatography

1. Dialysis membranes with a molecular weight cutoff of 3.5 kDa.
2. Equilibration buffer: 50 m*M* formate, 0.01% Tween-20, pH 4.0. Storage at 4°C.
3. Elution buffer: 50 m*M* formate, 0.01% Tween-20, 1 *M* NaCl, pH 4.0. Storage at 4°C.
4. Mono S cation-exchange column (e.g., 50 mm × 5 mm; Amersham Pharmacia Biotech).

2.2.3. RP-HPLC

1. Equilibration buffer: 0.1% trifluoroacetic acid (TFA) in H_2O (Millipore, Bedford, MA).
2. Elution buffer: 0.1% TFA, 80% acetonitrile in H_2O.
3. C_8 Aquapore RP-300 column (e.g., 220 mm × 2.1 mm; PerkinElmer, Norwalk, CT).

2.3. Protein Identification

2.3.1. Amino Acid Sequence Analysis by Edman Degradation

2.3.1.1. NH_2-Terminal Amino Acid Sequence Analysis

1. Amino acid sequencer (e.g., Model 477A/120A or 491 cLC; Applied Biosystems Inc., Foster City, CA).
2. Biobrene Plus™ (Applied Biosystems Inc.)
3. Glass-fiber filter (Applied Biosystems Inc.)
4. Transfer buffer for semidry electroblotting: 50 m*M* Tris-HCl, 40 m*M* glycine, 0.04% SDS (w/v), 20% methanol (v/v) in H_2O, pH 9.2. Storage at 4°C.

5. Semidry blot apparatus.
6. Polyvinylidene difluoride (PVDF) membrane (ProBlott membrane, Applied Biosystems Inc.).
7. Coomassie blue (Serva).

2.3.1.2. Generation of Peptide Fragments for Internal Sequencing

- Chemical Cleavage

1. Formic acid (100%).
2. Freshly made blocking mixture: 20 mg *o*-phthalaldehyde (Fluoropa®, Pierce, Rockford, IL), 50 µL β-mercaptoethanol and 10 mL acetonitrile.
3. RP-HPLC buffers (*see* **Subheading 2.2.3.**).
4. RP-HPLC column (e.g., C_8 Aquapore RP-300 column; 50 mm × 1 mm; PerkinElmer).

- Proteolytic Digestion

1. Sequencing-grade endoproteinase, e.g., Lys C (Roche Molecular Biochemicals, Mannheim, Germany). Storage at 4°C.
2. Digestion buffer (2X concentrated), e.g., for Lys C: 50 m*M* Tris-HCl, pH 8.5, 2 m*M* EDTA. Storage at 4°C.
3. Freshly made 0.25 *M* dithiothreitol solution in H_2O.
4. RP-HPLC buffers (*see* **Subheading 2.2.3.**).
5. RP-HPLC column (e.g., C_8 Aquapore RP-300 column; 50 mm × 1 mm; PerkinElmer).

2.3.1.3. Modification of Cysteine Residues for Detection by Sequence Analysis

- *In Situ* Modification

1. Freshly made reduction/alkylation mixture: 2 µL of tri-*n*-butylphosphine + 5 µL of 4-vinylpyridine in 100 µL acetonitrile.

- Modification in Solution

1. Freshly made reduction buffer: 200 m*M* Tris-HCl, pH 8.4, 100 m*M* DTT, 1% SDS.
2. Alkylation reagent: 6 *M* acrylamide in H_2O. Storage at 4°C.
3. Methanol (100%).
4. Methanol (20%) in H_2O.
5. TFA (0.1%) in H_2O.
6. ProSorb cartridge (Applied Biosystems Inc.).

2.3.2. Mass Spectrometry

1. Mass analyzer (e.g., ES/ion trap Esquire LC; Bruker Daltonik, Bremen, Germany).
2. ZipTip$_{C18}$ micro columns (Millipore).
3. Wetting solution: 50% acetonitrile in H_2O.
4. Sample preparation solution (5X): 2.5% TFA in H_2O.
5. Equilibration solution and wash solution: 0.1% TFA in H_2O.
6. Elution solution: 50% acetonitrile, 0.1% TFA in H_2O.

2.4. Chemical Synthesis of Chemokines

2.4.1. Chain Assembly

1. Automated peptide synthesizer (e.g., Model 433A, Applied Biosystems Inc.).
2. Amino acids with an Fmoc-protected α-amino group and protected side chains: Asn, Cys, Gln: trityl; Ser, Thr: *t*-butyl; Asp, Glu: *t*-butyl ester; Lys: *t*-butyloxycarbonyl; Arg: 2.2.5.7.8-pentamethylchroman-6-sulfonyl (Pmc).
3. 4-Hydroxymethyl-phenoxymethyl-copolystyrene-1% divinylbenzene (HMP) resin. Storage at 4°C.

2.4.2. Cleavage of the Peptide from the Resin and Deprotection

1. Freshly made cleavage mixture: 0.75 g crystalline phenol, 0.25 mL 1,2-ethanedithiol, 0.5 mL thioanisole, 0.5 mL H_2O, 10 mL TFA.
2. Bottle of nitrogen gas.
3. Two TFA resistant round-bottom flasks.
4. Cold methyl *t*-butyl ether (MBTE). Storage at 4°C.
5. Medium-porosity glass filter.
6. RP-HPLC buffers (*see* **Subheading 2.2.3.**).
7. Preparative RP-HPLC column: Resource RPC 3 ml column (Amersham Pharmacia Biotech) or 100 mm × 8 mm C_{18} Delta-Pak™ (particle size 15 µm; pore size 30 nm) column (Waters, Millipore) or 250 mm × 10 mm Aquapore octyl column (PerkinElmer).

2.4.3. Folding of the Peptide

1. Freshly made folding buffer: 1 m*M* EDTA, 0.3 m*M* oxidized glutathione, 3 m*M* reduced glutathione, and 1 *M* guanidinium chloride in 150 m*M* Tris-HCl, pH 8.7.
2. RP-HPLC buffers (*see* **Subheading 2.2.3.**).
3. RP-HPLC column (e.g., C_8 Aquapore RP-300 column; 220 mm × 2.1 mm; PerkinElmer).

3. Methods

3.1. Protein Concentration

3.1.1. Adsorption to Controlled Pore Glass

1. Conditioned medium (1–3 L; neutral pH) is magnetically stirred with CPG beads (1/30 v/v) for 2 h at 4°C in spinner flasks (3–10 L).
2. The CPG beads are allowed to sediment and the supernatant is decanted.
3. The CPG beads are washed once with PBS, pH 7.4 (500–1000 mL) and once with 10 m*M* glycine-HCl, pH 3.5 (250–500 mL) by manually stirring the beads for 5 min and subsequently decanting the supernatant after sedimentation of the beads.
4. To eluate proteins from the beads, CPG is manually stirred twice with 0.3 *M* glycine-HCl, pH 2.0 (25–50 mL) for 5 min at room temperature. The supernatant (eluate) is collected by pipeting after sedimentation of the beads.

5. To further improve recovery, CPG is magnetically stirred twice with 0.3 *M* glycine-HCl (25–50 mL), pH 2.0 for 30 min at 4°C. The eluate is collected by pipeting after sedimentation of the beads.
6. All eluates are pooled, filtered through a sintered glass filter to remove remaining CPG beads, and stored at –20°C until further processing.

3.1.2. Adsorption to Silicic Acid

1. Conditioned medium (1–5 L; neutral pH) is magnetically stirred with silicic acid (10 g/L) for 2 h at 4°C in a spinner flask (5–20 L) or by means of a large stirring device.
2. To sediment the silicic acid, the medium is centrifuged for 15 min at 1350*g* in 1-L centrifuge bottles. The supernatant is decanted.
3. The silicic acid is washed, once with PBS, pH 7.4 (500–1000 mL) and once with PBS containing 1 *M* NaCl (250–500 mL). Therefore, the silicic acid is magnetically stirred for 10 min at 4°C and subsequently centrifuged for 15 min at 1350*g*. The supernatant is decanted.
4. To elute acid labile proteins, the silicic acid is magnetically stirred in PBS containing 1.4 *M* NaCl and 50% ethylene glycol (50–100 mL) for 30 min at 4°C (four times). The silicic acid suspension is centrifuged for 15 min at 1350*g* and the eluate is collected.
5. Further elution to recover acid stable proteins is achieved by magnetically stirring the silicic acid with 0.3 *M* glycine-HCl, pH 2.0 (50–100 mL) for 15 min at 4°C (four times). The suspension is centrifuged (15 min, 1350*g*) and the eluate is collected.
6. The eluates are pooled and further concentrated by dialysis (3.5 kDa cutoff membranes) against 20 m*M* Tris-HCl pH 7.4 supplemented with 15% polyethylene glycol.

3.2. Chemokine Purification

3.2.1. Heparin-Sepharose Affinity Chromatography

1. The CPG eluate or the concentrated silicic acid eluate is extensively dialyzed (3.5 kDa cutoff membranes) against 50 m*M* Tris-HCl, 50 m*M* NaCl, pH 7.4.
2. The dialyzed CPG or silicic acid eluate is loaded onto a heparin-Sepharose column, equilibrated with 50 m*M* Tris-HCl, 50 m*M* NaCl, pH 7.4.
3. To remove weakly bound proteins, the heparin-Sepharose column is washed with the equilibration buffer (100 mL).
4. The proteins are eluted with a linear NaCl gradient (0.05–2 *M*) in the equilibration buffer (20 mL/h; 5-mL fractions).
5. The protein concentrations in the fractions are determined by a Coomassie blue G-250 protein assay (***5***), using BSA as a standard.
6. The biological activity and purity of the proteins in the fractions are determined in the Boyden microchamber chemotaxis test and by SDS-PAGE, respectively.

3.2.2. Cation-Exchange Chromatography

1. Fractions derived from heparin-Sepharose chromatography which contain the peak of chemotactic activity are dialyzed against 50 m*M* formate, 0.01% Tween 20, pH 4.0 to remove salt and to adjust the pH (*see* **Note 4**).
2. The dialyzed fractions are loaded on a Mono S column, equilibrated with 50 m*M* formate, 0.01% Tween 20, pH 4.0.
3. To remove weakly bound proteins, the Mono S column is washed with the equilibration buffer (20 mL).
4. Proteins are eluted with a linear NaCl gradient (0–1 *M*) in the equilibration buffer (1 mL/min; 1-mL fractions).
5. Absorbance at 280 nm is measured on-line as a parameter for the protein concentration (*see* **Note 5**).
6. The biological activity and the purity of the proteins in the fractions are determined in the Boyden microchamber chemotaxis test and by SDS-PAGE, respectively.

3.2.3. RP-HPLC

1. Fractions derived from cation-exchange chromatography containing chemotactic activity are loaded on a C_8 Aquapore RP-300 column, equilibrated with 0.1% TFA (v/v) in H_2O.
2. The proteins are eluted with a linear acetonitrile gradient (0–80%) in the equilibration buffer (0.4 mL/min; 0.4-mL fractions).
3. Absorbance at 220 nm is measured on-line as a parameter for the protein concentration (*see* **Note 5**).
4. The biological activity and the purity of the proteins in the fractions are determined in the Boyden microchamber chemotaxis test and by SDS-PAGE, respectively.

3.3. Protein Identification

3.3.1. Amino Acid Sequence Analysis by Edman Degradation

Once pure chemokine is obtained, NH_2-terminal amino acid sequence analysis can be performed to reveal the identity of the purified protein. The presence of contaminants at low concentrations compared to the chemokine does not interfere with the identification of the protein of interest (*see* **Note 6**).

NH_2-terminal amino acid sequence analysis by Edman degradation is performed on a pulsed liquid phase protein sequencer with on-line detection of phenylthiohydantoin (PTH)-amino acids. Edman degradation is a cyclic process, consisting of a coupling, cleavage, and conversion step, in which amino acids are successively removed from the NH_2-terminus of the peptide chain, converted to their PTH-derivative, and identified chromatographically (*see* **Fig. 1**). After each degradation cycle, the remaining protein obtains a new NH_2-terminal amino acid, which is susceptible for the next Edman degradation step.

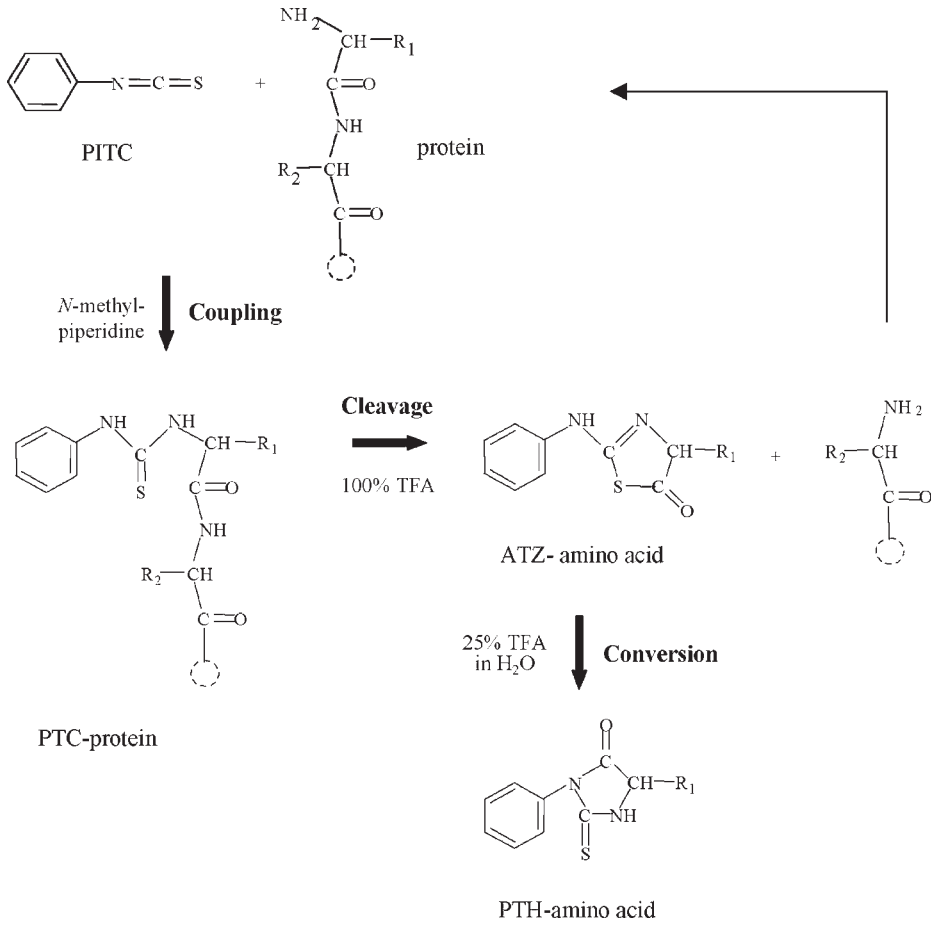

Fig. 1. NH_2-terminal sequence analysis by Edman degradation. PITC reacts with the free NH_2-terminus of the protein to form a PTC–amino acid. This derivatized amino acid is cleaved by addition of 100% TFA, yielding an ATZ–amino acid and a protein with a new NH_2-terminal amino acid, ready for the next degradation step. Aqueous TFA converts the ATZ–amino acid into a PTH–amino acid, which is on-line detected by RP-HPLC.

During the coupling step in the reaction cartridge of the sequencer, phenylisothiocyanate (PITC) reacts with the free NH_2-terminal amino group on the peptide, resulting in the formation of a phenylthiocarbamoyl (PTC)-protein. The derivatized amino acid is cleaved from the peptide by addition of TFA. The resulting anilinothiazolinone (ATZ) amino acid is extracted with *n*-butylchloride and transferred to the conversion cartridge, where it is trans-

formed to the more stable PTH amino acid. After dissolution in 20% acetonitrile, the PTH amino acid is identified by RP-HPLC.

Because of the high sensitivity of the currently available protein sequencers, 3 pmol of protein are sufficient for the identification of 20 amino acids. However, maximally about 50 residues can be determined in one sequencing run. Thus, to obtain the complete amino acid sequence of proteins consisting of more than 50 amino acids, fragmentation (chemical or enzymatic) of the protein and subsequent separation of the fragments by RP-HPLC prior to sequencing is necessary. By cleavage of the chemokine and subsequent sequencing of overlapping peptide fragments, the complete sequence of a chemokine can be obtained.

Fragmentation of the protein is also required when the protein is NH_2-terminally blocked for sequence analysis. For example, the monocyte chemotactic proteins MCP-1 to 4 all possess an NH_2-terminal pyroglutamic acid, resistant to the first chemical reaction of the Edman degradation. These chemokines contain a single Asp-Pro sequence that can be chemically cleaved by formic acid. Because the NH_2-terminal fragment is blocked for sequencing, the COOH-terminal fragment can be sequenced after cleavage without prior separation of the two fragments.

The presence of cysteine residues in proteins cannot be directly detected because of the formation of an unstable amino acid derivative after Edman degradation. However, its presence is often obvious from the absence of any detectable signal. Nevertheless, the presence of cysteines can be confirmed by alkylation of these residues prior to Edman degradation (***6,7***).

3.3.1.1. NH_2-Terminal Amino Acid Sequence Analysis

1. An NH_2-terminally blocked protein must be fragmented by chemical digestion or enzymatic proteolysis prior to sequencing (*see* **Subheading 3.3.1.2.**). If cysteine residues have to be determined, the protein can be treated with an alkylating agent (*see* **Subheading 3.3.1.3.**).
2. A porous glass-fiber filter is placed in the reaction cartridge, pretreated with Biobrene Plus™, and cycled through Edman chemistry to reduce contaminants. The pure protein sample is loaded on the glass-fiber filter and dried (*see* **Note 6**).
3. The NH_2-terminal sequence is determined by automated sequencing with on-line detection of PTH-amino acids by RP-HPLC.

3.3.1.2. Generation of Peptide Fragments for Internal Sequencing

- Chemical Cleavage of Chemokines

1. The protein sample is concentrated to 10–20 µL and incubated for 50 h at 37°C with 3 vol of 100% formic acid.
2. If the protein is NH_2-terminally blocked and contains a single Asp-Pro cleavage site, the protein can be loaded onto the sequencer directly after cleavage. Only the COOH-terminal fragment will be sequenced.

3. However, if the sample is an NH_2-terminally accessible protein with a single Asp-Pro cleavage site, the protein is treated with a mixture of 20 mg *o*-phthalaldehyde, 50 µL β-mercaptoethanol and 10 mL acetonitrile, prior to sequencing, to block the NH_2-terminal fragment for sequence analysis (***8***) (*see* **Note 7**).
4. If more than one Asp-Pro cleavage site is present in the protein, the different peptide fragments are separated by RP-HPLC prior to loading on the sequencer (*see* **Subheading 3.2.3.**).

- Proteolytic Digestion of Chemokines

1. The protein sample is concentrated to 15 µL and incubated at 37°C for 18 h with 60 µL of H_2O, 75 µL of 2X digestion buffer (composition depends on the enzyme and is described by the manufacturer), and the endoproteinase (concentration of 1/20 to the protein substrate).
2. The sample is incubated for 1 h at 37°C with DTT to reduce intramolecular disulfide bonds allowing the separation of the fragments by RP-HPLC.
3. The peptide fragments are separated by RP-HPLC prior to loading on the sequencer (*see* **Subheading 3.2.3.**).

3.3.1.3. Modification of Cysteine Residues for Detection by Sequence Analysis

- *In Situ* Modification

1. The protein sample is loaded on the sequencer as usual (*see* **Subheading 3.3.1.1.**).
2. Twenty microliters of the reduction/alkylation mixture is added to the sample in the reaction cartridge.
3. An additional, first reaction cycle is introduced in which the reaction cartridge is equilibrated with *N*-methylpiperidine and subsequently washed with *n*-butylchloride and ethylacetate to remove the reduction/alkylation mixture.
4. The next reaction cycles are the same as usual.

- Modification in Solution

1. The protein is lyophilized, dissolved in 10 µL of the reduction mixture, and incubated for 30 min at 70°C.
2. After incubation, 40 µL of H_2O is added to the sample as well as 25 µL of an alkylating reagent, i.e., 6 *M* acrylamide solution. The mixture is incubated for 45 min at 37°C in the dark.
3. Methanol is added to the sample at a final concentration of 10%. Subsequently, the sample is desalted in a ProSorb cartridge (*see* **Note 8**).
4. After the membrane is air-dried, it is punched out of the ProSorb cartridge and loaded onto the sequencer (*see* **Subheading 3.3.1.1.**).

3.3.2. Mass Spectrometry

The molecular mass is a highly specific characteristic of a protein. Mass spectrometry (MS) permits mass determinations of proteins with an accuracy far superior to SDS-PAGE. Only a few picomoles of protein are necessary to

obtain its molecular mass with an accuracy of ± 0.01-0.1%. Therefore, MS is often used to verify the amino acid sequence of chemokines, to reveal micropolymorphisms or to determine posttranslational modifications (NH_2-terminal processing).

A mass spectrometer generates gas phase ions from a sample, separates the ions according to their mass-to-charge (*m/z*) ratio, and records a spectrum of their abundancies. The most important methods to generate gas phase ions are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ES). For MALDI MS, a ultraviolet (UV) absorbing matrix is mixed with the analyte and dried to form protein-doped matrix crystallites. Upon irradiation with a pulsed UV laser in the mass spectrometer, the crystallites are rapidly sublimated, which leads to the formation of gas-phase protonated molecular ions from the matrix/analyte substrate.

For ES MS, the solution of solvent and analyte is sprayed through a very fine needle into the spray chamber of the mass spectrometer. Owing to the high-voltage electrostatic field in the spray chamber, fine highly charged droplets are formed at the tip of the needle. A countercurrent flow of heated drying gas evaporates the solvent from the droplets, resulting in an additional increase of surface charge density. When the field created by the ions at the surface of the droplet exceeds the surface tension, bare multiply charged gas-phase-analyte ions are emitted from the droplet and are directed to the mass filter for the separation of the ions.

Frequently used methods for the separation and the detection of ions according to their mass-to-charge ratio are time-of-flight (TOF), quadrupole, and ion trap techniques. MALDI is generally combined with TOF detection. Ions are accelerated in an electrostatic field and are subsequently allowed to fly through a fixed-distance field-free region before they are recorded by the electric signal generated upon impact on a detector. The TOF depends on their *m/z* value. A highly charged ion will obtain more kinetic energy in an electrostatic field compared to a lower charged ion with the same mass. Having identical kinetic energy, small molecular ions move faster than large molecular ions.

Separation of ions by a quadrupole mass filter is achieved by creating an oscillating electric field between four parallel metal rods, the quadrupole. At a given oscillation frequency and amplitude of the potentials applied to the rods, ions of just one *m/z* value are allowed to pass. By scanning at different amplitudes, a spectrum can be recorded.

The ion trap consists of a ring electrode between two endcap electrodes. Holes at the center of the endcaps allow ions to pass in and out the trap. The entrance endcap is held at ground potential, whereas a high-voltage radio frequency (RF) potential and an auxiliary voltage potential are applied to the ring

and the exit endcap, respectively. Depending on the level of the RF voltage, the field can trap ions of a particular mass range. The quadrupolar field in the ion trap induces an oscillatory harmonic motion of the ions. The frequency of the oscillation is principally determined by the *m/z* value of the ion and the RF amplitude. When the amplitude of the RF voltage is progressively increased, ions with successively larger *m/z* values take up energy very quickly and are ejected from the trap. The ion detector produces an electrical current recording the mass spectrum. Because the ion trap is a storage device, it is possible to accumulate weak signals over an extended period of time.

Peptide or protein mixtures can be analyzed by mass spectrometry without prior purification. However, nonprotein or nonpeptide contaminants may prevent mass analysis because they interfere with molecular-ion production or obscure the signals corresponding to the analyte of interest. For example, in ES, salts and detergents may act as charge scavengers, thereby decreasing the yield of analyte molecular ions. Therefore, the sample preparation is very important. Here, the protocol for ES/ion trap MS is described.

1. Salts and detergents present in the sample are removed prior to injection on the mass spectrometer (*see* **Note 4**). Therefore, the sample is first diluted in the sample preparation solution to obtain a final TFA concentration of 0.1–1% at a pH < 4.0. Subsequently, the sample is loaded on a $ZipTip_{C18}$, a 10-µL pipet tip with a bed of chromatography media fixed at its end. The tip is prewetted with wetting solution and washed twice with equilibration solution prior to loading of the sample. Finally, the proteins are eluted with the elution solution.
2. The parameters for mass analysis (e.g., target mass) can be adjusted according to the mass expected to obtain a stronger signal.
3. The eluate is loaded on the mass spectrometer and the data acquisition is started.
4. The data are analyzed using special software programs.

3.4. Chemical Synthesis of Chemokines

Chemical synthesis of chemokines can be used as a fast alternative for the molecular cloning and expression of recombinant proteins or the expensive and labor-intensive purification of natural chemokines from conditioned media of cells. By solid-phase peptide synthesis using Fmoc (9-fluorenylmethoxycarbonyl) chemistry on a model 433A peptide synthesizer, milligram amounts of chemokine can be produced within a few weeks. Solid-phase peptide synthesis consists of five steps: chain assembly on a resin, cleavage of the peptide from the resin and removal of the side-chain protecting groups, purification of the crude peptide, additional chemical modification of the peptide (e.g., formation of disulfide bonds), and finally biochemical and biological characterization of the folded and purified peptide.

During solid-phase peptide synthesis, the peptide is synthesized from the COOH-terminus, which remains attached to a resin, toward the NH_2-terminus

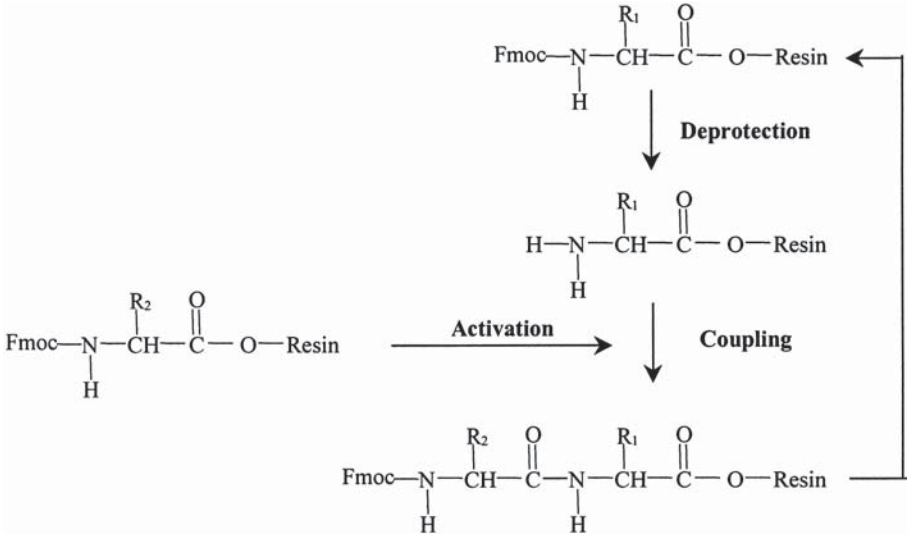

Fig. 2. Synthesis of peptides by Fmoc chemistry. After coupling of the first COOH-terminal amino acid to the HMP-resin, the Fmoc protection group is removed from the resin-coupled amino acid. The next Fmoc-protected amino acid is activated and coupled to the resin–peptide chain. The deprotection, activation, and coupling steps are repeated for each amino acid.

(*see* **Fig. 2**). The reactive amino group of each amino acid used for synthesis is protected by an Fmoc group. Reactive side-chain groups are also protected (*see* **Subheading 2.4.1.**).

First, the COOH-terminal amino acid is coupled to the HMP (4-hydroxymethyl-phenoxymethyl-copolystyrene-1% divinylbenzene) resin. Therefore, the protected amino acid must be activated prior to addition to the resin. Two equivalents of the protected amino acid react with 1 equivalent of DCC (dicyclohexylcarbodiimide), which results in the formation of a symmetric anhydride and a DCU (dicyclohexylureum) precipitate. Before the activated COOH-terminal amino acid is added to the resin in the reaction cartridge, 0.1 equivalent of a coupling catalyst, DMAP (dimethylaminopyridine), is added to the resin. After coupling of the first amino acid to the resin, remaining free hydroxyl groups on the resin are capped by addition of benzoic anhydride in the presence of DMAP. The Fmoc group on the coupled amino acid is removed by 20% piperidine in *N*-methylpyrrolidone (NMP). Before the next amino acid is transferred to the reaction vessel, this amino acid is first dissolved in NMP and activated by addition of 1 equivalent of HBTU [2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and 1 equivalent of HOBt

(1-hydroxybenzotriazole) in DMF (*N,N*-dimethylformamide), whereas the growing peptide in the reaction vessel is treated with 1.5 equivalent of DIEA (diisopropylethylamine) in NMP to initiate the activation. In the coupling step, the COOH-group of the amino acid reacts with the NH_2-group of the peptide on the resin. The synthesis of chemokines is mostly perfomed on a 0.1 mmol scale, which means that 10 equivalents of amino acid (1 mmol) are used for 1 equivalent of the growing peptide chain (0.1 mmol) on the resin. The free amino groups of the nonreacted peptides on the resin are capped by addition of acetic anhydride. This prevents the formation of peptides that lack internal amino acids. The resin is washed with NMP to remove nonreacted amino acids and the Fmoc group is cleaved off by 20% piperidine in NMP. The coupling, capping and Fmoc removal steps are repeated for each amino acid. When the last Fmoc group is removed from the NH_2-terminal amino acid of the peptide, the resin is subsequently washed with NMP and DCM (dichloromethane).

A 10-min coupling program and a series of four 2-min deprotection steps per cycle are used for the synthesis of chemokines. Coupling and deprotection efficiencies are assumed to be linked and are measured by monitoring the conductivity of the cleaved Fmoc group. Two additional deprotection steps (2 min and 30 min) for the current amino acid and two alternative coupling steps (twice 50 min) for the next amino acid are performed whenever the deprotection efficiency, determined by the conductivity measurements, is too low.

3.4.1. Chain Assembly

1. The amino acids are ranked according to the sequence to be synthesized. For each amino acid in the sequence, two flasks of amino acid (each containing 1 mmol) are placed in the synthesizer to be able to perform a conditional double coupling.
2. The peptide is synthesized on a 0.1 mmol scale using the Fastmoc program on a solid-phase peptide synthesizer.
3. When the synthesis is finished, the peptide resin is washed with NMP and DCM and is subsequently dried on the synthesizer.

3.4.2. Cleavage of the Peptide from the Resin and Deprotection

1. To obtain a cleavage mixture for 0.1–1.5 g dried peptide-resin, 0.75 g crystalline phenol, 0.25 mL EDT, 0.5 mL thioanisole, 0.5 mL distilled water, and 10 mL TFA are mixed in a round-bottom flask. The cleavage mixture is cooled on ice.
2. The resin-bound peptide (0.1–1.5 g) is placed in another round-bottom flask, which is subsequently cooled on ice and is degassed.
3. The cleavage mixture is added to the flask containing the cooled resin-bound peptide, which is then allowed to warm to room temperature.
4. The flask is stoppered and the reaction mixture is stirred at room temperature for 90 min.

5. To remove the resin, the reaction mixture is vacuum-filtered through a medium-porosity glass filter, directly into 30 mL cold MTBE, in which the protein will precipitate.
6. Approximately 1 mL TFA is added to the reaction flask to wash the remaining resin. This solution is also filtered through the medium-porisity filter into the cold MTBE suspension from the previous step.
7. The MTBE solution is centrifuged for 10 min at 1350*g* at 4°C. The supernatant is decanted and the precipitate is resuspended in 30 mL MTBE. This step is performed four times.
8. The peptide is dissolved in H_2O prior to lyophilization.
9. The crude peptide is purified on a RP-HPLC column (*see* **Subheading 3.2.3.**).

3.4.3. Folding of the Chemokine

1. The purified unfolded peptide is incubated with folding buffer for 90 min at room temperature.
2. The folded peptide is purified to homogeneity by C_8 RP-HPLC (*see* **Subheading 3.2.3.**).

4. Notes

1. The serum concentration in the induction medium is kept low to facilitate the purification of chemokines to homogeneity. The large abundance of proteins in serum would disturb the efficiency of the purification steps. However, serum cannot totally be omitted, as this would extremely reduce the chemokine production by the cells.
2. The recovery of chemokines from CPG is usually better than the recovery of chemokines from silicic acid. However, glass beads are more expensive compared to silicic acid. Therefore, adsorption to CPG beads is prefered for the concentration of medium from cultured cells, containing low serum concentrations. By treatment with 70% nitric acid, the noneluted proteins can be removed from the glass beads, which allows the CPG beads to be reused. Adsorption to silicic acid is prefered for concentration of media from stimulated buffy coats, containing high protein levels. The silicic acid is discarded after a single use.
3. The purity and the relative molecular mass of the proteins are determined by SDS-PAGE on discontinuous Tris-HCl/tricine gels under reducing conditions as described in **ref. *3***. This method gives a superior resolution for proteins with a molecular mass in the 5–20 kDa range, such as chemokines. The stacking, spacer, and separating gels contain 5% T (total percentage concentration of acrylamide and *bis*-acrylamide) and 5% *C* (percentage concentration of cross-linker relative to *T*), 10% *T* and 3.3% *C*, and 13% *T* and 5% *C*, respectively. Relative molecular mass markers routinely used in our laboratory are phosphorylase *b* (M_r 92,500), BSA (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), lysozyme (M_r 14,400), and aprotinin (M_r 6,500). The proteins are visualized by the sensitive silver-staining procedure, allowing for the detection of 10 ng of protein.

4. The detergent Tween 20 is added to the buffers to prevent the purified chemokines from sticking to the column and to the tubes. Once added, it is very hard to remove Tween 20 from the samples. However, this is absolutely necessary prior to analysis on a mass spectrometer.
5. The peptide bonds and aromatic compounds of proteins are measured at 220 nm and 280 nm, respectively.
6. The protein sample is checked for purity by SDS-PAGE. If contaminants are still present in large amounts, the proteins are electroblotted on a PVDF membrane after gel electrophoresis and visualized by Coomassie blue staining. After destaining and washing the membrane five times with H_2O, the membrane is air dried and the relevant protein band is excised to load on the sequencer.
7. The reaction cartridge is equilibrated with *N*-methylpiperidine and subsequently wetted with the *o*-phthalaldehyde reaction mixture, which is delivered from the reagent bottle to the reaction cartridge. The cartridge is washed with ethyl acetate and *n*-butylchloride and double coupling times are programmed in the first cycle following the *o*-phthalaldehyde treatment.
8. The PVDF membrane is first wetted with 100% methanol. After loading of the sample, the membrane is washed once with 20% methanol, once with 0.1% TFA and three times with H_2O.

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7

Receptor Isolation and Characterization

From Protein to Gene

Daniela Novick and Menachem Rubinstein

1. Introduction

Affinity chromatography as it is known today, namely, the concept and the immense power of biorecognition as a means of purification, was introduced in 1968 by Cuatracasas, Wilchek, and Anfinsen ***(1)***. This technique is used in 60% of all purification protocols ***(2)***. Almost any given biomolecule that one wishes to purify has an inherent recognition site through which it recognizes a partner molecule. If one of these partners is immobilized on a polymeric carrier, it can be used to selectively capture the biomolecule of interest. Isolation of a protein by affinity chromatography is a very effective technique. It provides the protein in a pure state, enabling its identification by partial sequencing, either by mass spectrometry or by N-terminal microsequencing. Upon completion of the human genome project, a partial protein sequence is enough for retrieving its complete mRNA and hence its complete protein sequence.

Ligand affinity chromatography provides a comprehensive solution for isolation and characterization of most receptors and binding proteins. Any receptor or binding protein can be captured on its immobilized ligand, provided that there is sufficient affinity and a high specificity of interaction with the ligand. In many cases, there is more than one type of receptor or binding protein for a given ligand. Examples include the two-membrane-associated tumor necrosis factor (TNF) ***(3)*** receptors and the two-membrane-associated interleukin (IL)-1 receptors ***(4)***, each derived from a different gene. In other cases, a single receptor gene may generate more than one mRNA splice variant, yielding several receptor species [e.g., IFNAR2b and its longer counterpart IFNAR2c ***(5,6)***].

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In addition to membrane-associated receptors, circulating ligand-binding proteins are quite common. One group of binding proteins consists of soluble forms of cell surface receptors. Such soluble receptors were identified for several hormones and growth factors, including insulin, somatotropin (growth hormone), insulin-like growth factor (IGF), and epidermal growth factor (EGF). Among cytokine receptors, a soluble form of the IL-2 receptor (soluble *Taq*) was identified in cell culture supernatants and then reported to be present in body fluids of normal individuals ***(7)***. These soluble receptors correspond structurally to the extracellular ligand-binding domain of the cell surface receptor, hence retaining their ligand-binding properties. Some of these soluble receptors are produced by proteolytic cleavage of their cell surface receptor counterparts, some from alternatively spliced mRNA, and some by both mechanisms. Studies performed in our laboratory and in other laboratories revealed the presence of many soluble cytokine receptors in body fluids, including the receptors for IL-6, IFN-γ, TNF-α, IL-2, IL-4, IFN-α/β, and IL-13 ***(5,8–14)***. Based on these studies, it became apparent that there are soluble receptors for most, if not all, cytokines in the circulation.

Another group of circulating binding proteins are derived from different genes and therefore are not homologous to the cell surface receptors with which they share ligands. Examples of such proteins include osteoprotegerin ***(15)***, cytokine-like factor-1 ***(16)***, and IL-18 binding protein ***(17)***. Ligand affinity chromatography has successfully been employed for isolating and identifying most of the above soluble receptors and binding proteins. In some cases, isolation of these binding proteins and their sequencing preceded and enabled cloning or identification of their genes ***(5,10,17)***.

This chapter describes the methods used in our laboratory for the purification, characterization, and molecular cloning of soluble cytokine receptors, binding proteins, and cell surface receptors. With the use of these methods and availability of pure and active ligand, on the one hand, and sufficient amounts of the source of the receptor to be isolated, on the other hand, successful receptor isolation is virtually guaranteed.

2. Materials

2.1. Receptor Isolation

1. Affi-Gel 10, or any other suitable preactivated carrier for ligand immobilization (Bio-Rad, Hercules, CA).
2. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 6.5 m*M* Na_2HPO_4, pH 7.4, if necessary, 0.02% NaN_3.
3. Benzamidine.
4. 0.5 µm membrane (e.g., Pellicon Cassette, Millipore, Bedford, MA).
5. 10 kDa cutoff membrane (PTGC 10, Millipore).

6. Hypotonic buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 22 TIU/mL aprotinin. Additional protease inhibitors such as pepstatin and leupeptin may be included.
7. Vortex shaker.
8. Tris-sucrose buffer: 20 m*M* Tris-HCl, pH 7.4, 0.25 *M* sucrose, 1 m*M* PMSF.
9. Ultratorax homogenizer.
10. Gauze.
11. HEPES buffer: 50 m*M* HEPES, pH 7.5, 1 m*M* PMSF, 20 TIU/mL aprotinin and other antiproteases, and 0.02% NaN_3.
12. Liquid nitrogen.
13. Solubilization buffer: 1% Triton X-100, 10 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 1 m*M* PMSF, 20 TIU/mL aprotinin, and other antiproteases.
14. Low pH buffer: 25 m*M* citric acid, pH approx 2.5, 1 m*M* benzamidine, 0.01% NaN_3.
15. High pH buffer: 25 m*M* Na_2CO_3, pH approx 11.0, 0.5–3 *M* NaCl, 1 m*M* benzamidine, 0.01% NaN_3.
16. 1 *M* Na_2CO_3.
17. 1 *M* Tris-HCl, pH 9.5.
18. Acetic acid (3 *M* and 5%).

2.2. Receptor Characterization

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
2. Fixing solution: 50% methanol, 10% acetic acid.
3. 10% glutaraldehyde.
4. Ethanol.
5. 30% NaOH.
6. Ammonia.
7. Silver nitrate.
8. 1% citric acid.
9. 37% formaldehyde.
10. Acetone.
11. PD-10 columns (Amersham-Pharmacia, Piscataway, NJ).
12. Gelatin.
13. 0.5 *M* phosphate buffer, pH 7.5.
14. Chloramine-T.
15. Na[^{125}I].
16. $NaHSO_3$.
17. KI.
18. Bovine serum albumin (BSA), 1% in water.
19. 20% trichloroacetic acid (TCA).
20. Disuccinimidyl suberate (DSS).
21. Dimethyl sulfoxide (DMSO).
22. Stopping solution (cross-linking): 1 *M* Tris-HCl, pH 7.5, 1 *M* NaCl.

3. Methods

3.1. Receptor Isolation Procedures

3.1.1. Preparation of a Ligand-Affinity Column

Successful receptor isolation depends on availability of a pure ligand (*see* **Note 1**), which retains its binding capability upon immobilization to an affinity resin. Affinity resins suitable for chromatography of proteins usually consist of derivatized agarose. There are several types of chemistries and spacers of various lengths that may be useful to avoid steric hindrance. Preactivated carriers are more convenient to work with. They are provided by several suppliers, who also include detailed procedures for ligand immobilization and performance of the chromatographic procedure (Amersham-Pharmacia, Bio-Rad, Sigma, Pierce an,d others).

The following procedure uses Affi-Gel 10 (Bio-Rad). This carrier is based on the active N-hydroxysuccinimide ester, which is relatively stable and reacts rapidly with primary (and secondary) amines of proteins, such as N-terminal sites and ε-amino groups of lysine). Affi-Gel 10 is suitable for coupling proteins having isoelectric points from 6.5 to 11. Proteins with isoelectric points below 6.5 are better coupled to Affi-Gel 15.

1. Couple 2–5 mg of highly purified (preferably homogeneous) ligand (*see* **Note 2**) to Affi-Gel 10 (0.5–1 mL) according to manufacturer's instructions. Make sure that the protein solution does not contain primary amines such as Tris-HCl, glycine, cell culture media, or ammonium salts. Usually, about 80–90% of the ligand is immobilized.
2. Store the resulting gel at 4°C in PBS containing 0.02% NaN_3. Stability of the immobilized ligand upon repeated use of the column is not predictable. In most cases, it may be used for chromatography numerous times.
3. Test the stability of the ligand by a bioassay following treatment with high and low pH. This will enable the choice of the most suitable elution conditions. Elution at a pH value that inactivates the ligand may still be employed for small-scale procedures, provided that the gel is rapidly neutralized following elution.

3.1.2. Preparation of Crude Receptor Extracts

Successful receptor isolation also depends on a suitable and sufficient source (*see* **Note 3**) of the receptor to be isolated. Sufficient amounts will ensure a final yield of the isolated receptor, enabling its characterization (N-terminal amino acid sequencing, mass spectrometry, SDS-PAGE, evaluation of biological activity, and so on) and antibody generation (*see* **Note 4**). Isolation of membrane-bound receptors requires a preliminary step of membrane isolation, followed by solubilization with a detergent**.** A number of mild detergents may be employed in the solubilization of cytokine receptors. Triton X-100 is an

example for a nonionic detergent; CHAPS, which is more effective for membrane solubilization, is an example of a zwitterionic detergent.

Isolation of soluble receptors does not require the use of detergents and hence is simpler to perform. Soluble receptors may be isolated from cell culture supernatants, from plasma, serum, or urine. Urinary proteins are derived from small amounts of plasma proteins, which pass through the kidney. Because the kidney retains high-molecular-weight proteins more effectively, the urine of healthy individuals is enriched with proteins < 65 kDa, including most soluble receptors. The low proportion of very high-molecular-weight proteins in the urine facilitates its concentration and makes it a convenient source for isolation of serum proteins with a molecular weight below 65 kDa.

3.1.2.1. Preparation of Crude Urinary Proteins

1. Collect urine from healthy donors in the presence of a protease inhibitor (e.g., 1 m*M* benzamidine) and 0.02% NaN_3. Store at 4°C (all procedures should be done at 4°C to minimize degradation).
2. Filter pools of urine (at least 100 L per batch) through a 0.5-µm membrane (e.g., Pellicon Cassette, Millipore).
3. Concentrate the clear filtrate 500–1000-fold by tangential ultrafiltration, using a membrane of 10 kDa cutoff (e.g., PTGC 10 membrane, Millipore). Remove the remaining small molecules by washing with PBS containing 0.02% NaN_3 and 1 m*M* benzamidine.
4. Keep the concentrated preparation of urinary proteins frozen at –20°C.

3.1.2.2. Cell Culture Supernatants

Cell culture supernatants may be concentrated using the same procedure as used for the concentration of urinary proteins ***(18,31)***. However, cells must be quickly removed, e.g., by centrifugation, as they tend to release proteases. Also, the presence of serum (e.g., fetal bovine serum) limits the degree of concentration, as it is not practical to work with serum concentrations exceeding 100%. Therefore, if possible, it is recommended to use a minimal amount of serum as a cell culture additive or employ serum substitutes.

3.1.2.3. Membrane-Associated Receptors From Cultured Cells ***(19)***

Perform all procedures at 4°C.

1. Wash cells (at least 3×10^{10} per batch) with PBS. Collect the cells by centrifugation (1500*g*, 7 min).
2. Disperse the cell pellet and suspend the cells in hypotonic buffer.
3. Mix vigorously on a Vortex shaker and allow standing for 5 min.
4. In order to clarify the lysate and remove debris, spin at three different stages: 700*g* for 5 min (discard pellet), 3500*g* for 10 min (discard pellet), and 40,000*g* for 1 h. Collect the pellet from the last spin.
5. Solubilize membranes as described in **Subheading 3.1.2.5.**

3.1.2.4. Membrane-Associated Receptors from Tissues

This procedure is a modification of the procedure described by Hock and Hollenberg ***(20)***. We successfully used it with human term placenta.

1. Separate a fresh placenta (approx 350 g) from its amniotic sac and cut into pieces of about 20 g.
2. Mince the pieces in a meat grinder into saline and wash thoroughly with saline.
3. Add 2 vol of Tris-sucrose buffer and homogenize by, e.g., Ultratorax homogenizer (five times, 30 s, setting 5).
4. Filter the homogenate through two layers of gauze and spin the filtrate (600*g*, 10 min) to remove debris.
5. Clarify the supernatant by centrifugation at 10,000*g* for 30 min.
6. Adjust the supernatant to a final concentration of 0.1 *M* NaCl and 0.2 m*M* $MgSO_4$ and spin at 48,000*g* for 40 min. Collect the pellet.
7. Suspend the pellet in an equal volume of HEPES buffer.
8. Freeze quickly in liquid nitrogen.
9. Solubilize membranes as described under **Subheading 3.1.2.5.**

One average-size placenta yields approx 450 mg of membranes (30 mg/mL).

3.1.2.5. Solubilization of Membranes

1. Suspend either intact, PBS washed cells (30 mL of cell pellet) or cell membranes (30 mg/mL, 15 mL) in an equal volume of a solubilization buffer.
2. Incubate for 1 h with occasional shaking.
3. Centrifuge (10,000*g*, 15 min).
4. Collect the supernatant and clarify by ultracentrifugation (100,000*g*, 4°C, 60 min).
5. The resulting supernatant is ready for ligand affinity chromatography.

3.1.3. Ligand Affinity Chromatography

Perform all steps at 4°C. The procedure described is optimized for soluble receptors. For solubilized membrane-bound receptors add 0.1% Triton X-100 or another detergent of choice to all the buffers.

1. Wash the affinity column with PBS.
2. Spin (10,000*g*, 15 min) the crude preparation containing soluble or solubilized receptor (the load fraction) prior to loading on the column. Set aside an aliquot for assays.
3. Apply the load fraction (*see* **Note 5**) to the column at a flow rate of 15–20 mL/h.
4. Collect the effluent fraction and keep for assays.
5. Wash the column (at a gravitation rate) with 250 column volumes of PBS containing 0.5–1 *M* NaCl. Collect the first and the last 10 column volumes separately for assays.
6. Elute the receptor with either low pH buffer or with a high pH buffer. Low pH buffer was found in most cases to be more effective than high pH buffer. If the

ligand is sensitive to low pH, elution at a high pH should be tried. In such cases, elution may be improved by the addition of high salt (1–3 *M*). Collect 10 fractions of one column volume each.

7. Immediately neutralize the elution fractions (e.g., with 1 *M* Na_2CO_3 or with 1 *M* Tris-HCl, pH 9.5, in the case of the low pH elution buffer and with 3 *M* acetic acid in the case of the high pH elution buffer). Calibrate the neutralization volumes needed to neutralize the elution buffer of choice: check the pH of the first three elution fractions (use 1 µL on pH indicator paper) and note that most probably the first elution fraction will be at neutral pH, the second will need half the neutralization buffer volume, and only the third will need the entire volume as determined by the calibration.
8. Wash the column immediately with PBS and store in PBS containing 0.02% NaN_3.
9. Determine the protein content in all the samples from the various purification steps. Alternatively, when the amount of pure protein is limited, protein content can be estimated following SDS-PAGE and silver staining of the gel.
10. Keep eluted fractions at 4°C. Repeated freezing and thawing is not recommended. Stability studies should be performed for the determination of the best storage conditions.

3.1.4. Additional Purification Steps

In most cases, an additional step of purification is required for obtaining the receptor in sufficient purity for further characterization. If the contaminating proteins differ very much in size from the protein of interest, they may be resolved by size exclusion chromatography (using, e.g., Superose 12, or Superdex 75, Amersham-Pharmacia) ***(5)***. As the volume loaded on size exclusion columns is limited, eluted fractions from the ligand affinity chromatography column should first be concentrated. Ultrafiltration in small devices (e.g., Ultrafree MC cutoff 10 kDa, Millipore) is recommended to minimize losses by adsorption to walls.

Alternatively, proteins may be further resolved following ligand affinity chromatography by reversed-phase (RP) high performance liquid chromatography (HPLC) ***(21)***. Not all proteins will resist the rather harsh conditions of RP-HPLC and some of them may not elute well from the column. However, the very high resolution of RP-HPLC makes this procedure very attractive. Use columns that have the largest pore size possible (100–300 Å). Octyl (C_8) chains offer a good compromise between columns with excessive hydrophobicity (C_{18}) and those exhibiting poor coverage of the silica surface (C_4). Use gradients of either acetonitrile or 1-propanol as the organic modifier and 0.1–0.3% trifluoroacetic acid (TFA). The organic solvent should be removed immediately after completion of the fractionation by evaporation (e.g., vacuum centrifugation) and residual TFA neutralized with a mild base, e.g., triethano-

lamine. We used Aquapore RP-300, 4.6 × 30 mm cartridge columns (Brownlee Labs, Perkin Elmer, Norwalk, CT) to further resolve the soluble IL-6 receptor ***(8)***, as well as many other proteins.

3.2. Receptor Characterization Procedures

3.2.1. SDS-PAGE

As a routine, run SDS-PAGE with the tested samples both under non-reducing and reducing conditions (e.g., DTT to a final concentration of 25 m*M*). Proteins isolated from urine are expected to be smaller than 65 kDa, thus 10% or 12% acrylamide gels are recommended. Membrane-associated receptors are usually larger than 65 kDa, requiring 10–7.5% acrylamide gels to achieve a satisfactory separation. Include in your gel samples from each purification step diluted according to the method of staining to be used. Optimal silver staining of the gel is achieved if the protein content does not exceed 100 or 200 ng per band. If the protein content of the elution fractions was not determined, load aliquots of 40 µL/lane. Always include in your gel a control lane containing your sample buffer, as the reducing agent tends to give artifacts at around 67 kDa upon silver staining ***(22)***.

3.2.2. Silver Staining of a Mini-Gel (8 × 6 cm)

Although many commercial kits and silver staining protocols exist in the market, the following protocol ***(23)*** had proven to be the best in our hands:

Note: Do not use autoclaved water, as the AgNO3 will precipitate. Use highly purified water (e.g., from a Milli-Q water purification system; Millipore) to prepare all your solutions and for all the washings of your gel.

1. Fix the gel overnight in methanol (50%), acetic acid (10%) solution (200 mL).
2. Wash the gel (four times, 15 min each) with water.
3. Fix the gel with glutaraldehyde (10%, 125 mL) for 30 min. Note that glutaraldehyde commercial stock is 25%.
4. Wash the gel (six times 10 min each) with water.
5. Freshly prepare a silver stain solution for 1-2 mini gels as follows: 105 mL water, 14.7 mL ethanol, 0.25 mL NaOH (30%), 1.5 mL NH_4OH. Weigh 0.8 g $AgNO_3$ (Analytical grade) and dissolve in 4 mL water. Add the silver solution drop wise with stirring into the above mixture. Keep in the dark until used.
6. Soak the gel in the silver stain solution (10 min).
7. Wash the gel with water four times, 15 min each wash.
8. Develop the gel (5–30 min) in the following freshly prepared developer solution: 179 mL water, 20 mL ethanol, 1 mL citric acid (1% stock), 0.1 mL formaldehyde (37%).
9. Stop the developer by brief washing with water (twice). Some protocols suggest a final rinse in water with 5% acetic acid.

Use a glass container rinsed well in highly purified water and then in acetone. Use gloves and do not touch the gel with your fingers.

All steps should be performed on a shaker to prevent the gel from sticking to the glass container.

3.2.3. Cross-Linking of the Purified Protein to Its Ligand

3.2.3.1. Labelling of Ligands with [^{125}I]

The simplest and most effective way to label proteins is by the Chloramine-T technique ***(24)***, which labels proteins at their tyrosine residues. If the protein is sensitive to oxidation, the Bolton and Hunter technique ***(25)***, based on acylation of amine residues, provides gentler labeling conditions.

In order to minimize any oxidation-induced damage to ligand-binding capability, the following modification of the Chloramine-T method should be used. All solutions are freshly prepared at room temperature and the labeling is performed on ice. Labeling is more efficient if the protein concentration is higher than 100 µg/mL (preferably 1 mg/mL).

1. Equilibrate a disposable size exclusion chromatography column (5–10 mL bed volume, e.g., prepacked Sephadex G-25, PD-10 column) with 25 mL PBS containing 0.25% gelatin, 0.02% NaN_3 (equilibration buffer).
2. Mix 1–25 µg of protein in a volume of 25–100 µL PBS (or another buffer compatible with the Chloramine-T technique) with an equal volume of 0.5 *M* phosphate buffer, pH 7.5 [A].
3. Mix 25 µL Chloramine-T solution (1 mg/mL in H_2O) with 1 mCi of Na[^{125}I] in 10 µL for 20 s [B].
4. Add [A] to [B] and incubate for 20 s.
5. Stop the reaction by the addition of 50 µL of $NaHSO_3$ (5 mg/mL, 50 µL) and KI (5 mg/mL, 50 mL) for 2 min.
6. Separate free from bound iodine using the size exclusion column. For optimal separation, let the column run until the bed is drained, apply the iodination mixture, allow the column to drain, add 1 mL equilibration buffer, let drain, and repeat five more times. Collect six fractions of 1 mL each into polypropylene tubes. The iodinated protein elutes in fractions 3 and 4. (Monitor with a Geiger counter.)
7. Count 2 µL from each fraction in a gamma counter. Typically, almost no counts are observed in the first two elution fractions. Up to 2–8 × 10^5 cpm/µL are obtained in fractions 3 and 4. Fractions 5 and 6 contain no more than 5% of the labeled protein.
8. Store the radiolabeled ligand at 4°C.

Check the extent of iodinated protein by TCA precipitation:

1. Mix 2 µL of each of the eluted fractions 3 and 4 with 100 µL of 1% BSA, add an equal volume of 20% TCA, and incubate for 20 min on ice.

2. Spin at 13,000*g* for 2 min.
3. Separate the supernatant from the precipitate and read both in a gamma counter.

Determine the counts in the precipitate as a percentage of the total counts in the 2 µL sample tested. Usually over 90% are TCA precipitable, providing a labeling level of approx 10^8 cpm/µg.

3.2.3.2. Cross-Linking of a Labeled Ligand to the Purified Soluble Receptor

This procedure is performed on ice. Avoid buffers containing free amines (e.g., culture media, Tris-HCl, glycine).

1. Mix an aliquot (40 µL) of the affinity-purified receptor peak fraction (chosen according to the silver staining of the gel) with the iodinated ligand ($0.5–5 \times 10^6$ cpm) for 1 h.
2. Add freshly prepared disuccinimidyl suberate (DSS, Pierce, 20 m*M*, dissolved in DMSO and kept at room temperature) to a final concentration of 1–2 m*M*. Incubate the mixture for 20 min on ice (see below for considerations for choice of cross-linkers).
3. Stop the reaction with a solution of 1 *M* Tris-HCl, pH 7.5, 1 *M* NaCl to a final concentration of 100 m*M*.
4. Add sample buffer (containing a reducing agent) and load on a 7.5% SDS-PAGE.

Alternatively, the cross-linked product can be immunoprecipitated or immunoaffinity purified ***(26)*** using specific antibodies to the ligand and then loaded on the gel.

There are many types of cross-linkers, which are comparable in their efficiency. Cross-linkers may be either water-soluble or insoluble. In terms of chemistry, two groups were used successfully, the activated *N*-hydroxysuccinimid esters and the imidoesters. Imidoesters (e.g., dimethyl suberimidate·HCl) are water-soluble and relatively inexpensive. The simple *N*-hydroxysuccinimid esters such as DSS need to be dissolved in a water-miscible solvent (DMSO, DMF, dioxan, and so on). Some of the *N*-hydroxysuccinimid esters are available as water-soluble sulfonated molecules. However, these are much more costly. All cross-linkers are sensitive to humidity, therefore should be stored under desiccation and also need to be dissolved immediately before use.

3.2.4. Determining the Affinity of a Soluble Receptor to Its Ligand

To date, most of the soluble receptors identified were found to function as inhibitors with the exception of the soluble receptor to IL-6, which is an agonist ***(27)***. At lower concentrations, these same soluble receptors may function as stabilizers of the ligand as was reported for IL-4 and TNF-α ***(28,29)***. High-affinity binding of the ligand with its receptor and slow off rate of this binding

strongly indicates that the receptor acts as an inhibitor rather than a carrier protein. The IL-18 binding protein ***(17)*** is an example of such a unique high affinity (K_d = 466 pM) soluble receptor with an association rate constant of 1.38×10^6 $M^{-1}s^{-1}$ and a markedly reduced dissociation rate constant of $6.43 \times 10^{-4}s^{-1}$, thus inhibiting the biological activity of its ligand at equimolar ratios ***(30)***.

Measuring the association and dissociation rate constants and hence the affinity of the soluble receptor to its ligand is best done using surface plasmon resonance (BIAcore, Uppsala, Sweden). This method is based on the use of disposable chips containing a gold leaf layer, to which activated dextran is attached on one side. The chip is placed in the BIAcore system, which couples a microfluidic system to the dextran side and applies a polarized light beam to the gold side. Changes in the mass of proteins attached to the dextran side are accurately measured as changes in the angle of surface plasmon resonance (angle of no reflection).

To perform the analysis, one protein is immobilized onto the BIAcore chip while the counter protein is analyzed at several concentrations. In our hands, immobilizing soluble receptors rendered them inactive, whereas immobilized cytokines retained their binding properties. The BIAcore system is very sensitive, enabling analyses to be performed at protein concentrations around 1 μg/mL.

1. Immobilize the ligand to an individual channel in a BIAcore sensor chip as recommended by the manufacturer. Usually low levels of binding (500–1000 resonance units) are suggested to minimize errors due to re-binding at the dissociation phase.
2. Perform binding-dissociation experiments at several concentrations of the soluble receptor as recommended by the manufacturer. Analyze the binding constants and kinetics of binding of the soluble receptor preparation to its ligand accordingly.

3.2.5. Bioassay of the Soluble or Solubilized Receptor

The goal of protein purification is to identify its function. Therefore, demonstrating the biological activity of an isolated protein is an essential step in this process. Because biological activity is a very broad term, a variety of procedures are used for its measurement. Generally speaking, bioassays of soluble receptors are based on their ability to modulate the biological activity of their respective ligands. In vitro bioassays using cell cultures are essential due to the limited yield of the isolated receptor. Analysis with the BIAcore system may also be used for determining whether the soluble receptor retains its ligand-binding activity, but only a bioassay will show if the soluble receptor acts as an antagonist, competing with the cell-surface receptor, or as an agonist, e.g., in the case of the soluble IL-6 receptor.

A simple bioassay with a reliable read-out should be chosen. Examples include the inhibition of the antiviral activity of IFNs in vitro ***(5)***, inhibition of

the induction of IFN-γ by IL-18 for measuring IL-18BP ***(17)***, or inhibition of cytotoxicity to cells by soluble receptors for TNF (TBPI and TBPII) ***(10,31)*** (*see* **Note 6**).

3.2.6. Protein Sequence Analysis

Sequence analysis of isolated proteins is performed either by mass spectrometry or by N-terminal sequencing using the Edman procedure. Mass spectrometry is more sensitive than N-terminal sequencing enabling analysis at subpicomole levels. With the completion of the genome project mass spectrometry is preferable, as all potential human protein sequences are contained in the database. The Edman procedure provides the N-terminal sequence of an isolated protein or its fragments. About 1–10 picomoles of protein or peptide may be analyzed by modern protein microsequencers (e.g., Model 475; Applied Biosystems Inc.). For analysis, the protein should be either purified to homogeneity or resolved on SDS-PAGE followed by electroblotting onto a PVDF membrane and staining. As a rule, sequencing proteins from solution is more efficient. Protein fragmentation (CNBr, trypsin, etc.) may be used if the N-terminus is blocked.

3.3. Further Structural Characterization of the Isolated Receptor

A partial protein sequence of an isolated receptor enables the determination of its complete structure by homology searching of the various DNA databases. A comprehensive set of databases and search tools is available at the NCBI web site (http://www.ncbi.nlm.nih.gov). These databases and tools may also be used for identifying related proteins or homologous proteins in other species. More sensitive search tools, e.g., those based on the Smith–Watermann algorithm, may be used for identification of proteins having a less obvious homology. A very rapid tool (Biocellerator; Compugen) is available at The Weizmann Institute bioinformatics web site (http://bioinformatics.weizmann.ac.il). Databases of expressed sequence tags (ESTs) are very useful for identifying mRNA (cDNA) sequences. However, many of these listings are rather incomplete. A very effective tool for obtaining full-length cDNA from a partial sequence is provided by Compugen (http://www.labonweb.com). For example following isolation of human IL-18BP and its partial N-terminal sequence, extensive use of such databases and tools facilitated its cloning, led to identification of the murine IL-18BP as well as a family of pox-virus encoded proteins that bind IL-18 and block its biological activity ***(17,32)***.

Searching genomic databases is just the first step. In many cases, a single gene generates numerous mRNA splice variants, whose relevance to biologically active proteins needs to be determined empirically. This requires complete sequencing of cDNA clones, either isolated from cDNA libraries or

obtained as individual clones from public collections (e.g., www.ATCC.org). RT-PCR and RNA blot hybridization must be used to indicate which of the mRNA molecules predicted to exist by the isolated cDNA clones is actually expressed.

The presence of a particular mRNA species does not serve as a proof that its corresponding protein is actually translated in vivo and is biologically active. Therefore, this protein must be expressed in order to compare its biological activity to that of the originally isolated protein. A good case in point is that of IFNAR2, the ligand-binding subunit of the type I IFN receptor. Three mRNA splice variants were identified: IFNAR2a, coding for a soluble IFNAR2, IFNAR2b, coding for a transmembrane receptor that has a short cytoplasmic domain ***(5)***, and IFNAR2c, coding for a transmembrane receptor that has a longer cytoplasmic domain ***(6)***. RNA blot analysis revealed that IFNAR2b was the major species at the mRNA level. However, the corresponding protein was barely detectable by immunoblotting and the recombinant protein bound IFNs but was unable to induce an antiviral response ***(33)***. As it turned out, the minor mRNA species IFNAR2c coded for the biologically fully active receptor subunit, whereas very little IFNAR2b protein is found in cells despite the high abundance of its mRNA ***(6)***.

3.4. Preparation of Antibodies

It is highly recommended to generate polyclonal and monoclonal antibodies to the purified proteins as soon as sufficient amount of homogeneous protein is available. Note that 5–10 µg of pure protein is enough for one injection of a rabbit and 1–5 µg is sufficient for one injection of a mouse. A good titer is obtained after four such injections. The antibodies will facilitate further characterization of the purified protein employing immunostaining, immunoblotting, and ELISA.

4. Notes

1. If possible, check by a bioassay that the ligand to be bound to the resin for ligand affinity column is active.
2. Use excess of ligand over protein to be purified in a minimal resin volume, in order to maximize yield and minimize nonspecific binding during the ligand affinity step.
3. Use sufficient amounts of a receptor source to ensure a reasonable final yield taking into account losses during the purification steps. Note that the level of most soluble cytokine receptors found so far in normal body fluids ranges from 0.5–5 ng/mL.

   If a cell surface receptor is to be purified, try to calculate the minimum amount of cells or tissue needed, based on the calculated number of these receptors per cell (e.g., from binding studies with a labeled ligand) and its apparent molecular weight (from cross-linking studies with a labeled ligand).

4. Use polypropylene or polyethylene (opaque) rather than polystyrene (transparent) tubes throughout the study in order to minimize protein losses by adsorption to surfaces. Also avoid unnecessary dilution of the protein solution and keep stocks of proteins at a high concentration.
5. Always spin (10,000*g*, 15 min, 4°C) the Load fraction prior to loading on the affinity column to avoid blocking of the column. A blocked column can be resuspended. If flow is still blocked transfer the resin to a new cartridge or use the resin in a batch adsorption and elution manner.
6. Some analyses (e.g., SDS-PAGE, ^{125}I-labeling) require pure proteins. When protein purity is not critical (e.g., bioassay) dilute the pure protein in diluents containing a carrier protein, e.g., 0.1–1% bovine serum albumin or culture medium containing 1–10% fetal bovine serum.

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8

Crystallization of Cytokine–Receptor Complexes

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1. Introduction

Crystallography is the most powerful method for determining the three-dimensional structure of complicated biological molecules. As the technology has improved, larger and more complicated structures involving previously unobtainable proteins have become available. Synchrotron radiation sources, fast and highly sensitive detectors, and cryogenic techniques have dramatically decreased the number and the size of crystals required for the analysis. Molecular biology techniques have allowed investigators to prepare milligram quantities of cytokines and their receptors that were previously extremely difficult to study because of their scarcity. Furthermore, mutations to remove naturally flexible or intramembranous domains make the preparation of specific cytokine-receptor complexes much more reasonable. However, the critical step is still the growth of crystals of macromolecules having sufficient size and quality to permit X-ray analysis.

The last few years have witnessed a marked increase in the number of cytokine–receptor complexes examined crystallographically. **Table 1** attests to these recent successes. Even though a cursory look at **Table 1** will show that there is relatively little in common among the conditions used to crystallize cytokine–receptor complexes, some common principles are apparent. We will focus on these common principles, as well as provide guidelines for experimentation.

2. Materials

Perhaps the most important requirement for successful protein crystallization is highly purified protein. In the case of cytokine–receptor complexes, the complex must be homogeneous, which is usually achieved by size exclusion chromatography (*see* **Notes 1** and **2**). Almost all of the complexes listed in

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Table 1
Crystal Structures of Cytokine/Cytokine Receptors Complexes[a]

Complex	Ligand: receptor stoichiometry	Diffraction resolution (Å)	Expression system	Purification method	Protein solution	Precipitant
EPO:EPOR ***(4,5)***	1:2	1.9	Human EPO expressed in *E. coli*; human EPOR (1–249) expressed in *P. pastoris*	Size exclusion with Superdex 75	10 mg/mL	32% PEG 1500, 0.28 *M* $(NH_4)_2SO_4$, 0.1 *M* MES (pH 6.5)
EPO:EPOR ***(4,5)***	1:2	2.8	Human EPO expressed in *E. coli*; human EPOR (1–249) expressed in *P. pastoris*	Size exclusion with Superdex 75	10 mg/mL	15% PEG 1500, 0.2 *M* $CaCl_2$, 0.1 *M* MES (pH 6.5–7.0)
EMP1:EPOR ***(6, 7)***	1:1	2.8	Human EPOR (1-255) expressed in *E. coli*	Not reported	1–2 mg/mL	11% mono-methyl ether PEG-5000, 0.2 *M* imidazole malate (pH 7.7)
FGF1:FGFR1 ***(8)***	1:1	2.8	Human FGF1 and FGFR1 expressed in *E. coli*	Size exclusion with Superdex 200	1 mg/mL, 150 m*M* NaCl, 25 m*M* Tris-HCl (pH 7.5)	30% PEG 4000, 0.2 *M* Li_2SO_4, 0.1 *M* Tris-HCl (pH 8.5)
FGF1:FGFR2 ***(9)***	1:1	2.4	Human FGF1 and FGFR2 (D2 and D3) expressed in *E. coli*	Size exclusion with Superdex 75	10 mg/mL, 150 m*M* NaCl, 20 m*M* Tris-HCl (pH 8.0)	1.6 *M* $(NH_4)_2SO_4$, 10 m*M* Tris-HCl (pH 7.5)

FGF2:FGFR1 ***(10)***	1:1	2.8	Human FGF2 and FGFR2 expressed in *E. coli*	Size exclusion with Superdex 200	10 mg/mL, 150 m*M* NaCl, 25 m*M* Tris-HCl (pH 8.5)	1.6 *M* $(NH_4)_2SO_4$, 20% glycerol, 0.1 *M* Tris-HCl (pH 8.5)
FGF2:FGFR2 ***(8)***	1:1	2.2	Human FGFR2 (D2 and D3) expressed in *E. coli*	Size exclusion	10 mg/mL, 150 m*M* NaCl, 25 m*M* Tris-HCl (pH 7.5)	10–15% PEG 4000, 10% isopropanol, 0.1 *M* HEPES-NaOH (pH 7.5)
GCSF:GCSFR ***(11,12)***	2.2	2.8	Human recombinant GCSF; mouse GCSFR (CRH region) expressed in baculovirus	Size exclusion with Superdex 200	0.1 *M* NaCl, 10 m*M* MES (pH 6.0)	1.2 *M* $(NH_4)_2SO_4$ (pH 7.5), 5% 1,4-dioxane
GH:GHR ***(1,13)***	1:2	2.8	Human GH and GHR (1–238) expressed in *E. coli*	Size exclusion with Sephadex G75-120	4 mg/mL	40%$(NH_4)_2SO_4$, 1% MPD, Tris-HCl (pH 7.5)
GH(G120R):GHR ***(14,15)***	1:1	2.6	Human GH(G120R) and GHR (1–238) expressed in *E. coli*	Phenylsuperose column	4.2 A_{280} U/mL, 120 m*M* NaCl, 20 m*M* sodium acetate (pH 5.5)	30% $(NH_4)_2SO_4$, 1% MPD, 0.1 *M* sodium acetate (pH 6.0); sitting drops
GH(G120R):PRLR ***(14,16)***	1:1	2.9	Human GH(G120R) and PRLR (1–211) expressed in *E. coli*	Phenylsuperose column	8.5 A_{280} U/mL, 125 m*M* NaCl, 50 u*M* $ZnCl_2$, 25 m*M* Tris-HCl (pH 7.5)	30% PEG 3400, 0.1 *M* sodium acetate (pH 6.0); sitting drops

(continued)

Table 1 (continued)

Complex	Ligand: receptor stoichiometry	Diffraction resolution (Å)	Expression system	Purification method	Protein solution	Precipitant
IFN-γ:IFN-γRa ***(17,18)***	1:2	2.9	Human IFN-γ and IFN-γRa (9–231) expressed in *E. coli*	Size exclusion with Superose 75	13 mg/mL	1.6 *M* ammonium phosphate in 0.1 *M* Tris-HCl (pH 8.8)
IFN-γ:IFN-gR ***(19)***	1:1	2.0	Human IFN-γ mutant and IFN-γR (1-229) expressed in *E. coli*	Size exclusion with S-100	8 mg/mL, 10 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.5)	8% PEG 8000, 0.1 *M* Tris-HCl (pH 8.5)
IFN-γ:IFN-γR ***(20)***	1:2.5	3.0	Human IFN-γ expressed in *E. coli*; human IFN-γR expressed in baculovirus	Preparative electrophoresis on native gels	20 mg/mL	Precipitant 14–16% PEG 8000, 1% β-octyl glucoside, 0.1 *M* Tris-HCl (pH 8.0); microbatch crystallization under paraffin oil
IL-1b:IL-1R ***(21)***	1:1	2.5	Human IL-1β and IL-1R (1–315) expressed in *E. coli*	Size exclusion with Superdex 75	5 mg/mL	1.8 *M* $(NH_4)_2SO_4$, 0.1 *M* MES (pH 6.0)

IL-2:IL-2R ***(22)***	1:1	3.5	Human IL-2 expressed in *E. coli*; human IL-2R mutant expressed in eukaryotic cells	IL-2R desialylated	10–15 mg/mL, 0.1 *M* ammonium acetate (pH 6.1)	1.7–1.8 *M* $(NH_4)_2SO_4$, 0.1 *M* imidazole (pH 7.5–8.4)
IL-4:IL-4Ra ***(23,24)***	1:1	2.3	Human IL-4 and IL-4Ra expressed in *E. coli*	Size exclusion with Sephacryl S-200	10 mg/mL, 120 m*M* NaCl, 25 m*M* sodium acetate (pH 5.0)	20% ethanol, 5% MPD, 0.1 *M* Tris-HCl (pH 8.0)
IL-10:IL-10R1 ***(25)***	2:4 (two dimers and four monomers)	4.5	Human and viral IL-10 expressed in *E. coli*; human IL-10R1 expressed in myeloma cells	Size exclusion with Superose 12; IL-10-R1 deglycosylated	16 mg/mL, 150 m*M* NaCl, 10 m*M* sodium phosphate (pH 6.9)	12% PEG 6000, 0.1 *M* sodium citrate (pH 5.6), 0.1 *M* Li_2SO_4.
NGF:TrkA-d5 ***(26)***	1:2	2.2	Recombinant human NGF; human TrkA expressed in *E. coli*	Size exclusion with S-75	10 mg/mL in 0.1 *M* NaCl, 0.1 *M* bicine (pH 8.5)	24% PEG 3350, 0.1 *M* citric acid (pH 5.0)
OPL:PROR ***(27)***	1:2	2.3	OPL and rat PROR expressed in *E. coli*	Size exclusion with S-100	9 mg/mL, 0.1 *M* NaCl, 0.05 *M* Tris-HCl (pH 7.5)	15% PEG 4000, 15% 2-propanol, 1% MPD, 0.1 *M* MES (pH 5.6)
TGFβ-1:FKBP12 ***(28)***	1:1	2.6	Human TGFβ-1 and human FKBP12 expressed in *E. coli*	Size exclusion with Superdex 75	20 mg/mL in 10 m*M* $MgCl_2$	1.40–1.53 *M* $(NH_4)_2SO_4$, 0.5 *M* NaI, 0.1 *M* Tris-HCl (pH 8.3–8.6)

(continued)

Table 1 (continued)

Complex	Ligand: receptor stoichiometry	Diffraction resolution (Å)	Expression system	Purification method	Protein solution	Precipitant
TNF-β:TNFR ***(29,30)***	1:3	2.85	Human TNF-β expressed in *E. coli*; human TNFR expressed in baculovirus	Size exclusion with Superose 12	20 mg/mL, 0.025 *M* NaCl, 0.01 *M* HEPES (pH 7.0)	15% PEG 4000, 0.2–0.4 *M* $MgCl_2$, 2% β-octyl-glucoside, 0.1 *M* sodium cacodylate (pH 5.5)
VEGF:Flt-1 ***(31)***	1:1	1.7	Human VEGF and human FLT-1 (129–229) expressed in *E. coli*	Size exclusion with S-200	7 mg/mL, 0.02 *M* Tris-HCl (pH 7.5)	30% PEG 4000, 0.2 *M* $(NH_4)_2SO_4$, 0.15 *M* Tris-HCl (pH 8.5)

[a]All crystals were grown with the hanging drop method unless specifically noted.

Table 1 were purified by this technique. Another potential problem is glycosylation, because some cytokines and receptors are normally glycosylated. Most of the proteins listed in **Table 1** were cloned and expressed in *Escherichia coli*, which produces unglycosylated protein. However, in at least two instances, the investigators used enzymatic procedures to deglycosylate the protein, and, in both cases, this seemed to yield better crystals. Most crystallization trials are carried out at a protein concentration of 5–10 mg/mL (*see* **Note 3**). Proteins may be conveniently concentrated with Centriprep centrifugal filter devices, which are available with various molecular weight cutoffs.

Relatively few primary reagents are needed for protein crystallization (see **Notes 4–6**). For screening initial crystallization conditions, the most useful tools are the various sparse matrix crystallization screening kits manufactured by Hampton Research (Laguna Niguel, CA; www.hamptonresearch.com). There are a number of these that are commercially available, but the three most popular are Crystal Screen, Crystal Screen 2, and MembFac. The Hampton Crystal Screen tests 50 conditions, whereas Crystal Screen 2 and MembFac each test 48 sets of conditions. Thus, with as little as 150 µL of protein solution, one can screen a large number of conditions with a fairly high probability of success. Although these broad screens are not always successful in yielding usable crystals, they usually provide a starting place for further screening.

For optimization after initial conditions are identified, the primary reagents required are ammonium sulfate and polyethylene glycol (PEG) of various molecular weights (*see* **Note 7**). The crystallization medium is almost always buffered; typical buffers include acetate, citrate, MES, HEPES, and Tris-HCl. Occasionally, other ions may also be added; the most common additives are various salts of chloride and sulfate.

By far the most common technique for protein crystal growth is vapor diffusion, generally in the form of hanging drops. Most experiments are set up using 24-well Linbro plates (Linbro model 76-0033-05) designed for cell culture. This technique also requires silicone grease and siliconized round cover slips. Glass cover slips may be purchased and siliconized using an organosilane solution such as Prosil 28, or siliconized cover slips may be purchased from Hampton Research. Solutions may be dispensed with micropipet tips or a 10-µL microsyringe.

3. Methods

1. Prepare the cytokine–receptor complex by mixing stoichiometric amounts of ligand and receptor (*see* **Note 1**).
2. Separate the intact complex from uncomplexed ligand or receptor by size exclusion chromatography (*see* **Note 2**).

3. Concentrate the protein solution to 5–10 mg/mL (*see* **Note 3**).
4. Prepare and dispense 1 mL of your various reservoir solutions into the wells of a Linbro plate (*see* **Note 6**).
5. Apply a thin layer of grease to the rim of each well in the Linbro plate.
6. Using a microsyringe, dispense 1–5 µL of your protein solution onto a siliconized cover slip (*see* **Note 8**).
7. Rinse the syringe chamber one or two times using distilled water.
8. Dispense 1–5 µL of your reservoir solution onto the cover slip and gently mix with the tip of the syringe.
9. Invert the cover slip and place it over the well containing your precipitating agent. Be sure that the seal is complete so that the chamber is airtight.
10. Place the tray in a constant temperature chamber (*see* **Note 9**).
11. Evaluate the crystallization trays at least every other day with a stereoscopic microscope (*see* **Note 10**).
12. If seeding is required, the following technique is suggested. To obtain seeds, crystalline aggregates or small crystals may be crushed with a needle and stirred (*see* **Note 11**).
13. Streak a fresh drop with the needle. Small perfect crystals will often grow along the needle trail through the drop.
14. Wash these single crystals in a stabilizing solution with a slighter lower concentration of precipitant than is present in the well.
15. Transfer the seed with as little liquid as possible to 2–4 µL drops containing the complex and the precipitant at a slightly lower concentration than was used to obtain the original seed crystals.
16. Equilibrate these drops against 1 mL of the precipitant.

4. Notes

1. Preparation of the cytokine–receptor complex obviously infers highly purified quantities of both the ligand and receptor. Stoichiometric mixing is most likely to yield success, but in cases where the stoichiometry is not known with certainty, the two proteins may simply be mixed in a 1:1 ratio. Successful formation of a growth hormone–receptor complex with 1:2 stoichiometry was accomplished, even though the initial mixing ratio was 1:1 ***(1)***.
2. A common procedure in all the reported cytokine–receptor complexes is some type of chromatography, e.g., size exclusion chromatography, to separate the complex from unliganded cytokine or receptor. Because crystallization infers regular arrangement of identical units, any contamination will make the growth of usable crystals less likely. Furthermore, some macromolecules have a strong tendency to aggregate, so the addition of a non-ionic detergent may help ensure monodispersity.
3. Although crystals have been grown from solutions containing 1–20 mg/mL, the most common concentration range is 5–10 mg/mL.
4. The hanging drop method is quite straightforward. In a typical experiment, 2–10 µL protein drops are suspended over a 1-mL reservoir solution. The reservoir solution is comprised of a precipitating agent that is buffered at a particular pH. The

protein drop contains 1–5 μL of protein solution and usually an equal volume of the reservoir solution. The two solutions are usually dispensed with a 10 μL microsyringe, mixed on a siliconized cover slip, and then inverted over the well. An airtight chamber is formed by placing a small bead of silicone grease around the circumference of the cover slip prior to inversion. Thus, the starting concentration of precipitating agent in the reservoir solution is twice that in the protein drop. The concentration gradient causes the aqueous solution in the protein drop to be slowly transferred through the vapor phase to the more concentrated reservoir solution until the two are in equilibrium. Crystal growth is induced as the protein is concentrated in the drop.

5. The Hampton Crystal Screen tests 50 crystallization conditions, whereas Crystal Screen 2 and MembFac each test 48 sets of conditions. Thus, with only about 150 μL of protein solution, one can screen a large number of conditions with a fairly high probability of success.
6. One of the most attractive features of a hanging drop experiment in Linbro plates is the opportunity to do a two-dimensional grid search for refining crystallization conditions. Typically, the two parameters that are varied are pH and the primary precipitating agent. For example, one might screen six pH values at intervals of 0.2 and four concentrations of ammonium sulfate at intervals of 0.2 *M*.
7. The most popular precipitating agents were PEG of various sizes (average molecular weight 1500–8000) and ammonium sulfate. PEG was used for 13 complexes, and ammonium sulfate was used in 8 complexes. The only other primary precipitating agents were ammonium phosphate and ethanol (one example each). *See* **Table 1**.

   The most common salt additive was NaCl, which was used in more than half of the cytokine–receptor complexes. This probably has more to do with the fact that proteins are usually eluted from columns with a NaCl-containing solution rather than any special properties of NaCl in the crystallization mixture. Others that were occasionally used include $CaCl_2$, Li_2SO_4, $(NH_4)_2SO_4$, $MgCl_2$, NaI, and $ZnCl_2$. The most common organic additive was 2-methyl-2,4-pentanediol (MPD; four complexes); others used were β-octylgluoside (two complexes) and glycerol, 1,4-dioxane, isopropanol, and propanol (one each).

   Although almost any buffer may be used, we have found that phosphate buffer is usually a poor choice, because many phosphate salts are insoluble and may crystallize, giving the crystallographer a transient feeling of success. Furthermore, many heavy metals used in preparing derivatives will precipitate in the presence of phosphate. According to **Table 1**, Tris is by far the most popular buffer, and MES is the second most commonly used. For trials at lower pH, the favorite buffers appear to be acetate and citrate.
8. Although micropipet tips may be used to dispense the solutions, we have found that the microsyringe is easier to use and accurately delivers the amount you desire. Furthermore, the fine tip of the needle allows gentle mixing of the drop.
9. In a typical laboratory the temperature in the room may vary by ±2°C. Although this may not be crucial, some proteins require less variation for optimal crystal growth. All experiments should be performed at 4°C and room temperature,

because the processes of nucleation and crystal growth may differ radically at these two temperatures.

10. Even if no crystals are obtained, the presence and amount of precipitated protein can generally be used to guide further experiments. Experiments should not be discarded as long as the drops are still liquid, because crystals have been observed only after several months in some cases.
11. Unfortunately, sometimes crystals only grow as aggregated plates and needles. In order to grow large single crystals suitable for X-ray diffraction, a macroseeding technique may be used. One such technique used to grow large perfect crystals has been described in detail in the crystallizations of a ubiquitin conjugating enzyme (UBC1) ***(2)*** and interleukin-10 (IL-10) ***(3)***. For example, UBC1 crystals were originally grown from solutions containing 35% saturated ammonium sulfate in 0.05 *M* MES buffer (pH 6.7). After 2–3 d at room temperature, aggregates of orthorhombic crystals with dimensions up to $0.5 \times 0.2 \times 0.1$ mm were obtained. To obtain seeds, crystalline aggregates were crushed with a needle and stirred; a fresh drop was then streaked with the needle. A number of small perfect crystals generally grew along the needle trail through the drop. These single crystals ($0.05 \times 0.02 \times 0.03$ mm) were washed in a stabilizing solution of 30% saturated ammonium sulfate in 0.05 *M* MES buffer, pH 5.5. They were then transferred with as little liquid as possible to 2-µL drops containing 18 mg of protein/mL and 25% saturated ammonium sulfate in 0.05 *M* MES, pH 5.5. Introduction of the seed crystals into the drops was accomplished by using a 0.5-mm capillary and a micromanipulator. These drops were equilibrated against 1.0 mL of 25% saturated ammonium sulfate in 0.05 *M* MES, pH 5.5. After 2–4 d, large plate-like crystals with dimensions up to $0.6 \times 0.4 \times 0.2$ mm were obtained. With careful seeding technique, only one crystal grew in each drop. This technique has been used a number of times in our laboratory with various protein crystals.

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9

Biosensor Analysis of Receptor–Ligand Interactions

Sheng-Jiun Wu and Irwin Chaiken

1. Introduction

1.1. The Biosensor as a Unifying Methodology for Measuring Cytokine–Receptor Interactions

Cytokines trigger growth factor responses through interaction with cell surface receptors. Determining the quantitative properties of cytokine–receptor interactions can help lead to mechanistic understanding of receptor activation as well as strategies to inhibit the interactions and, consequently, antagonize activation. For many cytokine systems, molecular tools are available for mechanism-revealing interaction analysis. These tools can include wild-type and mutant forms of cytokine, soluble domains of the multiple receptor subunits, phage-displayed forms of cytokine and receptor (often obtained for combinatorial epitope mapping and antagonist design studies), and membrane-incorporated forms of receptors such as in vesicles or pseudoviral particles. In the face of such tools and promise of useful new mechanistic knowledge through their study, a key need remains unifying interaction analysis methodologies, techniques which can provide a reasonable level of kinetic and thermodynamic rigor and can be applicable across the broad range of molecular forms that incorporate cytokine and receptor. One such methodology that has significant promise is optical biosensor kinetics analysis. This technology has successfully been applied in cytokine-receptor interactions, such as interleukin 2 (IL2) ***(1,2)***, interleukin 5 ***(3–7)***, and GM-CSF ***(8,9)***.

1.2. Principles of Optical Biosensors

Optical biosensors monitor molecular interactions in real time (*see* **Fig. 1**). Measurements can be made using molecules in their native states, without the need for radioisotopic, fluorescent, or enzymatic labeling. Applications of optical biosensors include determination of biomolecular specificity, concen-

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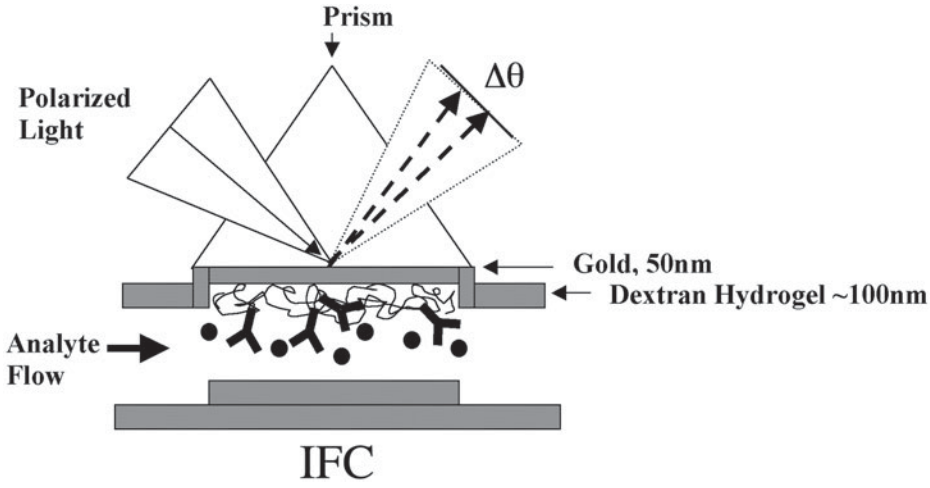


Fig. 1. Schematic diagram of optics and signal generation of the BIAcore SPR biosensor. Polarized light is shone on the sensor surface "mirror" side. Θ Is the angle at which resonance occurs at time = 0 and is detected as an extinction of light. As mass accumulates at the sensor surface, ΔΘ is monitored. The light source, the detector, the glass prism, and the sensor surface (top part of diagram) constitute the transducer block. The flow cell component is referred to as the integrated microfluidic cartridge (IFC). Reproduced with permission from Canziani et al. ***(10)***.

tration, affinity, kinetics, cooperativity, and relative binding patterns. Broadly, optical biosensors transduce the accumulation of mass at a surface into an optical signal. Coating the sensor surface with purified ligands confers selectivity. Optical biosensors can detect a stable signal by binding as little as femtomoles of soluble interactant (analyte) to immobilized ligand over seconds to hours. At least three types of optical biosensors have been introduced. They are BIAcore surface plasmon resonance (SPR) biosensors (Uppsala, Sweden), IAsys resonant mirror sensors (Affinity Sensors, Cambridge, UK), and KinExA kinetic exclusion assay instrument (Sapidyne Instruments, Boise, ID). A Lunascan sensor based on fiber optic technology is in development (Luna Analytics, Blacksburg, VA). Other optical biosensors are in development. BIAcore biosensors are currently the most widely used and are described below.

1.2.1. The Sensor Chip

The sensor chip of the BIAcore technology is a signal transducer consisting of three layers: a glass support, a 50-nm-thin gold film, and a surface matrix. The gold film is chosen for SPR response. A layer of dextran matrix on which ligand can be immobilized is bound covalently to the gold film through a linker

layer. In BIAcore, the continuous flow of analyte contacts the dextran matrix surface of the chip in the flow cell, while the gold film is illuminated from the glass side. SPR is generated through the interaction of light energy with the gold film ***(10)***. Flow minimizes the thickness of the diffusion layer and maximizes the contact between analyte and immobilized ligand at the sensor surface.

1.2.2. The SPR Detection System

Surface plasmon resonance has been applied for the analysis of molecular interaction in BIAcore instruments. This methodology monitors interactions between two molecules, such as protein–protein, protein–sugar, protein–lipid, and protein–DNA. The SPR response is detected on a real-time basis by using a wedge of incident light and a fixed array of detectors. Detection of binding and dissociation events that lead to increase and decrease, respectively, of mass concentration at the surface of the sensor chip is the direct consequence of refractive index change at the interface between the solution and the surface. In SPR, a change in refractive index leads to altered refraction of incident polarized light, leading in turn to a change in angle of absorbance maximum by plasmon electrons of the gold film of the sensor chip at the water interface ***(11–13)***. Hence, the shift in angle can be correlated with the mass concentration of the analyte molecule bound at the surface layer ***(14)***. A sensorgram is a recording of changes in the resonance signal as a function of time.

The processed resonance signal is given in resonance units (RU), where 1000 RU represents a shift in resonance angle of 0.1° and corresponds to a change in surface concentration on the sensor chip of about 1 ng/mm^2 for proteins ***(15)***. The high signal-to-noise (SNR) ratio achieved by SPR permits binding of molecules as small as 200 Daltons to be detected. Kinetic models have been applied successfully to interactions of molecules as small as 15 kDa ***(16)***.

1.2.3. Integrated Microfluidic Cartridge (IFC)

BIAcore system uses a continuous flow system, by which reagents, buffers, and samples are delivered to the sensor chip surface (through a precision liquid-handling system). The motor-driven syringe pumps are designed for buffer flow and autosampler functions. Liquid samples are delivered to the sensor surface through the IFC. The IFC is pressed into contact with the sensor chip. Channels in the IFC are managed through microcomputer-controlled pneumatic microvalves. In consequence, switching between analyte and running buffer can be automatically operated through the instrument software. In BIAcore X (semiautomatic system), sample is injected manually into the IFC injection port. In BIAcore 2000 or 3000 systems (fully automated), sample can be automatically mixed, diluted, and delivered to the sensor surface by programming the instrument. Current instruments provide either two or four cells on the sen-

sor chip surface depending on the IFC design in different instrument models. The technology to combine the liquid-handling system of the IFC with the SPR detection system makes the real-time analysis of molecular interactions possible with very small amounts of sample.

2. Materials

2.1. The BIAcore System

1. A bench-top BIAcore instrument.
2. An associated personal computer.
3. Sensor chip CM5 (*see* **Note 1**).

2.2. Running Buffers

All running buffers should be filtered through a 0.22-μm filter and thoroughly degassed before use.

1. HBS-EP buffer: 10 m*M* HEPES, pH 7.4, 0.15 *M* NaCl, 3 m*M* ethylenediaminetetraacetic acid (EDTA), 0.005% Surfactant P20, recommended as standard running buffer in BIAcore.
2. Phosphate-buffered saline (PBS) pH 7.4, containing 0.005% Tween-20 or Surfactant P20.

2.3. Reagents for Immobilization

1. 0.2 *M N*-ethyl-*N*'-(dimethylaminopropyl)carbodiimide (EDC).
2. 0.05 *M N*-hydroxysuccinimide (NHS).
3. 10 m*M* sodium acetate buffer (pH ranged from 4.0 to 5.5), used to prepare ligand immobilization solutions (*see* **Subheading 3.1.1.**).
4. 1 *M* ethanolamine-hydrochloride, pH 8.5.

2.4. Regeneration Solution

1. 10 m*M* HCl.
2. 10 m*M* glycine-HCl, pH 2.5.
3. 50 m*M* NaOH.
4. 1 *M* NaCl.

3. Methods

Biosensor analysis of the cytokine–receptor interactions involves two types of molecules (**Fig. 2**). One of the interacting molecules, defined as the ligand, is immobilized on the surface of the sensor chip. The other molecule, the analyte, is injected into the IFC and flows over the chip surface. Based on the immobilization methods, two protocols for simple two-component interactions are described: direct covalent coupling (*see* **Subheading 3.1.**) and oriented immobilization / capturing method (*see* **Subheading 3.2.**) (*see* **Fig. 3**). A three-component interaction system is cited in **Note 2**.

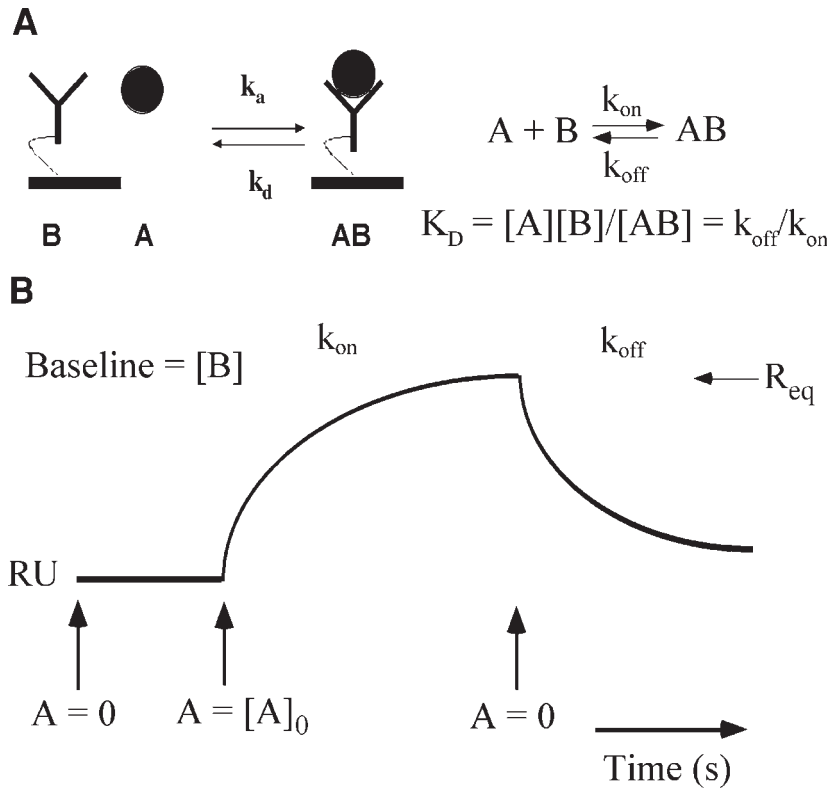


Fig. 2. The 1:1 interaction binding model and a binding sensorgram. **(A)** Interactants at biosensor surface. *A* represents the analyte in solution phase; *B* represents the immobilized ligand. The kinetic model and affinity constant, K_d, are described at right side of the scheme. **(B)** A continuous plot of binding signal as a function of time results in the binding sensorgram. (Figure adapted from Rux, A., personal communication.)

3.1. Direct Covalent Coupling of Ligand

3.1.1. Preparation of Ligand

The purity of cytokine or receptor used as the ligand should be 90% homogeneous or greater (*see* **Note 3**). Any additives containing primary amine groups or strong nucleophiles, e.g., Tris-HCl buffer, sodium azide, must be removed before use. In addition, from commercial products, BSA, added as a stabilizer, should be removed as well. For electrostatic preconcentration to take place, ligand should have a net positive charge. Choice of pH for immobilization can be made if the p*I* of the specific ligand is known. For p*I* 3.5–5.5, use 0.5 pH unit below p*I*; for p*I* 5.5–7.0, use 1 pH unit below p*I*; for p*I* > 7.0, use pH 6.0.

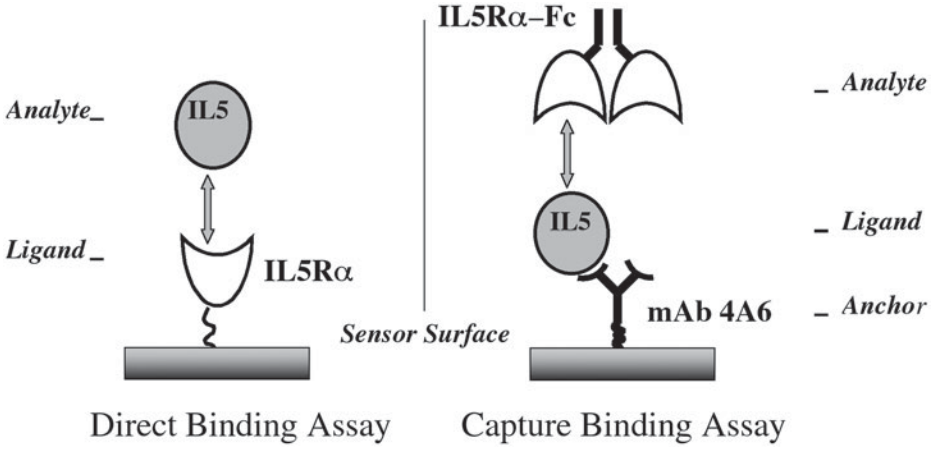


Fig. 3. Examples of orientation options used for biosensor kinetics analysis of IL-5 interaction with its receptor α-subunit. **Left**: direct binding assay: IL-5Rα is immobilized covalently onto the sensor surface. Binding of IL-5 to receptor was detected by the change in refractive index at the surface due to the increase in mass. **Right**: capture binding assay: IL-5 was captured via monoclonal antibody 4A6 (MAb 4A6). The chimeric receptor IL-5Rα-Fc (or soluble IL5Rα) interacting with IL-5 molecule was detected as described in direct binding assay. Figure adapted from Chaiken et al. ***(17)***.

3.1.2. Immobilizing Ligand Using Amine Coupling Method

1. Dock sensor chip CM5 in the BIAcore instrument. Initiate sensorgram recording with continuous flow buffer (HBS-EP or PBS containing Tween-20) at a flow rate of 5 µL/min using the BIAcore control software.
2. Activate the carboxyl groups on the dextran surface with a 7-min pulse of 0.05 *M* NHS/0.2 *M* EDC (*see* **Note 4**).
3. Inject a 7-min pulse of ligand solution (typically 10–200 µg for cytokine or receptor). The amount of immobilized ligand can be adjusted using the "Manual injection" mode to control the length of the ligand pulse (*see* **Note 5**).
4. Deactivate excess reactive carboxyl groups on the chip surface with a 7-min pulse of 1 *M* ethanolamine hydrochloride, pH 8.5.
5. To remove noncovalently bound material from the sensor surface, high salt solution and/or high- or low-pH regeneration buffer are used. Inject 25 µL of 10 m*M* HCl or 10 m*M* glycine-HCl, pH 2.5, at the flow rate 100 µL/min. Repeat this regeneration step two to three times (*see* **Note 6**).
6. One flow cell in the same sensor chip is used as a blank control. This blank flow cell is crucial to test if there is nonspecific binding of analyte to the sensor chip. Therefore, a flow cell needs to be activated with NHS/EDC and exposed to BSA or other "control" protein to the same level as the ligand. Follow **steps 1–5** above.

3.1.3. Detecting Ligand-Analyte Interaction

1. Analyte concentrations chosen around the value of dissociation constant K_d will be a starting point. Generally, analyte concentrations ranging from 1 to 100 nM in a series of five or six dilutions minimum will cover the K_d values from 2×10^{-10} to 2×10^{-7} *M*. The homogeneity of analyte should be as high as possible (*see* **Note 3**).
2. To minimize the bulk effect, running buffer should be used as the solvent to prepare the analyte (i.e., use PBS containing Tween-20 to dilute cytokine or receptor).
3. The amount of the analyte needed for each injection could be in the range of 50–100 µL. A flow rate of 30 µL/min is recommended for pilot experiments (*see* **Note 5**).
4. After the end of each analyte concentration measurement, regeneration of the ligand surface is important to ensure reliability of subsequent measurements. To wash away noncovalently bound analyte from the sensor surface, high-salt solution and/or high- or low-pH regeneration buffer are used. Inject 25 µL of 10 m*M* HCl or 10 m*M* glycine-HCl, pH 2.5, at a flow rate of 100 µL/min. Repeat this regeneration step one or more times (*see* **Note 6**).
5. Repeat the injection and regeneration cycles for different concentrations of analyte to obtain sensorgrams for cytokine-receptor interactions.
6. Analysis of the kinetic data for cytokine-receptor interactions is described under **Subheading 3.3**.

The prototype biosensor analysis is shown in **Fig. 4**.

3.2. Oriented Immobilization/Ligand Capture Method

Ligand-capturing methods (**Fig. 3**) can enhance both the specificity and sensitivity of the binding interaction. A capturing molecule is first immobilized on the sensor chip. The ligand is not covalently bound to the surface. The critical requirement for an analyte is that it binds to ligand at a site, which is independent of the anchoring site. There are several applications of capture methods, for example, immobilized monoclonal antibody capturing specific antigen ***(5,18,19)***; immobilized streptavidin capturing biotinylated ligand; immobilized anti-*His*-tagged antibody capturing *His*-tagged fusion protein; and immobilized anti-GST antibody capturing GST fusion protein. This method also can be used as an *in situ* affinity purification of ligand, so that ligand from crude preparations can be selectively anchored. In capture techniques, the anchoring molecule can orient the ligand to bind to analyte with increased specificity vs directly immobilized ligand, as the ligand will be more homogeneously oriented (presumably with binding site exposed) by the anchor than it would by random direct immobilization. In addition, because the ligand is captured in each cycle, inactivation effects caused by exposure of ligand to harsh regeneration conditions can be avoided. Here, we describe the capture protocol using immobilized monoclonal antibody as anchor.

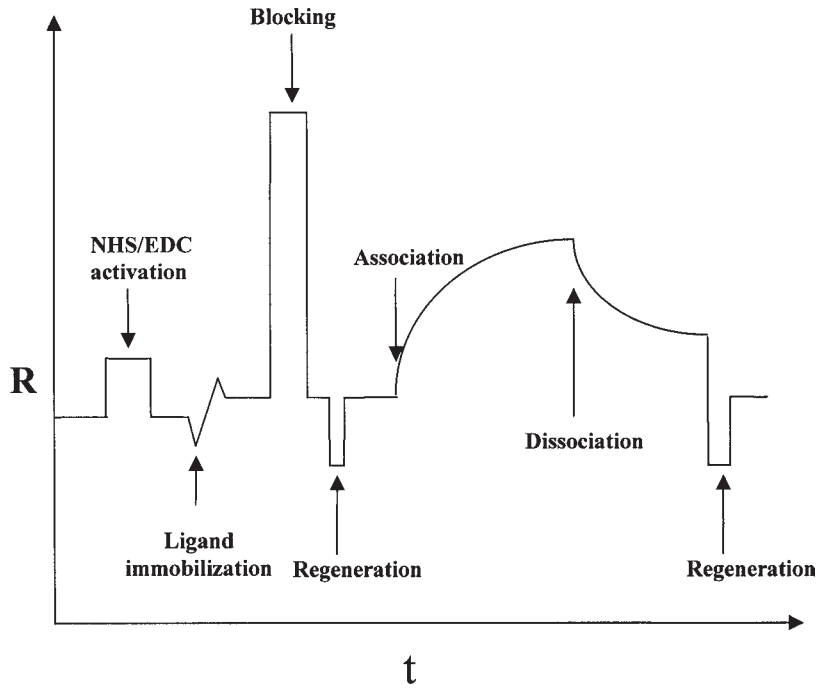


Fig. 4. Prototype of biosensor sensorgram. This diagram shows the stages of activation, ligand immobilization, blocking, regeneration, analyte binding curve (association and dissociation of analyte to ligand), and regeneration of sensor surface for next cycle of binding. Reproduced from Canziani et al. ***(10)***.

3.2.1. Immobilizing Monoclonal Antibody Using Amine Coupling Method

1. Dock sensor chip CM5 in the BIAcore instrument. Initiate the sensorgram using the BIAcore control software, with HBS-EP buffer and a flow rate of 5 µL/min.
2. Aliquot the immobilization reagents (NHS, EDC, and ethanolamine) for the amine coupling method as described under **Subheading 3.1.2.**
3. Dilute the monoclonal antibody to 30 µg/mL with 10 m*M* sodium acetate buffer, pH 5.0. Start the immobilization procedure as described under **Subheading 3.1.2.**, **steps 2–6**. Use 10 m*M* glycine-HCl, pH 2.5, in multiple regeneration steps.

3.2.2. Capturing Ligand on the Sensor Surface

1. Dilute the ligand (cytokine, e.g., IL-5) to 50–100 µg/mL with HBS-EP buffer.
2. Inject ligand solution at a flow rate of 5 µL/min using "Manual injection." The same amount of ligand anchored can be adjusted in each cycle.

3.2.3. Detecting Anchored Ligand and Analyte Interaction

1. Inject analyte at a flow rate of 30 µL/min to detect ligand–analyte binding.
2. After the association–dissociation cycle, the ligand is removed in the regeneration step.
3. Start a new cycle and repeat the capture, binding, and regeneration steps for different concentrations of analyte to obtain sensorgrams of cytokine–receptor interactions.

3.3. Kinetic Data Analysis

Biosensor binding data can be used for both qualitative and quantitative analysis. Evaluation of rate and affinity constants of these interactions is intrinsically informative to investigate cytokine–receptor interaction mechanism. Both kinetic parameters and binding affinities can be derived from the sensorgrams. This section focuses on experimental aspects of rate and affinity constant determination using the simplest 1:1 interaction model. A comprehensive treatment of the mathematics of kinetic measurements and details of how to use the BIAevaluation Software supplied with BIAcore can be found in the User's Manual.

3.3.1. Basic Kinetic Analysis

The 1:1 binding model between analyte and ligand, equivalent to the Langmuir differential equation ***(20)***, takes the immobilized ligand as a solid-phase component, and assumes that rapid mixing of the analyte from bulk phase to the sensor surface layer is a first-order surface adsorption process.

Kinetic analysis can be performed as described in detail by Karlsson et al. ***(21)***. A two-component model for a biosensor interaction may be described as

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB \tag{1}$$

where A is the analyte and B is the immobilized ligand. The rate equation for the formation of AB complex is given by

$$d[AB]/dt = k_{on}\,[A][B] - k_{off}\,[AB] \tag{2}$$

In Biosensor experiments, eq. 2 can be expressed in terms of the SPR signal as

$$dR/dt = k_{on}\,[A](R_{max} - R) - k_{off}\,R \tag{3}$$

which can be rearranged to eq. 4,

$$dR/dt = k_{on}\,[A]\,R_{max} - (k_{on}\,[A] + k_{off}\,)R \tag{4}$$

where dR/dt is the rate of change of the SPR signal; $[A]$ is the concentration of analyte; R_{max} is the maximum binding signal in RU; R is the SPR signal in RU at time t.

For the dissociation phase, when $[A] = 0$, eq .3 reduces to

$$dR/dt = - k_{off} R \tag{5}$$

3.3.2. Linear Transformation of Sensorgram Data

The interactions between cytokines and their receptors have been analyzed by BIAcore biosensors. In one configuration, single-chain IL-5 (scIL-5) was immobilized on the sensor chip (**Fig. 5A**). Sensorgrams were obtained with injections at various concentrations of soluble human IL-5Rα (shIL-5Rα). The data in **Fig. 5A** were analyzed by linear transformation according to the least-squares fit. In the association phase, the term ($k_{on}[A] + k_{off}$) in eq .4 is defined as k_s. Hence, k_s values can be obtained from the slopes of dR/dt vs R plots at various values of $[A]$ (**Fig. 5B**), and k_{on} can be subsequently determined from the slope of k_s versus $[A]$ (**Fig. 5C**). In the dissociation phase, eq. 5 can be rearranged to

$$\ln(R_0/R_n) = k_{off}(t_n - t_0) \tag{6}$$

where R_0 is the response at t_0, the beginning of the dissociation (or wash) phase. Thus, k_{off} can be obtained as the slope of the $\ln(R_0/R_n)$ vs $t_n - t_0$ (**Fig. 5D**). On this basis, the dissociation constant K_d was determined from the ratio k_{off}/k_{on}.

3.3.3. Nonlinear Curve Fitting

Alternatively, kinetic parameters can be derived from sensorgram data by fitting the experimental curves to the integrated rate equations ***(22–24)***. BIAevaluation Software provides global fitting algorithms for evaluating kinetic data. This analysis fits both association and dissociation data simultaneously at a series of analyte concentrations. The global fitting provides the best set of parameters that fits the whole data set. Sensorgrams for this analysis are shown in **Fig. 6**. Kinetic association and dissociation rate constants were obtained by global fitting of the data to a simple 1:1 binding model. Although using nonlinear curve fitting is straightforward, it is important to note that a proposed model is a tool for interpretation of data, and a reasonable fit of data to a model does not necessarily prove that the model is a correct representation of the interaction mechanism at the sensor surface. A complete use of biosensor binding data should include fitting to rate equations of various interaction models and consequent kinetic analysis.

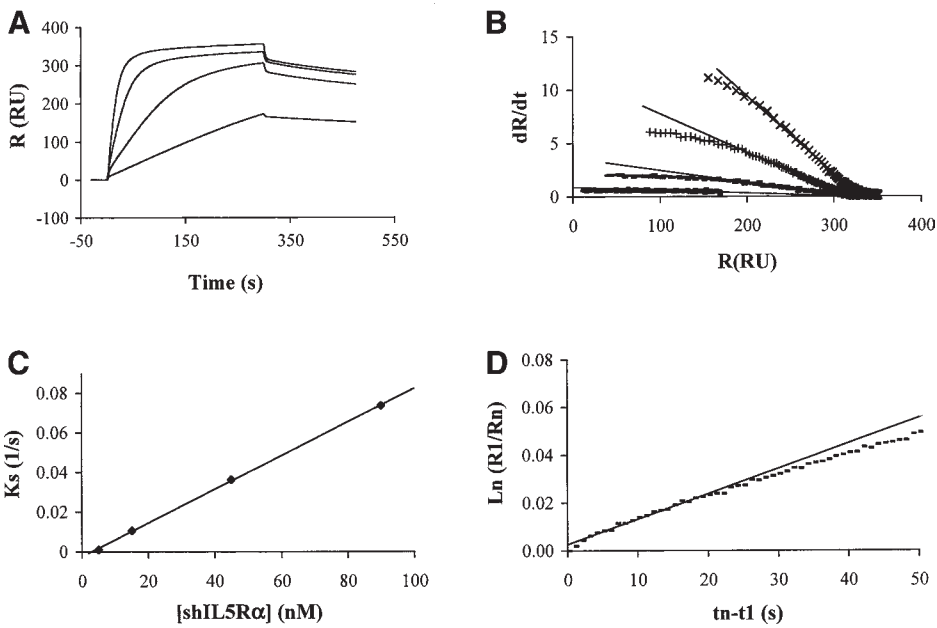


Fig. 5. Example of linear transformation of sensorgram data from a study ***(6)*** of *Escherichia coli*-expressed scIL-5 and its interaction with IL-5R-α. The binding interaction of IL-5 with its receptor. **(A)** Sensorgram overlays showing binding by various concentrations of soluble IL-5Rα (5, 15, 45, and 90 n*M*) to IL-5. IL-5 was captured by MAb 4A6. **(B)** Calculation of on-rate constant for interaction of IL-5 with IL-5Rα. The association phases of the sensorgrams in **A** were replotted as the slope of the curve at a given time vs relative response at the time. The straight lines show the best fit of the data to a straight line. The slopes of these lines give values for k_s at each concentration. **(C)** Plot of k_s vs concentration. The slope of the line through these points gives the association rate constant. **(D)** Determination of dissociation rate constant k_{off}. The dissociation phase of the sensorgram at 90 n*M* IL-5Rα in **A** was replotted according to eq. 6 (using R_1 and t_1 in place of R_0 and t_0) as ln (response at time 0 of dissociation/response at time n) vs time. The straight line shows the best-fit line for the first 50 s of dissociation. The slope of the line gives the dissociation constant.

3.4. Steady-State Binding Analysis

Sensorgram data can be used to evaluate equilibrium binding constants. For equilibrium or steady-state determinations, use a range of analyte concentrations to bind 20–80% of the surface ligand. Ensure that *R* reaches its steady-state value (*R*eq) at any given [*A*], that is, to an *R* value at which no further increase in signal is detected as a function of time. In this analysis, the steady-

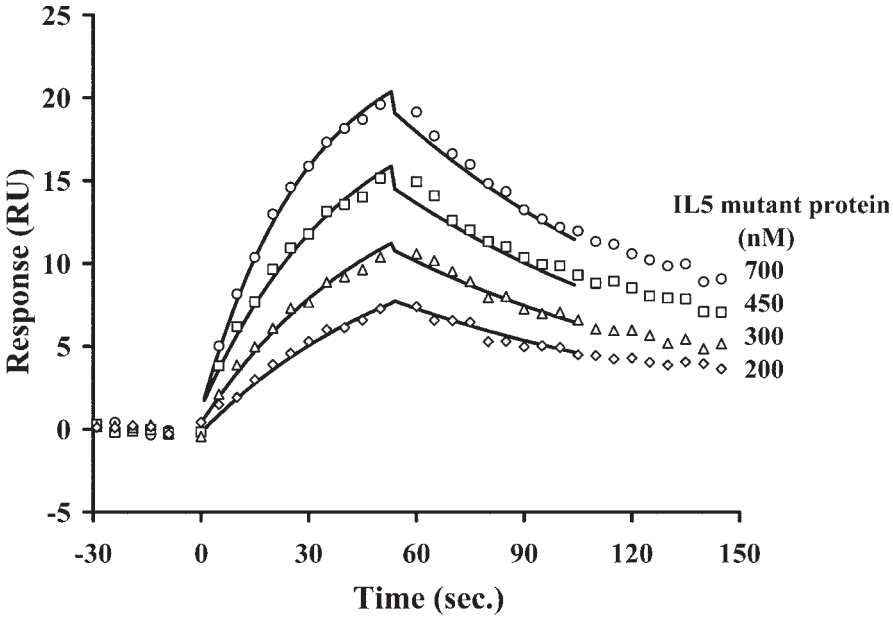


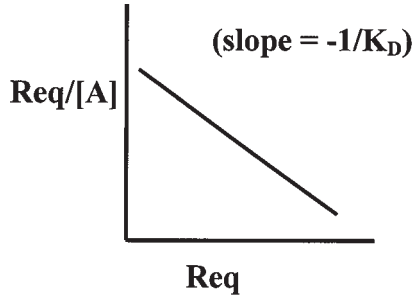
Fig. 6. Representation of global fitting algorithms for evaluating kinetic data using BIAevaluation Software. Sensorgram overlays for IL-5 mutant binding to IL-5Rα-Fc at different concentrations of analyte. The analyte concentrations are shown at the right of sensorgram. The sensorgram for IL-5 mutant protein binding directly to the immobilized IL-5Rα-Fc on the sensor chip, surface density 2000 RU. The rate constants for binding to IL-5Rα-Fc were calculated by globally fitting the association and dissociation phases of the sensorgrams to a 1:1 Langmuir model, $A+B \rightleftharpoons AB$, as described in **Subheading 3.** [Figure taken with permission from Plugariu et al. ***(7)***].

state amount bound, at each of a set of analyte concentrations, is determined by the plateau R value reached during sample injection, when $dR/dt = 0$ ***(24)***. The steady-state values of R (Req in **Fig. 2**) provide data that can be used to determine equilibrium affinity constants of the receptor–ligand interaction (**Fig. 7**). Affinity constants, K_a or K_d, also can be derived from the ratio of the kinetic rate constants as given by

$$K_a = k_{on}/k_{off} \quad \text{or} \quad K_d = k_{off}/k_{on} \tag{7}$$

where K_a is the equilibrium binding constant, and K_d is the equilibrium dissociation constant.

If the sensorgram data do not fit to the simple 1:1 model, other non–1:1 models may be tested. This may occur because the interaction is more complex



$$\mathbf{Req/[A] = - Req/ K_D + Rmax/ K_D}$$

Fig. 7. Scatchard plot analysis of K_d from steady-state response values for the binding of analyte (*A*) to immobilized ligand.

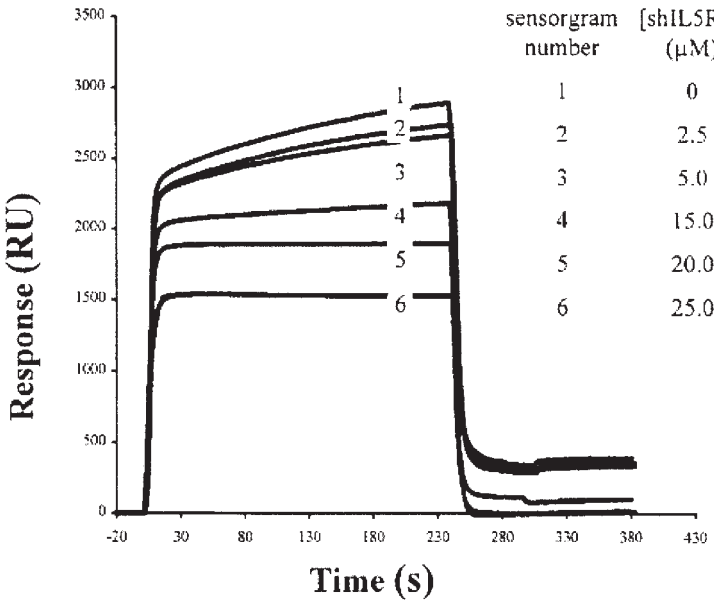
or artifacts distort the variation of response. Mechanistic conclusions from biosensor data, in particular, which model describes the macromolecular interaction, should be drawn cautiously. In addition, technical factors may complicate the data analysis (*see* **Note 7**).

3.5. Competition Binding Analysis

Two kinds of competitive binding experiments can be performed in BIAcore instruments. In one, "surface competition," two analytes compete for the same immobilized ligand. In the other, "solution competition," analyte can bind to either immobilized ligand or ligand in solution. In competition binding analysis, it is essential that two analytes compete for the same binding site on the immobilized ligand, or ligands in two phases compete for the same binding site on the analyte.

3.5.1. Surface Competition Binding

This approach can be used for rapid affinity ranking of different mimetics. For example, IL-5 receptor α chain can be immobilized on the sensor surface. A peptide or other test molecule of interest, mixed with IL-5, can compete with the IL-5 for binding to the receptor α chain. A decrease in the RU response in the binding phase is indicative of a competitor. Quantitative evaluation of the biosensor binding data can provide rate constant estimates for competitor. This approach is useful for low-molecular-weight competitors, because the binding signal will be low compared to that for IL-5.



sensorgram number	[shIL5Rα] (μM)	net bound (RU) after 60s wash
1	0	415
2	2.5	364
3	5.0	326
4	15.0	127
5	20.0	7
6	25.0	0

Fig. 8. Optical biosensor analysis of IL-5 phage competition binding to immobilized IL-5Rα-Fc. 14,000 resonance units of IL-5Rα-Fc were immobilized directly on the sensor chip. Single chain IL-5 phage at a titer of 6×10^{13} CFU/mL was preincubated with IL-5Rα (0–25 μ*M*) and then passed over the IL-5Rα-Fc surface for 4 min. The abrupt change of resonance signal at the beginning and end of association phases is due to differences in refractive index between the running buffer and phage samples. Inset, table showing the net amount of signal bound, after buffer wash of the sensor surface, vs the amount of IL-5Rα competitor. [Taken from Wu et al. ***(6)***].

3.5.2. Solution Competition Binding

In this assay, the analyte is mixed with the competitor in solution and allowed to reach equilibrium. The method can be referred to as an inhibition assay, because the competitor concentrations are known and the inhibition of binding of the analyte to the surface can be measured by BIAcore sensorgrams. Competition curves can be obtained from the plots of response (RU) vs concentrations of competitor. IC_{50}s can be derived from these competition curves. An example for this application is shown in **Fig. 8**. The shIL-5Rα protein was immobilized on the sensor surface. Single chain IL-5 (ScIL-5) phage samples, in which a scIL-5 was fused in frame with phage coat protein pIII and hence displayed on the phage surface, were preincubated at varying concentrations of sIL-5Rα and passed over the sensor surface ***(6)***. After washing with running

buffer, the net amount of phage bound to the immobilized shIL-5Rα decreased proportionately as the competitor concentration increased.

4. Notes

1. Sensor chip CM5, in which the matrix consists of non-crosslinked carboxymethylated dextran covalently attached to the gold film on the sensor surface, is the most widely used sensor chip for BIAcore instruments. BIAcore AB also provides other sensor chips for different applications. Those sensor chips include SA, streptavidin coated, for binding of biotinylated ligand; NTA, preimmobilized NTA nickel chelate, for capture of histidine-tagged ligand; HPA, hydrophobic surface coated for membrane biochemistry studies; and other pioneer chips that are described in the manufacturer's instructions.
2. A three-component biosensor-based interaction assay has been developed to investigate IL-2 binding recruitment of its receptor subunits ***(1,2)***. Analysis was carried out to compare the binding of IL-2 to biosensor surfaces containing the α-subunit, the β-subunit, or both subunits together. A detailed kinetic analysis indicated that the high-affinity binding results from capture of IL-2 by a preformed complex of IL-2R α and β subunits. Another phase of this study was the analysis of IL-2R complexes that contained the IL-2Rγ subunit. Sensorgrams were obtained by injection of the soluble form of IL-2Rγ, with or without IL-2 over an IL-2Rβ surface. This study indicated IL-2 was required for IL-2Rγ to bind IL-2Rβ. An IL-5 related three-component assay also has been developed and used to reveal the assembly of IL-5 with its two receptor subunits shIL-5 Rα and β_c ***(28)***.
3. A homogeneous ligand sample is essential for the direct immobilization method to simplify the evaluation of binding sensorgrams and to enable correct interpretation of rate constants. A heterogeneous ligand population may be caused by denaturation or partial unfolding during sample purification and immobilization. If the purity of ligand is a concern, the affinity capture method can be used to enhance ligand homogeneity. If the analyte samples are produced from recombinant techniques, heterogeneity may be introduced by posttranslational modifications. Empirically, aggregation of analyte may result in multivalent binding to the ligand. From our experience, GST fusion proteins are easily aggregated and cause multiphase sensorgrams, hence, they should be avoided. Using the appropriate buffer and gel filtration prefractionation may alleviate aggregation problems.
4. There are at least five immobilization methods available for covalently attaching ligands or capturing molecules on the surface of carboxymethylated dextran sensor chips. The immobilization chemistries include amine coupling, ligand thiol coupling, surface thiol coupling, aldehyde coupling, streptavidin–biotin affinity capture, and antibody–antigen capture (*see* **ref. *10*** for details). The amine coupling method is used most generally as an initial approach. However, acidic ligands (pI < 3.5) are difficult to immobilize by amine coupling, and ligands

containing active sites with reactive side chains such as nucleophilic groups also are not amenable to direct coupling. In these cases, other coupling methods should be sought.

5. For different experimental purposes, the amount of immobilized ligand and flow rate for analyte injection are varied ***(25)***. In practice, the immobilization level for detecting ligand–analyte interaction or screening for binding partners can be reached in the range of 1000–5000 RU, at a flow rate of 10 µL/min. If the purpose is for affinity analysis, the amount of ligands immobilized can be 200–500 RU, at a flow rate of 5 µL/min. For analysis of interaction kinetics, the immobilized level should be in the range 50–150 RU, at a flow rate of 30 µL/min.
6. Regeneration should be complete to eliminate noncovalently attached ligand and to ensure that the amount of immobilized ligand will remain relatively constant throughout the repeated measurements. On the other hand, removing residual bound analyte must be accomplished without denaturing or inactivating the immobilized ligand. We found that a brief regeneration with a chaotropic agent offers the best approach (*see* **ref. *10***). The best choice is to use the mildest possible regeneration that will return signal to baseline. Increasing harshness in the regeneration procedure will reduce the lifetime of the immobilized ligand sensor surface.
7. Some often-observed limiting factors, which cause deviation from the 1:1 model of data evaluation, are mass transport, rebinding during the dissociation phase, conformational changes, ligand heterogeneity, and multivalent analyte ***(10)***. Mass transport is the movement of analyte from bulk solution to the sensor surface ***(26,27)***. If the interaction between analyte and ligand is rapid and mass transport relatively slow, the transport process becomes rate limiting. Observed binding and dissociation rates will be artifactually slow. Although the mass transport rate is increased at higher flow rates and the interaction rate between ligand and analyte is decreased at lower immobilization levels, mass transport limitation can be overcome by increasing flow rates and decreasing ligand immobilization levels. In practice, if varying the flow rates from 5 to 75 µL/min does not change the observed binding rates, mass transport can be concluded to be insignificant. BIAevaluation software provides models, including terms for mass transport, for fitting sensorgrams partially constrained by mass transport limitations.

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10

Analysis of SH2 Ligands and Identification of Sites of Interaction

Manuel Baca

1. Introduction

The molecular analysis of cell signaling pathways requires an understanding of what each component in the pathway is, the manner in which they interact, and how these interactions serve to propagate the signal. Cytokine signaling begins with the binding of an extracellular cytokine to a specific cell-surface receptor. This usually results in the activation of intracellular protein kinases, which phosphorylate protein substrates important for the propagation of a cell signal. One mechanism by which protein phosphorylation regulates the transmission of a cell signal is through the creation of docking sites recognized by other signaling proteins. Such sites promote the association between protein molecules leading to the formation of active signaling complexes. Src homology 2 (SH2) domains are protein modules of approx 100 amino acids that recognize and bind to specific phosphotyrosine-containing polypeptide sequences ***(1)***. SH2 domains are recruited to tyrosine-phosphorylated proteins in their normal physiological setting; however, short synthetic phosphopeptides derived from these cellular docking sites contain all of the necessary binding determinants to interact with SH2 domains. This fact has allowed the identification of SH2 binding sites on tyrosine-phosphorylated proteins based on the analysis of SH2 domains interacting with phosphopeptides. Structural and functional characterization of these interactions has led to the observation that a central phosphotyrosine (pY) residue is the defining feature of ligands recognized by all SH2 domains; however, specific binding of a particular SH2 domain to a tyrosine-phosphorylated protein is dependent on the primary sequence in the immediate vicinity of the pY residue, particularly the flanking sequence immediately C-terminal to the pY residue ***(2)***.

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Identification of SH2 domain docking site(s) can help elucidate the role that protein plays within a specific signaling cascade and the mechanism by which it exerts its function. Where an SH2-containing protein is known to act within a particular signal transduction pathway, potential docking sites can be identified by screening short tyrosine-phosphorylated peptides for their ability to interact with that protein. These peptides would represent the sequence surrounding individual tyrosine-phosphorylated residues within proteins involved in the signaling cascade. A qualitative screen, suitable for testing multiple phosphopeptides, is first used to test for interaction of the phosphopeptides with the SH2 domain. This screen is based on the ability of resin-immobilized phosphopeptides to capture soluble recombinant protein. If interaction is detected by this preliminary screen, more rigorous analysis of the interaction is performed by physical measurement of the phosphopeptide-SH2 binding affinity using a biosensor instrument. Analysis of the measured binding affinity can provide an indication of whether or not the interaction under examination is likely to be physiologically relevant based on whether it falls into the range expected (K_d 0.040–4.0 μM) for a typical SH2-phosphopeptide interaction. Finally, the specificity and relevance of the SH2-phosphopeptide interaction can be further addressed by comparative binding studies. Comparison of the binding affinities of phosphorylated and nonphosphorylated forms of the peptide ligand, obtained using a competition binding assay, is used to confirm the phosphorylation-dependent nature of binding, whereas comparison of the affinities of different phosphopeptides allows for a quantitative ranking of how well each phosphopeptide binds to a particular SH2 domain. This information can be useful in terms of distinguishing which of several potential SH2 docking sites (represented by different phosphopeptides) is likely to be the most significant.

This chapter only covers the in vitro molecular analysis of potential SH2 docking sites. To verify the biological significance of any putative SH2 docking sites determined by these methods, it is important to follow up on these biochemical studies with appropriate cellular studies, for instance, by demonstrating a change in cell signaling when the critical tyrosine residue within a predicted SH2 docking site is mutated. To see a specific example of how these methods have been used to elucidate the docking site of an SH2-containing protein involved in regulating cytokine signaling, the reader is directed to the work of Nicholson et al. ***(3)***.

2. Materials

2.1. General Reagents

1. Synthetic phosphopeptides, either biotinylated or nonbiotinylated (*see* **Note 1**).
2. Recombinant SH2 domain protein (*see* **Notes 2** and **3**).

3. Dimethylsulfoxide (DMSO).
4. Streptavidin agarose resin (Pierce Chemical Co. cat. no. 20349, Rockford, IL).
5. 2 *M* guanidinium hydrochloride (GuHCl), 50 m*M* Tris-HCl, pH 8.0.
6. 4 *M* GuHCl, 50 m*M* Tris-HCl, pH 8.0.
7. 2X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (for Tris-glycine gels): 2 mL 10% w/v SDS, 2 mL glycerol, 2 mL 0.5 *M* Tris-HCl, pH 6.8, 2 mL water, 8 mg bromophenol blue. Fresh dithiothreitol (DTT) (to 10 m*M* final concentration) is added immediately before use.
8. Coomassie stain: 1.0 g Coomassie brilliant blue R-250, 400 mL methanol, 100 mL glacial acetic acid, 500 mL water.
9. Destain: 250 mL methanol, 100 mL glacial acetic acid, 650 mL water.
10. 6 *M* GuHCl, 50 mM Tris-HCl, pH 8.0.
11. 10 m*M* Tris-HCl, pH 7.5 containing 0.1% v/v Tween-20.
12. Phosphate-buffered saline (PBS) containing 0.1% v/v Tween-20 (PBS/Tween).

2.2. Biosensor Instrumentation

The quantitative analyses of SH2 domain–phosphopeptide interactions described in this chapter are performed on a biosensor instrument using surface-plasmon-resonance detection. This technology, which measures molecular interaction kinetics in real time, is well suited to the study of protein complexes, which display rapid association and dissociation kinetics as is typical for SH2 domain-phosphopeptide interactions ***(4)***. The methods reported here are written for a BIAcore 2000 biosensor instrument (BIAcore International AB, Uppsala, Sweden), used in conjunction with streptavidin-coated biosensor chips (SA5, Biacore). Alternate biosensors can be used; however, if the instrument is not capable of monitoring multiple immobilized ligand channels simultaneously, the analysis of binding to specific vs nonspecific peptide ligands would need to be measured in separate runs.

3. Methods

3.1. Immobilization of Biotinylated Phosphopeptides onto Streptavidin-Agarose Resin

1. Design phosphopeptides corresponding to known or potential tyrosine phosphorylation sites on intracellular proteins involved within a particular signaling pathway. These peptides should be 15 residues in length and contain 7 amino acids in either direction from the central pY residue. Synthesize/order biotinylated derivatives of these peptides (*see* **Note 1**).
2. Solubilize 0.5 mg of each biotinylated phosphopeptide in 25 µL DMSO, then dilute with 500 µL of 2 *M* GuHCl, 50 m*M* Tris-HCl, pH 8.0.
3. Transfer the solubilized peptides to approx 200 µL of streptavidin-agarose resin from which the supernatant has been removed. (There is no need to prewash the resin.)
4. Mix resin/phosphopeptide slurry at room temperature for 1 h.

5. Centrifuge resin and remove supernatant.
6. Wash the resin twice with 1 mL 4 *M* GuHCl, 50 m*M* Tris-HCl, pH 8.0 and then twice again with 1 mL PBS/Tween, carefully removing the supernatant between each wash.
7. Resins can be stored at 4°C in PBS/Tween containing 0.02% w/v sodium azide. Under these conditions, they should be stable for at least 1 mo.

3.2. Qualitative Analysis of SH2-Phosphopeptide Interactions Using Immobilized Phosphopeptide Resins

1. To 20-µL aliquots of each phosphopeptide resin, or to unmodified streptavidin-agarose resin, add 25 µg of purified recombinant SH2-containing protein (*see* **Note 2**) made up to a total volume of 1 mL with 10 m*M* Tris-HCl pH 7.5/0.1% Tween-20. Incubate with gentle shaking for 1 h at room temperature.
2. Centrifuge to pellet resin, then carefully remove supernatant. Wash each resin sample twice with 1 mL cold PBS/Tween, carefully removing the supernatant after each wash.
3. To each resin sample, add 25 µL of SDS sample buffer containing 10 m*M* DTT and heat at 100°C for 3 min. Remove supernatants and analyze by SDS-PAGE. Stain gel with Coomassie blue then destain to reveal any protein captured by the resin samples (*see* **Note 4**).
4. Phosphopeptides, which specifically interact with the SH2 protein, will capture significantly more protein (visible as a band on the Coomassie-stained gel) relative to phosphopeptides which do not (*see* **Fig. 1**). The unmodified streptavidin-agarose resin result serves as a control in case the protein binds nonspecifically to resin. Interacting peptides, as identified by this method, are then further analyzed (as described below) to quantitatively determine the affinity of interaction.

3.3. Quantitative Analysis of SH2-Phosphopeptide Interactions

3.3.1. General Biosensor Procedures

The following instructions refer to the use of a BIAcore 2000 (BIAcore) surface plasmon resonance instrument. If an alternate instrument is being used for these analyses, the methods may need to be modified as appropriate.

1. Dock a streptavidin-coated biosensor chip (SA5, BIAcore).
2. Prime the system with PBS/Tween buffer.
3. Prepare a 1-µg/mL solution of the biotinylated phosphopeptide(s) (200 µL) to be immobilized in H_2O. Inject a 35-µL sample of peptide over the chip at a flow rate of 5 µL/min. A target immobilization density of approx 100 resonance units should be sought. If necessary, adjust the conditions to achieve this level of immobilized peptide (*see* **Note 5**).
4. In addition to immobilizing any specific SH2-binding phosphopeptide, a nonspecific phosphopeptide should also be immobilized on a separate channel on the same chip to serve as a negative control (*see* **Note 6**).

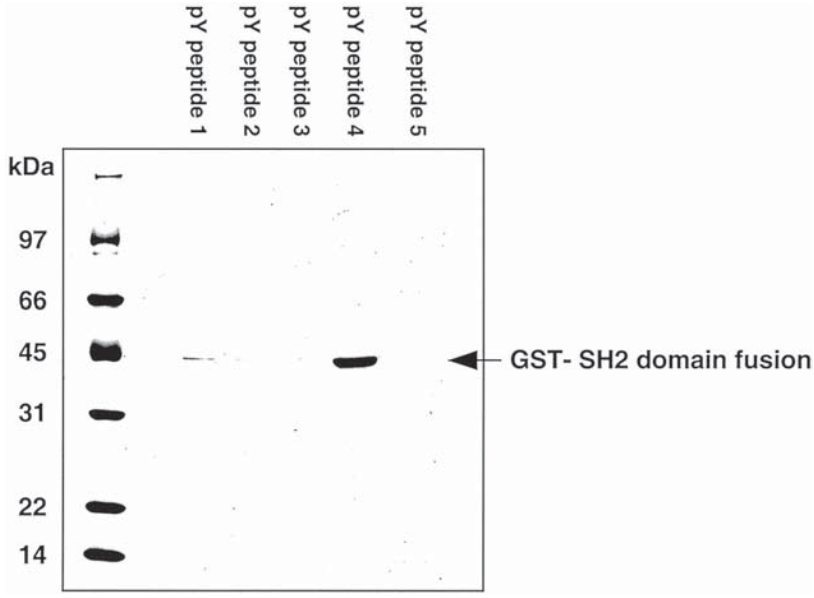

Fig. 1. Specific interaction of an SH2 domain with a phosphopeptide ligand; 25 µg samples of a recombinant GST-SH2 domain fusion protein were incubated with five different immobilized phosphopeptides. Following washing of the resin and elution of the bound protein, samples were analyzed by SDS-PAGE on a 12% polyacrylamide gel. Only phosphopeptide 4 showed any appreciable interaction with this SH2 domain as detected by this analysis.

5. SH2 protein samples to be analyzed by biosensor are diluted to the appropriate concentration in the biosensor running buffer (PBS/Tween) and injected over the chip at a flow rate of 15 µL/min. Typically, 30–150 µL is injected depending on the time taken to reach saturation binding.
6. Following a binding measurement, the phosphopeptide binding surface is regenerated by an injection of 6 *M* GuHCl, 50 m*M* Tris-HCl, pH 8.0 (25 µL at 50 µL/min), followed by a needle wash with PBS/Tween running buffer to remove residual GuHCl (*see* **Note 7**).
7. Binding profiles are analyzed using the BIAevaluation software (BIAcore), and the signal obtained for the nonspecific phosphopeptide channel is subtracted from that of the specific channel(s) to correct for nonspecific binding.

3.3.2. Determination of SH2-Phosphopeptide Binding Constants

The binding affinity for an SH2-phosphopeptide interaction is calculated from an analysis of biosensor binding profiles obtained by injecting the soluble SH2 protein over the immobilized phosphopeptide surface at a range of con-

centrations. A Scatchard-type analysis of the resulting data is used to calculate the dissociation binding constant (K_d) for the interaction.

1. Inject a 5-μ*M* sample of the SH2-containing protein onto the biosensor chip to confirm that the protein binds to the phosphopeptide as expected. Allow for a total injection time of 10 min, or until the level of bound protein no longer increases over time (i.e., until equilibrium binding is attained). At this concentration of protein (i.e., 5 μ*M*), a robust signal should be expected for a typical SH2 protein–phosphopeptide interaction.
2. Inject a 10-μ*M* sample of the SH2 protein as described above. Determine which of the two following scenarios applies:
   a. If the equilibrium binding level increases by *more* than 5% (after background subtraction), test twofold-higher concentrations of protein until the increase in equilibrium binding is approx 5% between successive samples.
   b. If the equilibrium binding level increases by *less* than 5%, (after background subtraction), test twofold-lower concentrations of protein until the increase in equilibrium binding is approx 5% between successive samples.
3. Prepare a twofold serial dilution series of the SH2-containing protein by diluting into PBS/Tween (eight samples total). The two highest protein concentrations in this series should be those that result in an approx 5% difference in equilibrium binding level (i.e. ,as determined in **step 2**). Analyze these samples on the biosensor (*see* **Note 8**), ensuring the injection time is sufficiently long to allow protein binding to reach equilibrium (typically 10 min is sufficient).
4. Analyze each binding profile and determine the equilibrium response values (RU). Plot RU against RU/concentration and use a Scatchard-type analysis, as illustrated in **Fig. 2**, to calculate K_d by determining the negative inverse of the slope.
5. Repeat experiments detailed in **steps 3** and **4** to verify that the experimentally derived K_d can be determined reproducibly (*see* **Note 9**).
6. Compare the calculated K_d with that reported for other SH2-phosphopeptide interactions ***(5)***. Typically, K_d values for SH2 domains binding to phosphopeptides derived from physiologically relevant docking sites are in the range 0.040–4.0 μ*M*.

3.3.3. Competition Analysis of SH2-Phosphopeptide Interactions

In order to verify that the binding of a tyrosine-phosphorylated peptide(s) to an SH2 domain is dependent on the phosphorylation state of the peptide, comparison of the binding affinities of phosphorylated vs nonphosphorylated forms of the same peptide are measured in a solution competition assay using the biosensor instrument. This assay measures the ability of a soluble peptide to compete with binding of SH2 protein to an immobilized phosphopeptide. This assay can also be used to rank the relative binding affinities of different phosphopeptides for the same SH2 domain.

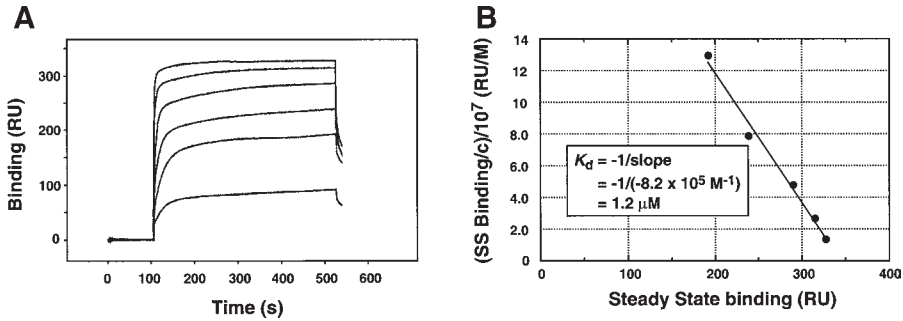

Fig. 2. Calculation of SH2 domain–phosphopeptide binding affinities. Data is shown for the interaction between the N-terminal SH2 domain from SHP-2, and the immobilized phosphopeptide biotin–STASTVE(pY)STVVHSG derived from the cytokine receptor gp130. **(A)** Biosensor analyses of a twofold serial dilution series of the SH2 domain (0.75–24 μ*M*) binding to the immobilized phosphopeptide. **(B)** Scatchard analysis of the data shown in **A**. Steady-state (i.e., equilibrium) binding values for the five highest concentrations of SH2 protein were plotted against the ratio of steady-state binding to SH2 concentration (*see* **Note 14**). The data are fitted to a linear equation, and the dissociation binding constant K_d is calculated as the negative value of the inverse of the slope. The *x*-axis intercept represents R_{max}, the saturation level of steady-state protein binding at infinite protein concentration.

1. Prepare a sample of the SH2 protein at a fixed concentration approximately equal to the K_d for binding to the immobilized phosphopeptide (as determined in **Subheading 3.3.2.**, **step 4**). Use PBS/Tween as a sample diluent.
2. Inject sample over the biosensor chip and analyze the binding profile after correcting for background nonspecific binding. Record the level of bound SH2 protein at a fixed time (*t*) within the sensorgram (*see* **Note 10**). This is R_t^0, the BIAcore response value at time *t* in the absence of soluble competing peptide.
3. Prepare samples containing the SH2 protein (at the same concentration used in **step 1**) and soluble competing peptide (nonbiotinylated) at 0.2, 2.0, or 20 μ*M* concentrations. Use PBS/Tween as a sample diluent.
4. Inject samples over the biosensor chip and analyze binding profiles after correcting each for background nonspecific binding.
5. Record the level of bound SH2 protein for each peptide concentration at the same time *t* as used in **step 2**.
6. Compare the level of SH2 protein bound in the presence vs absence of competing peptide. Estimate the concentration of competing peptide required to reduce the level of SH2 protein binding to $0.5R_t^0$ (i.e., the IC_{50}). If the IC_{50} appears to fall outside of the range 0.2–20 μ*M*, test for inhibition at higher or lower concentrations of soluble peptide as appropriate until a rough estimate of the IC_{50} is deter-

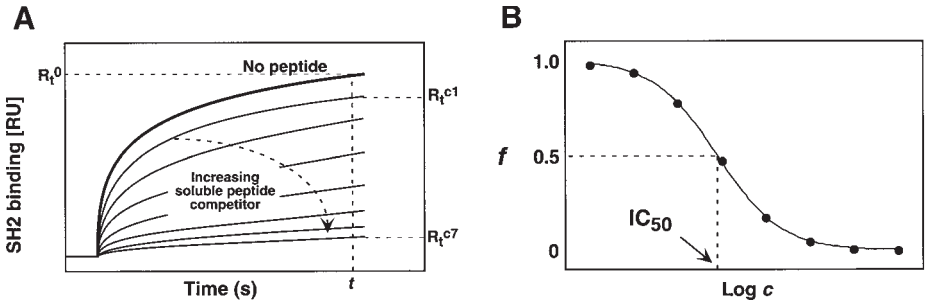

Fig. 3. Determination of relative binding affinities. **(A)** Binding of a subsaturating amount of SH2 protein to an immobilized phosphopeptide is measured in the absence or presence of increasing amounts of a soluble competing peptide. The amount of SH2 protein that binds to the immobilized phosphopeptide is recorded at an arbitrary time point for each sample (R_t^c; R_t^0). **(B)** The fraction (*f*) of protein bound in the presence vs absence of soluble peptide is calculated, and this data is plotted against the log of the soluble peptide concentration (*c*). The relative binding affinity (IC_{50}) is calculated as described in the text, and corresponds to the concentration of soluble peptide required to inhibit 50% of the SH2-immobilized phosphopeptide binding.

mined (*see* **Note 11**). This approximate value is used to define the appropriate peptide concentration range that should now be used to more accurately determine the IC_{50}.

7. Set up a fourfold dilution series of the competing peptide in PBS/Tween (eight samples total) such that the concentration range evenly spans the IC_{50} estimate determined in **Subheading 3.3.3., step 6**. Each sample should again contain the same fixed concentration of SH2 protein as used previously (*see* **Notes 12** and **13**).
8. Inject samples over the biosensor chip and analyze binding profiles. Record the level of bound SH2 domain at time *t* for each peptide concentration (R_t^c) (*see* **Fig. 3A**).
9. Using the data for each sample containing soluble peptide, calculate the ratio R_t^c / R_t^0. This gives the fraction (*f*) of protein bound to the immobilized phosphopeptide in the presence of soluble competing peptide. Values of *f* will range from 0 (total inhibition of binding by the soluble peptide) to 1.0 (no inhibition of binding).
10. Plot the fractional binding data as a function of the log of the soluble peptide concentration (**Fig. 3B**). A displacement binding curve is generated by fitting data to the equation $f = 1/[1+(c/IC_{50})m]$, where *c* is the soluble peptide concentration, *m* is a curvature constant, and IC_{50} is the concentration of competing peptide required to inhibit binding to the immobilized peptide by 50%.

4. Notes

1. Biotinylated peptides should be chemically synthesized with a specific biotin residue on the amino terminus only.

2. The recombinant protein need not represent the full-length molecule and can be limited to the active SH2 domain. Good yields of soluble recombinant His6- or GST-tagged SH2 domains can generally be obtained by expression in *Escherichia coli*.
3. It is important to determine accurately the concentration of the protein. The two most reliable methods are by amino acid analysis or by measurement of the absorbance at 280 nm ***(6)***. Other methods such as the BCA colorimetric assay ***(7)*** can be used as a guide.
4. The percentage acrylamide gel chosen should be appropriate to the molecular weight of the SH2-containing protein being analyzed.
5. The level of peptide immobilized can be most readily modulated by changing the concentration of the peptide stock. If the peptide has a low p*I*, electrostatic repulsion by the negatively charged chip surface can result in poor immobilization. In this event, try preparing the peptide stock in 20 m*M* sodium acetate, pH 5.0 buffer rather than unbuffered H_2O.
6. Select a phosphopeptide that showed no binding in the qualitative assay (*see* **Subheading 3.2.**) as the nonspecific control.
7. Alternatively, 0.1 *M* sodium hydroxide can be used in place of the 6 *M* GuHCl buffer as a chip-regenerating reagent. This has the advantage that the needle wash step is not required.
8. Although it is common to run a dilution series on a BIAcore instrument in an automated fashion, care should be taken if the protein is "sticky" and prone to adsorbing to plastic surfaces (particularly at low concentrations of protein). If a freshly diluted sample gives a significantly different biosensor signal to one diluted several hours beforehand, only freshly diluted samples should be analyzed to minimize errors in affinity determinations.
9. K_ds determined by BIAcore analysis can be prone to errors caused by mass transport effects, i.e., a local depletion in the concentration of soluble protein at the surface of the chip caused by limitations in the rate of analyte diffusion. This tends to be more of a problem for large proteins, but its possible effects can be tested for by comparing sensorgrams obtained at normal (15 µL/min) vs high (75 µL/min) flow rates. If the initial binding rate does not increase more than 5–10% at the higher flow rate, the mass transfer effect can be ignored.
10. Choose any arbitrary time within the sensorgram after protein binding commences where the signal is at a reasonable level (ideally ≥ 200 RU). Unlike the data used for determining K_d, binding does not need to have reached equilibrium.
11. Caution should be used that the solubility limit of the competing peptide is not exceeded.
12. As this assay measures a relative binding affinity (IC_{50}) rather than the true dissociation constant (K_d), the affinity of a soluble, nonbiotinylated form of the immobilized phosphopeptide should also be measured as part of an experimental set of IC_{50} determinations. The relative IC_{50}s of other peptides are then compared to this value.
13. If the SH2 protein is prone to inactivation by adsorption, BSA (0.05% w/v) can be included in the assay buffer and/or samples left overnight at 4°C prior to analy-

sis so that the rate of inactivation becomes slow as a function of time. In addition, samples lacking soluble peptide competitor should be run at regular intervals through the course of the BIAcore analysis to monitor and correct for any change in the level of active protein.

14. At lower concentrations of protein, the plot tends to be curved due to the sensorgrams failing to reach equilibrium. Use only co-linear points in calculating the K_d.

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11

Assays for Antiviral Activity

Anthony Meager

1. Introduction

It is well known that interferons (IFNs) have antiviral activity ***(1,2)***. In fact, the definition of interferon springs from this observation. Although it is reported that other cytokines, e.g., tumor necrosis factor (TNF) ***(3)***, may exert antiviral effects, biological assays based on antiviral activity, or antiviral assays (AVA), were originally developed solely for potency estimations of IFNs ***(4,5)***. The development of AVA started more than 40 yr ago and has continued and progressed with the discovery and characterization of IFN. Although many IFNs are now known to have multiple activities, e.g., antiviral, antiproliferative, immunostimulatory ***(1,2)***, the AVA remains one of the most widely used methods of measuring IFN potency ***(4,5)***. This chapter is therefore devoted to describing the methodology underlying AVAs (*see* **Subheading 3.2.**), and includes tips on how to best carry these out in the laboratory.

In humans and mammals, the IFN family is molecularly heterogeneous (***6***). Human IFN contains at least three types of acid-stable IFNs, known as α, β, and ω, and one type of acid-labile IFN, known as γ ***(1,2,6,7)***. Most mammals also possess these IFN types, but ruminants have a further acid-stable type called τ, which has a role in establishing pregnancy (***8***). In humans, IFN-α is molecularly heterogeneous, being comprised of 12 molecularly distinct, but closely related, species known as subtypes ***(2,6)***. The latter are usually signified by a number following the a symbol, i.e., α_1, α_2, α_4, α_5, and so on. However, there is only one species of human IFN-β, -ω, and -γ ***(2,6,7)***. In comparison, mammals other than humans may possess multiple IFN-β and -ω subtypes as well as multiple IFN-α subtypes, but in all species examined, only one IFN-γ molecule has been found ***(2,6,7)***. From a molecular point of view, the three-dimensional (3-D) structures of IFN-α, -β, -τ, and -ω are related, each protomer containing five α-helices connected by loops which fold up into

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a compact α-helical bundle ***(9,10)***. In contrast, although it contains similar α-helical features, IFN-γ is structurally unrelated to IFN-α, -β, -τ, and -ω, the active molecule being a dimer of two identical, noncovalently bound, intertwined protomers ***(11)***.

In nature, IFN genes are rarely expressed unless triggered by potentially pathogenic events such as viral infections ***(12)***. In most cases, stimulation leads to the expression of multiple IFN types. For example, viral stimulation of peripheral blood leukocytes, in particular, dendritic cell precursors, leads to the expression and secretion of a mixture of IFN-α subtypes, IFN-β, and IFN-ω, of which approx 90% is α, 2% is β, and 8% is ω, and which has been designated *leukocyte IFN* ***(12)***. In contrast, viral stimulation of human fibroblasts yields a mixture of IFN in which IFN-β predominates, and which has been designated *fibroblast IFN* ***(12)***. Thus, although currently it is possible to produce each individual IFN type or subtype by rDNA technology and work with these as single molecular species, the vast majority of samples generated from stimulated cells will contain mixtures of IFN, often in varying, uncertain proportions, which are difficult to purify. Although it is perfectly possible to measure the activity/potency of such samples in AVA, the heterogeneity of IFN contained in them has led to some difficulties in the calibration of AVA (*see* **Notes 1** and **2**).

There are a variety of cell/virus systems and assay designs that can be used to construct AVA (*see* **Notes 3** and **4**). However, not all cell lines are suitable, particularly some tumor cell lines that have defective IFN response systems (***16***). The latter are not protected from lysis by viruses such as VSV, even in the presence of relatively high concentrations of IFN ***(16)***. In all types of AVA it is necessary to quantify the inhibitory activity of IFN on viral propagation. The *virus yield reduction* assay ***(4,12)***, which involves infectivity (plaque) assays of virus progeny obtained from cells treated with each serial dilution of IFN, can be informative and quantitative, but is highly tedious to perform. A more convenient and simple procedure is to measure the capacity of IFN to protect cells against the cytopathic effect (CPE) of a lytic virus over a range of IFN concentrations ***(4,5,12,13)***. This type of AVA is known as the cytopathic effect reduction (CPER) assay, and currently is very widely used in 96-well microtiter plate formats. The basic procedure is as follows: after seeding wells of microtiter plates with sufficient numbers of cells of an adherent *IFN-sensitive* cell line to form confluent monolayers, serial dilutions of IFN preparations are incubated with the cells to induce the antiviral state, following which the cells are challenged with a cytolytic virus until maximum CPE is manifested in control, untreated cells. At this point, viable IFN-protected cells are stained with a vital dye (*see* **Note 5**) to determine CPE levels and, following spectrophotometric measurements of dye absorbance, to plot dose–response curves and cal-

culate potency of IFN preparations in terms of units of antiviral activity. AVA, such as CPER assays, are, by nature, sensitive to cell culture and general assay conditions and are therefore inherently variable.

These assays require a well-defined representative reference IFN preparation that can first be used to act as a positive control, which serves to qualify the performance of individual assays ***(13,14)***. After the reference IFN has been assigned a universally recognized unitage of biological activity, it can be secondly and importantly used as a primary standard to calculate the potencies of other preparations of the same material and, in certain instances, thereby be used to establish in-house working standards. The latter can then be used to calibrate further assays, so preserving the stock of the primary standard ***(13,14)***.

2. Materials

1. Tissue culture plates (Falcon cat. no. 3075 96-well or equivalent).
2. Plastic culture dishes, 9-cm (Sterilin or equivalent).
3. Pipetters with tips (Gilson Pipetman or equivalent).
4. Multichannel pipets with tips (Titertek or equivalent).
5. Laminar-flow biological safety hood (Envair class II cabinet or equivalent).
6. CO_2 incubator set at 37°C and set at 5% CO_2 (Forma Scientific or equivalent).
7. Water bath set at 37°C (Techne Tempette Junior or equivalent).
8. Absorbent paper towels (Kimnet Reinforced Cloths or equivalent).
9. Enzyme-linked immunosorbent assay (ELISA) plate reader (Titertek Multiskan Plus or equivalent).
10. Whatman No. 1 filter paper.
11. Inverted light microscope.
12. Reference reagent/laboratory standard: international standard, national standard, or laboratory reference reagents for the particular IFN to be tested. Store in freezer set at –20°C or below. A list of available standards is given in **Table 1**.
13. Supplemented RPMI-1640 medium with antibiotics containing 7% fetal calf serum (RPMI1640/7% FCS): RPMI-1640 stock solution (450 mL), L-glutamine, 200 m*M* sterile (5 mL), sodium pyruvate, 100 m*M* sterile (5 mL), FBS (heat inactivated at 56°C for 30 min) (35 mL), penicillin and streptomycin (10,000 U/mL and 10 mg/mL, respectively) (5 mL). Store at 2–8°C; expiration is 4 wk from the date of preparation.
14. Phosphate-buffered saline (PBS), six-salt (8000 mg/L NaCl, 200 mg/L KCl, 1150 mg/L Na_2HPO_4, 200 mg/L KH_2PO_4, 100 mg/L $CaCl_2$,120 mg/L $MgSO_4$), pH 7.0.
15. IFN sensitive cell line, e.g., 2D9 human glioblastoma cell line (***15***). 2D9 cell suspension at 5×10^5 cells/mL. A number of other recommended or widely used cell lines are listed in **Table 2**.
16. An appropriate cytopathic virus, e.g., encephalomyocarditis virus (EMCV) ***(4,5)***. Virus titer should be greater than 5×10^8 plaque forming units (PFU)/mL. Several other viruses may be used for AVA and these are listed in **Table 2**. Suitable combinations of cell line and virus are also indicated.

Table 1
World Health Organization (WHO) International Standards for Interferons

Preparation	Product code	Status	Potency/ampule (IU)
Human IFN			
IFN-α leukocyte*	94/784[b]	1st IS	11000
IFN-α_1 rDNA*	83/514[b]	1st IS	8000
IFN-$\alpha_{1/8}$ rDNA*	95/572[b]	1st IS	27000
IFN-α_{2a} rDNA*	95/650[b]	2nd IS	63000
IFN-α_{2b} rDNA*	95/566[b]	2nd IS	70000
IFN-α_{2c} rDNA*	95/580[b]	1st IS	40000
IFN-α_{n1} lymphoblastoid*	95/568[b]	2nd IS	38000
IFN-α_{n3} leukocyte*	95/574[b]	1st IS	60000
IFN-α consensus rDNA*	94/786[b]	1st IS	100000
IFN-ω rDNA	94/754[b]	1st IS	20000
IFN-β fibroblast	Gb23-902-531[c]	2nd IS	15000
IFN-γ rDNA	Gxg01-902-535[c]	2nd IS	80000
Murine (mouse) IFN			
IFN-α/β	Gu02-901-511[c]	2nd IS	10000
IFN-α	Ga02-901-511[c]	1st IS	12000
IFN-β	Gb02-902-511[c]	1st IS	15000
IFN-γ	Gg02-901-533[c]	1st IS	1000
Rabbit IFN			
IFN-α/β	G-019-902-528[c]	1st IS	10000
Chick IFN			
IFN-β	67/18[b]	1st IS	80

*Note: In 1999, several **new** WHO International Standards for human IFN-α preparations were established by the Expert Committee for Biological Standards as replacement standards. These are listed below:

IFN-α preparation	Current WHO International Standard	Discontinued standard[a]
IFN-α leukocyte	94/784	69/19 and Ga23-902-530
IFN-α_1 (D)	83/514	—
IFN-$\alpha_{1/8}$	95/572	—
IFN-α_{2a}	95/650	Gxa01-901-535
IFN-α_{2b}	95/566	82/576
IFN-α_{2c}	95/580	—
IFN-α_{n1} lymphoblastoid	95/568	Ga23-901-532
IFN-α_{n3} leukocyte	95/574	—
IFN-α consensus	94/786	—

[a]These standards are no longer distributed.

[b]These standards are available from The National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts. EN6 3QG, U.K.

[c]These standards are available from The research Resources Branch, The National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Bethesda, MD 20205.

Table 2
Antiviral Assay Cell/Virus Combinations

Cell line	Virus	IFN
Madin-Darby bovine kidney cell line (MDBK)	Vesticular stomatitis virus (VSV)	Human, bovine, etc.
Human lung carcinoma cell line (A549)	VSV or Encephalomyocarditis virus (EMCV)	Human
Human amniotic cell line (WISH)	VSV, EMCV, Semliki forest virus (SFV) or Sindbis virus	Human
Human cervix carcinoma cell line (HeLa)	EMCV or VSV	Human
Human larynx carcinoma cell line (Hep2)	EMCV, VSV, or mengovirus	Human
Human foreskin diploid fibroblast cell line (FS4, FS71)	VSV, EMCV, or mengovirus	Human
Human amnion-derived cell line (FL)	VSV, EMCV, mengovirus, or Sindbis virus	Human
Human glioblastoma cell line (2D9)	EMCV	Human
Murine fibroblastic cell line (L929)	VSV or EMCV	Murine

17. An appropriate vital stain, e.g., Naphthol blue black (NBB) solution (Aldrich Chemical Co. or equivalent) ***(4,5)***. This stain solution contains 0.05% NBB in 9% acetic acid with 0.1 *M* sodium acetate: NBB (0.5 g), glacial acetic acid (90 mL), sodium acetate (0.1 *M*) (8.2 g), distilled water to 1.0 L.
    The stain solution is prepared by stirring on a magnetic stirrer for 1 h, and the resulting solution is filtered through Whatman No. 1 filter paper. It is stored in a tightly stoppered bottle at room temperature.
18. An appropriate fixative solution, e.g., acidified formalin. This contains approx 4.0% formaldehyde in the above (*see* **item 17**) acetic acid–sodium acetate buffer: formalin (formaldehyde 40%) (100 mL), glacial acetic acid (90 mL), sodium acetate (0.1 *M*) (8.2 g), distilled water to 1.0 L. Store in a tightly stoppered bottle at 4°C.
19. Stain elution solution, e.g., for NBB use 0.1 *M* NaOH solution: sodium hydroxide (NaOH) (4 g), distilled water to 1.0 L. Store at room temperature.

3. Methods

3.1. Use of IFN Standards and Sample Preparation

1. For the testing of IFN, use the appropriate WHO International Standard for IFN (*see* **Table 1**) as the primary calibrant and, subsequently, well-calibrated laboratory standards of the appropriate IFN. For example, for the testing of Roferon A

(human recombinant IFN-α2a), use 95/650 2nd WHO International Standard for human IFN-α2a as the primary calibrant, and subsequently well-calibrated laboratory standards of human recombinant IFN-α2a.

2. Dilute IFN standards, i.e., International Standards, laboratory standards, in RPMI-1640/7% FCS on the basis of their assigned potency such that this initial, off-plate, dilution is appropriate for the subsequent dilution series for the titration of antiviral activity in the assay.
3. IFN test samples. If the potency is known, e.g. ,from a manufacturer's estimate, dilute IFN sample in RPMI-1640/7% FCS (*see* **Subheading 2., item 13**) as per **Subheading 3.2.** If the potency is unknown, prepare a series of 10-fold dilutions (i.e., 1:10, 1:100, 1:1,000, and so on) of the test sample in RPMI-1640/7% FCS.

3.2. Test procedure

The procedure is to be carried out in laminar-flow biological safety hood. The following is an example of how an AVA is set up.

1. Label the outside of 96-well microtiter plates with the type of assay and plate number and the date of test. Use columns 1 and 12 for controls, i.e., cell controls, cells only, no IFN, no EMCV; virus controls, cells, no IFN, plus EMCV. Use columns 2–11 for standard/reference preparation and test samples (*see* **Note 6**). Use a format diagram to label what samples and what dilutions are applied.
2. To each well of a 96-well plate, add 100 µL of RPMI-1640/7% FCS using a multichannel pipet.
3. Add 100 µL of reference preparation (standard) to row A, columns 2 and 3. The reference preparation should be included on all plates in the assay.
4. Add 100 µL of test samples to row A, columns 4 and 5, columns 6 and 7, columns 8 and 9, columns 10 and 11, i.e., duplicates of each test sample.
5. Using a multichannel pipet set at 100 µL mix the contents of the wells of row A by withdrawing and ejecting half of the contents several times. Transfer 100 µL in the row A vertically to the wells of row B. Mix contents and transfer 100 µL to the wells of row C. Continue diluting as above in rows D, E, F, G and H. Finally, discard 100 µL from the wells of row H. This procedure gives the dilution series 1:2, 1:4, 1:8, 1:16, 1:32, and so on. Repeat the procedure with fresh tips for each plate in the assay.
6. Pour cell suspension of 2D9 cells (***15***), which has been adjusted to contain approx 5×10^5 cells/mL of RPMI-1640/7% FCS, into a 9-cm sterile plastic Petri dish. Add the cell suspension from the Petri dish using a multichannel pipetter set at 100 µL to each well of all 96-well plates included in the tests. This addition will make dilutions of IFN in wells twofold greater, i.e., 1:4, 1:8, 1:16, and so on. Shake/agitate dish occasionally to keep cells evenly suspended. Alternatively, the 2D9 cells can be added to empty 96-well plates (as above) and preincubated for 24 h to form confluent monolayers before transferring dilutions from the 96-well plates set up in **steps 2–5**. A further alternative is to actually make the standard and test sample dilutions in the wells of plates preseeded with cells to

form confluent monolayers. However, this step is only recommended if the cells used are very adherent and are not removed by the mixing action of the multichannel pipetter used for making dilutions.

7. Incubate the plates for about 24 h in an incubator set at 37°C and 5% CO_2.
8. At this stage, using an inverted microscope, check that monolayers of 2D9 cells are confluent, show relatively even distribution of cells, and are healthy.
9. Remove most of the RPMI-1640/7% FCS in wells by flicking out and blotting on a paper towel.
10. Dilute EMCV stock with fresh RPMI-1640/2% FCS to give a concentration of approx 3×10^7 PFU/mL. Note, each plate requires approx 10 mL of diluted virus, plus 5–10% extra volume. Add this from a 9-cm sterile plastic dish using a multichannel pipet set at 100 µL to all wells including virus controls, but excluding cell controls. Add approx 100 µL of RPMI-1640/2% FCS without virus to each of the cell control wells.
11. Return plates to incubator set at 37°C and 5% CO_2 for approx 24 h.
12. Examine plates microscopically to check that EMCV has effected a strong cytopathic effect (CPE) in the virus controls. The time interval for maximum CPE may vary from one assay to the next because of inherent variation of 2D9 cells to virus challenge over a given period of continuous cultivation. If the CPE is not fully developed, prolong incubation until this is so.
13. Remove most of medium from wells by flicking out into a container with chloros. Add PBS to each well using a multichannel pipet set at 200 µL. Flick out PBS into chloros. Add NBB solution to each well using a multichannel pipet set at 150 µL. Stain cells for approx 30 min at room temperature.
14. Flick out NBB solution into chloros solution. Add approx 150 µL of fixative solution and leave cell monolayers to fix for 10 min at room temperature. Flick out fixative solution into chloros and wash cell monolayers by immersing assay plates in a plastic box containing running tap water.
15. Flick out tap water and superficially dry plates with paper towels. Dry the assay plates at 20–37°C until all moisture has evaporated.
16. Add 150 µL 0.1 *M* NaOH to each well using a multichannel pipet set at 150 µL. Elute stain by gentle agitation of the plates or knocking them against the palm of the hand. Make sure the stain is evenly distributed in all wells before making spectrophotometric readings.
17. Read absorbance at 610–630 nm in the same plates using an ELISA-type reader (e.g., Titertek Multiskan or equivalent). A well or column of wells containing no cells and approx 150 µL of 0.1 *M* NaOH may be used as a blank, or simply use a well or column of wells in an empty plate as a blank.

3.3. Data Analysis

Results of the CPE reduction assay generally fit a sigmoidal dose–response curve, when the IFN concentration (the log of the reciprocal of the IFN dilution) is plotted vs stain absorbance (*see* **Subheading 3.2., step 17.**) (***4***,***5***). Using the linear portion of the curve, calculate the concentration of interferon in the

sample by comparing the responses for test and reference solutions, using the statistical methods for a parallel line assay. Alternatively, titers may be roughly calculated using an end point method, as follows:

1. Plot IFN concentration (reciprocal of dilution) vs stain absorbance (**Subheading 3.2., step 17.**) for the IFN reference preparation and all IFN test samples. Use only the linear portion of the curve to estimate a titer of any IFN preparation/sample. (The titer is the IFN concentration interpolated at the arbitrarily defined endpoint of the response measured, i.e., the median absorbance on the scale of stain-uptake measurements that range between those of the uninfected cell controls and the virus-infected controls not treated with IFN). The IFN titer corresponding to the endpoint can be obtained graphically from semilog plots or by linear regression calculations, using only those points that fall within the linear portion of the curve.
2. Correct the IFN titers of test samples expressed in observed or laboratory units (LU)/mL to the assigned titer (potency) of the IFN reference preparation, which if an international or national standard will have its potency expressed in international units (IU). Use the following equation:

Titer of IFN test sample in IU/mL = [observed titer of IFN test sample (LU/mL)]/[observed titer of IFN ref prep (LU/mL)] × assigned titer of IFN ref prep (IU/mL)

4. Notes

1. Whenever possible, IFN activity (potency) should be reported with reference to an appropriate WHO international standard (IS) and expressed in international units (IU). WHO IS for many types and subtypes of human IFN, together with several other animal IFN (**Table 1**), have been established from data provided by the expert participants of WHO international collaborative studies (***13***,***14***). These WHO IS are the result of considerable effort to prepare and establish appropriate reference materials for IFN research and for assigning potencies to clinical IFN products and, therefore, represent a valuable resource. International standards, as primary standards with potency assigned in IU, are used to calibrate working standards (in-house or national standards), which are well characterized and as close as possible in purity and form to the IS. That the IS and the working standard contain identical or very similar IFN is necessary for compliance with the central tenet of biometric validity of assays. "When two or more IFN preparations are being compared, they must behave identically in the assay for the assay to be truly valid." In this case, where the IS serves as primary reference preparation the working standard must behave as if it were a more concentrated or more dilute solution of the IS preparation. Stated differently, if like is compared with like in the same assay system, under the same conditions, then any difference in the measured response from the assay system should reflect only the difference in concentration. Dose–response lines for the IS and the working standard must be parallel for biometric validity. Usually, the IS and working standard are com-

pared in assays performed on at least five separate occasions and the geometric mean potency of the working standard, expressed in IU, assigned to it (***4***). The working standard should then be used to routinely calibrate all further assays to estimate the potency in IU of IFN preparations containing the same IFN as the working standard. In this way, stocks of ampoules of the IS are conserved. [It has to be emphasized that the potency of IFN cannot be expressed in molar concentrations or mass units because molarity/mass is a measure of the physical quantity of material and, thus, does not reflect its biological activity. For example, the potency of an IFN preparation held under different conditions may vary significantly as a function of time without any change in molarity/mass. It is imperative, therefore, to express potency in IU of biological activity.]

Although a fairly extensive range WHO IS for human IFN now exists (**Table 1**), it has not been practicable to prepare an IS for every human IFN preparation, e.g., for each of the 12 IFN-α subtypes. Where an IS is not available, e.g., for human IFN-α_{10}, calibration of assays should be made with the IS for the human IFN preparation to which IFN-α_{10} shows most similarity. In this case, an IS for a single human IFN-α subtype, e.g., IFN-α_{2b}, should be chosen and not one for a mixture of IFN-α subtypes, e.g., leukocyte IFN. The converse will be true when establishing a working standard for a leukocyte IFN preparation.

2. WHO IS for IFN are ampuled freeze-dried preparations that have demonstrated long-term thermal stability. However, once reconstituted, the resultant solutions are not guaranteed to maintain their full activity, particularly at temperatures above 4°C. Therefore, aliquots of the reconstituted IS solution should be stored frozen at –70°C until required. After thawing an aliquot, it should be used only once for calibration and then discarded. For the working standard, a suitably large volume should be subdivided into appropriately sized aliquots and frozen at –70°C. Plastic containers or siliconized glass containers are recommended for this purpose because most IFN are hydrophobic proteins that can adsorb to unsiliconized borosilicate glass surfaces, leading to potential losses of activity.

   Clinical grade or commercially supplied IFN products are usually relatively easy to handle. They are normally formulated with excipients, e.g., human serum albumin, to stabilize the IFN activity and thus have long shelf lives. If freeze-dried, they can be reconstituted and aliquots frozen at –70°C if necessary. Frozen liquid preparations are best kept frozen at their recommended storage temperature. The only problem with clinical grade IFN products from an assay point of view is that they are very highly potent. Their assigned potencies are usually 1.0 million IU (MIU) or greater and, therefore, they require considerable dilution before titration in AVA (*see* **Subheading 3.2.**). The accuracy of the IFN potency determination in the AVA is therefore subject to the accuracy with which the off-plate dilution can be made. This may involve several 10-fold dilution steps. Appropriate volumes of dilution medium, normally cell culture medium containing bovine serum, should be chosen and dilution at each stage carried out with micropipets calibrated for precise delivery of the dilution volume.

   In contrast, samples obtained from stimulated cell cultures or clinical samples,

including sera, plasmas, cerebral spinal fluid (CSF), bronchial lavage fluids (BLF), and so on, are generally more difficult to handle. Often the IFN present will be of low concentration, may be comprised of a mixture of IFN types and/or subtypes, and contain other cytokines and substances that have modulating effects on the sensitivity of AVA. Serum samples for example may contain antiviral substances other than IFN, which act additively or synergistically with IFN. Additionally, serum samples may contain growth or cytotoxic factors for cells, which act to induce proliferation and cell death, respectively. Such ingredients can therefore make it difficult to decide whether an antiviral effect manifested in the AVA is a result of IFN or another substance. In some cases, it may be possible to judge whether it is IFN or not by the slope of the dose–response line, which should be close to that of the working standard. However, in other cases, serum components may alter the slope of the dose–response line and thus apparently disguise the presence of IFN. One way to confirm that IFN is really present is to use a neutralizing antibody to IFN (***21***), although a guess will probably have to be made as to what specificity (anti-α?, -β?, -γ?) is required owing to the uncertain nature of the IFN presumed to be present.

3. It is important to ensure that the cell line used for AVA (*see* **Subheading 3.2.**) is really suitable for its purpose. If starting for the first time to perform AVA, try to obtain from a reliable source one of the cell lines recommended in **Table 2**. Several cellular characteristics are crucial for good and reproducible performance. First, the cell line should be healthy and, if possible, free of mycoplasma contamination. A frozen stock of a low-passage number must be established in order that, if and when changes in IFN sensitivity occur, a return to low-passage cells can be undertaken. A cell-passaging schedule should be established and rigorously adhered to in order that the cells used for AVA are obtained from confluent monolayer cultures grown on a consistent basis. Second, the cells should be fully adherent and spread out in culture to form uniform monolayers; cell lines that clump or aggregate or slough off cells into the medium should be avoided. In connection with this characteristic, cell lines that can be prepared as single-cell suspensions following trypsinization are better for distribution into wells of microtiter plates than those that aggregate.
4. The virus and cell combinations that work well for AVA are shown in **Table 2**. However, it is often the case that experimental conditions need to be tested thoroughly in order that the read-outs of AVA are satisfactory. Thus, an empirical approach is advised. It is important to establish the "dose" of challenge virus that efficiently kills the cells and the time required for this to happen. For example, human 2D9 glioblastoma cells (***15***) are killed by EMCV in 20–24 h, whereas human A549 lung carcinoma cells (***4***) are killed in 30–48 h by the same dose of EMCV. There are three critical factors that largely determine the sensitivity of AVA: (a) Cell density; (b) multiplicity of infection (MOI) of the challenge virus; (c) time after viral challenge at which AVA are terminated. For example, if the cell density is low and the MOI is high, the sensitivity to IFN will be lower than if the cell density were high and the MOI were low. However, there are limits,

and if the MOI is too low, then CPE will not fully develop and the assay may become "unreadable," i.e., the dose–response line will be very shallow. If the AVA are left much longer than the time period required for maximal CPE to develop, then usually the assay sensitivity will diminish because the virus will gradually destroy cells that are protected by the weakest concentrations of IFN. Therefore, it is best to try and balance those conditions in order to produce an equitable trade-off between viral killing of cells and sensitivity.

The incubator temperature and its pH are also factors that can influence the rate of viral growth and cell killing. These should be controlled as completely as possible and maintained unchanged over long periods so that AVA can be performed consistently on a routine basis.

Another factor that should be considered is whether the cell line used produces its own IFN in response to the challenge virus. If this occurs to any extent, then cell killing by the virus may be reduced or even eliminated. Cells not protected by IFN, i.e., virus controls, may show only partial CPE even after long incubation periods. The IFN induced by the challenge virus may act additively, or even synergize, with the "exogenous" IFN to produce variable and/or abnormal results. Such variation is unwanted and, therefore, virus and cell combinations should be carefully chosen to avoid induction of "endogenous" IFN.

5. The majority of AVA are still terminated by staining and fixing cell monolayers (*see* **Subheading 3.2., steps 13–15**). Several vital stains, i.e., those that stain only, or preferentially, live cells, are available. The violet dyes, such as crystal or gentian violet prepared as 1–2% solutions in 20% ethanol/PBS, are often used for this purpose, but tend to be very messy ***(4,5)***. For this reason, we recommend naphthol blue black (alternatively called amido black or amido blue black) dissolved in sodium acetate–acetic acid buffer (NBB solution) for staining followed by cell fixation with acidified formalin solution.

   At the point of termination of AVA, cells in the cell controls should appear totally alive and healthy, whereas cells in the virus controls should appear dead. Usually, with dead adherent cells, they remain attached to the plastic bottom of the well. However, subsequent washing and staining procedures may variably dislodge these dead cells. It is evident that, where dead cells are more or less completely removed by washing/staining, the final stain uptake will be very low (the well will look almost clear), whereas if the dead cells remain mostly attached, then there will be a stained background appearance to the well (most "vital" stains seem to stain dead cells to some degree). Some cell lines, e.g., Hep2, A549 (**Table 2**), tend to give high backgrounds in the virus controls because of the stickiness of dead cells. These high backgrounds may affect the slope of the dose–response lines and, therefore, adversely impact on the outcome of AVA. To avoid high backgrounds, cell lines such as 2D9, the dead cells of which are removed by washing/staining, should be used (*see* **Subheading 3.2., steps 13–15** and **Table 2**).

   Once cell monolayers or the remaining viable cells are stained, it is possible to "read" the plates visually and CPE is generally assessed by a + and – score.

Although it is possible to gage relative activities by this method, there will be a major subjective element involved and, therefore, we recommend that spectrophometric methods for determining absorbances be used. The latter generate data that lend itself more readily available to classical methods of analysis and computation of results (*see* **Subheading 3.3.**).

Staining methods are by no means perfect and require washing steps that often remove cells. To avoid the possibility of cell loss, use can be made of the direct addition of chemicals that are converted by cellular activities to colored products. The classical example of this is the metabolic reduction of tetrazolium salts such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in viable cells to a colored (purple) formazan product (***17***). In the case of MTT, its formazan product is insoluble, but may be released by acidified sodium dodecyl sulfate (SDS) and absorbances read at 570–590 nm. However, the MTT procedure takes several hours to give evenly distributed color in wells and, therefore, we prefer to use MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium, inner salt) ***(18)***. MTS is taken up by live cells and, in the presence of electron acceptors such as phenazine methosulfate (PMS) or menadione sodium bisulfite (MSB; soluble vitamin K_3), is metabolically reduced to a soluble red formazan product, which diffuses out of the cells into the medium and whose absorbance can be determined at 492 nm. Addition of MTS (1.0 mg/mL) plus PMS (75 μ*M*) or MSB (60 μ*M*) solution requires minimal manipulation and absorbances may be read after 1–2 h incubation at 37°C without further interventions. In practice, the MTS method only works satisfactorily for cells that are efficiently killed by the challenge virus, e.g., 2D9 by EMCV, because if any live cells remain, they will produce red formazan into the medium, i.e., a high background will be generated and the dose–response line will be correspondingly shallow. However, we and others have shown that, with suitable virus and cell combinations, the MTS method of processing AVA gives as good dose–response data as staining methods and even better sensitivity ***(19)***. We, therefore, recommend the MTS method for routine processing of AVA, and especially when titrating very low concentrations of IFN.

6. The nonuniformity of 96-well microtiter plates and the variable accuracy of multichannel micropipets can introduce untoward biases, which can contribute to uneven responses across plates. In many cases, the outer wells of 96-well microtiter plates appear to give the most uneven responses, either as a result of uneven spraying of wells in manufacture or because of the exposure of cells in these outer wells to more rapid variations in temperature and pH. Some investigators therefore opt to use only the inner 60 wells for titrations, even though this means that fewer samples can be titrated on each plate and that the number of serial dilutions of each sample may be more restricted. Nevertheless, we have often found that, even if wells in columns 1 and 12 are excluded, that titrations performed in the wells of column 2 and 11 are still more likely to produce results that are "outliers" than columns 3–10. If wells in columns 2 and 3 are used as duplicates, then we frequently find that the sensitivity of cells to IFN is higher in

column 3 compared with column 2. Sensitivity appears to increase further to columns 6 and 7, i.e., the middle columns, and then fall off again on the right side of the plate toward column 11. This may not happen in all circumstances, but the likely manifestation of these "plate effects" should be borne in mind when deciding the positions of samples and standards. Ideally, when several preparations are tested, they should be titrated on several plates such that the positions of individual samples relative to the standard are varied. The position of the standard should also be moved from plate to plate. An international study to assign potencies to candidate tumor necrosis factor alpha (TNF-α) standards found that designs in which each preparation is tested on several plates in any assay with independent serial dilutions of the preparation on each plate generally gave more accurate and reproducible estimates of potency (***20***). Complete randomization of samples in terms of plate positions is usually not feasible, but efforts should be made to carefully design assays to permit more valid use of classical methods of analysis. One way of checking intraassay variability and thus the validity of results in each assay is, using independent dilutions, to titrate one preparation twice, e.g., as coded duplicates, say A and H, where H is identical to A except for code. The ratio of the potency of A to that of H should approach 1.0. Large deviations from 1.0 indicate that biases have been introduced or occurred in the assay and may compromise its overall validity.

In general, the slopes of dose–response lines generated in AVA are sigmoidal, but are steep (*see* **Subheading 3.3.**). This means that going from fully IFN-protected live cells to unprotected dead cells occurs over a relatively narrow IFN concentration range. In turn, this translates into having only a few data points in the linear portion of the dose–response line and potential difficulties in comparing the slopes of dose–response lines for individual samples. Slopes should be parallel if the calculated results are to be valid. Therefore, assay conditions and design should be set so that the number of data points in the linear portion is maximized. This may be done by using smaller serial dilutions, e.g., 1:1.5, rather than 1:2, of IFN test samples or sometimes by reducing the MOI of the challenge virus. Alternatively, stain methods for processing AVA may be substituted by formazan methods (*see* **Note 5**), which may generate less steep slopes.

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12

Assays for Cytotoxicity

Anthony Meager

1. Introduction

Among cytokine families, the tumor necrosis factor (TNF) superfamily stands out as the one that contains several members with cytotoxic activity ***(1–3)***. The best characterized is the prototypic member TNF-α, which is cytotoxic to a range of tumor cells, but not to most types of normal cells ***(1,4)***. However, there are now known to be around 20 molecularly related members of the TNF superfamily, several of which induce programmed cell death, i.e., apoptosis, in particular types of normal and tumor cells ***(1–3)***. The nomenclature for the members of the TNF superfamily was becoming complicated; individual members were given different names, e.g., Fas ligand, CD95 ligand, APO-1 ligand, by the competing research groups involved in their discovery. Recently, a nomenclature committee has adopted and promoted a simplified naming system; each member is designated tumor necrosis factor super family, TNFSF, with a numerical suffix. Thus, for example, TNF-α becomes TNFSF2 and Fas ligand becomes TNFSF6 (*see* **Table 1** for the full list).

Of the approx 20 members of the TNFSF, only one, TNFSF1 (TNF-β, lymphotoxin-α) is a truly secreted cytokine ***(1,4)***. The rest are cell membrane bound, but in some cases, the C-terminal ectodomains are enzymatically cleaved almost in their entirety to form soluble, trimeric, biologically active molecules. For example, the soluble active form of TNFSF2 (TNF-α) is produced by enzymatic cleavage by a disintegrin metalloproteinase from the external face of the cell membrane ***(5,6)***. For each of the TNFSF members there is one or more cell surface membrane receptors ***(1–4)***. These receptors, of which more than 30 have been characterized, are molecularly related and together constitute the tumor necrosis factor receptor super family (TNFRSF). Many individual receptors were originally named according to the TNFSF molecule(s) they bound, e.g., TNF-R1, TNF-R2, or according to CD nomen-

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Table 1
TNF Ligand and Receptor Superfamily Members[a]

Ligands	Receptors
LT-α(TNF-β)/**TNFSF1**	TNF-R1/**TNFRSF1A** & TNF-R2/**TNFRSF1B** TNF-Rrp or LT-βR/**TNFRSF3**
TNF-α/**TNFSF2**	TNF-R1/**TNFRSF1A** & TNF-R2/**TNFRSF1B**
LT-β/**TNFSF3**	TNF-Rrp or LT-βR/**TNFRSF3**
OX40.L/**TNFSF4**	OX40 (CD134)/**TNFRSF4**
CD40.L/**TNFSF5**	CD40/**TNFRSF5**
Fas.L (APO-1L)/**TNFSF6**	Fas (CD95)/**TNFRSF6** & DcR3/M68/*TR6*
CD27.L (CD70)/**TNFSF7**	CD27/**TNFRSF7**
CD30.L (CD153)/**TNFSF8**	CD30/**TNFRSF8**
4-1BB.L/**TNFSF9**	4-1BB (CD137)/**TNFRSF9**
TRAIL (APO-2.L)/**TNFSF10/***TL2*	DR4/**TNFRSF10A/***TR4*&DR5/**TNFRSF10B/***TR7* DcR1/**TNFRSF10C/***TR5*(nonfunctional) & DcR2/**TNFRSF10D/***TR10* (nonfunctional) OPG/**TNFRSF11B/***TR1*
TRANCE (OPGL)/**TNFSF11/***TL9* **TNFRSF11B/***TR1*	TRANCE R/**TNFRSF11A** & OPG/
TWEAK (LTK)/**TNFSF12/***TL8*	TWEAK-R/**TNFRSF12A/***TR3*
APRIL (TALL-2)/**TNFSF13/***TL3*	TACI/**TNFRSF13/***TR18*
BlyS (TALL-1)/**TNFSF13B/***TL7*	TACI/**TNFRSF13B/***TR18*, BCMA/**TNFRSF17/***TR19* & BAFF-R/**TNFRSF13C**
LIGHT/**TNFSF14/***TL5*	TNF-Rrp (LTβR)/**TNFRSF3** & HVEM**TNFRSF14/***TR2*
VEGI/**TNFSF15/***TL1*	DR3/**TNFRSF12?/***TR3?*
GITRL/**TNFSF18/***TL6*	GITR/**TNFRSF18/***TR11*
TNF-epsilon/**TNFSF?/***TL3*	**TNFRSF?**
ED1/EDA/**TNFSF?/***TL10*	EDAR/**TNFRSF?/***TR17* & XEDAR/**TNFRSF?/***TR14*
TANGO-L/**TNFSF?**	TANGO 129/**TNFRSF20?/***TR12*
EMILIN/**TNFSF?**	**TNFRSF?**
TRAIN-L/**TNFSF?**	TRAIN-R(OAF65α/APO4/TROY)/ **TNFRSF19/***TR15*

[a]**Unassigned** receptors:- DR6/*TR9, TR13, TR16,* TACI-like/HPMK140/*TR20,* TR12-like/ HCFMV39/*TR21,* TR21-like/HBHMF28/*TR22.*

clature, e.g., CD27, CD30, CD40, or according to whether they contained a "death-inducing domain" in their cytoplasmic domain, e.g., death receptor 3 (DR3), and so on ***(1–4)***. Under the new nomenclature system, in keeping with

the TNFSF system, they have been designated TNFRSF, each with a numerical suffix (*see* **Table 1** for full list and ligand receptor pairing). Following activation of receptors by cognate soluble- or membrane bound-TNFSF ligand binding, intracellular signaling pathways are activated leading to biological end-effects, such as cell death, proliferation, or differentiation ***(1–4)***. Those receptors containing a death domain are most closely associated with pathways leading to cell death, although this result is not always manifested because of cellular protection mechanisms (*see* below).

Assays for cytotoxicity determinations of cytotoxic TNFSF ligands, such as TNFSF2 (TNF-α), were developed in the 1970s ***(7)***. Such assays are of relatively simple design, requiring only exposure of susceptible cells to the cytotoxin (CTX) and then measurement of cell death after a fixed incubation period ***(7,8)***. CTXs are known to induce cell death by necrotic and apoptotic mechanisms ***(9)***. Necrosis is characterized by cell swelling, destruction of cellular organelles, and cell lysis, whereas in apoptosis caspase pathways are activated, cells shrink, apoptotic bodies are formed, and, in most cases, specific internucleosomal DNA fragmentation occurs ***(9)***. Cytotoxic cytokines, such as TNFSF2, can induce either necrotic or apoptotic cell death depending on the cells and conditions used ***(9)***. However, it is clear that TNFSF2 (TNF-α)-mediated cytotoxicity does not require RNA or protein synthesis. In fact, when TNFSF2 is combined with either a transcription inhibitor, e.g., actinomycin D, or a translation inhibitor, e.g., cycloheximide, cytotoxicity is increased considerably ***(7,8)*** and even cells that are resistant to TNFSF2 alone can in some instances succumb to cell death when such combinations are applied. These observations suggest that certain cellular proteins have a protective effect against TNFSF2-mediated cytotoxicity. However, the mechanisms leading to cell death appear complex and may be specific to certain cell phenotypes. One likely cause of cytotoxicity is the TNFSF2-induced production of reactive oxygen radicals by mitochondria ***(9)***. Thus, free radical scavengers, such as glutathione, and enzymes, such as mitochondrial manganous superoxide dismutase (MnSOD) ***(10,11)***, which catalyze the conversion of these toxic ions to harmless molecules, probably mediate resistance to TNF in some cases. Bcl-2 protein of the inner mitochondrial membrane, an inhibitor of apoptosis, has also been shown to protect against TNFSF2-mediated cytoxicity, possibly by regulation of an antioxidant pathway ***(12,13)***. Overall, susceptibility to cytotoxicity is probably to a large part governed by a cell's capacity to resist any TNFSF2-induced increase in oxidants. However, TNFSF2 and other cytotoxic members of the TNFSF, such as TNFSF6 (FasL) and TNFSF10 (TRAIL), induce receptor-activated procaspase-8 activation, leading to further activation of effector caspase pathways and apoptosis ***(14,15)***. Although highly regulated, this cell death–inducing mechanism may be of similar importance to the mitochondrial

oxidative mechanism. Decoy receptors DcR1/TNFRSF10C and DcR2/TNFRSF10D for TRAIL/TNFSF10, appear to block TRAIL/TNFSF10 signaling by competing with DR4/TNFRSF10A and DR5/TNFRSF10B ***(15)***. The inhibitory molecule cFLIP blocks both FasL/TNFSF6 and TRAIL/TNFSF10 cytotoxicity by preventing the recruitment and activation of the initiator caspase-8. In addition, some tumor cell lines express caspase-8 protein poorly or not at all, and are therefore resistant to TRAIL/TNFSF10-induced apoptosis ***(16)***.

Cytotoxic TNFSF members have generally been quantified by potency tests of in vitro cytotoxic activity ***(7,8,17)***. In practice, this means that susceptible cell lines (*see* **Note 1**) are incubated with serial dilutions of a test TNFSF preparation for a set period of time and then the residual cell viability is estimated by appropriate means. Historically, murine cell lines were the first to be identified as being suitably sensitive for TNFSF-induced cytotoxicity assays (*see* **Subheading 3.2.**) and thus useful for estimating potency. For example, the mouse L-929 fibroblast cell line was the first cell line to be shown to be highly susceptible to both human and murine TNFSF2 (TNF-α)-induced cytotoxicity and has remained a popular choice for cytotoxicity assays ***(7,18)***. The additional finding that its susceptibility to TNFSF1 and TNFSF2 was greatly increased by including one or more metabolic inhibitors (*see* **Note 2**), e.g., actinomycin D (AMD), mitomycin C, or emitine, has led to the development of more sensitive assays (*see* **Subheading 3.2.**) ***(7,8,18)***. Another advantage of using those inhibitors is that they also speed up the cytotoxic processes leading to shorter assay duration. Since the development of cytotoxicity assays based on L-929, a variety of other cell lines, including murine LM ***(19)***, WEHI 164 clone 13 ***(20)***, and EMT-6 ***(21)*** lines and human HeLa ***(22)***, U-937, and KYM-1 ***(23–25)*** lines, have also been found to be useful for this purpose. However, their usefulness in assays is normally restricted to particular TNFSF members. For example, KYM-1 is most sensitive to human TNFSF2, relatively less sensitive to human TNFSF1 and TNFSF10, and completely insensitive to TNFSF6 ***(24,26)***. Therefore, other cell lines are required to assay the cytotoxicity of TNFSF6. A range of T-cell lines, including Jurkat and CEM, and the Hodgkin's lymphoma cell line HDLM-2 are sensitive to apoptosis by TNFSF6 (FasL) and may be used for assays ***(27)***. Besides KYM-1, many T- and tumor cell lines are sensitive to TNFSF10 (TRAIL)-induced apoptosis. Some melanoma cell lines that were resistant to TNFSF2 and TNFSF6 have been reported to be sensitive to TNFSF10, with apoptosis being increased in the presence of cycloheximide or actinomycin D ***(28)***. Adenovirus E1A oncogene expression has also been shown to sensitize tumor cell lines to TNFSF10-mediated killing ***(29)***.

2. Materials

1. Tissue culture plates (Falcon cat. no. 3075 96-well or equivalent).
2. Plastic culture dishes, 9 cm (Sterilin or equivalent).
3. Pipetters with tips (Gilson Pipetman or equivalent).
4. Multichannel pipets with tips (Titertek or equivalent).
5. Laminar-flow biological safety hood (Envair class II cabinet or equivalent).
6. CO_2 incubator set at 37°C and set at 5% CO_2 (Forma Scientific or equivalent).
7. Water bath set at 37°C (Techne Tempette Junior or equivalent).
8. Absorbent paper towels (Kimnet Reinforced Cloths or equivalent).
9. Enzyme-linked immunosorbent assay (ELISA) plate reader (Titertek Multiskan Plus or equivalent).
10. Phosphate-buffered saline (PBS), six-salt (8000 mg/L NaCl, 200 mg/L KCl, 1150 mg/L Na_2HPO_4, 200 mg/L KH_2PO_4, 100 mg/L $CaCl_2$, 120 mg/L $MgSO_4$), pH 7.0–7.4
11. Supplemented RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) medium with antibiotics containing 7% fetal calf serum (FCS) (RPMI-1640/7% FCS): 450 mL RPMI-1640 or DMEM stock solution, 200 m*M* sterile L-glutamine, 5 mL of 100 m*M* sterile sodium pyruvate, 35 mL FCS (heat inactivated at 56°C for 30 min), 5 mL of 10,000 U/mL penicillin, and 10 mg/mL streptomycin. Store at 2–8°C; expiration is 4 wk from the date of preparation.
12. Appropriate cell lines for the TNFSF being tested: mouse L-929, WEHI164 clone 13, human KYM-1 for TNF-β/TNFSF1 and TNF-α/TNFSF2 from most species; human HDLM-2, Jurkat, or CEM cell lines for FasL/TNFSF6; Jurkat or human colon carcinoma cell lines, e.g., HT-29, for TRAIL/TNFSF10. KYM-1 and derived clonal lines may be also used for TRAIL/TNFSF10 and TWEAK/TNFSF12 (*see* **Note 1**).
13. Reference reagent/laboratory standard: international standard, national standard, or laboratory reference reagents for the particular TNFSF to be tested. Store in freezer set at –20°C or below.
    The following WHO reference materials ***(18)*** are available:
    - **87/650**, 1st WHO international standard for human TNF-α/TNFSF2, 40,000 international units (IU) per ampule.
    - **87/640**, 1st WHO international reference reagent for human TNF-β/TNFSF1, 160,000 units (U) per ampule.
    - **88/532**, NIBSC reference reagent for mouse TNF-α/TNFSF2, 200,000 U per ampule.

    Note: There are as yet no available reference materials for human FasL/TNFSF6 or for human TRAIL/ TNFSF10.
14. Appropriate stain solutions, e.g., naphthol blue black (NBB) solution (Aldrich Chemical Co. or equivalent). The stain solution contains approx 0.05% NBB in 9% acetic acid with 0.1 *M* sodium acetate ***(30)***: 0.5 g NBB, 90 mL glacial acetic

acid, 8.2 g of 0.1 *M* sodium acetate, distilled water to 1.0 L. The stain solution is prepared by stirring on a magnetic stirrer for 1 h, with the resulting solution filtered through Whatman No. 1 filter paper. It is stored in a tightly stoppered bottle at room temperature.

15. Fixative solution. This contains approx 4.0% formaldehyde in the above (*see* **item 14**) acetic acid-sodium acetate buffer ***(30)***: 100 mL formalin (formaldehyde 40%), 90 mL glacial acetic acid, 8.2 g of 0.1 *M* sodium acetate, distilled water to 1.0 L. Store in a tightly stoppered bottle at 4°C.
16. Sodium hydroxide solution (0.1 *M* NaOH): 4 g sodium hydroxide, distilled water to 1.0 L. Store at room temperature.

3. Methods

3.1. Use of TNFSF Standards and Sample Preparation

For the testing of cytotoxic TNFSF, use, where available, the appropriate WHO International Standard or Reference Reagent (*see* **Subheading 2., item 13**) as the primary calibrant and, subsequently, well-calibrated laboratory standards of the appropriate TNFSF. For example, for the testing of human and murine TNFSF2 (TNF-α), use 87/650 1st WHO International Standard for human TNFSF2 (hTNF-α) ***(18)*** and 88/532 NIBSC reference reagent for murine TNFSF2 (mTNF-α) ***(18)***, respectively, as the primary calibrants and, subsequently, well-calibrated laboratory standards of human and murine TNFSF2 (TNF-α). For the testing of TNFSF1 (LT-α), use 87/640 WHO reference reagent for human recombinant TNFSF1 (LT-α) ***(18)***. For other TNFSFs, prepare laboratory standards (*see* **Note 3**).

1. Dilute TNFSF standards, i.e., International Standards (*see* **Subheading 2., item 13**), reference reagents, laboratory standards, in cell growth medium, e.g., DMEM or RPMI-1640/7% FCS (*see* **Subheading 2., item 11**), on the basis of their assigned potency such that this initial, off-plate, dilution is appropriate for the subsequent dilution series for the titration of cytotoxicity in the assay (*see* **Note 4**). If the assay is one in which cells are also treated with a metabolic inhibitor, e.g., actinomycin D, then this should be included in the cell growth medium at the appropriate concentration.
2. TNFSF test samples. If the potency is known, e.g., from a manufacturer's estimate, dilute TNFSF sample in cell culture medium as per **step 1**. If the potency is unknown, prepare a series of 10-fold dilutions (i.e., 1:10, 1:100, 1:1,000, and so on) of the test sample in cell culture medium. If necessary, include metabolic inhibitor in cell growth medium used for dilution.

3.2. Test Procedure

This procedure is to be carried out in laminar-flow biological safety hood. The following is an example of how a cytotoxicity assay is set up.

1. Label the outside of 96-well microtiter plates with the type of assay and plate number and the date of test. Use columns 1 and 12 for controls, i.e., cell controls, cells only, no TNFSF; cytotoxicity positive controls, cells plus dose of TNFSF that induces maximal cytotoxicity. Use columns 2–11 for standard/reference preparation and test samples. Use a format diagram to label what samples and what dilutions are applied (*see* **Note 5**).
2. To each well of a 96-well plate, add 100 µL of DMEM or RPMI-1640/7% (*see* **Subheading 2., item 11**) FCS with or without metabolic inhibitor, e.g., actinomycin at 2.0 µg/mL, using a multichannel pipet (*see* **Note 5**).
3. Add 100 µL of appropriately diluted (*see* **Note 4**) reference preparation (standard) (*see* **Subheading 2., item 13**) to row A, column 2 and 3. The reference preparation should be included on all plates in the assay.
4. Add 100 µL of appropriately diluted (*see* **Note 4**) test samples to row A, columns 4 and 5, columns 6 and 7, columns 8 and 9, columns 10 and 11, i.e., duplicates of each test sample.
5. Using a multichannel pipet set at 100 µL, mix the contents of the wells of row A by withdrawing and ejecting half of the contents several times. Transfer 100 µL in the row A vertically to the wells of row B. Mix contents and transfer 100 µL to the wells of row C. Continue diluting as above in rows D, E, F, G, and H. Finally, discard 100 µL from the wells of row H (*see* **Note 5**). This procedure gives the dilution series 1:2, 1:4, 1:8, 1:16, 1:32, and so on. Repeat the procedure with fresh tips for each plate in the assay.

3.3. Cell Preparation

3.3.1. Adherent Cells

Monolayers of these should be prepared the day before addition of standard and test sample dilutions. Pour cell suspension of L929 cells (or other TNFSF-sensitive adherent cell line, e.g., KYM1-D4 clone 21; *ref.* ***25***), which has been adjusted to contain approx 2×10^5 cells/mL of DMEM/7% FCS (*see* **Subheading 2., item 11**) into a 9-cm sterile plastic Petri dish (*see* **Note 6**). Add the cell suspension from the Petri dish using a multichannel pipettor set at 100 µL to each well of all 96-well microtiter plates required for the assays. Incubate at 37°C overnight to form monolayers. Using a multichannel pipet set at 100 µL, transfer the well contents from the microtiter dilution plate(s) (*see* **Subheading 3.2., step 5**) in a stepwise fashion from the largest dilution (row H) to the smallest dilution (row A). This addition results in a further 1:2 dilution of standard and sample dilutions across the assay plate (*see* **Note 7**).

3.3.2. Nonadherent Cells

1. Spin down cells from a suspension culture by low-speed centrifugation and resuspend in fresh cell growth medium, RPMI-1640/7%FCS (*see* **Subheading**

2., item 11), at 2×10^5 cells/mL (*see* **Note 6**). Add this suspension at 100 µL/well to all wells of microtiter plates included in the assays. This addition results in a further 1:2 dilution of standard and sample dilutions across the assay plate.
2. Incubate the plates for about 24 h in an incubator set at 37°C and 5% CO_2 (*see* **Note 7**).
3. At this stage, using an inverted microscope, check the cells for appearance of cytotoxicity. In contrast to cells in "cell controls," which should remain healthy looking, the cells in the cytotoxicity positive controls should be mostly or completely dead. The wells containing serial dilutions of TNFSF standard or test samples should contain cells with varying degrees of cytotoxicity, i.e., a portion that are dead, and a portion that are viable, depending on TNFSF concentration. If the cytotoxicity is not fully developed in the positive controls, prolong the incubation until this is so.
4. Remove most of the culture medium in wells by flicking out into chloros and blotting on a paper towel. Add PBS to each well using a multichannel pipet set at 200 µL (*see* **Note 8**). Flick out PBS into chloros.

3.4. Staining or Processing Cells for Cytotoxicity Measurements

There are several alternative methods to do this and below are three examples (*see* **Note 9**).

3.4.1. Staining Viable Cells

1. Add NBB solution (*see* **Subheading 2., item 14**) to each well using a multichannel pipet set at 150 µL.
2. Stain cells for approx 30 min at room temperature. *(Crystal violet can also be used, but tends to be very messy and, hence, the recommendation to use NBB.)*
3. Flick out NBB solution into chloros solution.
4. Add approx 150 µL of fixative solution (*see* **Subheading 2., item 15**) and leave cell monolayers to fix for 10 min at room temperature.
5. Flick out fixative solution into chloros and wash cell monolayers by immersing assay plates in a plastic box containing running tap water (*see* **Note 8**).
6. After drying, add 150 µL 0.1 *M* NaOH (*see* **Subheading 2., item 16**) to each well using a multichannel pipet set at 150 µL.
7. Elute stain by gentle agitation of the plates or knocking them against the palm of the hand. Make sure the stain is evenly distributed in all wells before making spectrophotometric readings at 610–630 nm. This method works well for fully adherent cell lines such as L-929 (*see* **Note 10**).

3.4.2. Insoluble Formazan Method

Staining methods are by no means perfect and require washing steps that often remove cells (*see* **Note 8**). To remove the possibility of cell loss, use can be made of the direct addition of chemicals that are converted by cellular activities to colored products. The classical example of this is the metabolic reduction of tetrazolium salts such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphe-

nyl tetrazolium bromide (MTT) in viable cells to a colored (purple) formazan product ***(31)***. In the case of MTT, its formazan product is insoluble, but may be dissolved by acidified sodium dodecyl sulfate (SDS) ***(24)***. Therefore, at the end of the incubation to produce cytotoxicity:

1. Add 10 µL of MTT (5.0 mg/mL in PBS) to all wells and incubate at 37°C for 1 h.
2. Add 25 µL of 10% SDS in 0.1 *M* HCl to all wells and incubate for at least 4 h at 37°C.
3. Check that the released formazan is evenly dispersed in the wells and read at 570–590 nm. This method works fine for cell lines that are partially adherent such as WEHI 164 clone 13.

3.4.3. Soluble Formazan Method

The MTT procedure takes several hours to give evenly distributed color in wells and, therefore, use of a water-soluble tetrazolium compound, such as 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium, inner salt (MTS) ***(32)***, to yield a soluble formazan product may be preferred. MTS is taken up by live cells and, in the presence of electron acceptors such as phenazine methosulfate (PMS) or menadione sodium bisulfite (MSB; soluble vitamin K_3), is metabolically reduced to a water-soluble, red, formazan product, which diffuses out of the cells into the medium and whose absorbance can be determined at 492 nm.

1. Add 50 µL of MTS (1.0 mg/mL) plus 75 µ*M* PMS or 60 µ*M* MSB solution to all wells.
2. Read absorbances at 492 nm after 1–2 h incubation at 37°C without further interventions ***(25)***. If this method is employed, improvement in color development may be made by using a cell growth medium, e.g., RPMI-1640, that does not contain phenol red. This method works well for nonadherent cell lines in general and is also good for KYM-1 and its derivatives ***(25)***.

3.5. Data Analysis

Results of the cytotoxicity assay generally fit a sigmoidal dose–response curve, when the TNFSF concentration (the log of the reciprocal of the TNFSF dilution) is plotted vs stain or formazan absorbance. A useful approach for comparison of test samples with standards is parallel line analysis. The slopes of the linear portions of the test and standard dose–response curves should be parallel for biometric validity. Using the linear portion of the curve (*see* **Note 5**), calculate the concentration of TNFSF in the sample by comparing the responses for test and reference solutions, using the statistical methods for a parallel line assay ***(18,33,34)***. Alternatively, titers may be roughly calculated using an end point method, as follows:

1. Plot TNFSF concentration (reciprocal of dilution) vs absorbance for the TNFSF reference preparation and all test samples. Use only the linear portion of the curve to estimate a titer of any TNFSF preparation/sample. (The titer is the TNFSF

concentration interpolated at the arbitrarily defined endpoint of the response measured, i.e., the median absorbance on the scale of measurements that range between those of untreated cell controls and cytotoxicity positive controls.) The TNFSF titer corresponding to the end point can be obtained graphically from semilog plots or by linear regression calculations, using only those points that fall within the linear portion of the curve.

2. Correct the TNFSF titers of test samples expressed in observed or laboratory units (LU)/mL to the assigned titer (potency) of the TNFSF reference preparation, which, if an international or national standard is available, will have its potency expressed in international units (IU). Use the following equation:

Titer of TNFSF test sample in IU/mL = [observed titer of TNFSF test sample (LU/mL)]/[observed titer of TNFSF ref prep (LU/mL)] × assigned titer of TNFSF reference preparation (IU/mL)

4. Notes

1. It is important to ensure that a cell line used for cytotoxicity assays is really suitable for its purpose. If starting to perform cytotoxicity assays for the first time, try to obtain from a reliable source one of the cell lines recommended under **Subheading 2., item 12.** Several cellular characteristics are crucial for good and reproducible performance. The cell line should be healthy and, if possible, free of mycoplasma contamination. A frozen stock of a low passage number must be established in order that, if and when changes in TNFSF sensitivity occur, a return to low-passage cells can be undertaken. A cell-passaging schedule should be established and rigorously adhered to in order that the cells used for cytotoxicity assays are obtained from cultures grown on a consistent basis. In the case of adherent cell lines, the cells should be fully adherent and spread out in culture to form uniform monolayers; cell lines that clump or aggregate or slough off cells into the medium should be avoided. In connection with this characteristic, cell lines that can be prepared as single-cell suspensions following trypsinization are better for distribution into wells of microtiter plates than those that aggregate.
2. It is often the case that experimental conditions need to be tested thoroughly in order that the sensitivity of cytotoxicity assays is satisfactory (*see* **Subheading 3.2.**). Thus, an empirical approach is advised. It is important to establish the "dose" of TNFSF that efficiently kills the cells and the time required for this to happen. For example, in the absence of AMD, murine L-929 cells are killed by TNFSF2 (TNF-α) in approx 48 h. In contrast, in the presence of AMD, L-929 cells are killed by TNFSF2, generally at a lower dose than that required for cell killing in the absence of AMD, in 18–24 h ***(7,8)***. The majority of human KYM-1 rhabdomyosarcoma cells are killed in 5–10 h by similar doses of TNFSF2 without AMD, but for unknown reasons some cells are spared and either are growth inhibited or can grow and divide. In such cases the point at which assays are terminated represents not only cytotoxic effects but also antiproliferative effects ***(24)***. In the event, this combined manifestation of TNFSF activity probably will

not matter too much when purified recombinant preparations are being compared, but may be relevant for impure preparations that contain other cytokines, whose activity may also regulate cell proliferation. Obviously, any TNFSF-induced antiproliferative effect is negated in the presence of a metabolic inhibitor such as AMD, which by itself rapidly inhibits cell growth and division. It should however be pointed out that the reduced metabolism of AMD-treated cells leads to reduced stain uptake and tetrazolium conversion to formazan products and, therefore, to less strong absorbances generally and, as a consequence, to shallower dose–response curves. In the case of human KYM-1 cells, AMD is itself significantly toxic at low concentrations and, therefore, we developed a variant KYM-1 cell line, designated KD4c21, which is much more resistant to the toxicity of AMD and can be used to measure the cytotoxicity of TNFSF1 and TNFSF2 ***(25)***. However, the metabolism of KD4c21 is greatly reduced so that satisfactory cytotoxicity assays can only be achieved if MTS is used to process the cells ***(25)***. It is evident that cell lines may vary greatly in their response to TNFSFs and to any metabolic inhibitors included in assays to enhance assay sensitivity. Therefore, the effects of metabolic inhibitors should be fully investigated in each cell line used and optimal concentrations of inhibitors in combination with TNFSFs established empirically (*see* **Subheading 3.2., step 2**).

3. Ideally, TNFSF cytotoxic activity (potency) should be reported with reference to an appropriate WHO international standard (IS) and expressed in international units (IU). However, at this point in time, there is only a WHO IS for human TNFSF2 (TNF-α) and a WHO reference reagent for human TNFSF1 (LT-α/TNF-β) available for cytotoxicity assay calibration (*see* **Subheading 2., item 13; ref. *18***). WHO IS and/or reference reagents for other members of the TNFSF have not yet been developed. Where an IS or reference reagent is not available, e.g., for human TNFSF6 (FasL), calibration of assays should be made with a well characterized and stable in-house or laboratory standard of the same material.
   International standards, as primary standards with potency assigned in IU, are used to calibrate working standards (in-house or national standards), which are well characterized and as close as possible in purity and form to the IS. That the IS and the working standard contain identical or very similar TNFSF2 (TNF-α) is necessary for compliance with the central tenet of biometric validity of assays ***(34)***. "When two or more TNFSF preparations are being compared, they must behave identically in the assay for the assay to be truly valid." In this case, where the IS serves as primary reference preparation, the working standard must behave as if it were a more concentrated or more dilute solution of the IS preparation. Stated differently, if like is compared with like in the same assay system, under the same conditions, then any difference in the measured response from the assay system should reflect only the difference in concentration ***(34)***. Dose–response lines for the IS and the working standard must be parallel for biometric validity. Usually, the IS and working standard are compared in assays performed on at least five separate occasions and the geometric mean potency of the working standard, expressed in IU, assigned to it. The working standard should then be

used to routinely calibrate all further assays to estimate the potency in IU of TNFSF2 preparations containing the same TNFSF2 as the working standard. In this way stocks of ampules of the IS are conserved. *(It has to be emphasized that the potency of TNFSF2 cannot be expressed in molar concentrations or mass units since molarity/mass is a measure of the physical quantity of material and, thus, does not reflect its biological activity. For example, the potency of a TNFSF2 preparation held under different conditions may vary significantly as a function of time without any change in molarity/mass. It is imperative therefore to express potency in IU of biological activity.)*

4. The WHO IS for TNFSF2 (TNF-α) is an ampuled freeze-dried preparation, which has demonstrated long-term thermal stability ***(18)***. However, once reconstituted, the resultant solutions are not guaranteed to maintain their full activity, particularly at temperatures above 4°C. Therefore, aliquots of the reconstituted IS solution should be stored frozen at –70°C until required. After thawing an aliquot, it should be used only once for calibration and then discarded. For the working standard, a suitably large volume should be subdivided in appropriately sized aliquots and frozen at –70°C. To prevent any adsorption of TNFSF2 (or other TNFSF) to container walls, plastic tubes or siliconized glass vials are recommended.

   Clinical grade or commercially supplied TNFSF products are usually relatively easy to handle. They are normally formulated with excipients, e.g., human serum albumin, to stabilize the TNFSF activity and thus have long shelf lives. If freeze-dried, they can be reconstituted and aliquots frozen at –70°C if necessary. Frozen liquid preparations are best kept frozen at their recommended storage temperature. The only problem with clinical grade TNFSF products from an assay point of view is that they are very highly potent. Their assigned potencies are usually 1.0 million IU (MIU) or greater and, therefore, they require considerable dilution before titration in cytotoxicity assays. The accuracy of the TNFSF potency determination in these is therefore subject to the accuracy with which the off-plate dilution can be made (*see* **Subheading 3.2.**). This may involve several 10-fold dilution steps. Appropriate volumes of dilution medium, normally cell culture medium containing bovine serum, should be chosen and dilution at each stage carried out with micropipets calibrated for precise delivery of the dilution volume.

   In contrast, samples obtained from stimulated cell cultures or clinical samples, including sera, plasmas, cerebral spinal fluid (CSF), bronchial lavage fluids (BLF), and so on, are generally more difficult to handle. Often the TNFSF present will be of low concentration, may be comprised of a mixture of TNFSF members, and may contain other cytokines and substances that have modulating effects on the sensitivity of cytotoxicity assays. Serum samples, for example, may contain substances other than TNFSF, which act additively or synergistically with TNFSF. Additionally, serum samples may contain growth or cytotoxic (non-TNFSF) factors for cells, which act to induce proliferation and cell death, respectively. Such ingredients can therefore make it difficult to decide whether a

cytotoxic effect manifested in the assay is caused by TNFSF or another substance. In some cases, it may be possible to judge whether it is a TNFSF or not by the slope of the dose–response line, which should be close to that of the working standard. However, in other cases, serum components may alter the slope of the dose–response line and, thus, apparently disguise the presence of TNFSF. One way to confirm that a particular TNFSF is really present is to use a specific neutralizing antibody, although in many cases a guess will probably have to be made as to what specificity is required because the identity of the TNFSFs contained in the sample may not be known.

5. The nonuniformity of 96-well microtiter plates and the variable accuracy of multichannel micropipets can introduce untoward biases, which can contribute to uneven responses across plates. In many cases, the outer wells of 96-well microtiter plates appear to give the most uneven responses, either due to uneven spraying of wells in manufacture or due to the exposure of cells in these outer wells to more rapid variations in temperature and pH. Some investigators therefore opt to use only the inner 60 wells for titrations, even though this means that fewer samples can be titrated on each plate and that the number of serial dilutions of each sample may be more restricted. Nevertheless, we have often found, even if wells in columns 1 and 12 are excluded, that titrations performed in the wells of column 2 and 11 are still more likely to produce results that are "outliers" than columns 3–10. If wells in columns 2 and 3 are used as duplicates, then we frequently find that the sensitivity of cells to TNFSF2 (TNF-α) is lower in column 3 compared with column 2. Sensitivity appears to decrease further to columns 6 and 7, i.e., the middle columns, and then rise again on the right side of the plate toward column 11. This may not happen in all circumstances, but the likely manifestation of these "plate effects" should be borne in mind when deciding the positions of samples and standards. Ideally, when several preparations are tested they should be titrated on several plates such that the positions of individual samples relative to the standard are varied. The position of the standard should also be moved from plate to plate. An international study to assign potencies to candidate TNF-α standards found that designs in which each preparation is tested on several plates in any assay with independent serial dilutions of the preparation on each plate generally gave more accurate and reproducible estimates of potency ***(33)***. Complete randomization of samples in terms of plate positions is usually not feasible, but efforts should be made to carefully design assays to permit more valid use of classical methods of analysis. One way of checking intraassay variability and thus the validity of results in each assay is, using independent dilutions, to titrate one preparation twice, e.g., as coded duplicates, say A and H, where H is identical to A except for code. The ratio of the potency of A to that of H should approach 1.0. Large deviations from 1.0 indicate that biases have been introduced or occurred in the assay and may compromise its overall validity ***(33)***. In general, the slopes of dose–response lines generated in cytotoxicity assays are sigmoidal, but are steep. This means that going from fully viable to dead cells occurs over a relatively narrow TNFSF concentration range. In turn, this trans-

lates into having only a few data points in the linear portion of the dose–response line and potential difficulties in comparing the slopes of dose–response lines for individual samples. Slopes should be parallel if the calculated results are to be valid ***(34)***. Therefore, assay conditions and design should be set so that the number of data points in the linear portion is maximized. Using smaller serial dilutions may do this, e.g., 1:1.5 rather than 1:2, of TNFSF test samples. Alternatively, stain methods for processing cytotoxicity assays may be substituted by formazan methods (*see* **Subheadings 3.4.2.–3.4.3.**), which may generate less steep slopes.

6. In all cases, cell density is an important factor in determining assay sensitivity. The amount of cytotoxicity manifested is inversely proportional to cell density, i.e., the higher the cell density, the lower the degree of cytotoxicity. Thus, relatively low cell densities are recommended to yield sensitive assays. However, if cell density is reduced too far, then final absorbances are correspondingly reduced and the dose–response curves will become very shallow and therefore more difficult to analyze.
7. The incubator temperature and its pH are also factors that can influence the rate of cell killing. These should be controlled as completely as possible and maintained unchanged over long periods so that cytotoxicity assays can be performed consistently on a routine basis.
8. At the point of termination of cytotoxicity assays, cells in the cell controls should appear totally alive and healthy, whereas cells in the cytotoxicity positive controls should appear dead. Usually with dead adherent cells, they remain attached to the plastic bottom of the well. However, subsequent washing and staining procedures may variably dislodge these dead cells (and also some live cells). It is evident that where dead cells are more or less completely removed by washing/staining that the final stain uptake will be very low (the well will look almost clear), whereas, if the dead cells remain mostly attached, then there will be a stained background appearance to the well (most "vital" stains seem to stain dead cells to some degree). Some cell lines, e.g., L-M, tend to give high backgrounds in the positive controls because of the stickiness of dead cells. These high backgrounds may affect the slope of the dose–response lines and therefore adversely impact on the outcome of cytotoxicity assays.
9. The majority of cytotoxicity assays are still terminated by staining and fixing cell monolayers. Several vital stains, i.e., those that stain only, or preferentially, live cells, are available. The violet dyes, such as crystal or gentian violet prepared as 1–2% solutions in 20% ethanol/PBS, are often used for this purpose, but tend to be very messy (***30***). For this reason, we recommend naphthol blue black (alternatively called amido black or amido blue black) dissolved in sodium acetate–acetic acid buffer (*see* **Subheading 2., item 14**, NBB solution) for staining followed by cell fixation with acidified formalin solution (*see* **Subheading 2., item 15**).
10. Once cell monolayers or the remaining viable cells are stained, it is possible to "read" the plates visually, and cytotoxicity is generally assessed by a + and – score. Although it is possible to gage relative activities by this method, there will

be a major subjective element involved and therefore we recommend that spectrophometric methods for determining absorbances be used. The latter generate data that lend themselves more readily available to classical methods of analysis and computation of results (*see* **Subheading 3.5.**). Staining methods are by no means perfect and require washing steps that often remove cells. To remove the possibility of cell loss, use can be made of the direct addition of chemicals that are converted by cellular activities to colored products. The classical example of this is the metabolic reduction of tetrazolium salts such as MTT in viable cells to a colored (purple) formazan product ***(31)***. In the case of MTT, its formazan product is insoluble, but may be released by acidified SDS and absorbances read at 570–590 nm (*see* **Subheading 3.4.2.**). However, the MTT procedure takes several hours to give evenly distributed color in wells and therefore we prefer to use MTS ***(32)***. MTS is taken up by live cells and, in the presence of electron acceptors such as PMS or MSB (soluble vitamin K_3), is metabolically reduced to a soluble red formazan product (*see* **Subheading 3.4.3.**), which diffuses out of the cells into the medium and whose absorbance can be determined at 492 nm ***(32)***. Addition of MTS (1.0 mg/mL) plus PMS (75 µ*M*) or MSB (60 µ*M*) solution requires minimal manipulation and absorbances may be read after 1–2 h incubation at 37°C without further interventions ***(25)***. In practice, the MTS method only works satisfactorily for cells that are efficiently killed by TNFSFs, because if any live cells remain, they will produce red formazan into the medium, i.e., a high background may be generated and the dose–response line will be correspondingly shallow. However, we, and others, have shown that, with appropriate combinations of metabolic inhibitor and cells, the MTS method of processing cytotoxity assays gives as good as dose–response data as staining or the MTT method and possibly even better sensitivity ***(25)***.

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Assays for Chemotaxis

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1. Introduction

Leukocytes play an important role in inflammation and cancer. They are attracted to the place of injury by chemotactic factors, e.g., plasma complement factors C5a and C3a, and chemotactic cytokines or chemokines, which are produced locally at the site of infection. Chemokines are produced by a variety of cell types including tumor cells. Leukocytes, selectively attracted by chemokines, will modulate the inflammatory response (phagocytosis) and affect tissue remodeling and tumorigenesis by the chemokine-induced release of proteases and other metabolites ***(1)***.

To study the chemotactic activity of factors that attract different leukocytic cell types in vitro, two methods are used in our laboratory: chemotaxis through micropores (Boyden chamber) and chemotaxis under agarose. Chemotaxis through micropores is currently performed in a 48- or 96-well microchamber. The number of cells that migrate through micropores of a precise size is evaluated. In the agarose assay, the effective migration distance of cells under an agarose gel is used as a parameter for chemotactic activity. The chemotaxis test through micropores is more sensitive, but also more labor intensive than the agarose assay.

Granulocytes and mononuclear cells (source for neutrophils or eosinophils and monocytes or lymphocytes, respectively) can be isolated from human peripheral blood. To study monocyte or lymphocyte chemotaxis in the microchamber, myelomonocytic THP-1 cells or ESb-MP lymphoma cells ***(2)***, respectively, can be used as an alternative.

Leukocyte extravasation in rabbit skin can be examined in vivo by measuring the local accumulation of intravenously injected ^{111}indium-labeled leukocytes after intradermal injection of various leukocyte chemotactic factors.

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2. Materials

2.1. Chemotaxis In Vitro

1. Phosphate-buffered saline (PBS; Life Technologies, Paisley, Scotland): 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, 1.15 g/L Na_2HPO_4, pH 7.2; store at 4°C.
2. Hydroxyethyl starch solution, 6%: Plasmasteril (Fresenius AG, Bad Homburg, Germany).
3. Ficoll-sodium metrizoate solution: Lymphoprep (Life Technologies); density = 1.077 g/mL.
4. Hanks' balanced salt solution (HBSS) supplemented with 1 mg/mL human serum albumin (HSA): HBSS (Life Technologies) + 1 mg/mL HSA; store at 4°C.
5. HSA; store at 4°C.
6. Türk's solution: acetic acid gentian violet solution (Merck, Darmstadt, Germany).
7. 3.6% NaCl solution.
8. HBSS + 1 mg/mL HSA + 2 m*M* ethylenediaminetetraacetic acid (EDTA); store at 4°C.
9. Colloidal superparamagnetic microbeads conjugated with monoclonal mouse antihuman CD16 Ab or CD14 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany); store at 4°C, protected from light.
10. Depletion column (Miltenyi Biotec); store dry, protected from light.
11. Magnetic cell separator: VarioMACS (Miltenyi Biotec).
12. Dulbecco's minimum essential medium (DMEM; Bio Whittaker Europe, Verviers, Belgium): store at 4°C.
13. Fetal calf serum (FCS; Bio Whittaker Europe): store at 4°C.
14. 0.5% Trypane blue solution (Fluka AG, Buchs, Switzerland).
15. 4.5% NaCl solution.
16. Microchamber, 48-well (Neuro Probe, Cabin John, MD).
17. *N*-Formylmethionylleucylphenylalanine: fMLP (Sigma, St. Louis, MO); store at <0°C.
18. Polyvinylpyrrolidone (PVP)-free and PVP-treated polycarbonate filters, 5 µm pore size (Nuclepore, Pleasanton, CA).
19. Human plasma fibronectin (Life Technologies): stock solution of 1 mg/mL in HBSS + 1 mg/ml HSA; store in aliquots at –20°C.
20. Fixative solution Hemacolor 1 (Merck).
21. Diff-Quick: color reagent red and blue, Hemacolor 2 and 3, respectively, (Merck).
22. Microscope with 500× magnification.
23. 10X concentrated Eagle's minimum essential medium with Earle's salts (Life Technologies); store at 4°C.
24. 7.5% sodium bicarbonate solution.
25. 200 m*M* glutamine, store at 4°C.
26. Agarose: Indubiose A37 (Sepracor/IBF, Villeneuve-la Garenne, France).
27. Methanol.

2.2. Granulocyte Migration In Vivo

1. Sodium pentobarbitone: 60 mg/mL Nembutal® (Abbott, Paris, France); 1:1 diluted with saline.
2. 0.9% saline: sterile pyrogen-free NaCl solution.
3. Acid-citrate-dextrose (ACD) (Baxter, Lessines, Belgium); store at 4°C.
4. 6% hydroxyethyl starch solution: Plasmasteril (Fresenius AG).
5. Isotonic Percoll: nine parts 100% Percoll (Amersham Pharmacia Biotec, Uppsala, Sweden) and one part 1.5 *M* NaCl solution; store at 4°C.
6. Citrated plasma: To prepare rabbit citrated plasma, collect 9 mL of rabbit blood in 1 mL of citrate solution (3.8% sodiumcitrate in sterile pyrogen-free water). Collect plasma after centrifugation: 15 min at 1500*g* and 15 min at 4000*g*; store at <0°C.
7. May-Grünwald and Giemsa stain (Merck).
8. $^{111}InCl_3$: 2 mCi in 0.2 mL sterile pyrogen-free 0.04 *M* hydrochloric acid (Amersham Pharmacia Biotech); store at 4°C; owing to the short half-life of $^{111}InCl_3$, it can be used only for 1 wk.
9. 2-mercaptopyridine-*N*-oxide (MERC) (Sigma): stock solution of 4 mg/mL in PBS; store at 4°C in the dark.
10. Prostaglandin E_2: PGE_2 (Sigma): stock solution of 1 mg/mL in ethanol; store at <0°C.
11. Calcitonin gene-related peptide (CGRP) (Sigma): stock solution of 2×10^{-5} *M* in saline; store at <0°C.
12. 2.5% Evans blue dye solution, in saline (Sigma).

3. Methods

3.1. Chemotaxis In Vitro

3.1.1. Isolation and Preparation of Leukocytes and THP-1 Cells

3.1.1.1. Isolation of Granulocytes and Mononuclear Cells (*see* **Note 1**)

Granulocytes and mononuclear cells are used as a source for neutrophils or eosinophils and monocytes or lymphocytes, respectively. They can be isolated from fresh human peripheral blood that is collected in a heparinized tube.

1. Mix 20 mL of blood with 20 mL of PBS and 20 mL of hydroxyethyl starch solution in a cylinder. Place the cylinder at 37°C for 30 min to allow sedimentation of erythrocytes.
2. Collect the supernatant and centrifuge for 10 min at 400*g* at 4°C.
3. Wash the cell pellet with PBS.
4. Resuspend the cell pellet in 10 mL of PBS. Load this cell suspension carefully onto 30 mL of Ficoll-sodium metrizoate solution. Centrifuge for 30 min at 4°C at 400*g* *without the brake on*. This step will separate the granulocytes and the remaining erythrocytes from the mononuclear cells.

5. Remove the layer of mononuclear cells and wash these cells with PBS. Resuspend the cells in 1 ml HBSS supplemented with 1 mg/mL HSA (HBSS + 1 mg/mL HSA). Count the mononuclear cells after staining with Türk's solution. Dilute the cell suspension in HBSS + 1 mg/mL HSA to an appropriate cell concentration. These mononuclear cells (containing 5–10% monocytes) can be used as a source for monocytes in the chemotaxis assays without further fractionation (*see* **Note 2**).
6. Discard the supernatant after removal of the mononuclear cells and wash the cell pellet (which contains granulocytes and remaining erythrocytes) with PBS.
7. Resuspend the cell pellet in 24 mL of double-distilled water for 30 s to lyse the remaining erythrocytes. Add 8 mL of a 3.6% NaCl solution to make the medium isotonic. Centrifuge for 10 min at 4°C at 400*g*.
8. Wash the cell pellet with PBS and resuspend the pellet in 1 mL HBSS + 1 mg/mL HSA. Count the cells after staining with Türk's solution. Dilute the granulocytes in HBSS + 1 mg/mL HSA to an appropriate cell density. This cell suspension consists of more than 95% of neutrophils and is used to study neutrophil chemotaxis.

3.1.1.2. Isolation of Eosinophils

Eosinophils are isolated from the purified granulocyte fraction (*see* **Subheading 3.1.1.1.**) by depletion of the neutrophils. Therefore, paramagnetic microbeads conjugated with antibodies against the neutrophilic cell marker CD16 (= negative selection) are used.

1. Resuspend the granulocytic cell pellet in 50 µL of HBSS + 1 mg/mL HSA + 2 m*M* EDTA per 5×10^7 total cells.
2. Add 50 µL of MACS CD16 microbeads per 5×10^7 total cells, mix and incubate for 30 min at 6–12°C (*see* **Notes 3** and **4**).
3. Add HBSS + 1 mg/mL HSA + 2 m*M* EDTA to a volume of 1 mL per 5×10^7 total cells.
4. Load the cell suspension on top of a depletion column, washed with buffer and placed in the magnetic field of a MACS separator (*see* **Notes 5** and **6**).
5. Let negative cells pass through the column and wash the column with 3–5 column volumes of HBSS + 1 mg/mL HSA (*see* **Note 7**).
6. Centrifuge the eluted cells for 10 min at 400*g* at 4°C.
7. Count the cells after staining with Türk's solution and resuspend the cells at the appropriate concentration in HBSS + 1 mg/mL HSA.

3.1.1.3. Isolation of Lymphocytes

Lymphocytes are isolated from the purified mononuclear cell fraction (*see* **Subheading 3.1.1.1.**) by depletion of monocytes. Therefore, paramagnetic microbeads conjugated with antibodies against the monocytic cell marker CD14 (= negative selection) are used.

1. Resuspend the pellet of mononuclear cells in 80 µL of HBSS + 1 mg/mL HSA + 2 m*M* EDTA per 10^7 total cells.
2. Add 20 µL of MACS CD14 microbeads per 10^7 total cells, mix and incubate for 15 min at 6–12°C (*see* **Notes 3, 4,** and **8**).
3. Add HBSS + 1 mg/mL HSA + 2 m*M* EDTA to a volume of 500 µL per 10^8 total cells.
4. Load the cell suspension on top of a depletion column, washed with buffer and placed in the magnetic field of a MACS separator (*see* **Notes 5** and **6**).
5. Let negative cells pass through the column and wash the column with 3–5 column volumes of HBSS + 1 mg/mL HSA (*see* **Note 9**).
6. Centrifuge the eluted cells for 10 min at 400*g* at 4°C.
7. Count the cells after staining with Türk's solution and resuspend the cells at the appropriate concentration in HBSS + 1 mg/mL HSA.

3.1.1.4. Preparation of THP-1 Cells

The myelomonocytic cell line THP-1 can be used as an alternative for mononuclear cells to measure monocyte chemotactic activity in the microchamber. THP-1 cells are grown in stationary flasks (175 cm^2, Nunc, Roskilde, Denmark) in DMEM (50 mL/flask) supplemented with 10% FCS. When cells are grown to a concentration of 1.5×10^6 cells per ml, they are subcultivated (1:3 split ratio). Cells are used in the chemotaxis assay on the second day after subcultivation:

1. Centrifuge the cell suspension at 20°C at 250*g* for 10 min.
2. Resuspend the cell pellet in HBSS + 1 mg/mL HSA. Count the cells and determine the viability after staining with 0.5% trypane blue solution/4.5% NaCl solution (4/1 v/v) (*see* **Note 10**).
3. Dilute the cell suspension in HBSS + 1 mg/mL HSA to the appropriate density.

3.1.2. Chemotaxis Assays

3.1.2.1. Chemotaxis Through Micropores

As a parameter for chemotactic activity, the number of leukocytes migrated through pores of a precise size is evaluated in this assay. This technique was introduced by Boyden ***(3)***: a polycarbonate filter is placed in a chamber to create two compartments; the lower compartment is filled with test sample, positive or negative control, and the upper compartment is filled with cells. This technique can be performed in a multiwell microchamber (48-well microchamber, consisting of a bottom plate, a top plate and a silicone gasket; *see* **Fig. 1**) ***(4)***.

1. Prepare dilutions of the test samples and the positive control in HBSS + 1 mg/mL HSA (*see* **Note 11**).

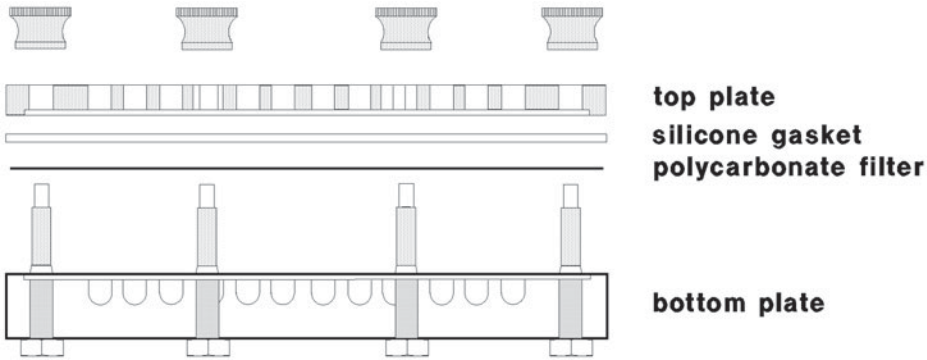

Fig. 1. Chemotaxis through micropores. The assay is performed in a 48-well microchamber, consisting of a bottom and top acrylic plate, sealed by a silicone gasket. The lower wells (containing samples or controls) are separated from the upper wells (containing cells) by a polycarbonate filter, through which the cells can migrate.

2. Add 27 µL of test sample or control (positive or negative) to the wells of the lower compartment of the 48-well chamber (*see* **Notes 12** and **13**).
3. Put the polycarbonate membrane on the bottom plate using forceps (*see* **Note 14** and **Table 1**).
4. Place the silicone gasket on the membrane.
5. Screw the top plate on the bottom plate.
6. Add 50 µL of cell suspension at the appropriate density (*see* **Table 1**) to each well of the top plate.
7. Incubate the chamber in a 5% CO_2-incubator at 37°C for 45 min (neutrophils), 1 h (eosinophils), 2 h (monocytes and THP-1 cells), or 4 h (lymphocytes) (*see* **Note 15**).
8. Dismount the microchamber unit and remove the filter (*see* **Note 16**).
9. Put the filter for 2 min in a fixative solution (Hemacolor 1) and stain the filter with Diff-Quick (2 min in color reagent red, Hemacolor 2 and 2 min in color reagent blue, Hemacolor 3). Rinse the filter with water to remove excess of color reagents. Place the filter on a microscope slide and let dry. Count the migrated cells (adherent at the lower surface of the filter) microscopically in 10 immersion oil fields for each well (magnification 500×). Calculate the average of cells migrated to the test samples and controls, respectively.
10. Determine the chemotactic index of test samples (*see* **Note 17**).

3.1.2.2. Chemotaxis Under Agarose (*see* Note 18)

In the agarose assay ***(5)***, the migration distance of cells under agarose toward the test sample is used as a parameter for chemotactic activity. This method has been applied in our laboratory to study neutrophil and monocyte chemotaxis. The assay is performed in agarose plates, that are prepared the day before.

Table 1
Experimental Conditions to Measure Chemotaxis of Different Cell Types in the Microchamber

Cell type	Cell density	Cells/chamber	Membrane	Incubation time (min)
Neutrophils	1×10^6 cells/mL (granulocytes)	2.4×10^6	PVP-free, 5 µm	45
Eosinophils	2×10^6 cells/mL (eosinophils)	4.8×10^6	PVP-free, 5 µm	60
Monocytes	2×10^6 cells/mL (mononuclear cells)	4.8×10^6	PVP-treated, 5 µm	120
THP-1 cells	1×10^6 cells/mL (THP-1 cells)	2.4×10^6	PVP-treated, 5 µm	120
Lymphocytes	10×10^6 cells/mL (lymphocytes)	24×10^6	Fibronectin-coated, 5 µm	240

3.1.2.2.1. Preparation of 10 Agarose Plates

1. Preparation of solution A: Mix 7 mL of FCS, 7 mL of 10X concentrated Eagle's minimum essential medium with Earle's salts, 0.7 mL of 7.5% sodium bicarbonate, 0.35 mL of 200 m*M* glutamine, and 20 mL pyrogen-free distilled water and warm up to 48°C.
2. Preparation of solution B: Boil 0.63 g of agarose in 35 mL of distilled water until complete dissolution. Allow this solution to cool to 48°C.
3. Pour solution B into solution A.
4. Pour 6 mL of this solution into tissue culture plates (diameter 6 cm, Nunc) and allow them to cool.

These plates can be stored in the refrigerator for a few days.

Before use of the agarose plates, six series of three wells (3 mm internal diameter, 3 mm interspace) have to be punched in the agarose gel in each plate:

1. Cut six series of three wells in the gel, using a template to align the wells as shown in **Fig. 2** and a stainless-steel punch with inside bevel.
2. Remove the agarose cores with a pipet using vacuum (*see* **Note 19**).
3. Incubate the plates at 37°C in a 5% CO_2-incubator until samples and cells are prepared.

*3.1.2.2.2. Assay Procedure (**Fig. 2**)*

1. Prepare dilutions of test samples and the positive control in HBSS + 1 mg/mL HSA (*see* **Note 11**).
2. Add 10 µL of dilution buffer to the inner wells and 10 µL of test sample dilution, positive or negative control to the outer wells (*see* **Note 13**).
3. Add 10 µL of cell suspension (3×10^7 and 1×10^8 cells/ml for granulocytes and mononuclear cells, respectively) to the center well.
4. Incubate the agarose plates in a 5% CO_2-incubator during 2 (neutrophils) or 3 h (monocytes) (*see* **Note 20**).
5. Fix the cells with methanol (3 mL/plate).
6. Determine the effective migration distance (the migration distance to the test sample minus the migration distance to the dilution buffer) by microscopical scoring (magnification 50×).
7. Determine the titration end point of samples tested at multiple dilutions (*see* **Note 17**).

3.2. Granulocyte Migration In Vivo

Granulocyte chemotactic activity can be evaluated in vivo as the local accumulation of intravenously injected ^{111}In-labeled granulocytes in response to intradermal injection of inflammatory mediators in the rabbit skin ***(6)***. The activity of various chemotactic agents can be determined in parallel.

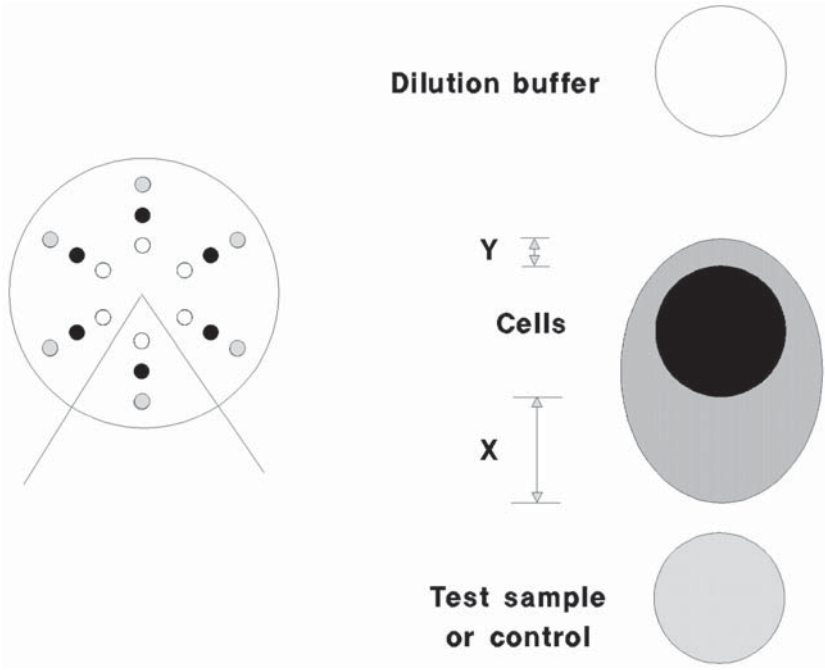

Fig. 2. Chemotaxis under agarose. The assay is performed in tissue-culture dishes containing an agarose gel. Six series of three wells are punched in the gel. The inner and outer wells are filled with dilution buffer and test samples or controls, respectively; the center wells are filled with cells. The effective migration distance (*X*-*Y*), being the migration distance toward the test sample (*X*) minus the migration distance toward the dilution buffer (*Y*), is used as a parameter for chemotactic activity.

3.2.1. Preparation of ^{111}In-Labeled Granulocytes

1. Cannulate the carotid artery of an anaesthethized (30 mg/kg iv sodium pentobarbitone) donor rabbit (male New Zealand White rabbits, 2.5–3 kg) with an 18-gage catheter (*see* **Note 21**).
2. Collect 72 mL of blood (8 times 9 mL) with a sterile plastic tubing in eight tubes containing 1 mL of acid-citrate-dextrose (ACD) and 5 mL of ACD 1:10 diluted with saline.
3. Add an equal volume of hydroxyethyl starch solution and incubate for 5 min at room temperature.
4. Centrifuge the tubes at 20*g* at room temperature for 15 min *without the brake on* to sediment the erythrocytes.

5. Centrifuge the platelet- and leukocyte-rich plasma for 10 min at 300*g* (brake maximal).
6. Collect the platelet-rich supernatant, except for the last 1 mL, which is used to resuspend the leukocyte-rich cell pellet.
7. Centrifuge the platelet-rich plasma twice at 1500*g* and 4000*g* for 10 min to prepare cell-free autologous plasma.
8. Dilute isotonic Percoll with citrated plasma to 63% and 80% (v/v) solutions.
9. Overlay 3 mL of the 80% Percoll solution carefully with 3 mL of the 63% Percoll solution.
10. Lay on top the leukocyte cell suspension (3–4 mL) and centrifuge at 1100*g* for 30 min at room temperature *without the brake on*.
11. Collect the layer of granulocytes at the 60/83% interface (*see* **Notes 22–24**).
12. Incubate during 5 min 20 µL of $^{111}InCl_3$ (50–200 µCi) with 40 µg 2-mercaptopyridine-*N*-oxide (MERC) in 100 µL PBS.
13. Add granulocytes (2×10^7 to 1×10^8 cells in 0.75 to 1.4 mL) to the ^{111}In-MERC complex, incubate for 15 min at room temperature.
14. Wash the labeled cell suspension by adding 10 ml of autologous cell-free plasma and centrifuge for 7.5 min at 1800*g* at room temperature.
15. Resuspend the cell pellet in 1 mL of autologous cell-free plasma. Repeat **step 14** twice.
16. Resuspend the cell pellet in 3 mL of autologous cell-free citrated plasma (*see* **Note 25**).

3.2.2. Estimation of the Accumulation of ^{111}In-Labeled Granulocytes in Rabbit Skin

1. Shave the back skin of a rabbit, anesthetized with sodium pentobarbital (30 mg/kg, iv).
2. Inject ^{111}In-labeled granulocytes into the recipient rabbit via the marginal ear vein through a 19-gage needle (*see* **Note 26**).
3. Inject after 15 min the chemoattractant (100 µL/site) intradermally in the back skin of the rabbit, each treatment having six replicates in one rabbit (*see* **Notes 27** and **28**).
4. Take a blood sample by cardiac puncture after 30–60 min and kill the rabbit by an overdose of sodium pentobarbitone (*see* **Note 29**).
5. Remove the back skin and excise the injection sites with a 17-mm diameter punch (*see* **Notes 30** and **31**).
6. Count the skin samples in a gamma counter (*see* **Note 32**).

4. Notes

4.1. Chemotaxis In Vitro

1. In the microchamber assay, the migrated cells can be identified morphologically during evaluation. This is more difficult in the agarose assay. The specificity of the latter rather depends on the purity of the isolated cells.

2. In the microchamber assay, lymphocytes do not adhere to the PVP-treated membrane used to test monocyte chemotactic activity, but drop to the bottom of the lower well. Lymphocytes do not migrate in the agarose assay.
3. Higher temperatures and longer incubation times may lead to unspecific binding of microbeads.
4. To avoid capping of antibodies on the cell surface during staining, cells have to be kept cold and cold solutions must be used.
5. Different types of depletion columns are available. The type of column that should be used depends on the amount of positive cells and is indicated by the manufacturer.
6. The magnetic separation of cells is performed at 4°C to keep good cell viability.
7. Negative cells are for 90% eosinophils.
8. Alternatively, lymphocytes can be isolated by positive selection using CD3 microbeads. However, the purity of lymphocytes obtained by negative selection was comparable to that of cells obtained by positive selection. Furthermore, by using negative selection, there is no risk of cell activation by binding of antibody.
9. Negative cells are for 80% lymphocytes.
10. The dead cells are blue after staining with trypane blue. The viability of THP-1 cells has to be at least 90% to perform the microchamber assay with success.
11. Chemokines show a bell-shaped dose–response curve. If samples are tested at supraoptimal concentrations, cell migration can be inhibited. Thus, testing samples at multiple dilutions is necessary.
12. Samples are tested in triplicate in each chamber to increase the reproducibility of the assay.
13. In each assay, negative and positive controls are tested in parallel with the samples to determine the spontaneous migration and to evaluate the responsiveness of the cells, respectively. Certainly in the case of freshly isolated leukocytes, the responsiveness can vary a lot depending on the blood donor. Dilution buffer is used as negative control; as positive control, *N*-formylmethionylleucylphenylalanine (fMLP) can be used for neutrophils and monocytes. One can also use chemokines as positive control, e.g., interleukin 8 (IL-8) ***(7)***, for neutrophils and monocyte chemotactic protein-3 (MCP-3) ***(8)***, for monocytes, THP-1 cells, eosinophils, and lymphocytes.
14. For neutrophils and eosinophils, polyvinylpyrrolidone (PVP)-free polycarbonate filters with pores of 5 µm are used, whereas for monocytes and THP-1 cells PVP-treated membranes with the same pore size are used. For lymphocytes, PVP-free polycarbonate filters are first coated for 24 h at 4°C with fibronectin (20 µg/mL) and washed with HBSS + 1 mg/mL HSA. Latter filters are coated to prevent migrating cells from dropping to the bottom of the lower well. The microchamber assay can also be used to study basophil chemotaxis ***(9)***.
15. The cells need time to migrate, but if the incubation time is too long, the spontaneous migration of the cells will further increase while the induced migration stops. This decreases the sensitivity of the assay.
16. Care should be taken to avoid changes in the orientation of the membrane (upside down and left side right). Good recording of which cells have migrated through

the membrane and where the different test samples are located in the multiwell chamber is necessary.

17. The chemotactic activity of the samples can be expressed as:
    a. The percentage of the number of migrated cells to the positive control for the microchamber assay or the percentage of the maximal migration distance for the agarose assay.
    b. The chemotactic index, being the number of cells migrated to the test sample divided by the number of cells migrated to the negative control for chemotaxis through micropores or the effective migration distance after subtraction of the random migration distance for chemotaxis under agarose;
    c. In units: if purified chemokine samples are tested in multiple dilutions, the potency can be expressed in U/mL, 1 U/mL corresponding to the half-maximal response obtained with the corresponding chemokine as control.
18. Chemotaxis under agarose is less sensitive (factor 10) than chemotaxis through micropores, but it has practical advantages:
    a. A lot of samples can be tested in different dilutions at the same time.
    b. The evaluation of the migration distance of cells under agarose is less labor intensive than the counting of cells that migrated through micropores.
19. When punching wells in the agarose gel, care needs to be taken to make no scratches on the surface of the tissue culture plates because this inhibits the migration of the cells. Also avoid lifting the gel and damaging the wells by removing the agarose cores by vacuum. Leakage of fluid from the gel into the wells due to storage must be prevented.
20. The agarose assay has the advantage that the migration distance can be evaluated microscopically before the cells are fixed. If the cells migrate slowly, the incubation time can be increased a little. The incubation has to be stopped before the cells migrate into the outer well and before the spontaneous migration is too pronounced, otherwise the sensitivity decreases.

4.2. Granulocyte Migration In Vivo

21. All the handlings have to be carried out at room temperature under sterile conditions and within approx 2.5 h to avoid activation of granulocytes during isolation.
22. Platelets, lymphocytes, and monocytes are located at the plasma/63% Percoll interface; erythrocytes are located on the bottom of the 80% Percoll layer.
23. 2×10^7 to 1×10^8 granulocytes can be recovered from 72 mL of blood. Differential staining (May-Grünwald Giemsa staining) revealed more than 98% purity for granulocytes.
24. Removal of remaining erythrocytes is not necessary because the amount of red cells left over is below 10% and red cell labeling yield under the used labeling conditions is only 2–3%.
25. Labeling yield (= percentage of the radioactivity taken up by the cells relative to the total amount of label added) and efficiency (percentage of the radioactivity

retained by the cells during the final washing procedure) can be determined by radioactivity counting on samples of cells (0.01–0.1 mL) and washing medium.

26. There is an initial fast phase of disappearance of labeled cells, probably by uptake into the marginal pool. Five minutes after injection, 40% of injected labeled granulocytes are found in the circulation. Half-life in the circulation of labeled cells after 5 min is 6.5 ± 1 h.
27. In the dorsal skin of a rabbit, up to 90 sites can be injected. All intradermal injections are given according to a balanced site pattern, injection order is based on a Latin-square design. Each animal serves as its own control.
28. Granulocyte extravasation in rabbit skin is based on a synergism between an increase in local blood flow on the arteriolar side and an increase in vascular permeability on the venular side. Therefore a vasodilator, like prostaglandin E_2 (PGE_2) (3×10^{-10} moles/site), or calcitonin gene-related peptide (CGRP) (10^{-11} moles/site), should be coinjected in the rabbit skin with the chemoattractants to increase the low basal blood flow.
29. The fraction of ^{111}In counts in plasma, not bound to the cell fraction, has to be less than 3% of the total blood radioactivity.
30. The injection sites can be visualized by iv injection of 2 mL of Evans blue dye solution (2.5%) before the start of the experiment.
31. Elimination of the remaining blood in the skin before punching out the injection sites is necessary to avoid positive counts from ^{111}In-labeled granulocytes in the peripheral blood circulation.
32. Results can be expressed as the number of labeled granulocytes by comparing skin sample ^{111}In counts with ^{111}In counts per cell in the preparation prior to intravenous injection.

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In Vitro and In Vivo Assays for High-Molecular-Weight Pegylated Muteins of Granulocyte Colony-Stimulating Factor

James F. Eliason, Nadine Tare, Sharon Bowen, Tomoaki Inoue, and Ikuo Horii

1. Introduction

The hematopoietic growth factor granulocyte colony-stimulating factor (G-CSF) has been in the clinic since 1986, when the clinical development of bacterially synthesized recombinant filgrastim began ***(1)***. A glycosylated form of G-CSF (lenograstim) has been approved for use in Europe and a bacterial recombinant mutein (nartograstim) has been approved in Japan. All forms of G-CSF are now commonly used for many types of neutropenia, including that resulting from anticancer therapies.

The hematopoietic growth factors were identified on the basis of their in vitro activities to induce colony formation by bone marrow cells in semisolid media ***(2)***. In serum-free media, G-CSF very selectively stimulates the formation of neutrophil colonies from progenitor cells in mouse bone marrow ***(3)***, although this specificity for neutrophil development is diminished when cultures contain serum. Such clonogenic assays are laborious and time consuming, taking one week or more, so development of factor-dependent cell lines has greatly facilitated quantitative functional assays in this field. These factor-dependent cells require the continuous presence of factor for survival as well as for proliferation. Thus, assays measuring either numbers of viable cells or DNA synthesis can be applied.

The primary effect of G-CSF in vivo is to increase the numbers of neutrophils, although macrophage numbers are also increased, whereas lymphocytes and platelets may be transiently depressed ***(4)***. The in vitro activities of hematopoietic growth factors are usually predictive for in vivo activities, but

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modifications of the native protein can alter activity. For example, removing sialic acids from glycosylated erythropoietin causes it to lose all activity in vivo while retaining in vitro activity *(5)*. The potency of G-CSF in vivo is limited because its half-life is short, meaning that it has to be administered once or twice a day. One approach to increasing the half-life is to modify specific amino acids that may impart instability. This approach was attempted in the design of nartograstim and resulted in a two- to threefold increase in activity *(6)*. However, the increased activity of this molecule is not sufficient to allow less frequent administration, so another approach was to increase the total molecular mass by adding polyethylene glycol (PEG). The primary site of elimination of proteins such as G-CSF is the kidney *(7)*, and the rate of elimination is inversely proportional to molecular mass *(8)*. Another important mechanism for elimination of hematopoietic growth factors is receptor-mediated endocytosis and degradation in the target cells *(9–12)*. This means that the effects of these factors are self-limiting based upon numbers of end cells present.

We tested a number of PEGylated conjugates of nartograstim (PEG-NTG) with different molecular sizes of PEG and different linkage chemistries in vivo and in vitro. As expected, the most important factor influencing in vivo activities in mice and cynomolgus monkeys was the overall molecular mass of the conjugates, whether by increasing the mass of PEG chains or by adding more PEG chains per protein molecule *(4,13)*. Surprisingly, there was an inverse relationship between molecular mass and activity in vitro, with the unmodified protein having the highest activity and the highest-molecular-weight conjugates having the lowest *(13)*. Because the in vitro activities are directly related to binding affinities of cytokines for their receptors, these results suggest that increased half-life is more important than high affinity binding for activity in vivo.

2. Materials

2.1. DNA Synthesis Assay

1. The basic nutrient medium for the thymidine uptake assay is RPMI-1640 supplemented with 2 m*M* L-glutamine and 80 µg/mL gentamycin. All tissue culture reagents should be stored at 4°C.
2. Fetal bovine serum (FBS).
3. G-NFS-60 murine leukemia cells (*see* **Note 1**).
4. Recombinant murine interleukin-3 (rmIL-3) for expanding cells (*see* **Note 2**).
5. 96-well flat-bottom microtiter tissue culture plates.
6. PEG-NTG diluted in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) (*see* **Note 3**).
7. For labeling the cells, tritiated thymidine is used. Because tritium-[^{3}H] is a β particle emitter, proper precaution must be taken.

2.2. Metabolic Assay

1. Preparation of amino acid–vitamin supplement stock solution.
   a. Weigh the following:

250 mg	L-alanine
500 mg	L-aspargine.H_2O
300 mg	L-aspartic acid
700 mg	L-cysteine
750 mg	L-glutamic acid
400 mg	L-proline
1100 mg	sodium pyruvate
0.25 mg	vitamin B-12
0.3 mg	biotin

   b. Dissolve each separately in 5 mL of water. L-Aspartic acid and L-glutamic acid require the addition of several drops of 5 *N* NaOH; L-cysteine needs several drops of concentrated HCl to dissolve.
   c. Mix the solutions together.
   d. Adjust the pH to 2.0.
   e. Bring the volume to 100 mL with water.
   f. Filter and store at –20°C in 10 mL aliquots.
2. The basic nutrient medium used for the metabolic assay is EF medium ***(14,15)***. This medium is not commercially available. It consists of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (containing 1000 mg/L glucose, without phenol red) and Ham's nutrient mixture F-12.
   a. Dissolve one package (for 1 L) of powdered DMEM containing 1000 mg/L glucose, without phenol red, and one package (for 1 L) of powdered Ham's nutrient mixture F-12 ingredients in approx 1.8 L of water (*see* **Note 4**).
   b. Add 4.76 g HEPES buffer.
   c. Add 3.20 g $NaHCO_3$.
   d. Add 10 mL of amino acid–vitamin supplement (*see* **Subheading 2.2., item 1**).
   e. Add 2 mL trace elements (1000X, CellGro, MediaTech, Inc., Herndon, VA).
   f. Add 13 µL α-thioglycerol.
   g. Add 160 µL gentamycin.
   h. Adjust pH to 6.9 with 1 *N* HCl. s
   i. If an osmometer is available, check osmolarity; adjust to 300 ± 10 mosm. If an osmometer is not available, bring to 2 L with water.
   j. Filter and store at 4°C protected from light.

2.3. Cell Culture and Assay

1. G-NFS-60 murine leukemia cells (*see* **Note 1**) cultured in EF medium. All tissue culture reagents should be stored at 4°C.
2. FBS.
3. Recombinant murine interleukin-3 (IL-3) for expanding cells (*see* **Note 2**)
4. 96-well flat-bottom microtiter tissue culture plates
5. PEG-NTG diluted in PBS supplemented with 1% BSA (*see* **Note 3**)

6. 3-(4,5-Dimethylthiazol-2yl)-2,3-diphenyltetrazolium bromide (MTT) diluted in PBS to 3 mg/mL.
7. Sodium dodecyl sulfate (SDS) 25% w/v in water with pH adjusted to 2.0.

2.4. In Vivo Assays

1. Five strains of mice, female or male, are used in these studies: C57Bl/6J, BALB/c, C3H/HeN, DBA/2, and C3H/HeJ mice (*see* **Note 5**). The mice should be 6–10 wk old and are housed in air-conditioned rooms with a 12-h light and dark cycle. They are given food and water *ad libitum*.
2. Cynomolgus monkeys (*Macaca fascicularis*) of either sex are used for the primate studies. The males should weigh 3.0–6.5 kg and the females 2.0–4.5 kg at the start of the experiments.
3. Sterile syringes and needles.
4. PEG-NTG diluted in PBS containing 1% autologous mouse or monkey serum (*see* **Note 3**).
5. Heparinized blood collections tubes.

3. Methods

3.1. In Vitro Assays

The development of factor-dependent cell lines that can be used for in vitro proliferation assays has provided great impetus for modern cytokine research. Factor-dependent cells require the presence of growth factor for their survival as well as for their proliferation. Without factor, the cells undergo apoptosis and die. At very low concentrations of factors, the cells survive but do not divide.

We have used two different assays to examine the effects of PEG-NTG, a cell proliferation assay, measuring uptake of tritiated thymidine into DNA and a metabolic assay using MTT, which is reduced in the mitochondria of viable cells. Each assay has its advantages and disadvantages. The thymidine uptake assay has a low background and a large range covering at least three or four logs of measurable values, so it is very robust. It involves radiolabeled thymidine and expensive harvesting and measuring equipment. The MTT assay measures viable cells, so does not depend on DNA synthesis and can potentially measure lower growth factor levels. It requires only a visible light microplate reader.

However, the range of measurable values is narrow, so careful preliminary experiments are necessary to determine the optimal cell concentration ranges for each cell line and incubation condition.

3.1.1. DNA Synthesis Assay

1. G-NFS-60 cells are passaged in RPMI-1640 supplemented with 2 m*M* L-glutamine, 10% (v/v) FBS, 80 µg/mL gentamicin, and 20 U/mL rmIL-3 (*see*

Note 2). Cultures are maintained in the log phase of growth either by the addition of fresh culture medium or by subculturing to about 5000 cells/mL every 3–4 d.
2. Test samples of PEG-G-CSF are adjusted in growth medium (RPMI-1640 containing 2 m*M* L-glutamine and 10% FBS) to be twice the highest concentration.
3. 200 µL of each sample are plated into the first well of each of four rows to give quadruplicate determinations.
4. 100 µL/well of growth medium are added into each of the remaining wells
5. Half the volume of sample (100 µL) is removed from the first row with a multichannel pipet and transferred to the medium in the second row.
6. The contents are drawn into the pipet several times to ensure thorough mixing and half the volume is transferred to the third row.
7. This process is continued to give 12 twofold dilutions.
8. The G-NFS 60 cells are washed three times in assay medium; viable cells are counted by trypan blue exclusion and are adjusted to give 5×10^5 cells/mL in assay medium.
9. Cells are plated in a volume of 100 µL/well, resulting in a final cell number of 5×10^4/well, with a final volume per well of 200 µL.
10. Each assay plate is prepared with a positive standard curve (*see* **Note 6**) using Neupogen rhG-CSF (Amgen, Thousand Oaks, CA) and a negative control (assay media alone).
11. Cultures are incubated at 37·C, 5% CO_2 in humidified air overnight.
12. Tritiated thymidine (0.5 µCi/well ^{3}H-Tdr) is added and the cultures are incubated for 6 h.
13. Cells are harvested onto filter mats using a Tomtec cell harvester (Tomtec, Hamden, CT).
14. ^{3}H-Tdr incorporation is determined by liquid scintillation spectrophotometry on a Betaplate Flatbed LSC counter (Wallac, Gaithersburg, MD).
15. Background counts are subtracted, means and standard errors of the quadruplicate wells are determined, curves are graphed, and units are calculated by linear regression (*see* **Note 7**).
16. Protein content is determined separately, and the results are normalized to the Neupogen rhG-CSF standard.

3.1.2. Metabolic Assay

1. G-NFS-60 cells are passaged in EF medium with 5% (v/v) FBS, 80 µg/mL gentamicin, and 20 U/mL rmIL-3 (*see* **Notes 2** and **8**). Cultures are maintained in the log phase of growth either by the addition of fresh culture medium or by subculturing to about 5000 cells/mL every 3–4 d.
2. Test samples of PEG-G-CSF are adjusted in growth medium (EF medium supplemented with 5% FBS) to be twice the highest concentration.
3. The first and last rows of each plate are filled with water. The wells of the first and last columns in the remaining rows of each plate are filled with 200 µL growth medium (*see* **Note 9**).
4. 200 µL of each sample are plated into the second wells of each of the next three rows to give triplicate determinations.

5. 100 µL/well of growth medium are added into each of the remaining wells.
6. Half the volume of sample (100 µL) is removed from the first row with a multichannel pipet and transferred to the medium in the second row.
7. The contents are drawn into the pipet several times to ensure thorough mixing and half the volume is transferred to the third row.
8. This process is continued to give 12 twofold dilutions.
9. The G-NFS 60 cells are washed three times in assay medium; viable cells are counted by trypan blue exclusion and are adjusted to give 1×10^5 cells/mL in assay medium
10. Cells are plated in a volume of 100 µL/well, resulting in a final cell number of 1×10^4/well, with a final volume per well of 200 µL.
11. Each assay plates is prepared with a positive standard curve (*see* **Note 6**) using Neupogen rhG-CSF in the remaining three rows of each plate.
12. Cultures are incubated at 3°C, 5% CO_2 in humidified air for 3 d.
13. 50 µL of MTT are added to each well and the plates are incubated for 4 h.
14. 50 µL of 25% SDS, pH 2.0, are added (*see* **Note 10**) and the plates are incubated overnight to dissolve the formazan crystals.
15. Measure absorbance at 540 nm, using a microplate spectrophotometer.

3.2. In Vivo Assays

3.2.1. Murine Assay

1. The cytokine preparations are administered subcutaneously in a total volume of 0.1–0.2 mL per mouse.
2. The PEG-G-CSF solutions are given once on d 0 and the nonconjugated proteins are given daily.
3. Groups of four animals are sacrificed at various times and blood samples are taken via the retro-orbital sinus or by cardiac puncture.
4. Total leukocytes, erythrocytes, and platelets are counted using a Coulter Counter IV (Beckman Coulter, Fullerton, CA) or a Technicon H*1 autoanalyzer (Bayer Diagnostics, Tarrytown, NY).
5. Differential counts of neutrophils and monocytes are determined on Wright-stained preparations or by using the autoanalyzer, and absolute numbers of cells of each type are calculated.

3.2.2. Primate Assay

1. To establish the baseline values for blood cell counts, blood is drawn from limb veins on d –7 and 0 before administration of the test compounds.
2. The PEG-NTG is injected subcutaneously in a volume of 0.5 mL/kg on d 0 at doses of 10 µg/kg to five monkeys, 30 µg/kg to three monkeys, and 100 µg/kg to three monkeys. Vehicle is also administered to three monkeys. The rhG-CSF preparation is administered subcutaneously on d 0–5 to thrr monkeys.
3. Blood samples are obtained after 8 h and on d 1–7, 10, 14, 21, and 28.
4. Total leukocytes, platelets and red cells are determined using a Technicon H*1 autoanalyzer.

5. Differential counts of neutrophils, eosinophils, basophils, monocytes, and lymphocytes are determined and absolute numbers of cells of each type are calculated by multiplying the leukocyte numbers by the percentages of each.

4. Notes

1. We have used an IL-3-dependent murine myelogenous leukemia cell line, NFS 60 ***(16)***, induced by wild mouse ecotropic virus, Cas-Br-MuLV, which is also G-CSF-dependent. This line was then selected for response to human G-CSF and lack of response to other human cytokines ***(17)*** giving G-NFS 60. Because G-CSF is not species specific, this line can be used to assay the biological activity of both human and mouse G-CSF.
2. Growing the cells in IL-3 rather than G-CSF keeps the G-CSF receptors empty, thereby enhancing sensitivity of the G-CSF assay.
3. The PEG-cytokine preparations are very stable when kept at high concentrations. However, at low concentrations they rapidly lose activity and, thus, they must be diluted in PBS or medium supplemented with carrier proteins.
4. Only tissue-culture-grade water, either purchased commercially (Sigma) or purified locally (e.g. Milli-Q, Millipore, Inc.), should be used to avoid endotoxin contamination.
5. Each strain of mouse has a different sensitivity to G-CSF. The C3H/HeJ strain are the least responsive of those we have tested, giving about a fivefold increase in neutrophil numbers at d 5–6 following subcutaneous injection of 10.8 µg/mouse of PEG-G-CSF. The C3H/HeN strain was slightly more responsive, having an eightfold increase. The BALB/c and C57Bl6 strains had intermediate sensitivities with about 12- to 14-fold increases under these conditions. DBA mice were the most sensitive responding with a 30-fold increase with the same dose. Therefore, higher dose levels must be used for C3H/HeJ mice. On the other hand, this strain has the advantage that the mice lack endotoxin receptors and so are insensitive to any contaminating endotoxin that may be in bacterial recombinant CSF preparations.
6. The positive G-CSF control is also used to normalize results between assays.
7. A standard curve of Neupogen demonstrates a detectable range from approx 2–2000 pg/mL. The curve is sigmoidal, with a linear region between approx 8 and 500 pg/mL. Calculations for specific activity and unit designation are performed in the linear region of the curve. One unit is defined as the concentration of factor that gives half-maximal activity in the assay.
8. Although other combinations of Dulbecco's medium and Ham's F-12 Nutrient Mixture will work, EF medium consisting of an enriched Dulbecco's medium, containing 1000 mg/L of glucose rather than 4500 mg/L and without phenol red, mixed 1:1 with Ham's F-12 gives the best results.
9. The outside wells in microtiter plates tend to dry faster than those in the middle of the plate giving anomalous results. We only use the inner 60 wells for the assays and fill the top and bottom rows with water to prevent further evaporation. The wells of the first and last columns are filled with medium alone and the average value for these wells is used as the background for subtractions from the results for assay wells in the same row.

10. The pH of the SDS solution is very important. The blue formazan color, formed by metabolic reduction in viable cells, is lost when the pH is too acidic and the crystals do not dissolve if the pH is too basic. Experiments should be run to determine the optimal pH for the growth medium used. We have compared EF medium to DMEM containing phenol red and without HEPES buffer. The optimal pH, giving the biggest absorbance difference between controls and wells containing cells, for DMEM was 1.2, whereas that for EF medium was 2.0, when measurements were made as soon as the plates were removed from the incubator. One of the advantages of using EF medium is that the difference between control and test wells is greater than when DMEM is used because the phenol red increases the background. The second advantage is that the readings are remarkably stable with time out of the incubator. The assay results with EF decreased only 14% after 72 h when SDS at pH 2 was used, and 16% at this time with pH 1 SDS. Assays with DMEM as the growth medium were much less stable, decreasing 80% when pH 1 SDS was used, 24% with pH 1.5, and 30% with pH 2.0.

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15

Development of a Mammalian Tet-On Expression Cell Line

Glucosylceramide Synthase Regulates TNF-α-Induced Apoptosis

Yong-Yu Liu and Myles C. Cabot

1. Introduction

Tumor necrosis factor-α (TNF-α) is one of the most pleiotropic of cytokines, acting as a host defense factor in myriad immunological and inflammatory responses and antitumor activity *(**1–3**)*. The cytotoxic effects of TNF-α are primarily mediated through TNF-R1 and the receptor-associated proteins, TNF-R1-associated death domain protein (TRADD) and Fas-associated death domain (FADD/MORT1) *(**3–5**)*. Ceramide generation and caspase activation represent potential regulation points of apoptotic signaling by TNF-α *(**6,7**)*. Increased ceramide formation via sphingomyelinase represents an early event in the apoptotic cascade of TNF-α *(**6,8,9**)*. In MCF-7 breast cancer cells, ceramide is one of the essential molecules in TNF-α-induced apoptosis *(**10–12**)*. Increased ceramide generation induced by the P-glycoprotein blocker PSC 833, restores TNF-α-induced apoptosis in KG1a leukemia cells *(**13**)*, whereas loss of ceramide production is a cause of cellular resistance to apoptosis induced by TNF-α *(**14**)*. Glucosylceramide synthase (GCS), converting ceramide into glucosylceramide *(**15**)*, exerts strong influence over cellular ceramide levels and is thus a major modulator of programmed cell death *(**16**)*. Introducing the *GCS* gene into TNF-α-sensitive MCF-7 cells greatly diminishes cellular response to TNF-α cytotoxicity *(**12**)*.

Here we describe a method for developing a stable cell line that inducibly expresses GCS, using a retroviral Tet-on system, to evaluate the influence of ceramide glycosylation on apoptotic signaling of TNF-α. The retroviral Tet-off/Tet-on vector is a highly regulatory, versatile mammalian expression system *(**12,17–22**)*. Regulation of inserted gene (*GCS*) expression in the multiple cloning site (MCS) under cytomegalovirus (CMV) promoter control, can be

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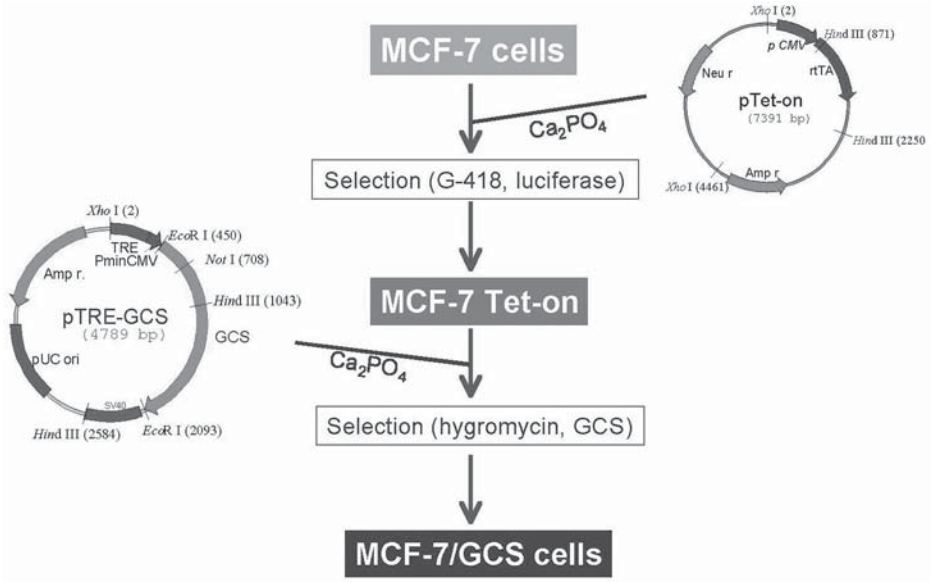

Fig. 1. Schematic for developing inducible Tet-on MCF-7/GCS cell line.

mediated simply by addition of doxycycline (**Fig. 1**). The pTet-on vector (pUHD17-1neo) expresses a doxycycline-controlled rtTA (reverse tetracycline transactivator), which is a fusion protein of a reverse Tet repressor (rTetR) and the C-terminal domain of protein 16 of herpes simplex virus, constitutively expressed under control of human CMV promoter (***17,21***; *see* **Note 1**). The pTRE vector (pUHD10-3) contains a MCS to accept any cDNA to be expressed, followed by an SV40 polyadenylation sequence (***19***). The promoter region upstream from the MCS contains a minimal human CMV promoter (P_{minCMV}) with heptamerized tet-operators. This promoter is silent in the absence of binding of rtTA to the tet-operators. However, when the rTetR of the rtTA binds to the tet-operators, the virion protein 16 domain of the rtTA can activate P_{minCMV} activity to a very high level, and switch on the expression of the target gene *GCS*. Binding of doxycycline to the rTetR domain of the rtTA can almost completely activate rtTA binding to the promoter (***17,21***; *see* **Note 2**).

2. MATERIALS

1. *Escherichia coli* XL1-Blue competent cells (Stratagene, La Jolla, CA) containing F' episome that confers cell resistance to antibiotics (ampicillin), and blue-white color screening (*lacIqZΔM15* gene).
2. SOC medium: Dissolve 2.0 g tryptone, 0.5 g yeast extract and 0.05 g NaCl in 80 mL H_2O, and adjust pH to 7.0 with 5 *N* NaOH. Make up to 98 mL with H_2O,

and sterilize by autoclaving for 20 min at 103.4 kPa on a liquid cycle. Prior to use, add 100 µL 1 *M* filter-sterilized $MgCl_2$, 100 µL 1 *M* filter-sterilized $MgSO_4$, and 10 µL 2 *M* filter-sterilized glucose.

3. LB–ampicillin medium: Dissolve 20 g Luria-Bertani (LB) broth base (Sigma, St. Louis, MO) in 800 mL of distilled H_2O, and adjust pH to 7.0 with 5 *N* NaOH. Make up to 1 L with H_2O, and sterilize by autoclaving for 20 min at 103.4 kPa on a liquid cycle. Add 50 mg of filter-sterilized ampicillin (50 mg/mL, stock solution), before using.
4. LB–ampicillin agar: 2% (w/v) agar in LB-ampicillin medium. Dissolve 20 g LB broth base (Sigma) in 800 mL of distilled H_2O, and adjust pH to 7.0 with 5 *N* NaOH. Make up to 1 L with H_2O and add 20 g select agar (Sigma), and sterilize by autoclaving for 20 min at 103.4 kPa on a liquid cycle. Add 50 mg of filter-sterilized ampicillin at about 56°C.
5. LB–ampicillin agar plate: Pour 25–30 mL of the melted LB–ampicillin agar into a Petri dish (100-mm). Once the agar sets, the plate can be stored inverted at 4°C.
6. Gel loading buffer (6X): 25 mg bromophenol blue, 25 mg xylene cyanol FF, 3 mL glycerol, up to 10 mL with H_2O.
7. TE buffer, pH 7.4: 10 mL 1 *M* Tris-HCl base, 1 mL 1 *M* ethylenediaminetetraacetic acid (EDTA) and 500 mL H_2O, adjust pH to 7.4 with 1 *M* HCl, and add H_2O to 1 L; TE buffer pH 8.0: 10 mL 1 *M* Tris-HCl base, 1 mL 1 *M* EDTA, and 500 mL H_2O, adjust pH to 8.0 with HCl, and add H_2O to 1 L.
8. TAE buffer (Tris-acetate): 10 mL 1 *M* Tris base, 1 mL 1 *M* EDTA, and 500 mL H_2O, adjust pH to 8.3 with acetate (approx 1 mL glacial acetic acid), and add H_2O to 1 L.
9. QIAGEN Plasmid Midi Kit (Valencia, CA): Buffer P1, RNase A, Buffer P2, Buffer P3, Buffer QC, Buffer GF, and QIAGEN-tip 100.
10. Agarose Gel DNA Extraction Kit (Roche, Basel, Switzerland): Silica matrix suspension (1 mL), agarose gel solubilization buffer (30 mL), nucleic acid binding buffer (100 mL), and wash buffer (20 mL).
11. DNA Ligation Kit (Stratagene, cat. no. 203003, –20°C): 10 m*M* rATP, pH 7.5 in sterile H_2O (4X 250 mL); pUC18 plasmid control DNA, *Bam*HI digested (10mg), T4 DNA ligase (4 U/mL); 10X ligase buffer [500 m*M* Tris-HCl, pH 7.5, 70 m*M* $MgCl_2$, 10 m*M* dithiothreitol (DTT)].
12. Mammalian transfection (MT) solutions: Mammalian transfection kit (Stratagene), MT solution 1, 2.5 *M* $CaCl_2$ (1.5 mL); MT solution 2, pH 6.95, 50 m*M* *N*,*N*-*bis*(2-hydroxyethyl)-2-aminoethanoesulfonic acid and buffered saline, 280 m*M* NaCl, and 1.5 m*M* Na_2HPO_4 (15 mL).
13. BCA Protein assay reagents: Freshly prepared from BCA protein assay kit (cat. no. 23225ZZ, Pierce, Rockford, IL). Add 1 part Reagent B (4% $CuSO_4 \cdot 5H_2O$), and 50 parts of Reagent A (BCA detection reagent) in test tube, mix well at room temperature.
14. Tet-on expression system (CLONETECH, Palo Alto, CA): the major characters of plasmids are listed below. pTRE-GCS is derived from pTRE response plasmid (pUHD10-3, 3.46 bp):

Name	Size (bp)	Mammalian selectable marker	Expressed protein	Diagnostic enzymes	Diagnostic fragments (kb)	GenBank access no.
pTet-on	7370	Neomycin	RtTA	*Xho*I, *Hin*dIII	2.91, 2.21,1.38, 0.94	U89930
pTRE-Luc	3146	None	Luciferase	*Xho*I, *Hin*dIII	2.21, 0.94	U89931
pTRE-GCS	4789	None	GCS	*Xho*I, *Not*I	4.08, 0.71	None
pTK-Hyg	5066	Hygromycin	n/a	*Eco*RI	2.32, 1.31, 1.04, 0.39	U40398

15. Luciferase assay: Lysis buffer, add 4 vol H_2O to 1 vol of cell culture lysis reagent (Luciferase Assay System, cat. no. E1500, Promega, Madison, WI), mix and aliquot.
16. Cell homogenization buffer: 50 m*M* Tris-HCl, pH 7.4, 1 µg/mL Leupetin, 10 µg/mL Aprotinin, 25 µ*M* phenylmethylsulfonyl fluoride (PMSF).
17. Enzymatic reaction buffer: 0.2 *M* phosphate buffer, pH 7.8, 4 m*M* EDTA, 20 µ*M* $MgCl_2$, 2 m*M* DTT.
18. UDP-glucose: 1 vol [^{3}H]UDP-glucose (40 Ci/mmol), 60 vol 5 m*M* UDP-glucose (Sigma), freshly prepared.
19. GCS liposomal substrate (freshly prepared before assay): Mix 4 m*M* C_6-ceramide (*N*-hexanoylsphingosine, LC Laboratories, Woburn, MA) with 2 mg/mL sulfatides (ceramide galactoside 3-sulfate, Matreya, Pleasant Gap, PA), and 11.3 mg/mL phosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids, Alabaster, AL) in 10-mL glass tube, and dry lipids under nitrogen stream. Add the same volume of H_2O, and homogenize by sonication, two times 30 s bursts on ice.
20. RPMI-1640 medium: 50 mL fetal calf serum (FCS) (HyClone, Logan, UT), 5 mL penicillin-streptomycin-glutamine (100X, 10,000 U/mL penicillin G sodium, 10 mg/mL streptomycin sulfate, 29.2 mg/mL L-glutamine, 10 m*M* sodium citrate, and 0.14% NaCl, Gibco-BRL) and 500 mL RPMI-1640 medium (Gibco-BRL, Invitrogen Corporation, Carlsbad, CA).
21. DMEM medium: 50 mL FBS, 5 mL penicillin-streptomycin-glutamine (100X), and 500 mL DMEM medium (high glucose, Gibco-BRL).
22. 50 mg/mL Genetecin (G-418, Gibco-BRL), 50 mg/mL Hygromycin B (Roche) and 50 mg/mL ampicillin (Sigma). Cultureware was from Corning Costar (Cambridge, MA).

3. Methods

3.1. Development of a Stable MCF-7 Tet-On Cell Line

As depicted in **Fig. 1**, it is essential to develop a stable Tet-on cell line in which overexpression of rtTA is profoundly induced by the addition of doxycycline (*see* **Notes 1** and **2**) (**Fig. 2**). After introduction of pTet-on vector (pUHD17-1neo) into MCF-7 cells, the transient transfection of pTRE-Luc and luciferase assay will be used to select MCF-7 Tet-on cell lines.

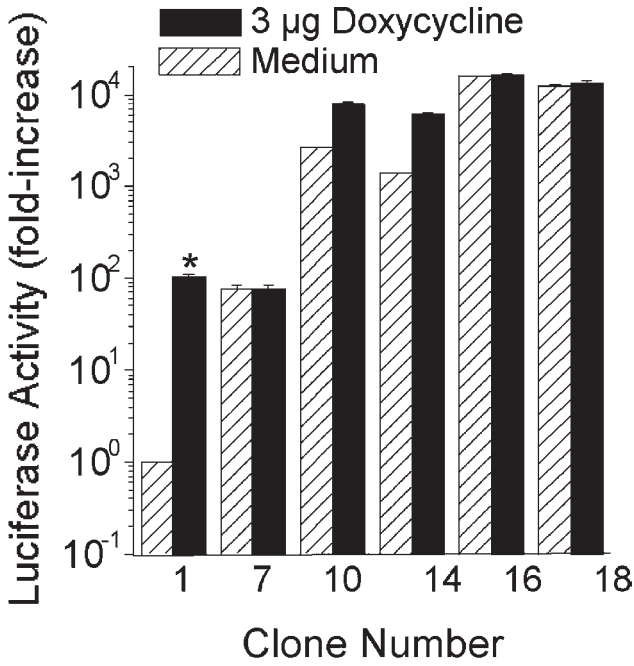

Fig. 2. Luciferase activity in MCF-7 pTet-on transfected cells. After transient transfection with pTRE-Luc plasmid, cells were incubated without or with doxycycline (3 µg/mL) for 48 h. Luciferase activity was analyzed as described in **Subheading 3.1.6.** Data presented as fold-increase, compared with MCF-7 wild-type cell transfected by pTRE-Luc plasmid.

3.1.1. Transformation

1. Thaw the *E. coli* blue XL1 competent cells on ice. Gently mix the cells by hand motion, and aliquot 100 µL of the cells into two prechilled 15-mL Falcon 2059 polypropylene tubes. (One tube is for the experimental transformation and one tube is for the control transformation.)
2. Prewarm LB-ampicillin agar plate to 37°C.
3. Add 2.0 µL β-mercaptoethanol (Strategene, provided with the competent cells) to each of the 100-µL aliquots of bacteria, giving a final concentration of 25 m*M* in each tube. Swirl the contents of the tubes gently, and incubate the cells on ice for 10 min, swirling every 2 min.
4. Adjust plasmid DNA concentration with sterile distilled H_2O, to 0.1 ng/µL for pUC18 control plasmid, and 1 ng/µL for pTet-on, pCG-2, pTRE-luc, pTRE, and pTK-Hyg plasmids. Add 1 µL of pTet-on DNA to one of the 100 µL of cells and swirl gently. Add 1 µL pUC18 plasmid to the control tube and swirl gently as well.
5. Incubate the cells on ice for 30 min. Preheat the SOC medium in a 42°C water bath for 30 min.

6. Heat-pulse the cells in a 42°C water bath for 30 s. Place the cells on ice immediately for 2 min.
7. Add 0.9 mL of preheated SOC medium, and incubate the tubes at 37°C for 1 h with shaking at 225 rpm.
8. Plate 50, 100, and 200 µL of each transformation mixture on LB–ampicillin agar plates using a sterile spreader.
9. Incubate the plates overnight at 37°C. Visualize clones in each plate, and store plates at 4°C, wrapping with paraffin film.

3.1.2. Minipreparation Plasmid DNA. Quantum Prep Plasmid Miniprep Kit (Bio-RAD, cat. no. 732-6100, Hercules, CA)

1. Retrieve a single colony from pTet-on plate using sterile inoculating loop, inoculate in 5.0-mL sterile LB–ampicillin medium, and grow to saturation in incubator at 37°C, with shaking (225 rpm).
2. Pour 1.5 mL of the culture into Eppendorf tube, and spin in microcentrifuge (14,000*g*) for 2 min. Store the remainder of the culture at 4°C.
3. Aspirate the supernatant, add 1.5 mL PBS and centrifuge again. Aspirate the supernatant and dry the pellet by inverting tube for 5 min.
4. Add 200 µL of the cell resuspension solution and vortex to resuspend pellet. Add 250 µL of the cell lysis solution and mix by gently inverting the capped tube about 10 times (do not vortex).
5. Add 250 µL of the neutralization solution and immediately mix by gently inverting the capped tube about 10 times (do not vortex). A visible precipitate should form.
6. Pellet the cell debris for 5 min in a microcentrifuge (12,000*g*).
7. Insert a spin column into one 2-mL collection tube supplied in the kit, and mix the Quantum Prep matrix by vortexing to suspend matrix.
8. Transfer the cleared lysate (supernatant) to the spin column, add 200 µL suspended matrix, then pipet up and down to mix. Centrifuge spin column for 30 s.
9. Remove the spin column from the 2-mL tube, discard the flow-through from the collection tube, and replace the spin column in the same collection tube. Add 400 µL of wash buffer to the column and centrifuge for 1 min. Discard the flowthrough and add another 400 µL of wash buffer, centrifuge for 2 min, to remove residual traces of ethanol of wash buffer in the column.
10. Remove the spin column and place in a sterile 1.5-mL microcentrifuge tube. For eluting the plasmid DNA, add 100 µL of TE buffer to the column, and centrifuge 1 min at 14,000*g*. Store the eluted DNA in a 1.5-mL tube at –20°C.
11. Measure DNA: add 4 mL eluted DNA sample and 196 mL H_2O in a 1.5-mL Eppendorf tube, and vortex. Measure OD absorbance at 260/280 nm on microspectrometer equilibrated with H_2O. The A_{260}/A_{280} ratio (≥1.8) indicates the purity of plasmid DNA. Calculate DNA concentration by:

$$OD_{260} \times 50 \times 50 / 1000 = OD_{260} \times 2.5 \ (\mu g/\mu L)$$

3.1.3. Restriction Mapping

The inserted *GCS* cDNA is cleaved with a variety of restriction endonucleases, either individually or in combination, and the resulting products are separated by agarose gel electrophoresis. Restriction endonucleases and their maps are deduced by computer analysis using Vector NTI 5.0.

1. Add 2 µg prepared plasmid DNA from each colony, 1 µL *Eco*RI endonuclease (25 U/µL, Stratagene), 1 µL reaction buffer (10X), and H_2O to 10 µL, in 1.5-mL microcentrifuge tube. Place 2 µg pCG-2 DNA in another tube, as a positive control.
2. Incubate tubes at 37°C for 60 min with shaking at 50 rpm.
3. Prepare 1% agarose gel: Add 0.4 g agarose (for routine use, Sigma), 40 mL TAE buffer, and 2 µL ethidium bromide (10 mg/mL stock solution) in 125-mL glass flask. Melt agarose in microwave oven, twice in 1-min periods, and pour and set agarose in gel tray with an eight-tooth comb insert (9 × 6.5 cm, Minicell System EC370M, Holbrook, NY).
4. Add 2 µL gel loading buffer (6X) in each tube, vortex, and spin down in microcentrifuge (5000 rpm, 20 s). Load 12 µL DNA mixture using gel-loading pipet tip and 8 µL kb DNA ladder solution (Stratagene) in the adjacent well.
5. Resolve the DNA fragments by electrophoresis, 60 V, 3 h; visualize DNA fragments under UV light, and document using Polaroid camera (f 1/1, 1/8 s).

3.1.4. Large-Scale Preparation of Plasmid DNA (Qiagen Plasmid Midi Kit)

1. Inoculate 0.2 mL of remaining culture (above) in a 1-L flask with 100 mL LB–ampicillin medium, and grow at 37°C for 16 h with vigorous shaking (250–300 rpm). The culture should reach a cell density of approx $3–4 \times 10^9$ cells/mL.
2. Harvest the bacterial cells by centrifugation at 6000*g* for 15 min at 4°C (6000*g* corresponds to 6,000 rpm with Sorvall GSA or GS3 or Beckman JA-10 rotors). Invert the open centrifuge tube on paper towel for 10 min.
3. Resuspend the bacterial pellets in 4 mL Buffer P1 containing RNase A and mix completely by vortexing.
4. Add 4 mL of Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.
5. Add 4 mL of chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 15 or 20 min. A fluffy white material forms, and the lysate becomes less viscous.
6. Invert the sample 4–6 times again, centrifuge at 20,000*g* for 30 min at 4°C. Promptly remove supernatant containing plasmid DNA. Centrifugation should be performed in nonglass tubes (e.g., polypropylene). A centrifugal force of 20,000*g* corresponds to 12,000 rpm in a Beckman JA-17 rotor or 13,000 rpm in a Sorvall SS-34 rotor. After centrifugation, the supernatant should be clear. Re-centrifuge the supernatant at the same conditions, and promptly remove the supernatant containing plasmid DNA.

7. Equilibrate a QIAGEN-tip 100 by applying 4 mL Buffer QBT. Allow the column to drain completely by gravity flow.
8. Apply the supernatant from **step 6** to the QIAGEN-tip and allow it to enter the resin by gravity flow. The supernatant should be loaded onto the QIAGEN-tip promptly.
9. Wash the QIAGEN-tip with 2X 10 mL Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow.
10. Elute DNA with 5 mL Buffer QF and collect the eluate in a 10-mL polypropylene tube. Store the eluate at 4°C.
11. Add 3.5 mL (0.7 vol) of room-temperature isopropanol to the eluted DNA; mix and centrifuge immediately at 15,000*g* for 30 min at 4°C. Carefully decant the supernatant.
12. Wash DNA pellet with 2 mL of room temperature 70% ethanol, and centrifuge at 15,000*g* for 10 min. Carefully decant the supernatant without disturbing the pellet.
13. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume TE buffer, pH 8.0.
14. Determine DNA concentration and quality by both UV spectrophotometry (*see* **Subheading 3.1.2.**), and agarose-gel electrophoresis (*see* **Subheading 3.1.3.**).

3.1.5. Stable Transfection of pTet-on in MCF-7 Cells

1. Grow human breast adenocarcinoma MCF-7 cells (National Cancer Institute, Bethesda, MD) in RPMI-1640 medium containing 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 584 mg/L L-glutamine. Cells are cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator, and subcultured weekly using trypsin-EDTA (0.05%, 0.53 m*M*) solution.
2. Place 10^6 cells in 100-mm dish with 10% FBS RPMI-1640 medium (10 mL), grow cells to about 20% confluence (about 18 h). Four dishes are required, two for pTet-on transfection, two for pWLneo plasmid control.
3. Rinse cells with DMEM medium (without FBS) once, and add 10 mL of 10% FBS DMEM medium, incubate at 37°C for 1 h.
4. Prepare transfection solution:
   a. pTRE-GCS: Tube 1: 10 µL pTet-on (10 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 440 µL H_2O. Tube 2: 20 µL pTRE-GCS (20 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 430 µL H_2O.
   b. pTRE: Tube 1: 10 µL pWLneo (10 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 440 µL H_2O. Tube 2: 20 µL pWLneo (20 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 430 µL H_2O.
5. Gently mix the transfection solution to ensure adequate suspension. Add this to culture drop-wise with 1-mL pipet-aid and swirl dish gently to distribute evenly.
6. Culture cells for 18 h at 37°C, 4% CO_2. Wash the cells with RPMI-1640 medium, and shift cells to 10% FBS RPMI-1640 medium containing 400 µg/mL G-418, and culture for 24 h.
7. Subculture cells (5×10^5 cells/dish) in 10% FBS RPMI-1640 medium containing 400 µg/mL G-418, and grow at 37°C. Change medium twice a week; colonies form in about 4 wk.

8. Identify each clone under the microscope by marking on the outside bottom of the dish. Pick single clone up using cloning cylinders (Bel-Art Products, Pequannock, NJ) and trypsin-EDTA. Place single clone of cells in 24-well plates with 10% FBS RPMI-1640 containing 400 µg/mL G-418, and grow until confluent (about 7 d).
9. Subculture cells in 12-well plates with 10% FBS RPMI-1640 medium.

3.1.6. Transient Transfection of pTRE-Luc in MCF-7-Tet-on Cells and Luciferase Activity Assay

1. Place each G-418 resistant clone of pTet-on MCF-7 cells in well of 6-well plates (4000 cells/well) with 2 mL 10% FBS RPMI-1640 medium, and grow for 18 h. MCF-7 cells transfected with pTRE-Luc are used as controls.
2. Shift cells to 10% FBS DMEM medium. After a 6-h incubation, add transfection solution containing 1.5 µg DNA of pTRE-Luc plasmid.
3. After culturing in 10% FBS DMEM medium for 18 h, switch cells to 2 mL 10% FBS RPMI medium without or with 3.0 µg/mL doxycycline, and grow for 48 h (*see* **Note 2**).
4. Rinse cells twice with 2 mL PBS, and add 200 µL cell culture lysis regent (Promega, Luciferase Assay Kit).
5. Scrape the attached cells with policeman and transfer to Eppendorf tube. Centrifuge in 10,000*g*, 4°C, 10 min. Transfer the supernatant to a fresh tube.
6. Measure the protein concentration of cell extract: Add 2 µL cell extract and 198 µL of BCA protein assay reagents in 96-well plate. Incubate for 30 min at 37°C, and measure absorbance at 570 nm using a microplate reader. Use H_2O as a blank control, and 2–20 µg BSA as a standard.
7. Measure luciferase activity: Add 100 µg cell extract (approx 10 µL), and 10 mL luciferase assay reagent (Luciferase Assay System, cat. no. E1500, Promega) to scintillation vial, mix, and incubate at room temperature for 1 min. Measure in scintillation counter, 2 min/vial.
8. To develop the doxycycline-induced GCS cell line, evaluate the degree of induction, choosing the clone with highest inducibility, such as Clone 1 (*see* **Fig. 2**), for MCF-7 Tet-on cell model.

3.2. pTRE-GCS Expression Vector Construction

3.2.1. Amplification of pCG-2 and pTRE Plasmid DNA

pCG-2 is a Bluescript II KS containing GlcT-1 (**ref. 23,** terminology for GCS) at the *Eco*RI site. Transform pCG-2 and pTRE into *E. coli* Blue XL1 competent cells (*see* **Subheading 3.1.2.**) and prepare pCG-2 plasmid DNA by large-scale preparation (*see* **Subheading 3.1.4.**).

3.2.2. Purification of Human GCS cDNA

1. Place 40 µL pCG-2 DNA (2 µg/µL), 5 µL *Eco*RI (25 U/µL) and 5 µL universal buffer in 1.5-mL Eppendorf tube.

2. Incubate tubes at 37°C for 1 h with shaking (40 rpm). Prepare 1% low-melting agarose gel: add 0.4 g agarose (low melting point, Sigma), 40 mL TAE buffer, and 2 µL ethidium bromide (10 mg/mL stock solution) to 125-mL glass flask. Melt agarose in a microwave oven for two 1-min periods; pour and set agarose in gel tray with four-tooth comb (9 × 6.5 cm, Minicell System EC370M).
3. Add 12 µL gel loading buffer, mix, and load gel.
4. Run electrophoresis with TAE buffer, pH 8.0, 60 V, for about 1 h, and observe the separated fragments under UV light.
5. After sufficient separation, cut out *GCS* DNA fragment (1.7 kb) with a sharp scalpel. Be careful to leave behind as much of the agarose gel as possible. Transfer to a preweighted 1.5-mL Eppendorf tube.
6. Add 300 µL of the agarose solubilization buffer per 100 µg of agarose gel.
7. Resuspend the silica suspension, and add 10 µL of the silica suspension to the sample. If the sample contains more than 2.5 µg DNA, increase the amount of silica suspension by 4 µL for each additional µg of DNA.
8. Incubate the mixture for 10 min at 56–60°C and vortex every 2–3 min.
9. Centrifuge in a microcentrifuge for 30 s at maximum speed and discard the supernatant.
10. Resuspend the matrix containing the DNA with 500 µL nucleic acid binding buffer (vial 3, green cap) on a vortex mixer. Centrifuge and discard supernatant as above.
11. Wash the pellet with 500 µL washing buffer. Centrifuge and discard supernatant as in **step 9**. Repeat this step once.
12. Remove all the liquid with a pipet, then invert the tube on an adsorbent tissue and dry at room temperature for 15 min.
13. Add 100 µL of TE buffer, pH 8.0 to elute DNA; vortex and incubate for 10 min 56 °C. Vortex every 2-3 min.
14. Centrifuge at maximum speed for 30 s; transfer the supernatant containing DNA to a fresh tube. Add another 100 µL of TE buffer to elute again.
15. Add 100 µL chloroform, and 100 µL phenol to extract DNA. After mixing, centrifuge tube at 6000*g*, 10 min, 4°C.
16. Transfer the upper phase to a fresh tube and add 50 µL 7.5 *M* ammonium acetate and 200 µL ethanol. Mix and set on ice for 10 min.
17. Centrifuge the tubes at 12,000*g* for 15 min, then carefully discard the supernatant.
18. Rinse the DNA pellets with 1 mL 70% ethanol, and centrifuge again. Carefully discard the supernatant and dry pellets in air for 10 min.
19. Resuspend pellet in TE buffer, pH 8.0. Determine DNA concentration and quality by both UV spectrophotometry (*see* **Subheading 3.1.2.**), agarose-gel electrophoresis (*see* **Subheading 3.1.3.**). Aliquot to 300 ng/µL, and store at –20°C.

3.2.3. Ligation and Transformation

1. Digestion of vector DNA: Add 1.0 µL (50 ng/µL) pTRE, 1.0 µL *Eco*RI (25 U/µL), 0.5 µL Universal buffer (10X), and 2.5 µL redistilled H_2O to microcentrifuge tube on ice. Mix and incubate for 20 min at 37°C.

2. Inactivate restriction endonuclease at 65°C, for 15 min.
3. Dephosphorylation: Add 0.7 µL dephosphorylation buffer (10X), and 1.0 µL alkaline phosphatase (shrimp, Roche, cat. no. 1758250), and incubate for 15 min at 37°C.
4. Inactivate alkaline phosphatase at 65°C, for 20 min.
5. Ligation: Add 0.5 µL (150 ng) purified GCS cDNA fragment, 1.0 µL ligation buffer (10X), 1 µL rATP, and 1 µL T_4 DNA ligase. Mix and incubate for 6 h at 12°C.
6. Add the ligation mixture to *E. coli* blue XL1 competent cells (*see* **Subheading 3.1.1.**, **step 3**), incubate the cells on ice for 30 min. Preheat SOC medium in a 42°C water bath for 30 min.
7. Heat-pulse the cells at 42°C for 30 s, and immediately place the cells on ice for 2 min.
8. Add 0.9 mL of preheated SOC medium, and incubate the tubes at 37°C for 1 h with shaking at 225 rpm.
9. Plate 50, 100, and 200 µL of each transformation mixture on LB–ampicillin agar plates using a sterile spreader.
10. Incubate plate overnight at 37°C. Visualize colonies in each plate, and store plates at 4°C, wrapping with paraffin film.

3.2.4. Identifying Sense-Orientation of GCS cDNA

This is analyzed with Vector NTI 4.0, and doubly checked by restriction enzyme digestion with *Hin*dIII, and *Xho*I plus *Not*I.

1. Reconstruct pTRE-GCS by Vector NTI, and analyze fragments with diagnostic restriction enzymes. Restriction endonucleases and respective fragments are listed as follows: *Eco*RI: 3.15, 1.64 kb; *Hin*dIII: 3.25, 1.54 kb; *Not*I and *Xho*I: 4.08, 0.71 kb.
2. Pick up a single clone from the LB–ampicillin plates in **Subheading 3.2.3., step 10**, inoculate in 5 mL LB–ampicillin medium, prepare plasmid DNA (*see* **Subheading 3.1.2.**).
3. Use restriction mapping by *Eco*RI to screen pTRE-GCS clones (*see* **Subheading 3.1.3.**), and mapping by *Hin*dIII, and *Not*I plus *Xho*I to identify transfection orientation of pTRE-GCS.

3.2.5. Preparation of pTRE-GCS Plasmid DNA

Grow the identified pTRE-GCS cells (*see* **Subheading 3.2.4.**, **step 3**) in LB-ampicillin medium; prepare the plasmid DNA (*see* **Subheading 3.1.4.**). Aliquot DNA, 10 mg/microfuge tube, and store at –20°C.

3.3. Development of MCF-7/GCS Cells

3.3.1. Preparation of pTK-Hyg Plasmid DNA

The pTK-Hyg vector, which has a hygromycin-resistant gene under control of the mouse β-globin promoter, was used to select the stable transfectants. Prepare pTK-Hyg DNA (*see* **Subheadings 3.1.1–3.1.6.**).

3.3.2. Stable Transfection of pTRE-GCS and pTK-Hyg in MCF-7 Tet-On Cells

The basic approach used to develop MCF-7GCS cells is similar to **Subheading 3.1.5.**, the only difference is described as follows.

1. Place 10^6 MCF-7 Tet-on cells (clone 1, *see* **Fig. 2**) in a 100-mm dish with 10% FBS RPMI-1640 medium (four dishes).
2. Transfection solution:
   a. pTRE-GCS: Tube 1: 10 µL pTRE-GCS (10 µg, 1 µg/µL), 10 µL pTK-Hyg (10 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 430 µL H_2O. Tube 2: 20 µL pTRE-GCS (20 µg, 1 µg/µL), 20 µL pTK-Hyg (20 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 410 µL H_2O.
   b. pTRE: Tube 1: 10 µL pTRE (10 µg, 1 µg/µL), 10 µL pTK-Hyg (10 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 430 µL H_2O. Tube 2: 20 µL pTRE (20 µg, 1 µg/µL), 20 µL pTK-Hyg (20 µg, 1 µg/µL), 50 µL MT solution 1, 500 µL MT solution 2, 410 µL H_2O.
3. Use 10% FBS RPMI-1640 medium containing 200 µg/mL hygromycin to select transfected cells.

3.4. Evaluation of GCS Expression

Radioenzymatic assay of glucosylceramide synthase is used to further select and identify the inducible MCF-7/*GCS* cells. Northern blot ***(22)***, RT-PCR ***(24)***, and Western blot ***(12)*** of GCS also can be used to evaluate GCS expression. The developed MCF-7/*GCS* cells display inducible overexpression of GCS activity (*see* **Fig. 3,** *see* **Note 2**), and resistance to TNF-α challenge (**Fig. 4**) (***12***, *see also* **Note 3**).

3.4.1. Partial Purification of Cellular Microsomal Membranes

1. Place 10^6 cells, hygromycin-resistant pTRE-GCS or pTRE transfected cells (*see* **Subheading 3.3.2.**, **step 3**) in 150-mm dish with 10% FBS RPMI-1640 medium. Divide into control, and doxycycline (3 µg/mL in medium) treated. Culture the cells at 37°C for 3 d.
2. Aspirate the medium, rinse cells with cold PBS, and harvest cells using EDTA-trypsin solution and centrifugation (2000 rpm, 4°C, 10 min) in table-top centrifuge.
3. Rinse cell pellet with 15 mL cold PBS, centrifuge again. Resuspend the pellets in 1 mL homogenization buffer.
4. Homogenize cells by sonication using 2 × 30 s bursts over ice, and centrifuge at 2500 rpm, 10 min at 4°C. Transfer the supernatant to ultracentrifuge tube, and spin at 4°C, 129,000*g* for 60 min.
5. Resuspend the microsomal pellets in 250 µL 10 m*M* Tris-HCl, pH 7.4, and homogenize by Dounce. Measure protein concentration (*see* **Subheading 3.1.6.**, **step 6**), aliquot, and store at –80°C. Enzyme is stable for 12 wk.

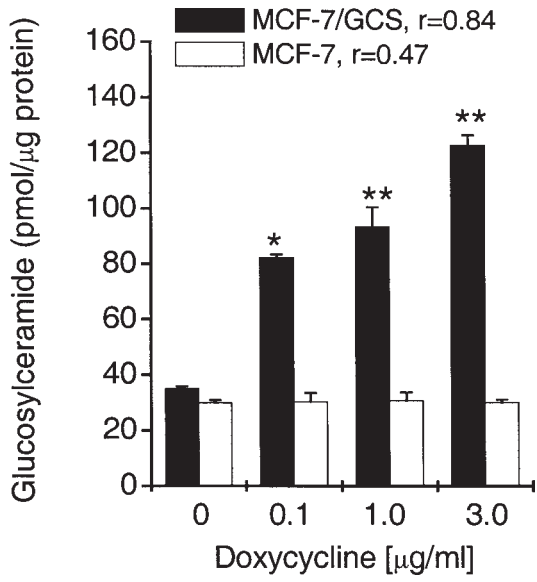

Fig. 3. Glucosylceramide synthase inducible activity in Tet-on MCF-7/GCS cells. Tet-on MCF-7/GCS cells were incubated for 48 h in medium containing the indicated concentrations of doxycycline. GCS activity was analyzed by radioenzymatic assay. *r*, correlation coefficient; $*p < 0.01$; $**p < 0.001$ compared with MCF-7 cells.

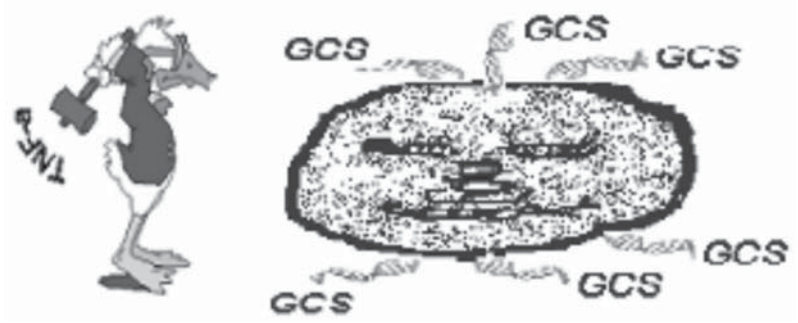

Fig. 4. Cartoon: Glucosylceramide synthase imparts cellular resistance to TNF-α. After introducing glucosylceramide synthase cDNA, cells survive TNF-α attack.

3.4.2. GCS Radioenzymatic Assay

1. Reaction solution: Add 30 µL microsomal protein (50 µg/tube), 100 µL reaction buffer, 50 µL liposomal substrate mixture, and 20 µL UDP-glucose solution to a 4-mL screw cap glass tube.

2. Incubate tubes in water bath for 60 min at 37°C with shaking (50 rpm).
3. Terminate the reaction by placing tubes on ice, add 0.5 mL isopropanol, 0.4 mL Na_2SO_4 (50 mg/mL), and mix on vortex.
4. Add 3 mL *t*-butyl methyl ether to each tube, and vortex for 20 s. Spin at 1500 rpm, for 10 min.
5. Transfer 500 µL of the upper phase to scintillation vial, add 4.5-mL EcoLume cocktail, vortex, and measure radioactivity in scintillation counter.

4. Notes

1. Be sure to obtain "inducible" MCF-7 Tet-on cells. This is essential. After introducing the pTet-on plasmid into cells, MCF-7 for example, there is about a 5% probability of obtaining overexpressing rtTA clones from G-418 resistant clones. The level of rtTA can be evaluated by the ratio of doxycycline-induced luciferase activity to uninduced luciferase activity. As shown in **Fig. 2**, doxycycline-induced luciferase activity is 100-fold higher than untreated (clone 1). Although Clone 16 has 10,000-fold higher luciferase activity compared with untransfected control cells, the luciferase activity in doxycycline-induced and doxycycline-free groups is similar. This suggests that the rtTA level in Clone 18 is close to zero. Transient transfection of pTRE-Luc and the luciferase activity assay should be done more than one time.
2. The inducing dose of doxycycline is dependent on the cell type. With the MCF-7/GCS cell line, 3 µg/mL doxycycline induces GCS overexpression (*see* **Fig. 3**), conferring resistance to TNF-a and adriamycin ***(12,22)***. In Mv1Lu cells (mink lung epithelial cells), 2 µg/mL doxycycline produces marked inducible efficacy ***(25)***. High-dose doxycycline is mildly cytotoxic. We recommend using several concentrations of doxycycline to screen induction of luciferase expression in pTRE-Luc transient transfections.
3. The *GCS* gene is introduced in MCF-7 Tet-on cells by co-transfection of pTRE-GCS and pTK-Hyg. Inducible overexpression of GCS depends on the functions of pTet-on and pTRE-GCS plasmids. The selections of G-418 and hygromycin do not guarantee the presence of pTRE-GCS in transfected cells. Using several different freeze-down methods, there is only about a 15% plating efficiency upon thawing. A new Tet vector, pTRE2HYG (Clontech) that includes the selection marker for hygromycin to replace pTRE and pTK-Hyg, allows for direct selection of cells that have been transfected with the response construct. We do believe however that it is best to finish the study using cells up to passage 20, after transfection, otherwise it may be necessary to subclone and reselect cells by GCS assay.

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16

Immunohistochemical Detection of Cytokines in Human Tissue Sections

Sandra Hulshof, Danny F. Dukers, Wim Vos, and Corline J. A. De Groot

1. Introduction

Cytokines, small soluble molecules, are secreted by many cells including cells of the immune system and the central nervous system (**Figs. 1** and **2**). Immunohistochemical detection of cytokines in tissues is hampered by the intrinsic properties of these molecules, i.e., their expression levels are usually very low, and they are released by the producing cells. These properties demand special attention regarding preparation of the slides as well as the immunohistochemical detection method. Upon cutting, the sections have to be fixed immediately in freshly prepared paraformaldehyde or air-dried and subsequently fixed in acetone to avoid diffusion of the secreted cytokines. Furthermore, a detection system should be used which can be enhanced to detect even low-level expression of the cytokines of interest.

Described here is a streptavidin-biotin complex-based method for the detection of cytokines in paraformaldehyde fixed, frozen tissues ***(1,2)***. In this method, antibodies are used that can be subsequently enhanced by the CARD method ***(3–5)***. This system includes quenching and blocking of potential background-inducing factors such as endogenous peroxidase and/or endogenous biotin. In addition, a basic protocol for immunohistochemical double labeling is described, which can be used for simultaneous detection of a cytokine and an antigen of interest for which an antibody is available with a different IgG subtype ***(6)***. The advantage of this protocol is its versatility and the ability to change the visualization method, fluorescent or chromogenic, but still use the same nonfluorescent-conjugated antibodies.

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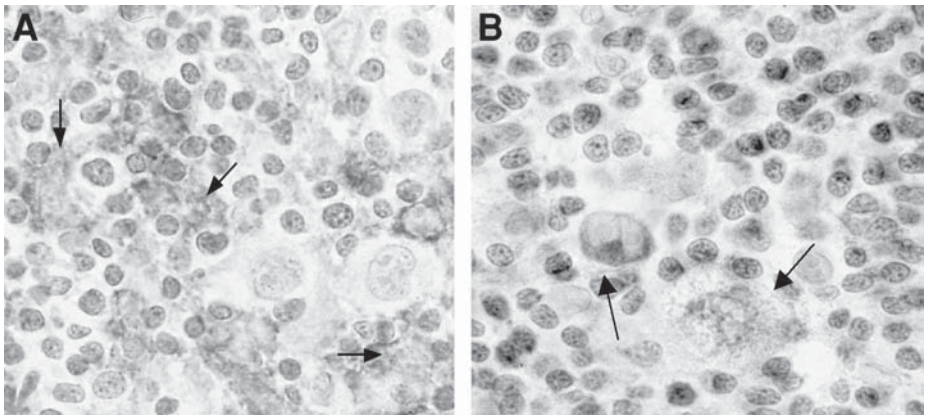

Fig. 1. Immunohistochemical detection of cytokines in Hodgkin's disease. **(A)** Expression of TGFβ is observed in macrophages as a brown cytosolic staining (arrows). **(B)** Strong granular staining of IL-10 is observed in neoplastic cells (arrows). Nuclear counterstaining with haematoxylin. Magnification **A**, **B**: ×560.

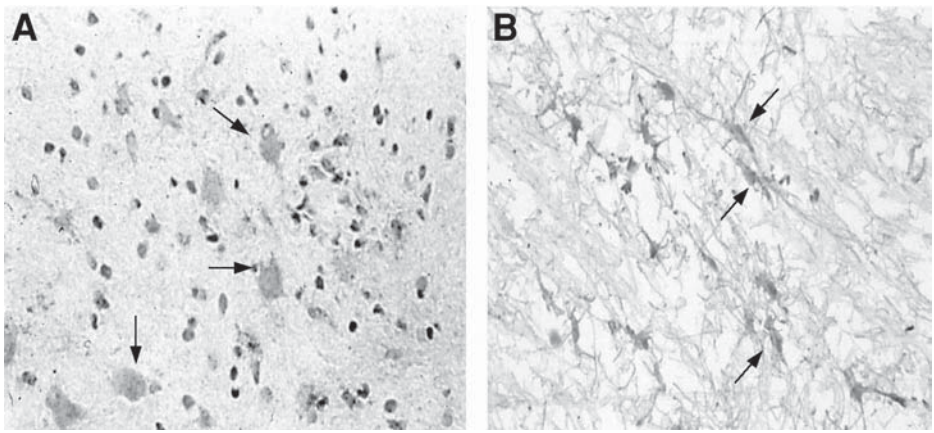

Fig. 2. Immunohistochemical detection of cytokines in multiple sclerosis (MS) brain lesions. **(A)** IL-10 expression is observed as a brown product in reactive astrocytes in a chronic active MS lesion (arrows). **(B)** Strong IL-4 staining is observed in reactive astrocytes in a chronic inactive MS lesion (arrows). Nuclear counterstaining with haematoxylin. Magnification **A**, **B**: ×360.

2. Materials

2.1. Immunohistochemistry

1. Paraformaldehyde-fixed cryostat tissue sections (*see* **Note 1**).
2. Phosphate-buffered saline (PBS, 0.01 *M*, pH 7.4).

3. PBS-S: PBS containing 0.1% saponin and 0.1% glucose (*see* **Note 2**).
4. PBS-BSA-S: PBS containing 1% bovine serum albumin (BSA), 0.1% saponin, and 0.1% glucose.
5. 1% hydrogen peroxide in PBS-S containing 0.02% NaN_3.
6. PBS-S containing 0.01% streptavidin.
7. PBS-S containing 0.01% D-biotin.
8. Blocking-buffer: Normal serum of the species in which the secondary antibody was raised is used diluted to 2–5% in PBS-BSA-S to block nonspecific binding sites.
9. Primary antibody diluted in PBS-BSA-S.
10. Biotinylated secondary antibody diluted in PBS-BSA-S.
11. Streptavidin-biotin complex-horseradish peroxidase (sABC-HRP; DAKO, Glostrup, Denmark).
12. Biotinylated tyramide (BT) in PBS-S containing 0.01% hydrogen peroxide.
13. Substrate solution 1: A stock solution of diaminobenzidine tetrahydrochloride (DAB) is prepared at 1 mg/mL in PBS, aliquoted into amounts of 5 mL, and stored at –20°C. Prior to use, add 45 mL of PBS to 5 mL of DAB-stock and add 35 µL of 30% hydrogen peroxide. This solution should be used within 1 h after preparation.
14. Hematoxylin.
15. 70, 96, and 100% ethanol.
16. Xylene.
17. Cover slips.
18. Depex-mounting medium (BDH Laboratory Supplies, Poole, England).

2.2. Additional Materials Immunofluorescence

1. Alkaline phophatase-conjugated, isotype specific, secondary antibody diluted in PBS-S.
2. Substrate solution 2: Rhodamine tyramide diluted 1:1000 in PBS containing 0.01% hydrogen peroxide.
3. 4',6'-Diamidino-2-phenylindole (DAPI) diluted 1:10 in PBS.
4. ELF-97 immunohistochemistry kit (E-6600, Molecular Probes Europe BV, The Netherlands; *see* **Note 3**).

3. Methods

3.1. Preparation of Tissue Sections

1. Cut cryostat tissue sections 4–6-µm thick and mount on poly-L-lysine coated or superfrost microscope slides.
2. Dry the tissue sections under a cold blow dryer for at least 30 min.
3. Use the tissue sections immediately or store them in a sealed box at –20°C (*see* **Note 4**).

3.2. Immunohistochemistry

1. Fix the tissue sections for 10 min at room temperature in 4% paraformaldehyde diluted in PBS containing 0.5% glucose (*see* **Notes 1** and **5**).

2. Rinse the sections in PBS-S. It is convenient to place the sections in a coplin jar during all washing steps. During incubation steps, place the slides horizontally in a moist chamber.
3. Inhibit endogenous peroxidase activity in the tissue sections by incubating in 1% H_2O_2/0.02%NaN_3 in PBS-S for 30 min (*see* **Note 6**).
4. Rinse the sections briefly in PBS-S.
5. Cover binding to endogenous biotin by incubating in 0.01% streptavidin in PBS-S for 20 min followed by washes with PBS-S (2 × 5 min) and an additional incubation in 0.01% D-biotin in PBS-S for 20 min (*see* **Note 6**).
6. Rinse the sections briefly in PBS-S.
7. Incubate the sections in blocking-buffer for 10 min to block nonspecific binding sites.
8. Remove excess of blocking-buffer from the slides with a tissue (*see* **Note 7**) and incubate the sections with the primary antibody diluted 2–5 µg/mL in PBS-BSA-S overnight at 4°C (*see* **Note 8**).
9. Rinse the sections in PBS-S for 15 min.
10. Remove excess of PBS-S with a tissue and apply the biotinylated secondary antibody diluted in PBS-BSA-S and incubate for 30 min.
11. Rinse the sections in PBS-S for 15 min.
12. Incubate the sections with sABC-HRP diluted 1:200 in PBS-BSA-S for 1 h (*see* **Notes 9** and **10**).
13. Rinse the sections in PBS-S for 15 min.
14. Incubate the sections in freshly prepared DAB substrate solution for 3–5 min. A brown reaction product will be deposited at the site of primary antibody binding.
15. Rinse the sections in running tap water for 5 min.
16. Counterstain lightly with hematoxylin for 1 min.
17. Dehydrate the sections by incubation at increasing concentrations of ethanol (70, 96, 100%), finally clear with xylene, and mount in Depex.

3.3. Double Label Immunofluorescence

1. Fix the tissue sections for 10–20 min at room temperature in 4% paraformaldehyde diluted in PBS (*see* **Note 5**).
2. Rinse the sections briefly in PBS-S.
3. Inhibit endogenous peroxidase activity in the tissue sections by incubating in 1% H_2O_2/0.02%NaN_3 in PBS-S for 30 min (*see* **Note 6**).
4. Incubate the sections with the first primary antibody diluted 2–5 µg/mL in PBS-BSA-S overnight at 4°C.
5. Rinse in PBS-S for 15 min.
6. Remove excess of PBS-S with a tissue and apply the biotinylated secondary antibody diluted in PBS-BSA-S and incubate for 30 min.
7. Rinse in PBS-S for 15 min.
8. Incubate the sections with sABC-HRP diluted 1:200 in PBS-BSA-S for 1 h (*see* **Notes 9** and **10**).
9. Rinse the sections in PBS-S for 15 min.
10. Incubate the sections in blocking buffer for 10 min to block nonspecific binding sites.

11. Incubate the sections with the second primary antibody diluted 2–5 µg/mL in PBS-BSA-S for 1 h.
12. Rinse in PBS-S for 15 min.
13. Remove excess of PBS-S and apply the alkaline phosphatase-conjugated isotype specific secondary antibody diluted in PBS-BSA-S and incubate for 30 min.
14. Rinse in PBS-S for 15 min.
15. Visualize the HRP by incubation in Rhodamine conjugated tyramide diluted 1:1000 in PBS containing 0.01% H_2O_2 for 10 min (*see* **Note 11**).
16. Rinse the sections in PBS-S for 15 min.
17. Visualize the alkaline phosphatase conjugate by ELF-97 according to the manufacturer's protocol (*see* **Note 3**).
18. Rinse in PBS-S for 15 min.
19. Nuclear counterstaining by incubation of the sections in DAPI diluted 1:10 in PBS for 3 min.
20. Rinse the sections briefly in PBS.
21. Mount the sections with ELF-97 mounting medium (component E in the ELF-97 immunohistochemistry kit), apply cover slip and store the slides in a closed slide box at 4°C (*see* **Note 12**).

3.4. Visualisation of Fluorescent-Stained Tissue

Fluorescent-stained tissue can be evaluated through a standard fluorescence microscope. The biotinylated antibody can be detected by the deposition of Rhodamine conjugated tyramide, which results in a red signal using a standard TRITC filter. The alkaline phosphatase conjugated antibody can be visualized through a standard DAPI filter. With this filter, the yellow–green signal appears very distinct against a black background and DAPI-positive nuclei.

4. Notes

1. We have also successfully used this method on acetone-fixed cryostat tissue. However, when using acetone-fixed tissue sections, saponin can be omitted from all solutions used in this protocol, i.e., PBS instead of PBS-S; normal serum, antibody, and sABC-HRP dilutions in PBS-BSA instead of PBS-BSA-S.
2. Saponin is added to keep the cells permeable so that optimal antibody binding to the antigen can take place. Addition of glucose to the solutions will increase the osmolarity and thereby tissue morphology will be preserved.
3. Molecular Probes enzyme-labeled fluorescence (ELF-97) immunohistochemistry kit contains a patented alkaline phosphatase substrate that yields a fluorescent precipitate resistant to photo bleaching. If endogenous alkaline phosphatase is expected to be present, for example, in blood vessels or kidney, levamisole can be added to the ELF substrate solution to a final concentration of 1 m*M*. Before use, this solution should be filtered through a 0.22-µm Millipore filter.
4. After storage at –20°C leave the slide box closed for at least 15 min so that the slides can adjust to room temperature before further use.

5. All incubation steps are carried out at room temperature unless stated otherwise.
6. Inhibition of endogenous peroxidase and/or endogenous biotin is recommended only when this is present at high amounts in the tissues used, i.e., endogenous peroxidase: erythrocytes, granulocytes, and mast cells; endogenous biotin: kidney, liver, breast tissues, and colon. Otherwise **steps 3–6** in this protocol can be omitted.
7. A pen for drawing a hydrophobic ring around the tissue sections is marketed by DAKO. It obviates the need to remove excess of buffer around the sections with a tissue after each step and reduces the amounts of antibody solution required.
8. Incubation of the primary antibody can occur either overnight at 4°C or 1 h at room temperature. However, when protein expression levels are low, an overnight incubation step is recommended. In addition, when primary antibodies are incubated overnight, they should be diluted 2:1 compared to primary antibody incubations 1 h at room temperature.
9. Prepare sABC-HRP, diluted 1:200, at least 30 min before use so that an enzyme complex can be formed. In addition, once the sABC-HRP has been prepared it can be stored for 3 h.
10. When very low protein expression levels are expected, additional steps are recommended to increase the signal intensity. However, enhancement of the signal intensity is not recommended for tissues derived from the central nervous system. To increase the signal intensity, these subsequent steps should be added to the immunohistochemistry protocol (**Subheading 3.2.**) following **step 11**:
    Incubate the sections with sABC-HRP diluted 1:500 in PBS-BSA-S for 30 min.
    a. Rinse the sections in PBS-S for 15 min.
    b. Incubate the sections in BT diluted 1:1000 in PBS-S containing 0.01% H_2O_2 for 10 min.
    c. Rinse the sections in PBS-S for 15 min.
    d. Incubate the sections with sABC-HRP diluted 1:200 in PBS-BSA-S for 30 min.
11. For the preparation of Rhodamine conjugated tyramide, mix tyramide-HCl and NHS-Rhodamine dissolved in *N*'-*N*'-dimethyl formamide to a dilution of 1–1.1, additionally add triethylamine as a buffer. Leave the mixture for at least 2 h and thereafter dilute in ethanol to a final concentration of 1 mg/mL fluorescent tyramide. Rhodamine conjugated tyramide can be stored in a closed box at 4–8°C for up to 8 mo. Prior to use dilute 1:1000 in PBS ***(4)***.
12. Fluorescent sections can be stored in a closed box at 4°C for several months with only minor loss of fluorescence.

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Detection of Cytokine- and Chemokine-Expressing Cells at the Single Cell Level

Karin Loré and Jan Andersson

1. Introduction

Methods for detection of cytokines and chemokines at a protein level in individual cells based on immunofluorescence or immunohistochemical ***(1–16)*** staining techniques have been developed in our laboratory. The difficulties in these procedures lie in the critical steps of fixation and permeabilization of the cells in combination with a very careful selection of suitable cytokine- or chemokine-specific antibodies.

In order to detect intracellular cytokines and chemokines, cells have to be permeabilized to allow the specific antibodies to penetrate the cell surface membrane, the cytosol, and the membranes of the Golgi–endoplasmatic reticulum complex. Cells, therefore, need to be fixed to stand the effects of the permeabilizing agent. An optimal combination of fixative and detergent preserves the cellular morphology and the antigenicity of intracellular proteins with minimal cell aggregation and cell loss. We achieved optimal results by combining a brief fixation with phosphate-buffered formaldehyde and a subsequent permeabilization of the cellular membranes by the detergent saponin. Aldehyde fixatives such as formaldehyde, paraformaldehyde, and glutaraldehyde form methylene bridges among amino, guanidyl, hydroxyl, carboxyl groups, and so on, in proteins and are therefore classified as cross-linking fixatives. We found that fixation by formaldehyde is gentle and retains a high degree of antigenicity. Many permeabilizing agents may interact with the cytokines/chemokines and change their structure so that antigenic sites become changed, or solubilize or extract the cytokines/chemokines so that the proteins are inaccessible. By permeabilizing the cells with saponin, it has been possible to stain for several cytokines and chemokines that were undetectable after treat-

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ment with other chemicals such as acetone, ethanol, methanol, Triton-X, or *n*-octyl-β-D-glucopyranoside. Saponin is a plant glycoside with high affinity for cholesterol. Saponin is thought to cause a reversible reaction by eluting cholesterol in the cellular membrane ***(17)*** and thereby permeabilizes the cells. It is thus crucial that saponin be present during all incubations and washes in the staining procedure. Because saponin interacts mainly with cholesterol, much of the morphology of the membrane structure of the cells treated with formaldehyde remains intact. However, the outlined protocols cannot be used for intranuclear staining, as saponin does not sufficiently permeabilize the nuclear cell membranes.

We found that monoclonal antibodies (MAbs) or antigen-affinity purified polyclonal antibodies are preferable for cytokine and chemokine staining. A combination of several MAb detecting different antigenic epitopes of a given cytokine and chemokine may also result in increased staining. One has to be aware that antibodies used for bioassays or enzyme-linked immunosorbent assay (ELISA) do not necessarily work well for intracellular immunostaining. We have screened several hundred supernatants from hybridomas that produce MAb in order to find the most suitable cytokine/chemokine specific MAbs for staining. Most of the cytokine/chemokine-specific antibody clones that we have successfully employed will not function if other fixatives or permeabilizing agents are used. During this screening procedure, we have observed that there has been a strong correlation between the biologic neutralizing capacity of cytokine/chemokine-specific MAbs and a distinct/clear staining signal obtained by the same MAb in our staining method. This may be because only intracellular cytokines and chemokines in producer cells will be detected, not the synthezised proteins that are bound to target cells via the specific receptors. A neutralizing MAb cannot bind to an epitope of the cytokine/chemokine protein that is occupied by the interaction with the specific receptor and thus is not available for detection. The identification of cytokine/chemokine-expressing cells detected by this method is therefore facilitated by the distinct characteristic intracellular morphology of the staining pattern generated by the cytokine/chemokine accumulation in the Golgi organelle. All cytokines/chemokines with the exception of the interleukin 1 (IL-1) family (including IL-18) have a leader sequence that directs secretion through the Golgi–endoplasmatic reticulum complex. The optimal concentration for most cytokine/chemokine-specific antibodies is 0.5–5 μg/mL, when used in our staining protocols for indirect immunofluorescent or immunocytochemical detection on slides. Direct detection may require a slightly higher concentration of antibodies. The concentration of the antibodies used in flow cytometry analysis is often lower than for staining on slides. However, titration of all antibodies is mandatory to obtain optimal results. The use of both overdiluted and overconcentrated antibody solutions will result in weak or negative staining. Excessively high concentra-

tions can also generate unspecific background staining. The methods described in this chapter were originally developed for identification of cytokines produced in smeared cells, but the same techniques can also be used with slight modification for the detection of cytokine/chemokine-expressing cells in tissue sections ***(18–24)***.

2. Materials

2.1. Preparation of Samples

2.1.1. Cell Stimulating Reagents

1. Phorbol-12-myristate-13 acetate (PMA) (Sigma, St. Louis, MO).
2. Ionomycin (Calbiochem Novabiochem Corp., San Diego, CA).
3. Lipopolysaccharide (LPS) (Sigma).

2.1.2. Cytokine/Chemokine Protein Transport Inhibitor

1. Brefeldin A (Sigma).

2.1.3. Microscope Slides

1. Adhesion slides Adcell (Erie Scientific Inc., Portsmouth, NH).
2. Adhesion slides (Bio-Rad Lab, Munich, Germany).

2.1.4. Tubes for Flow Cytometry

1. Falcon (Becton Dickinson Labware, Franklin Lakes, NJ).

2.1.5. Cell and Tissue Fixation Buffer

1. Formaldehyde (37% v/v Sigma) diluted in phosphate-buffered saline (PBS) to a final formaldehyde concentration of 2% (v/v) and adjusted to pH 7.4. Light sensitive, store at 4°C in the dark; prepare working dilution fresh prior to fixation. Do not use diluted solution for more than 7 d.

2.2. Immunohistochemical Staining

2.2.1. Wash Buffers

1. Wash buffer: 1X Earl's buffered salt solution (EBSS) (Gibco, Paisley, UK).
2. Wash buffer/saponin: 1X EBSS with 0.1% (w/v) saponin (Riedel-de Haen AG, Seelze, Germany) supplemented with 0.1 *M* HEPES buffer (Gibco). Adjust the pH of the wash buffers to 7.2–7.4 with NaOH.

2.2.2. Blocking Solutions

1. Endogenous peroxidase blocking buffer: 1% H_2O_2 (Sigma) in wash buffer/saponin.
2. Endogenous biotin blocking buffer: Avidin/biotin Blocking Kit (Vector Laboratories, Inc., Burlingame, CA). The solutions A + B included in the kit should be supplemented with 0.1% (w/v) saponin.

3. Serum blocking buffer:
   a. 2% Fetal calf serum (FCS; Gibco) in wash buffer/saponin.
   b. 1% FCS, 1% normal mouse serum (NMS; ICN Biomedicals, Inc., Aurora, OH), 1% normal goat serum (NGS; Dako, Glostrup, Denmark) in wash buffer/saponin.

2.2.3. Cytokine/Chemokine Specific Antibodies

An overview of antibodies suitable for staining human cells is given in **Table 1**.

2.2.4. Secondary Antibodies

1. Anti-mouse:
   a. Biotinylated goat anti-mouse IgG_1, IgG_{2A}, IgG_{2B} (Caltag Lab, Burlingame, CA).
   b. Flourescein isothiocyanate (FITC)-labeled goat anti-mouse IgG_1, IgG_{1A}, IgG_{2B} (Caltag Lab).
   c. Cy3-labeled goat anti-mouse IgG_1, IgG_{1A}, IgG_{2B} (Caltag Lab).
   d. AP conjugated goat anti-mouse IgG1, IgG_{2A}, IgG_{2B} (Southern Biotechnology Associates Inc, Birmingham, AL).
   e. FITC-conjuated goat $F(ab')_2$ anti-mouse immunoglobulins (Dako).
   f. Alexa™ 488 coupled goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR).
2. Anti-goat: Biotinylated swine anti-goat IgG (Caltag Lab).
3. Anti-rat:
   a. Biotinylated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).
   b. Biotinylated goat anti-rat IgG (Vector Laboratories Inc.).

2.2.5. Secondary/Tertiary Reagents

1. Vectastain Elite-ABC-peroxidase kit (Vector Laboratories Inc.).
2. Vectastain AEC AP kit (Vector Laboratories Inc.).
3. FITC-labeled streptavidin (Dako).
4. Alexa™ 488 labeled streptavidin (Molecular Probes Inc.).
5. Alexa™ 546 labeled streptavidin (Molecular Probes Inc.).

2.2.6. Substrates for Immunohistochemistry

1. Peroxidase substrates:
   a. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories Inc.).
   b. AEC (Vector Laboratories Inc.).
2. Alkaline phosphatase substrates:
   a. Vector Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories Inc).
   b. Alkaline Phosphatase Substrate kit (Vector Laboratories Inc.).
   c. Red Fuchsin (Dako).

2.2.7. Nuclear Counterstaining for Immunohistochemistry

1. Mayer's hematoxylin counterstain (1:1 v/v distilled water) (Sigma).

Table 1
Antibodies Suitable for Staining of Human Cells

Cytokine/ chemokine	Antibody clone	Isotype	Manufacturer
IL-1α	1277-89-7+	mouse IgG_1	Immunokontact, Bioggio, Switzerland
	BAF 200	biotin-goat IgG	R&D Systems, Minneapolis, MN
IL-1β	2-D-8+	mouse IgG_1	Immunokontact } mix
	1437.96-15	mouse IgG_1	Immunokontact } mix
	BAF201	biotin-goat IgG	R&D Systems
IL-1rα	1384-92-17-19+	mouse IgG_1	Biomedicals, Augst, Switzerland
	1398.93.9	mouse IgG_1	Biomedicals
	BAF280	biotin-goat IgG	R&D Systems
IL-2	MQ1-17H12	rat IgG_{2A}	BD PharMingen, San Diego, CA
	BAF202	biotin-goat IgG	R&D Systems
IL-3	BVD8-3G11+	rat IgG_1	BD PharMingen
	BVD3-IF9	rat IgG_1	BD PharMingen
IL-4	8D4-8	mouse IgG_1	BD PharMingen
	MP4-25D2	rat IgG_1	BD PharMingen
	BAF204	biotin-goat IgG	R&D Systems
IL-5	JES-39D10	rat IgG_{2A}	BD PharMingen
IL-6	MQ2-6A3	rat IgG_{2A}	PharMingen
	BAF206	bitoin-goat IgG	R&D Systems
	MQ2-13A5	rat IgG_1	BD PharMingen
IL-7	BVD10-11C10	biotin-rat IgG_{2A}	BD PharMingen
IL-8	NAP-1	mouse IgG_1	Immunokontact
	BAF208	biotin-goat IgG	R&D Systems
IL-10	JES3-19FI	rat IgG_{2A}	BD PharMingen
	JES3-12G8	rat IgG_{2A}	BD PharMingen
IL-12 (p70)	20C2	rat IgG	Hoffman-LaRoche, Nutley, NJ
	MAB 219 + MAB 619	mouse IgG_1	R&D Systems
	BAF219	goat	R&D Systems
IL-13	JES8-30F11	rat IgG_{2A}	DNAX, Palo Alto, CA
	JES10-5A2	rat IgG_1	BD PharMingen
	B-B13	mouse IgG_1	Diaclone, Besacon, France
IL-17	AF317NA	goat IgG	R&D Systems
MIP-1α	BAF270	biotin-goat IgG	R&D Systems
MIP-1β	BAF271	biotin-goat IgG	R&D Systems

(continued)

Table 1 (continued)

Cytokine/ chemokine	Antibody clone	Isotype	Manufacturer
RANTES	BAF278	biotin-goat IgG	R&D Systems
GM-CSF	BVD2-21C11	rat IgG_{2A}	BD PharMingen
	BVD2-5A2	rat IgG_{2A}	BD PharMingen
G-CSF	BVD13-3A5+	rat IgG_1	BD PharMingen
	BVD11-37G1	rat IgG_{2A}	BD PharMingen
TNF-α	mAb 1+mAb 11	mouse IgG_1	BD PharMingen
	BAF210	biotin-goat IgG	R&D Systems
TNF-β	LTX 21	mouse IgG_{2B}	Biosource Int., Louisville, KY
	MAB611	mouse IgG_1	R&D Systems
IFN-α	9-1-1	mouse IgG_1	BioNative, Umeå, Sweden
IFN-γ	DIK-1 + 7-B-6	mouse IgG_1	Mabtech, Nacka, Sweden
	7-B6-1	biotin-mouse IgG_1	Mabtech
Isotype control	mouse IgG_1		Dako, Gostrup, Denmark
Isotype control	rat IgG_{2A} (R35-95)		BD PharMingen

2.2.8. Mounting Media for Staining on Slides

1. Immunohistochemistry mounting medium: PBS-buffered glycerol (Kebo Lab, Stockholm, Sweden) 1:9 (v/v), and adjust pH to 7.4.
2. Fluorescence antifading mounting medium:
   a. Carbonate/bicarbonate buffered glycerol (1:1 v/v) containing 2% 1,4-diazobicyclo (2,2,2)octane (Sigma) and adjust pH to 7.4.
   b. FluorSave™ Reagent (Calbiochem-Novabiochem Corp, San Diego, CA).

2.3. Immunofluorescent Staining on Slides and in Tubes

1. Wash buffer B/saponin: 0.1% saponin in PBS supplemented with 0.5 *M* HEPES buffer.
2. Serum blocking buffers B:
   a. 1% human AB-serum (obtained from healthy blood donors from the blood bank) in wash buffer B/saponin.
   b. 1% NMS in wash buffer B/saponin.
   c. 1% FCS in wash buffer B/saponin.

3. Methods

3.1. Cell Stimulation and Preparation

3.1.1. Induction of Cytokines and Chemokines

1. Add 5 ng/mL PMA and 0.5 µ*M* ionomycin, or 0.1–1 µg/mL LPS to the cell culture.
2. Add brefeldin A (1–10 µg/mL) to the cell cultures 2–8 h prior to harvest if detection of cytokines/chemokines by flow cytometry is to be performed.
3. Incubate cells for defined periods (*see* **Note 1**).

3.1.2. Sample Preparation for Immunostaining on Slides

1. Harvest cells by cell scraper or pipeting and wash twice in V-bottomed tubes with PBS by centrifugation (250*g* for 5 min) to remove extracellular proteins, including cytokines and chemokines.
2. Resuspend cells at 1–5 × 10^6 cells/mL in PBS.
3. Transfer 10–15 µL of the cell suspension to each reaction well on the adhesion slide.
4. Allow the cells to adhere electrostatically in a monolayer for 10–20 min at room temperature. Check the cell density under the microscope. Add more cells if necessary. Prevent the cells from drying out.
5. Rinse the slides very gently with PBS to wash away nonadherent dead cells and debris.
6. Add 50 µL of 2% formaldehyde fixation buffer to each well to fix the cells. Incubate for 10–15 min at room temperature.
7. Rinse very gently several times with PBS to remove all formaldehyde.

The slides are now ready for staining. Slides can be dried and stored at –20°C for several months before being stained (*see* **Note 2**).

3.2. Immunohistochemical Staining on Slides

The staining must be carried out in a humidity chamber to prevent the wells from drying during the procedure.

1. Wash the slides by gently rinsing with wash buffer without saponin, using a plastic transfer pipet and glass Pasteur pipet. Wipe the surrounded area of the wells with a folded filter paper.
2. Incubate with 25 µL endogenous peroxidase blocking buffer per well for 30 min at room temperature to block the endogenous peroxidase activity in the cells. Repeat the procedure until no activity is left, indicated by no gas production, i.e., no bubbles. This step can be omitted if the cells are to be stained by nonperoxidase-based enzymatic methods. Rinse three times with wash buffer/saponin.
3. Add 15 µL of a serum blocking buffer, 1–3% serum in wash buffer/saponin, to block unbound surface area on the slide. Incubate for 10 min at 37°C.

4. Block endogenous biotin activity with the avidin/biotin blocking kit in a two-step procedure for 30 min in the presence of saponin. Follow the instructions from the manufacturer. Incubate each well with solution A (included in the kit) for 15 min. Wash the slides three times in wash buffer/saponin. Incubate in solution B (also included in the kit) for 15 min. Rinse three times with wash buffer/saponin. This step may not be necessary, as significant biotin activity does not exist in many cells types. However, the serum blocking procedure can result in some background activity.
5. Incubate the defined wells on the slides with 15 µL unlabeled or biotinylated cytokine/chemokine-specific antibody (0.5–5 µg/mL) diluted in wash buffer/saponin for minimum 30 min at 37°C. Rinse slides three times in wash buffer/saponin.
6. If secondary antibodies are required, incubate the slides for 30 min at 37°C with 15 µL of a biotinylated secondary appropriate antibody in wash buffer/saponin supplemented with 1% serum of the species the secondary antibody was produced in. Rinse the slides three times in wash buffer/saponin.
7. Prepare the avidin-enzyme complex reagent (Vectastain AEC-AP kit or Vectastain Elite ABC kit) according to the manufacturer's instructions and supplement with saponin (0.1% w/v) 30 min prior to **step 8**.
8. Incubate for 30 min with 15 µL of the avidin–enzyme complex reagent at room temperature in the dark. Rinse slides three times in wash/buffer/saponin.
9. Prepare the substrate according to the protocol given by the manufacturer without any supplementation of saponin. Incubate for approx 2–30 min depending on the substrate and the type of staining at room temperature in the dark. Monitor the reaction in the microscope to judge when the development is sufficient.
10. Stop the development of the color reaction by repeated washes in wash buffer.
11. Counterstain, if desired, with hematoxylin for 1–5 s. Wash three times in distilled water. Slides can then be dried, completely is not necessary, and mounted in mounting medium for immunohistochemistry. (Slides that need to be kept for long periods in optimal shape should be dehydrated in graded ethanol and mounted in special mounting medium). (*See* **Notes 3**, **5**, **6**, and **7**.)

3.3. Immunofluorescent Staining on Slides

Cytokine/chemokine-specific staining based on either biotinylated primary antibodies or unlabeled primary antibodies along with biotinylated secondary antibodies can also be developed by techniques based on immunofluorescence. The procedure is very similar.

1. Add 15 µL of one of the serum blocking buffers B containing wash buffer B/saponin and serum to prevent unspecific staining of the slide and of the cells. Incubate for 10 min at 37°C.
2. Block endogenous biotin activity with the avidin/biotin blocking kit in a two-step procedure for 30 min in the presence of saponin. Follow the instructions from the manufacturer. Incubate each well with solution A (included in the kit) for 15 min.

Wash the slides three times in wash buffer B/saponin. Incubate in solution B (also included in the kit) for 15 min. Rinse three times with wash buffer B/saponin. This step may not be necessary, as significant biotin activity does not exist in many cells types. However, the serum blocking procedure can result in some background activity.

3. Incubate each defined well on slides for 30 min at 37°C with 15 µL unlabeled or biotinylated cytokine/chemokine-specific antibody (0.5–5 µg/mL) diluted in wash buffer B/saponin. Rinse slides three times in wash buffer B/saponin.
4. Incubate cells for 30 min at room temperature with 15 µL of fluorophore-labeled secondary antibody or fluorophore-labeled streptavidin diluted in wash buffer B/saponin supplemented with 1% serum from the species in which the secondary antibody was produced. Rinse slides repeated times in PBS only.
5. Mount slides in antifading mounting buffer. Several fluorophores require a mounting medium including some antifading substance to reduce quenching in UV light. It is possible to store fluorophore-stained slides for long periods in the freezer, provided that they have not been mounted in mounting medium (*see* **Notes 4**, **5,** and **7**).

3.4. Immunostaining in Tubes for Flow Cytometry Analysis

1. Wash cells after harvesting twice in PBS by centrifugation (250*g*, 5 min). Discard the supernatant.
2. Fix the cells in 2% formaldehyde fixation buffer for 15 min.
3. Distribute cells to staining tubes for flow cytometry. Add 2 mL PBS/tube and wash by centrifugation.
4. Resuspend and wash cells in 2 mL wash buffer B/saponin.
5. Add 10 µL human AB-serum and incubate for 5 min at room temperature.
6. Add fluorophore conjugated cell marker–specific antibodies. Add fluorophore or biotinylated cytokine/chemokine-specific antibody. Incubate for 30 min, 4°C, in the dark. Wash in wash buffer B/saponin.
7. If required, add fluorophore-labeled streptavidin to the biotinylated cytokine/chemokine specific antibody. Incubate for 20 min 4°C, in the dark.
8. Wash twice in PBS only.
9. Analyze cells directly by flow cytometry or leave cells maximum 3 d in the refrigerator until analyzed.
10. One option is to put the stained cells on BioRad adhesion slides, let them sediment and dry. Mount with mounting antifading buffer and evaluate the cells by UV light microscopy (*see* **Note 8**).

3.5. Conclusion

The outlined techniques are optimal for single-cell detection of cytokine/chemokine-expressing cells at the protein level. The method of choice depends on the aim of the investigation in addition to the equipment and experience in the laboratory. We have found no differences in sensitivity between the immunofluorescent and the immunohistochemical techniques described ***(12)***.

4. Notes

1. The frequency of spontaneous cytokine-producing cells in blood mononuclear cells separated by gradient centrifugation from healthy blood donors is very low. Normally we find one cytokine-synthesizing cell out of 10,000 cells for the cytokines produced by lymphocytes and one cytokine-producing cell out of 100–1000 cells for the cytokines produced by cells in the monocyte lineage. However, the number of detectable cytokine-producing cells may increase significantly after exposure to low amounts of contaminating endotoxin in the culture medium or prolonged plastic adherence. Depending on the culture system, the cell type, and the purpose of the investigation, the cells may be in vitro stimulated prior to detection to induce cytokine and chemokine expression. PMA and ionomycin is a good combination to obtain rapid cytokine expression in mainly T-cells (***4***). PMA and ionomycin are esters that penetrate the cellular membrane without specific receptor binding, enter the nucleus, and turn on the cellular transcription factors unspecifically. A sufficient concentration is 5 ng/mL of PMA and 0.5 μ*M* of ionomycin. However, this is a very powerful stimulus that is harmful to the cells after long stimulation periods (>24 h). LPS or endotoxins are also often used, especially for stimulation of monocytes ***(6)***, dendritic cells ***(11,12,25)***, or B cells of murine origin. LPS activates cells through the CD14 receptor but recent reports show that the Toll-like receptor (TLR), particularly TLR2 (mouse) and TLR4 (human), are involved in the interaction with LPS ***(26–28)***. We use LPS extracted from *Escherichia coli* in quantities from 0.1 to 1 μg/mL for stimulation periods from 0.5 to 72 h. There are many different endotoxins commercially available and the cellular response can differ between them.
   A cytokine/chemokine transport inhibitor is required for detection of cytokine/chemokine expression by flow cytometry in order to increase the positive staining signal compared to background. Treatment with either carboxylic ionophore monensin ***(29–32)*** or brefeldin A ***(20,21)*** uncouples the Golgi organelle from the endoplasmatic reticulum, which leads to an inability to secrete proteins from the cell via the secretory pathway. The proteins are instead accumulated within the cytoplasm of the cells, which promotes an increased but diffusely distributed intensity of the staining signal. Brefeldin A (1–10 μg/mL) should be added together with the stimuli in the culture for 2–6 h prior harvest of cells. Brefeldin A is toxic to the cells after longer exposure.
2. Dead cells adhere very poorly, if at all, to the adhesion slides. They are therefore easily rinsed away. Stimulated and unstimulated cells, and all the different subgroups of leukocytes, will adhere equally well. Fixed cells are also difficult to smear on. Already fixed cells can be adhered to the wells by sedimentation and drying without subsequent washes. However, this may lead to unsuccessful staining.
3. The development of the cytokine stainings based on immunohistochemistry by adding a substrate to generate a color reaction must be monitored in the microscope in order to determine when the optimal color reaction is obtained. The DAB reaction will go to completion without causing significant background, while the reactions in the alkaline phosphatase (AP) systems need to be stopped

before background signals occur. Furthermore, in our experience, DAB (brown) gives the most distinct, reproducible, and intense stain in detecting cytokines and chemokines. Another peroxidase substrate, AEC (red), gives satisfactory results. However, in some cells and tissues or in double-labeling experiments, the AP-system may be preferred. We have found that Vector Blue AP substrate is the best among the AP substrates. However, the other substrates that we have listed work quite well.

4. Immunofluorescence techniques facilitate phenotypical identification of the cytokine/chemokine-producing cells by multiple color stainings ***(3,4,13,33)***. Similar approaches can be taken to visualize the production of several cytokines/chemokines in the same cell ***(4,5,18)***.
   It is important to choose a combination of antibodies that do not cross-bind to each other. A careful selection of antibodies, preferably from different species, but also different subclasses within the same species, should be used. Some cell surface antigens are highly expressed and directly fluorophore-labeled antibodies may therefore generate a sufficient signal. The antigen that is predominantly expressed should be stained with the weakest fluorophore and vice versa. It is possible to apply the antibodies simultaneously, but the system needs to be optimized. The antibodies may require different concentrations than when they are individually applied. The choice of fluorophores is also critical for the best distinction between antigens. Cross-bleeding between different colors can cause considerable problems. We prefer to use the fluorophores that are listed in the Materials section. Old antibodies can lose their conjugate, which results in a grainy staining due to contaminating fluorophore molecules. Optimal results for us have been obtained using FITC or Alexa 488 staining for the cell surface and Alexa 543 or Cy2 staining for the cytokine. Supplementation of blocking serum 0.1% BSA-C (Aurion BSA-C™, Wageningen, The Netherlands) in all buffers and antibody dilutions has been found to prevent unspecific background staining. The use of confocal microscopes significantly increases the advantage of evaluation of immunostaining for co-localization experiments.
5. The identification of cytokine- and chemokine-expressing cells in our methods has been greatly facilitated by the characteristic morphology of the staining pattern of intracellularly accumulated cytokines to the Golgi–ER complex. This staining pattern in the producer cells is drastically different from classical membrane signals seen for cellular membrane receptors. All studied cytokines and chemokines, except the members of the IL-1 family (including IL-18), have shown a local perinuclear staining reflecting the accumulation of the cytokines in the Golgi organelle of producer cells [*see* **Fig. 1(A),(B)**]. Staining with FITC-labeled reagents has always generated the most crisp and informative microscopy images with a high degree of resolution [*see* **Fig. 1(A)**]. At the early stage of cytokine/chemokine synthesis, a discrete staining localized close to the nucleus with a ring or a string formation is observed. Later, these perinuclear vesicles will form a larger perinuclear dot in the Golgi–ER complex. Some cytokines show an additional cytoplasmic and membrane staining together with the Golgi

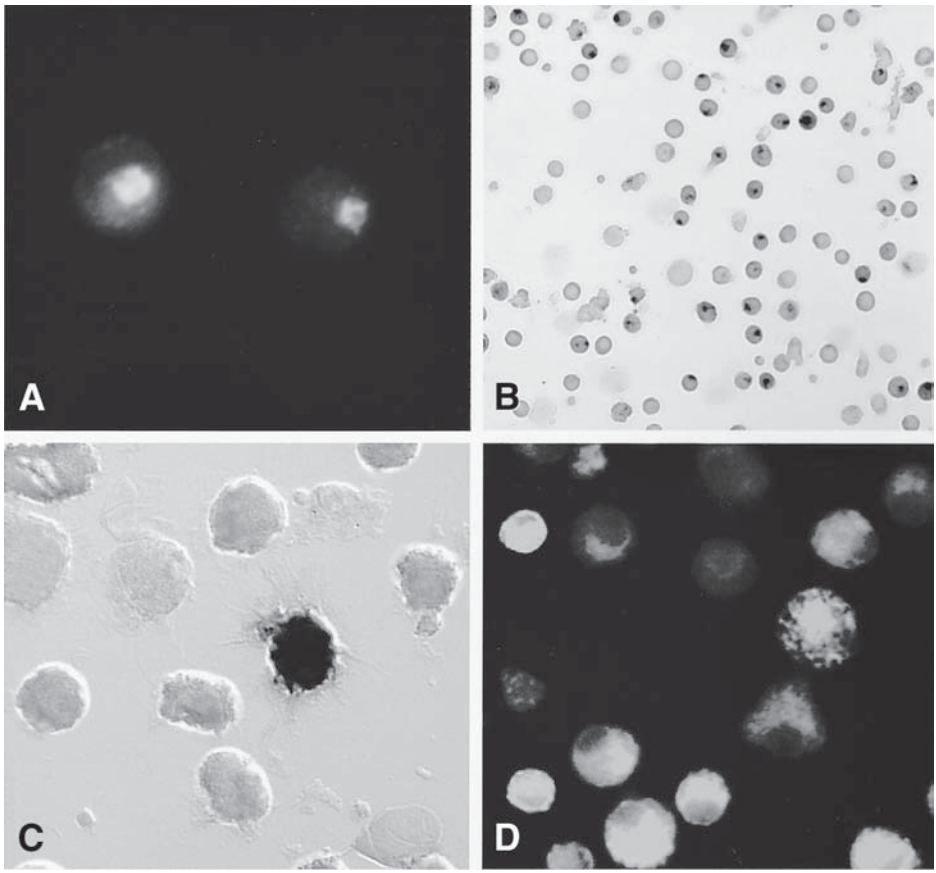

Fig. 1. Characteristics of intracellular staining of cytokine/chemokine expression. The dot-like feature of cytokines localized to the Golgi organelle in the cells is shown in IL-6 expressing in vitro derived dendritic cells **(A)** by immunofluorescence and in low power magnification IL-2 in PMA-ionomycin stimulated PBMCs **(B)** detected by immunohistochemistry. In contrast, dendritic cells **(C)** show a strong cytoplasmic localization of IL-1β after exposure to LPS detected by imunohistochemistry. DAB was used as the peroxidase substrate to generate a brown staining signal. Addition of the protein transport inhibitor brefeldin A to the cell culture leads to the inability to secrete cytokines and chemokines via the Golgi-endoplasmatic reticulum complex **(D)**. The proteins are instead accumulated in the cytoplasm generating the different staining signal shown by IL-8 expression in dendritic cells.

staining. This phenomenon is found particularly for cells producing TNF-α, TNF-β, IFN-γ, and IL-8. The typical feature of IL-2- and IL-6-producing cells is in contrast to a much more isolated Golgi staining with only rare cells expressing additional cytoplasmic and cell surface signals. The staining morphology of the

IL-1 family, IL-1α, IL-1β, IL-18, and late phases of IL-1ra expression, is different from the other cytokines and chemokines ***(5)***. IL-1 proteins lack a signal peptide that directs the proteins to the Golgi–ER complex. The staining appearance of IL-1 is therefore visualized as a strong nuclear and cytoplasmic expression [*see* **Fig. 1(C)**]. However, IL-1ra is secreted through Golgi in the early phase of the synthesis.

6. A more objective way to determine the frequency of cytokine/chemokine-expressing cells is to use computerized *in situ* image analysis. We have developed a special software routine for this application **(*8*)**. Images from the microscope are transferred to a Quantiment 550 IW image analyzer (Leica Cambridge Ltd., Cambridge, UK). The total cells are enumerated by the image analysis system using both the blue color and the morphology of the hematoxylin counterstained cells as a standard. Threshold values are initially defined for the determination of cytokine- and chemokine-producing cells as well as for nonproducing cells. The software program combines cell morphological, densitometric, and staining-specific color values in order to identify threshold values and morphometric criteria used for separating cytokine- and chemokine-expressing cells from nonproducing cells. The frequency of cytokine and chemokine expressing cells is assessed by examination of ≥1000 cells in at least 20 fields.
7. Control of the specificity of the cytokine/chemokine staining should be based on parallel studies of isotype MAb controls, staining with the second-step antibodies alone, and an abolished signal of the immunoreactivity by preabsorption of the specific primary antibody with the relevant recombinant cytokine or chemokine. We recommend a 4°C overnight incubation of the antibody and the corresponding recombinant protein at a 1:10 molar ratio and then addition of this complex to the cells instead of primary antibody alone in the staining to evaluate specificity of the antibody. Cytokine and chemokine transfected cell lines may also be used for assessment of specificity of antibodies.
8. Cells cultured for several days often gain autofluorescence, which changes the scatter properties revealed by flow cytometry and may complicate analysis based on immunofluorescence. We have observed this problem of autofluorescence in in vitro differentiated dendritic cells. The fluorescence intensity of the negative cells can therefore overlap the positive population. This creates a problem for precise quantification. Our experience is that detection of cytokines like IFN-γ, IL-2, and IL-4 in lymphocytes is the most convenient assay with this technique ***(12,34–42)*** (*see* **Fig. 2**).

   The transport inhibitor brefeldin A leads to the inability to secrete proteins from the cell. This results in a general cytosolic accumulation of the cytokine/chemokine proteins and the staining pattern is consequently not restricted to the Golgi organelle (*see* **Fig. 1D**). This enhanced staining signal is readily detected by flow cytometry. However, one has to consider that brefeldin A may activate the cells and cause some cytokine/chemokine expression above the negative controls. Monensin is also a potent activator of macrophages/monocytes that generates IL-1, TNF-α, and IL-8 production ***(13)***.

   In summary, the cytokine/chemokine-detection procedure for PBMCs by flow

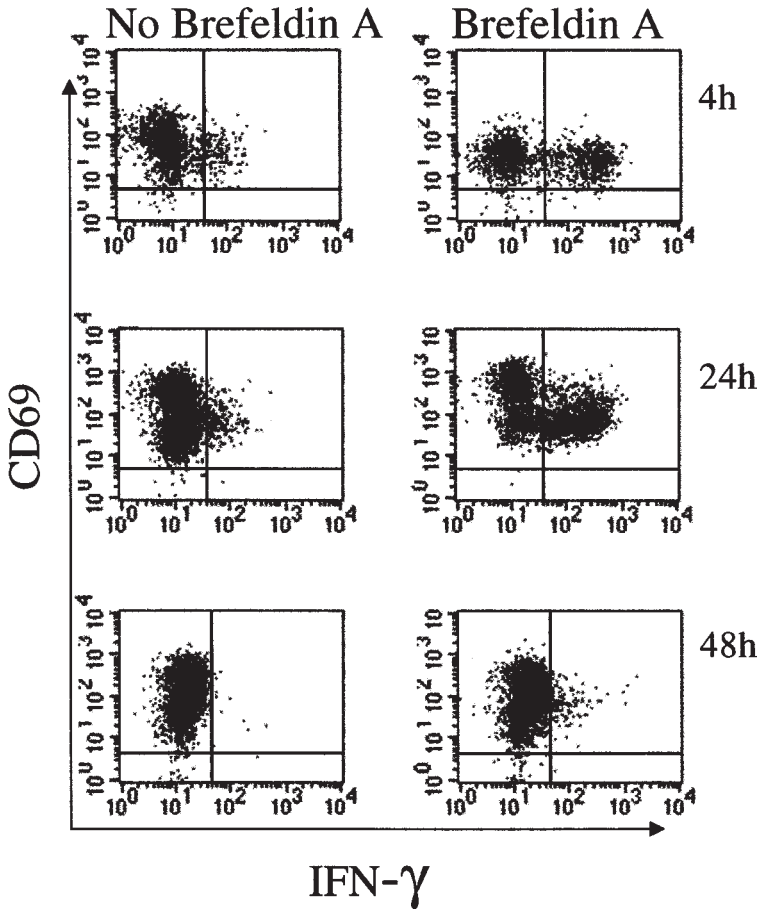

Fig. 2. IFN-γ expression in freshly purified PBMCs after PMA and ionomycin stimulation for different stimulation periods revealed by flow cytometry. Cells were cultured with or without brefeldin A. The cells were stained for IFN-γ, CD69, and CD3 simultaneously. The CD3+ T cells were gated and analyzed. The IFN-γ expression seen in the Golgi-compartments of the cells cultured without brefeldin A only resulted in a low number of positive cells. Addition of brefeldin A significantly enhanced the staining signals for IFN-γ expressing cells and higher frequencies of positive cells were detected. A significantly more distinct positive population of IFN-γ producing cells was found by cultivation with brefeldin A.

cytometry offers a quick efficient handling of high number of cells. Multiple color detection is more easily performed by flow cytometry and facilitates more complete understanding from the experimental analyses (*see* **Fig. 2**).

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18

Detection of Cytokine Signal Transduction "Cross-Talk" in Leukocyte Activation

Howard R. Petty

1. Introduction

Cytokines are a diverse group of proteins and glycoproteins that promote intercellular communication among leukocytes or between leukocyte and other cell types ***(1)***. Although most cytokines are soluble proteins, they can infrequently be found expressed at the cell surface. Cytokines are ligands for one or more cell surface receptors that mediate communication with intracellular signaling, metabolic, and gene regulation pathways. Cells respond to these intracellular signals by alterations in their growth, differentiation, or motility. However, during physiological activities, cytokines are not expressed individually, but rather as a variety of cytokine patterns. Factors that may contribute to cytokine expression patterns are: (a) their ability to be either stimulatory or inhibitory and (b) the presence of both positive and negative feedback pathways at many levels. It is likely that the tools of biocomplexity being developed for understanding the behavior of metabolic and neural networks will be also contribute to our understanding of cytokine networks in vivo (e.g., **ref. *2***).

One impediment to understanding the properties of cytokines in complex in vivo conditions is the staggering number of potential interactions among cytokines. Another fundamental problem is that cell responses to cytokines are often not additive; that is, two cytokines may individually have two different effects on cells, but the combination of the two cytokines has an entirely new third effect on cells. The commercial availability of large quantities of purified cytokines allow certain of their synergistic features to be examined in vitro. One approach to this problem is to look for changes in transmembrane chemical reactions, such as protein phosphorylation, in response to dual cytokine exposure. Another approach is to look at a change in cell function, such as superoxide or NO production, then work backward toward the metabolic and

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signaling apparatuses that drive these changes. In this chapter we discuss methods to detect superoxide, NO, NAD(P)H, and calcium changes in response to single or multiple cytokine exposure, which allow the identification of potential mechanisms of cytokine cross-talk in living cells.

1.1. Overview of Strategy

Cytokine synergy is generally measured as population averages. For example, one might measure the amount of superoxide production, marker release from target cells, or intracellular calcium levels. However, it should be clear that population averages are not representative of the events occurring at the level of single cells. For example, calcium is known to oscillate in cells ***(3)***, yet most published studies report a single calcium concentration. Moreover, the averages are generally performed over long periods of time (seconds to minutes). Oscillations in superoxide production, NO release, marker release from targets, and many other biochemical and physiological variables have been observed (for a review, *see* **ref. *4***). Thus, a great deal of information is lost during conventional spectrophotometric or physiological assays. To retain this information, single-cell microscopic assays must be performed. In addition to retaining spatiotemporal information generally lost when averaging over many cells and/or comparatively long periods of time (seconds to minutes), distinctions can be drawn between motile and resting cells. Furthermore, one can correlate in space and time the substrate (NADPH) and product (superoxide) of biochemical reactions. In this chapter I review the appropriate methods for detecting cytokine cross-talk using microscopic methods.

2. Materials

2.1. Cells

1. RAW264.7 macrophages are grown in RPMI-1640 (cat. no. 11835-030) containing 10% fetal calf serum (FCS) and 1% penicillin G/streptomycin/amphotericin B (PSA) (Invitrogen, Life Technologies, Grand Island, NY). For microscopy experiments, cells are grown for 24 h attached to glass coverslips in a humidified CO_2 incubator at 37°C ***(5)***.
2. Peripheral blood monocytes and neutrophils are obtained using two Ficoll-Hypaque solutions of different bouyant densities (Histopaque 1077 and 1119; Sigma, St. Louis, MO) and centrifugation. Cells are washed twice by centrifugation and then resuspended in HBSS (*see* **Note 1**).

2.2. Reagents

1. Murine and human cytokines can be obtained from R & D Systems (Minneapolis, MN). These should be stored as recommended by the manufacturer.
2. Diaminofluorescein-2 diacetate (DAF-2 DA) can be obtained from Daiichi Kagaku Yakuhin, Inc. (Tokyo, Japan) or Calbiochem (La Jolla, CA).
3. Indo-1 can be obtained from Molecular Probes (Eugene, OR) (*see* **Note 2**).

4. Dihydrotetramethylrosamine (H_2-TMR) can be obtained from Molecular Probes.
5. Cell buffers, such as HBSS, can be obtained commercially (Invitrogen, Life Technologies) (*see* **Note 3**).
6. Gelatin type B is obtained from Sigma. The primary purpose of the gelatin is to form a matrix that restricts of diffusion of fluorescent reporter compounds.

2.3. Microscopic Detection of Signals

Cells are examined using an axiovert fluorescence microscope (Carl Zeiss, New York, NY) with mercury illumination (HBO 100) and a quartz epifluorescence condenser and a quartz objective (*see* **Fig. 1**). Quartz components are used because they transmit light better in the ultraviolet region of the spectrum. A 100-W lamp is recommended because it produces four times the output of the HBO 50 lamp (*see* **Note 4**). Most of the excitation light provided by this lamp is concentrated in several discrete emission wavelengths. The intense lines near 365 nm are used to excite NAD(P)H. NAD(P)H autofluorescence is detected using 365DF20 and 455DF35 filters and a 405 long-pass dichroic mirror (*see* **Note 5**). NAD(P)H autofluorescence is a long-established tool in studying cell metabolic activity ***(6)***. Fluorescence levels are quantitated using a Hamamatsu (Bridgewater, NJ) photon-counting photomultiplier tube (PMT, model R928) held in a Products for Research (Danvers, MA) housing coupled to a D104B photometer (Photon Technology International, Lawrenceville, NJ). PMTs can differ dramatically in their spectral responsivities owing to the composition of the photocathode. Becuase several of the experiments described below study fluorescence near 400 nm, it is important that the PMT selected for these experiments have a reasonable efficiency near 400 nm. Some PMTs designed to operate at longer wavelengths may perform poorly for molecules emitting near 400 nm. The PMT is cooled by a Peltier system interfaced to a chilled water supply. Noise is substantially reduced using the cooled PMT, which is especially important in NAD(P)H experiments. In general, the system should be allowed to cool for at least 30 min prior to experiments. Cells are illuminated individually while held at 37°C by a temperature-controlled stage. Quantitative experiments are performed using a 40× objective to provide depth of field. Background photon count rates are taken from a neighboring area that contained no cells. Data from an integrated amplifier/discriminator system are captured by computer and analyzed using Felix software.

3. Methods

3.1. Gel Matrix Preparation for NO Detection

Hanks' gel matrices are prepared in a fashion similar to that previously described *(7)*. To detect NO release, fluid-phase gelatin is mixed with 15 μ*M*

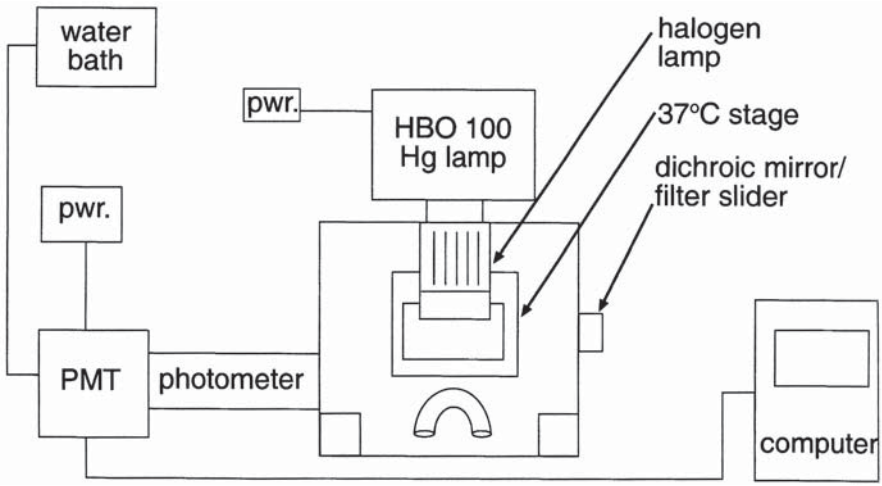

Fig. 1. Schematic arrangement of the instrument components. The microscope is viewed from the top (Pwr. = power supply; PMT = photomultiplier tube).

DAF-2 DA at 45°C and then allowed to cool to 37°C where it is a semisolid. DAF-2 DA has been previously shown to become fluorescent upon exposure to NO, but not other reactive species such as superoxide and hydrogen peroxide ***(8)***. Cells can be directly incorporated into the matrix by allowing the gelatin solution to cool to about 42°C, then gently placing a small volume of the solution on adherent cells. DAF-2 experiments should be performed using a fluorescein filter set.

3.2. Gel Matrix Preparation for Reactive Oxygen Metabolite Detection

To observe the production of reactive oxygen metabolites, 2% gelatin matrices containing 100 ng/mL dihydrotetramethylrosamine (Molecular Probes, Eugene, OR,) or 3 μ*M* hydroethidine (Sigma) are prepared at 45°C, then allowed to cool to 37°C as described above. Hydroethidine is used in conjuction with the indo-1 filter set. Dihydrotetramethyrosamine can be used with a rhodamine filter set. Previous studies have illustrated that these compounds show little or no fluorescence, but become fluorescent on exposure to reactive oxygen metabolites ***(9–12)***.

3.3. Cell Labeling with Indo-1

Cells (10^6/mL) in 1 mL of PBS are loaded with 1–2.5 μ*M* indo-1/acetoxymethyl (AM) for 1 h at room temperature or 30 min at 37°C. This step

is performed in the dark. Cells are washed by dilution into cold PBS then centrifuged at 400*g* for 10 min (*see* **Note 6**).

3.4. Cytokine Exposure

1. Cells are exposed to cytokines for various periods of time. Incubation times generally correspond to those observed in physiological studies. Specifically, interferon-γ requires an incubation time of 1 h with neutrophils or macrophages to discern an effect. However, other cytokines such as interleukin 2 (IL-2) and IL-6 act on interferon-γ-primed cells immediately. Some cytokines, such as IL-8, require an intermediate period of time (approx 30 min). Negative controls should also be included in experimental designs.
2. After cytokine treatment, the region of cells is encircled (approx 2×2 cm) with a small amount of stopcock grease. When a cover slip is gently attached to a microscope slide, the stopcock grease prevents crushing of the cells and evaporation of the medium.

3.5. Detection of Calcium Spikes/Detailed Description of Procedures

1. I recommend that individuals new to quantitative microfluorometry in the near UV begin with indo-1 experiments. This gives the experimenter valuable practice in setting up the instrument and performing such studies. Calcium experiments should definitely be mastered before attempting to perform NAD(P)H experiments.
2. To observe calcium spikes, a slide with adherent cells is scanned (*see* **Note 7**). Polarized cells are selected for detailed analysis.
3. To minimize background levels, the aperture in the back focal plane (between the sample and lamp) is reduced in size until it is just slightly larger than the cell (*see* **Fig. 2**). This is another important step in obtaining high-quality data and should not be overlooked. If necessary, the position (or focus) of the aperture should be adjusted to ensure that its edges are in focus at the same time that the cell is in focus.
4. Many microscopes are equipped with analyzers and sliders for differential interference contrast microscopy. These optical elements must be removed from the light path at this time (*see* **Note 8**).
5. The microscope is then adjusted to send the fluorescence emission to the photometer system. This will vary among microscope manufacturers and the microscope manual should be consulted for details. If the photometer system has an eyepiece, the position of the cell relative to the PMT should be confirmed. The light is then directed toward the PMT.
6. The output of the PMT amplifier/discriminator system is then captured by computer (*see* **Note 9**). The intensity is captured as a function of time. **Figure 3** shows a series of experiments illustrating calcium spikes in cells in response to various cytokine treatments. Using computer software, these data can be evaluated for their amplitude and frequency.

Fig. 2. A polarized cell is shown using DIC microscopy. Left panel shows a typical setup. Right panel shows the same cell after the aperture in the back focal plane has been stopped down (×1000).

3.6. Detection of NAD(P)H Oscillations

NAD(P)H autofluorescence is detected in the same fashion as that of indo-1 (*see* **Subheading 3.5.**), except that the cells are not labeled. If this proves difficult to perform, I recommend practicing with larger and brighter cells, such as tumor cells (*see* **Note 10**). Examples of the sinusoidal NAD(P)H oscillations of cells treated with several cytokine exposures are shown in **Fig. 3**.

3.7. Detection of Superoxide and NO Release

1. The doped gels for NO and superoxide release are prepared as described under **Subheadings 3.1.** and **3.2.**, respectively. These gel matrices provide an extracellular matrix-like environment that restricts the diffusion of the probes. Cells migrating through the gel or on the glass substrate surrounded by the gel can be observed.
2. Using the appropriate filter set, the image of the cell is relayed to the PMT as described under **Subheading 3.5.** Quantitative data are collected as a function of time. Owing to the weak fluorescence of the gel, it is important to reduce the size of the aperture as described in **Subheading 3.5.** ***(3)***. Examples of quantitative intensity traces detecting NO formation are shown on the right-hand side of **Fig. 3**.
3. Qualitative data on superoxide and NO release can be obtained by microphotography of the gels using an intensified charge-coupled device camera ***(13)***. This is because the gel stabilizes the position of the fluorescent reporters for a period of time sufficient to capture photomicrographs.

4. Notes

1. To obtain physiologically relevant and reproducible data, it is important to use a glucose-containing buffer. There are a number of papers published on the calcium oscillator of neutrophils and macrophages. Experiments demonstrating regular calcium spikes generally include glucose in the incubation medium. We have shown that regular NAD(P)H oscillations also require a supply of glucose.

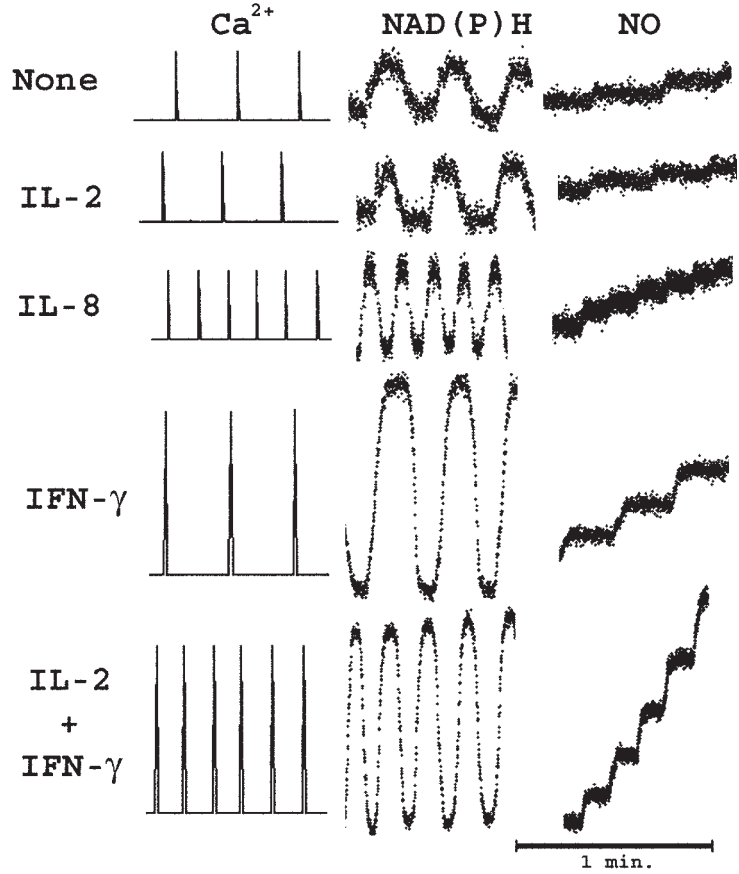

Fig. 3. PMT output data showing oscillations in calcium, NAD(P)H, and NO (columns 1 through 3, respectively) in response to various cytokine treatments. In the absence of cytokine treatment, polarized cells display calcium spikes and sinusoidal NAD(P)H oscillations with a 20 s period. Although IL-2 had no effect on cells, IL-8 doubled the frequency of calcium and NAD(P)H oscillations and substantially increased the rate of NO production. In contrast to the effect of IL-8, IFN-γ dramatically increased the amplitude of calcium and NAD(P)H oscillations and magnitude of NO release. Although IL-2 alone had no effect on cells, IL-2 plus IFN-γ had a dramatic synergistic effect on cells by increasing the amplitude and frequency of intracellular oscillators and dramatically increasing the rate of NO release. The time-scale (bar = 1 min) is shown on the lower right-hand side of the illustration.

2. After opening the bottle or during any type of storage, it is best to cover the probes with a layer of argon gas to prevent exposure to oxygen and potential degradation of the compound.

3. Many cell buffers contain phenol red as a pH indicator. Unfortunately, when experiments are conducted using high sensitivity detectors, phenol red is also fluorescent.
4. Carefully follow the manufacturer's guidelines. Never look directly at the high intensity source; its intensity and UV component can cause blindness. Similarly, always ensure that the filters are in place before looking into the microscope. Carl Zeiss, Inc. now produces an adjustable HBO100 lamp, which is very stable. The ability to adjust its intensity is useful in prolonging the lifetime of the bulb and in preventing photobleaching of the sample.
5. We have recently found that the AF filter series available from Omega Optical (Brattleboro, VT) offers superior performance in these experiments.
6. A common misconception is that if the fluorescence is weak, more indo-1 should be added to the sample. This is quite incorrect because indo-1, and all similar probes as well, are calcium buffers. By increasing the indo-1 concentration, indo-1 buffers the calcium changes it is intended to measure. In all cases, indo-1 should be carefully titered for the cells of interest. In my experience, the manufacturer's recommended concentrations are generally too high to be usefully employed with neutrophils.
7. Calcium spikes should not be confused with illumination spikes that can arise from old bulbs. Old bulbs can produce a jagged array of spikes whereas the calcium spikes are regular with a low background (*see* **Fig. 3**). Bulbs should be exchanged on a regular basis by keeping records of the number of hours of use. After about 200 h of use, one should be prepared to replace the bulb. Bulbs can, and do, occasionally explode after excessive use. Use the manufacturer's recommended bulbs, as other inexpensive bulbs may not match the required specifications.
8. As a general rule, every optical component inserted into the light path reduces the light intensity to one-half of its incoming intensity. Hence, components unnecessary for epifluorescence microscopy must be removed.
9. It is possible for stray room light to enter a microscope. Experiments should be performed in a darkened room. It may be advisable to reduce the intensity of computer monitors and other unnecessary light sources.
10. Inasmuch as NAD(P)H fluorescence is weak, it is essential to minimize other sources of fluorescence. For example, we have found that glass cover slips manufactured by Corning Glass Works absorb and emit light in a fashion similar to NAD(P)H, presumably caused by chromium inclusion in the glass. This problem has not been found in Swiss glass. Therefore, the optimal signal-to-noise ratio is obtained using Swiss glass or quartz slides and cover slips.

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19

Assay System for the Effect of Prostaglandin E_2 in the Determination of Polarized Cytokine Production

Etsushi Kuroda and Uki Yamashita

1. Introduction

The activation of $CD4^+$ T-cell subsets, Th1 and Th2, is one of the most important focuses of immunology. Th1 and Th2 are differentiated from naive T cells after antigenic stimulation by the influence of cytokines [interleukin 12 (IL-12)/interferon-γ (IFN-γ) in Th1 and IL-4/IL-13 in Th2] or costimulatory molecules (CD80/CD86) provided from antigen presenting cells (APC), and have several important immunological functions ***(1)***. Recently, several reports have shown that chemical mediators, such as prostaglandins (PG) and histamine, also affect Th1/Th2 cytokine production and Th differentiation ***(2–7)***. PGE_2 is one of prostanoids produced by action of the enzyme cyclooxygenase (COX) on arachidonic acid. PGE_2 is mainly produced by APC: monocytes, macrophages, and dendritic cells in the immune system ***(2)***. PGE_2 has a suppressive effect on Th1-related cytokine productions such as IL-12 and IFN-γ by T cells, NK cells, and APCs, and an enhancing effect on Th2 development and activation.

PGE_2 affects not only cytokine, but also Th1/Th2-related chemokine productions. The chemokine receptor CXCR3 is highly expressed on Th1, and interferon-inducible protein-10 (IP-10, CXCL10), that binds to CXCR3, induces chemotaxis of Th1, whereas CCR4 is preferentially expressed on Th2, and ligands for CCR4, macrophage-derived chemokine (MDC, CCL22), and thymus and activation-regulated chemokine (TARC, CCL17), promote migration of Th2 ***(8,9)***. Recently, we reported that PGE_2 upregulates MDC (Th2-related), but suppresses IP-10 (Th1-related) production ***(10)***.

Many reports and our observations indicate that PGE_2 affects Th1/Th2 activation at various stages of immune responses via modulation of cytokine or

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chemokine productions. In this chapter, we describe the assay system for determining the effect of PGE_2 on cytokine and chemokine productions by mouse spleen cells in vitro.

2. Materials

1. Mice (C3H/HeN, C57BL/6 or BALB/c), 7–8 wk old.
2. PGE_2 (Cayman Chemicals, Ann Arbor, MI) is dissolved in 100% ethanol. Store at –30°C. Make fresh as required.
3. NS-398 (COX-2 specific inhibitor) and indomethacin (COX-1 and COX-2 inhibitor) (Cayman Chemicals) are dissolved in 100% ethanol. Store at –30°C. Make fresh as required.
4. *Staphylococcus aureus* Cowan I (SAC). PANSORBIN cells (Calbiochem, San Diego, CA) are adjusted to 1% (w/v) in phosphate-buffered saline (PBS). Store at 4°C.
5. Lipopolysaccharide (LPS from *Eschrichia coli* serotype 055:B5, Sigma, St. Louis, MO) is dissolved in PBS.
6. Dibutyryl cAMP (dbcAMP: membrane permeable cAMP analog, Sigma) is suspended in PBS and stored at –30°C.
7. Culture plate (24-well culture plate, Falcon cat. no. 35-3047, BD Bioscience, Franklin Lakes, NJ).
8. Hank's balanced salt solution (Hanks, Gibco, Invitrogen, Carlsbad, CA).
9. Red blood cell lysing buffer: NH_4Cl (6.723 g) and Trizma base (2.0594 g) are dissolved in 1 L of distilled water. The pH of the solution is adjusted to 7.2 by addition of HCl.
10. Culture medium: RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS) and penicillin–streptomycin (Gibco).
11. Antigen for immunization: Ovalbumin (OVA, grade VI) and hemocyanin from Keyhole Limpet (KLH, chromatographically purified) (Sigma).
12. Bovine serum albumin (BSA, fraction V, Sigma).
13. Complete Freund adjuvant (CFA, Difco laboratories, Detroit, MI).
14. IL-12p70 enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA).
15. ELISA antibodies; capture antibodies (anti-mouse IL-4, clone BVD4-1D11, polyclonal anti-mouse/rat IFN-γ, Biosource International), detection antibodies (biotinylated anti-mouse IL-4, clone BVD6-24G2, biotinylated anti-mouse IFN-γ, clone XMG1.2, PharMingen, San Diego, CA).
16. Alkaline phosphatase-labeled streptavidin (Sigma) is dissolved in PBS containing 3% BSA. Adjust the concentration to 1 mg/mL. Store at –30°C.
17. DEM solution: 10 m*M* diethanolamine-HCl, pH 9.5, 0.5 m*M* $MgCl_2$. Store at room temperature.
18. *p*-Nitrophenyl phosphate (PNPP, Sigma). Store at 4°C.
19. Alkaline phosphatase substrate; PNPP is dissolved in DEM solution (1 mg PNPP/1 mL DEM solution). Make fresh as required.
20. IL-4, IFN-γ, MDC, IP-10/CRG-2 (R&D systems, Minneapolis, MN), anti-mouse CD3 antibody (Biosourse International), anti-mouse CD40 antibody (Serotec,

Kidlington, Oxford, UK), anti-mouse MDC antibody and anti-mouse CRG-2 antibody (Genzyme/Techne, Cambridge, MN) are dissolved in PBS and stored at –30°C.
22. ELISA plate (Nunc-Immuno Plate Maxisorp, Nunc, Roskilde, Denmark).
23. Peroxidase-conjugated rabbit anti-goat IgG antibody (MBL, Nagoya, Japan).
24. *o*-Phenylenediamine (OPD) is adjusted to 4% (w/v) in 100% methanol. Stable at –30°C for up to 1 mo.
25. Peroxidase substrate: (0.04% OPD, 0.01% H_2O_2 in 50 m*M* disodium hydrogen phosphate and 150 m*M* citric acid, pH 5.0).

3. Methods

3.1. Analysis of Suppressive Effect of PGE_2 on Cytokine Productions by Mouse Spleen Cells In Vitro

3.1.1. Spleen Cell Stimulation

1. Remove spleen from mouse, make cell suspension, and remove red blood cells by red blood cell lysing buffer.
2. Adjust the cell number to 3×10^6 cells/mL. Seed cells into a 24-well culture plate (*see* **Note 1**).
3. Add agents to the cells and culture them at 37°C for 12 h. The final concentration of the agents should be as follows: PGE_2, 1–100 n*M*; indomethacin or NS-398, 1 µ*M*; dbcAMP, 50 µ*M* (*see* **Note 2**).
4. Stimulate cells with SAC (final concentration 0.005–0.01%) and culture for an additional 24 h (*see* **Note 3**).
5. Collect cell-free culture supernatants by centrifugation at 250*g* for 10 min and use for ELISA.

3.1.2. Cytokine ELISA

1. Coat ELISA plates with 100 µL of diluted capture antibodies (1–2 µg/mL of cytokine antibodies in PBS) at 4°C overnight (*see* **Note 4**).
2. Wash wells with PBS four times and block them with 200 µL of culture medium at 37°C for 30 min.
3. Wash wells with PBS four times and add 100 µL of standard solution (5000, 2500, 1250, 625, 312, 156, and 78 pg/mL of IFN-γ, and 1000, 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL of IL-4) and samples to each well. Keep at 4°C overnight.
4. Wash wells six times with PBS containing 0.05% Tween-20, and add 100 µL of diluted detection antibodies (0.5–2 µg/mL in culture medium) to each well. Incubate at 37°C for 1 h (*see* **Note 5**).
5. Wash wells six times with PBS containing 0.05% Tween-20, and add 100 µL of diluted alkaline phosphatase-labeled streptavidin (1–2 µg/mL in PBS containing 0.05% Tween-20 and 1% BSA) to each well. Incubate at 37°C for 30 min (*see* **Note 5**).
6. Wash wells four times with PBS containing 0.05% Tween-20 and twice with PBS, and add 100 µL of alkaline phosphatase substrate to each well. The substrate in the wells will begin to turn yellow slowly.

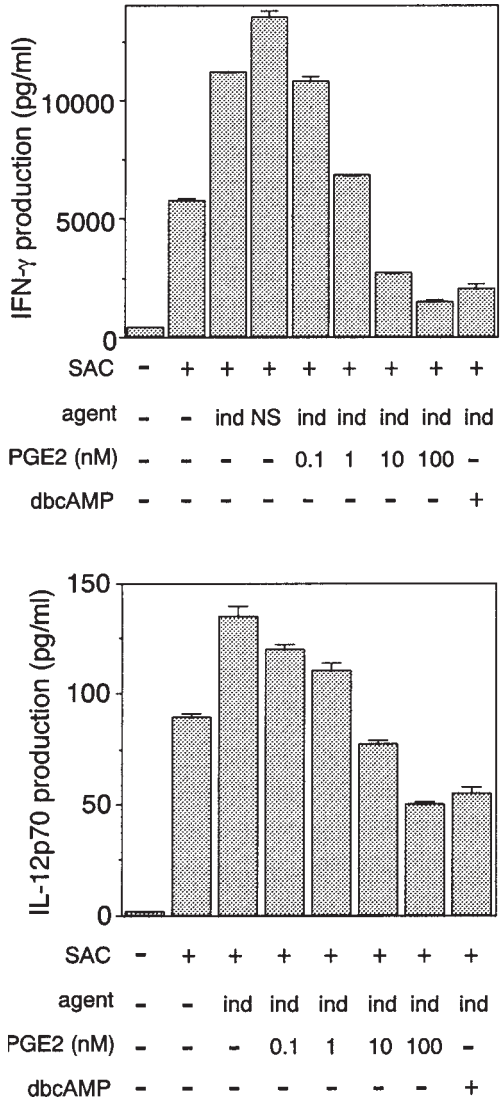

Fig. 1. Effect of PGE_2 or COX inhibitor on IFN-γ production by spleen cells from BALB/c mice.

7. Incubate plates for more than 30 min at 37°C (*see* **Note 6**).
8. Measure the absorbance at 405 nm.

A typical experimental result is shown in **Fig. 1**. PGE_2 suppressed IFN-γ and IL-12 production in a dose-dependent manner. This suppressive effect is

dependent on the intracellular accumulation of cAMP. On the other hand, the addition of NS-398 or indomethacin for the inhibition of prostanoids synthesis increased IFN-γ and IL-12 productions. There is a report that PGE_2 downregulates IL-12 and IL-6, but upregulates IL-10 production by macrophages ***(5)***.

3.2. Analysis of the Effect of PGE_2 on Antigen-Specific Th1/Th2 Cytokine (IFN-γ/IL-4) Productions In Vivo

1. Prepare antigen for immunization (e.g., KLH or OVA).
2. Administer agent (10–1000 pmol of PGE_2 or 0.5–5 nmol of NS-398/mouse) by ip injection into mice before the immunization (*see* **Note 7**).
3. Immunize mice with 50 μg antigen in emulsified CFA by im injection into the thigh (*see* **Note 7**).
4. Administer agent again by ip injection into mice 1 and 2 d after the immunization.
5. Immunize again with 50 μg antigen in emulsified CFA by im injection 7 d after the first immunization.
6. Fourteen days after the first immunization, remove spleen, prepare spleen cell suspension, adjust the cell number to 5×10^6 cells/mL, and seed cells into a 24-well culture plate. Stimulate cells with antigens (final concentration 10–100 μg/mL) at 37°C for 24–72 h (*see* **Note 8**).
7. Collect cell-free culture supernatants by centrifugation at 250*g* for 10 min and use for ELISA.

A typical experimental result is shown in **Fig. 2**. Administration of PGE_2 reduced antigen-specific IFN-γ production by spleen cells, whereas NS-398 treatment enhanced IFN-γ production. Antigen-specific IL-4 production had no effect on mice administered with PGE_2 or NS-398. We observed 1.2–2-fold enhanced antigen-specific IFN-γ production in NS-398-treated mice and 2–5-fold reduced IFN-γ production in PGE_2-treated mice. We do not use indomethacin in this assay. However, there is a report that enhanced Th1 development is observed by the oral administration of indomethacin in *Leishmania major*–infected BALB/c mice ***(11)***.

3.3. Analysis of the Effect of PGE_2 on Th1/Th2-Related Chemokine Productions by Mouse Spleen Cells In Vitro

3.3.1. Sample Preparation

1. Prepare spleen cells, adjust the cell number to 5×10^6 cells/mL, and seed cells into a 24-well culture plate. Culture cells with or without PGE_2 (final 100 n*M*), dbcAMP (final 50 μ*M*), IFN-γ (final 50 ng/mL), or IL-4 (final 10 ng/mL) in the presence of indomethacin (final 1 m*M*) at 37°C for 12 h (*see* **Note 9**).
2. Stimulate cells with LPS (final 10 μg/mL), anti-CD3 antibody (final 1 μg/mL), or anti-CD40 antibody (final 10 μg/mL) at 37°C for an additional 48 h (*see* **Note 10**).

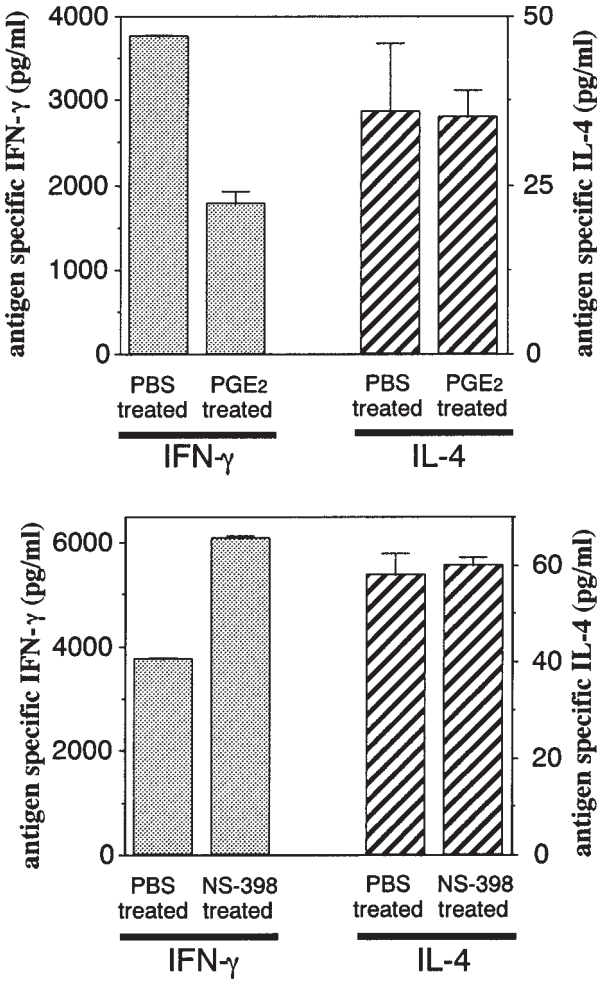

Fig. 2. Effect of PGE_2 or NS-398 on Th1/Th2 development in mice.

3. Collect cell-free culture supernatants after centrifugation at 250*g* for 10 min and use them for competitive ELISA inhibition assay as follows.

3.3.2. Chemokine Competitive ELISA Inhibition Assay

1. Coat ELISA plates with 100 µL of 250 ng/mL MDC in PBS at 4°C overnight (*see* **Note 11**).
2. Wash wells twice with PBS and block them with 300 µL of culture medium at 37°C for 30 min.
3. Mix well standard solutions (40,000, 20,000, 10,000, 5000, 2500, 1250, and 625 pg/mL of MDC in culture medium) or cell-free culture supernatants and an equal

volume of 400 ng/mL anti-MDC antibody in microtubes and incubate at 37°C for 1 h (*see* **Note 12**).
4. Transfer 200 µL of antibody-mixed standard or sample solutions to each well of the MDC-coated plate. Keep at 4°C for 18 h.
5. Wash wells six times with PBS containing 0.05% Tween-20 and fill them with 100 µL of peroxidase-conjugated rabbit anti-goat IgG antibody (diluted 1/2000 in PBS containing 0.05% Tween-20 and 1% BSA). Incubate at 37°C for 1 h (*see* **Note 12**).
6. Wash plates four times with PBS containing 0.05% Tween-20 and twice with PBS, and add ELISA substrate to each well. The substrate in the wells will begin to turn yellow.
7. Incubate at room temperature for 30 min (*see* **Note 13**).
8. Stop the enzyme reaction by the addition of 25 µL of 8 *N* H_2SO_4.
9. Measure the absorbance at 490 nm.

A typical assay data is shown in **Fig. 3A**. The detection limit of this assay is 1000 pg/mL. Assay for IP-10 is also performed by a similar method except for the antibody concentration. The anti-IP-10 antibody is used at a concentration of 100 ng/mL (*see* **Note 12**).

A typical experimental result is shown in **Fig. 3B**. PGE_2 upregulates Th2-related chemokine, MDC, production, but suppresses Th1-related chemokine, IP-10, production by spleen cells.

4. Notes

1. All mouse strains can be used in this assay. However, spleen cells from BALB/c mice are highly sensitive to the suppressive effect of PGE_2 on IFN-γ and IL-12 productions as compared to those from C3H/HeN and C57BL/6 mice *(8)*.
2. Stimulator (SAC) can be added with the agent at the same time. However, the efficiency of the suppression and the increase of cytokine production by PGE_2 are reduced. To obtain the strong effects of PGE_2, cells should be precultured with the agent for about 12 h. The amount of agent is also important. The maximum effects of the suppression of IFN-γ and IL-12 by PGE_2 or dbcAMP are found at 100 n*M* and 50 µ*M*, respectively. A sufficient amount of NS-398 and indomethacin is 1 µ*M*, as a higher amount (>10 µ*M*) is toxic for cells.
3. We also performed the same experiments using the T-cell mitogen concanavalin A and soluble anti-CD3 antibody. However, the indomethacin-induced increase and the PGE_2-induced suppression of IFN-γ production were not significant. We found similar results using Bacillus Calmette-Guéllin (BCG) instead of SAC. LPS stimulation is weaker than SAC and BCG, and low amounts of IFN-γ and no IL-12 production were detected.
4. Incubation at 37°C for 2 h may be allowable. An optimal concentration of capture antibodies should be determined by using preliminary titrations.
5. An optimal concentration of detection antibodies and alkaline phosphatase labeled-streptavidin should be determined by using preliminary titrations.

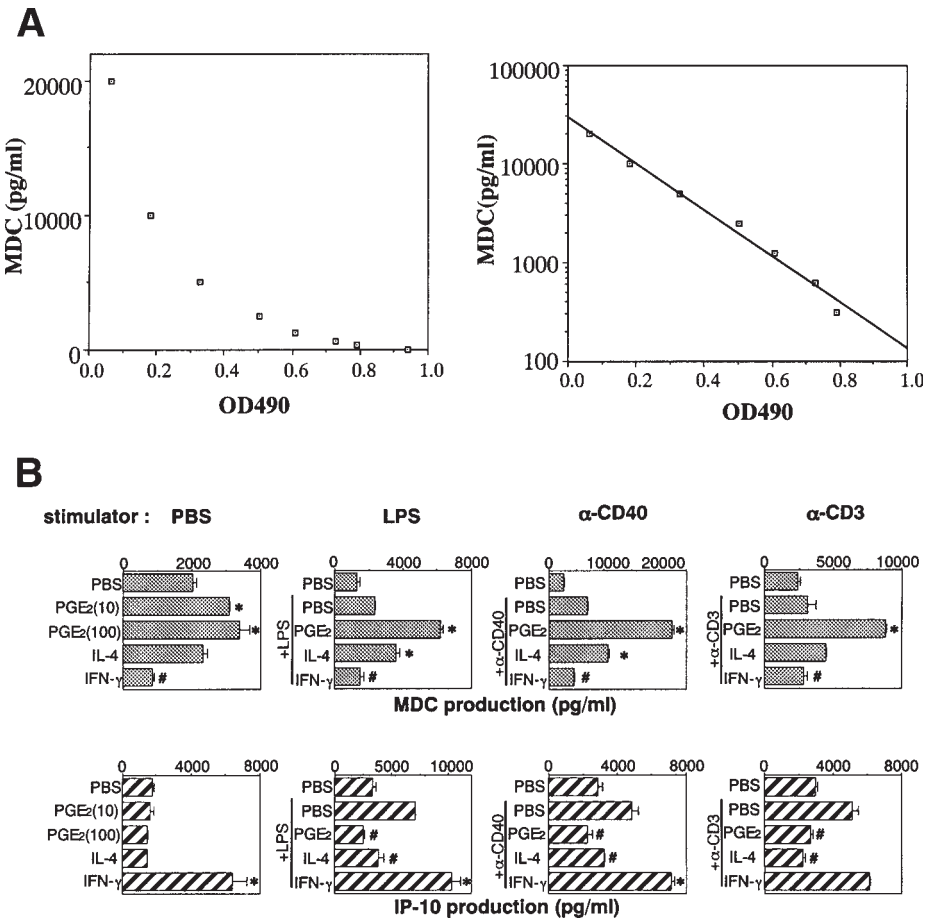

Fig. 3. **(A)** A typical data of MDC competitive ELISA inhibition. **(B)** Effect of PGE_2, IL-4, or IFN-γ on MDC/IP-10 production by spleen cells. *Significantly enhanced; #significantly suppressed. (Data from **ref. *10*** with permission of the American Association of Immunologists, Inc.).

6. It is desirable that the tops of standard wells (5000 pg /mL of IFN-γ or 1000 pg/mL of IL-4) result in an OD_{405} of more than 1.0.
7. Depending on one's purpose, the amounts of agents and antigens should be determined by preliminary experiments. If cells from draining popliteal lymph node are used, antigen should be injected into the footpad of mice.
8. If purified $CD4^+$ T cells are used, mitomycin C-treated spleen cells from nonimmunized mice as APC (5×10^5 cells/mL) should be added to $CD4^+$ T cells (1×10^6 cells/mL). In this system, antigen-specific IFN-γ production by $CD4^-$

cells plus APCs or APCs alone are not observed. The amount of IFN-γ in the supernatant reaches peak values in 48–72 h and decreased gradually after that.

9. Follow instructions in **Note 2**, to obtain the clear effects of cytokines or PGE_2. Cells should be precultured with agent for about 12 h. For the assessment of the effect of PGE_2 in chemokine production, it is desirable to add indomethacin or another COX inhibitor, because endogenously produced PGE_2 after the stimulation also serves as a modulator for chemokine production.
10. Anti-CD40 antibody is the strongest stimulator for MDC and IP-10 production, because both chemokines are mainly produced by APCs (macrophages and dendritic cells) ***(10)***. For CD40 stimulation, a stimulating antibody should be used (e.g., clone 3/23 of Serotec rat anti-mouse CD40).
11. Incubation at 37°C for 2 h may be allowable.
12. Antibody titers are different among each lot of antibodies. An optimal concentration of antibodies should be used as determined by preliminary titrations. If a higher background is observed, samples mixed with anti-chemokine antibody in microtubes are further incubated at 4°C overnight after 37°C incubation, or the enzyme-conjugated antibody is further diluted.
13. If the substrate in the well rapidly turns yellow, the enzyme reaction should be stopped within 30 min. It is desirable that 0 pg/mL of standard wells is at an OD_{490} of about 1.0.

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