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Preparation of antigens

3

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Virtually every diagnostic and research-oriented test in virology begins with an antigen that has been specially prepared for that test. For some tests, the antigen can simply be the supernatant fluid from the cell culture the virus was propagated in, with no regard to the maintenance medium used in culture or the storage conditions for the final product. For others, intact virus is required at the highest titers achievable, so that several freeze–thaw or sonication cycles are applied to the virus culture at complete (4^+) CPE to thoroughly disrupt all remaining cells containing virus; or, a particular viral product needs to be enriched in proportion to other products, but not purified in the biochemical sense. For still others, only a specific viral protein can be used in the test, so that it has to be purified, concentrated, and preserved while all other interfering viral products and medium components are removed. The procedures described in this chapter will accomplish all of these tasks.

The most general concept to begin with is also the most important – viz, the microbiological purity of the starting viral culture. Wherever possible, laboratories should be equipped with recirculating laminar-flow safety cabinets; all operations likely to produce aerosols of infectious materials should be carried out in the cabinet. Minimum requirements for protective clothing include a lab coat or gown and, for more hazardous operations, gloves and masks. During operations involving infectious materials, the workbench would be covered with disposable plastic-lined table soakers to prevent contamination. All work areas should be decontaminated with 0.5% hypochlorite solution. All discarded materials must be placed in discard pans and sterilised by standard procedures. One product should be worked with at a time in a very clean environment. All virus products should be certified to be free of viable bacterial, fungal, and mycoplasmal contamination by appropriate culturing in thioglycollate broth, trypticase soy broth, 5% sheep blood agar pour/streak plates, Sabouraud dextrose slants for yeasts, and mycoplasma biphasic medium. While these concepts may seem basic, many a lengthy piece of work has been erroneously interpreted or had to be discarded because the starting culture was contaminated and the test results were obfuscated by the contaminant.

In most cases, infected cell antigens are derived from cells previously infected at a high moi, i.e. 2–10 pfu $cell^{-1}$. For every virus antigen there should be a parallel 'normal antigen' to serve as the negative control in the test. Normal antigens are prepared by sham-inoculating cell cultures with negative culture material (without virus), and following these cultures through the entire virus culture and antigen preparation steps in exact parallel fashion.

Depending on the test and the day-to-day usage, many antigens can be preserved by adding thimerosal to the final product to a

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final concentration of 1:10,000, or sodium azide to a final concentraton of 0.1%.

Conditions for storing virus antigens must be monitored carefully. For some 4°C is adequate, others require storage at −20°C or −70°C. The potency of antigen batches should be regularly tested following thawing, usually by means of a serological test.

Special preparation of antigens is required for many diagnostic tests, for monoclonal or polyclonal antibody production, for many EIA and LA tests, etc. Many viruses and antigens have their own peculiarities based on the specific properties of each virus group; these can be found elsewhere in this book and in other reference works (Fields et al 1990; Hierholzer 1993; Hierholzer et al 1990; Lennette et al 1988; Schmidt and Emmons 1989).

Particular subsets of antigens can be prepared in similar ways to those outlined below; in some cases with previous treatment of infected cells with inhibitory drugs, e.g. inhibitors of DNA synthesis. This allows early and late antigens to be selected. Time of cell harvest also allows for a predominant antigen type to be selected. Non-glycosylated virus proteins can be prepared from cells treated with glycosylation inhibitors (e.g. tunicamycin and monensin). For the preparation of structural antigens the starting material may be purified virus, prepared as outlined in chapter 4.

Whole-cell antigen preparations

Total extracts of infected cells have a wide range of uses in virology. The simplest and most often used techniques for liberating virus from the host cells, for dislodging the host cells from the cell culture surface, and sometimes for disrupting virions to liberate particular antigens, are outlined below.

Freeze–thawing

1. At the appropriate time during the culture period, usually at 4+ CPE, lay the flasks or tubes in a −70°C or −80°C freezer such that the maintenance medium is covering all the cells.
2. Alternatively, wash the cell monolayer with phosphate buffered saline to remove maintenance medium and replenish with a similar volume of buffer before freezing.
3. Allow to freeze solid (the time will depend on the temperature and the volume).
4. Thaw under warm (but not hot) water or at room temperature. When enough ice has thawed to form a slush, use the slush to further dislodge the cells by striking the flask several times against the palm of the hand.
5. Repeat the freeze–thaw cycle at least twice more. For labile viruses, stop at two freeze–thaw cycles.
6. Clarify the final material by low-speed centrifugation (e.g. 1000 *g*, 30 min, 4°C). The supernatant is the antigen product, and can be further treated by any of the following procedures if necessary.

Sonication

1. At the appropriate time during culture, usually at 4+ CPE, the cultures may be either freeze–thawed as above but not clarified by centrifugation, or the cells scraped off the glass or plastic surface with a rubber policeman.
2. For some viruses (e.g. herpes viruses) the majority of antigens may remain intracellularly bound and a centrifugation step is necessary in order to remove infected cells from the supernatant. Low speed centrifugation is used to pellet the cells which are washed in PBS before resuspending in an appropriate volume of buffer.

   The cells/medium/virus mixture is then sonicated at sufficient energy to disrupt all the cells and release the virus, or with additional energy to disrupt the virions to release particular antigens. The energy level must be determined by pilot trials for the test procedure in use. Usually, 30–70 watts for 1–3 min with multiple pulses per minute will break the cells. For labile viruses such as CMV and RSV, a 60-second sonication at 60 watts output is sufficient to produce an enhanced antigen. **Note**: The manufacturer's instructions for the sonicator must be followed carefully, and all sonication steps must be done in an ice bath and in vessels which will not shatter. It is absolutely essential that the antigen does not become heated.

   For many viruses, sufficient energy can be created from the small

ultrasonic baths that are used to clean jewelry. These are usually cheap and easily obtainable. For all procedures, aerosols must be avoided.

3. Sonicated material is usually clarified by low speed centrifugation (as above). For some purposes a batch of 'soluble' antigen may be necessary and therefore virions will need to be removed. In this case the sonicated material is ultracentrifugated and the pellet discarded, the supernatant being a preparation of 'soluble' antigen which is particularly useful for immunodiffusion purposes.
   Solubilization of infected cell antigen can also be achieved by chemical treatment. This procedure is particularly useful for the preparation of antigens for PAGE/Western blotting techniques. Washed pelleted cells are solubilized by resuspending in RIPA buffer (1% NP-40, 0.1% SDS, 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4) then protein solubilizing buffer (0.125 M Tris-HCl pH 6.8; 4% (w/v) glycerol and, if required, a drop of bromophenol blue).

Alkaline glycine treatment, and others

High-titered intracellular antigens for CF, IHA, and RIA tests, such as for CMV, can be prepared by alkaline glycine extraction. In this procedure, the infected cell monolayer is scraped and the cells packed by centrifugation; then the cell pellet is mixed with 0.1 M glycine-buffered saline, pH 9.5, for 6 h at 37°C, followed by clarification at 600 *g* for 20 min (Cremer et al 1975; Schmidt and Emmons 1989). Alternatively, the infected cell monolayer is rinsed with 0.01 M PBS (1.096 g Na_2HPO_4 + 0.315 g $NaH_2PO_4.H_2O$ + 8.5 g NaCl + 1000 ml distilled water; pH 7.2; filter sterilized) before 4+ CPE, and is overlayered with a small amount of 0.05 M glycine-buffered saline (3.75 g glycine + 0.35 g NaOH + 8.5 g NaCl + 1000 ml distilled water; pH 9.0; 0.22 μ filter-sterilized). The cells are scraped thoroughly into this buffer, and the slurry is sonicated (70 watts, 1 min, 0°C), gamma-irradiated (0.5 MRads, 0°C), stabilized with 10% sorbitol (final concentration), preserved with 0.01% thimerosal, packaged, and tested (Chappell et al 1984).

Another simple antigen preparation, usually made for EIA tests, involves washing an infected cell monolayer with cold PBS, treating with 0.25% trypsin or trypsin/versene (see chapter 1), further washing of the dislodged cells with cold PBS to remove the trypsin, suspension of the cells in a buffer suitable for the test, sonication in an ice bath, and finally centrifugation at 1500 *g* for 15 min at 4°C to remove the cellular debris. This procedure has worked well for EIA antigens for CMV and VZV and for HA antigens for certain arboviruses (Ardoin et al 1969; Schmidt and Emmons 1989). IFA antigens can also be prepared with trypsin, utilizing the washed, suspended cells as the test antigen.

Fractionation of antigens by various extraction procedures (sub-unit preparations)

Tween-80/ether extraction

1. Obtain a virus culture as above, at 4+ CPE or maximum hemadsorption in companion flasks. Treat the cultures by freeze–thaw cycles, sonication, or scraping the cells from the flasks, and pool.
2. Clarify by centrifugation (1000 *g*, 30 min, 4°C).
3. Add Tween-80 slowly to 0.125% final concentration to disrupt the virus.
4. Add an equal volume of diethyl ether (reagent grade) to extract lipids, with constant mixing on a magnetic stirrer. Mix for about 1 h at ambient temperature or in the cold, depending on the virus. **Note:** Good ventilation and the absence of fire are mandatory!
5. Centrifuge to form a distinct ether layer (on top), which is aspirated away. Residual ether in the lower, aqueous layer is removed by slowly bubbling filtered nitrogen gas through the layer until no odor of ether can be detected.
6. The product may need to be clarified again by light centrifugation. This procedure makes excellent HA antigens for rubella, influenza, parainfluenza, mumps, and measles viruses, and CF antigens for influenza and parainfluenza viruses (Chappell et al 1984; Norrby 1962; Schmidt and Emmons 1989).

β-Propiolactone (BPL) extraction

1. Clarify culture by centrifugation (1000 *g*, 30 min, 4°C).
2. Add 1 M Tris in PBS [12.11 g Tris(hydroxymethyl)amino-methane + 100 ml PBS, pH 7.2 (see page 50); 0.22 μ filter-sterilized] to the virus supernatant in a 1:10 ratio (i.e., 1 ml Tris per 9 ml supernatant).
3. Add cold reagent-grade BPL, dropwise and with constant mixing at room temperature, to a final concentration ranging from 0.05% to 0.3%, depending on the virus and the antigen test system. Final concentration must be determined in pilot trials, but 0.3% is suitable to inactivate and extract CF antigens for herpesvirus 1 and 2, rotavirus, RSV, rabies, parainfluenza, measles, and echoviruses, polioviruses, and some other enteroviruses; 0.2% is sufficient for coronavirus CF antigens; 0.1% treats HA antigens of NDV and CF antigens of mumps virus, Coxsackie A

and B viruses, and some other enteroviruses; and 0.05% inactivates and extracts HA antigens of influenza, mumps, and parainfluenza viruses (Chappell et al 1984; Schmidt and Emmons 1989; Sever et al 1964). **Note:** Use gloves, and avoid eye and skin contact with BPL.

4. Let stand 4–18 h at 4°C, or place in a 37°C water bath for 2 h, with occasional mixing. Time and temperature must be determined in pilot trials.
5. Adjust to pH 7.3–7.4 as necessary.
6. Clarify by light centrifugation as above, and add preservative if desired. Thimerosal at 0.01% is generally preferred for CF antigens, while sodium azide at 0.1% usually has least interference in HA tests, but there is no solid rule for this. Test for potency.
7. The BPL extraction of CF and HA antigens from many Togaviridae (EEE, Ross River, Sindbis, VEE, WEE, and other formerly Group A arboviruses), Rhabdoviridae (VSV), Reoviridae (Colorado tick fever), Flaviviridae (Powassan), Bunyaviridae (California encephalitis virus, Trivitattus), and some other arboviruses, is done slightly differently. Included here, because of the need for the borate-saline buffer, is the CF antigen for the Arenavirus LCM and the HA antigen for the Poxvirus vaccinia. For these antigens, prepare 10% mouse brain suspensions or infected cell cultures in borate-saline [100 ml of 0.5 M H_3BO_3 + 80 ml of 1.5 M NaCl + 24 ml of 1.0 N NaOH, QS to 1000 ml with distilled water; pH 9.0; 0.22 μ filter-sterilized]. Homogenize the mixture for 1–3 min in a tightly-capped container in an ice bath, and allow to sit for 30 min. Then, add 1 M Tris in borate-saline [12.11 g Tris + 100 ml borate-saline, pH 9.0; 0.22 μ filtered] to 1/10 the volume of the original suspension. Slowly add cold BPL to achieve the desired final concentration, usually 0.3%, in the cold and with constant mixing. Hold the suspension at 4°C for 18–24 h, clarify by centrifugation at 1000–9000 *g* for 1 h at 4°C, and save the supernatant. If desired, wash the pellet with additional borate-saline one or more times and combine the supernatants. Thimerosal may be added to a final concentration of 0.01% for a preservative. Aliquot and store at −70°C or colder; then safety-test the antigen for complete loss of infectivity in appropriate host systems, and test the antigen for homologous CF and HA titers. **Note:** Hemagglutination by Togaviruses and Flaviviruses is often pH-dependent (e.g. Powassan at pH 6.1), so the HA antigen and the HA test may have to be prepared in buffers with the required pH.

   For Tween-80/ether and sucrose/acetone/BPL extractions of certain arbovirus HA and CF antigens, see Chappell et al (1984) and Schmidt and Emmons (1989).

Nonionic detergent extraction

A rapid means of separating, for example, virus envelope proteins from capsid and surrounding tegument is to treat with detergent. Such a treatment will also serve for the preparation of plasma membranes from infected cells. Nonidet P-40 (NP-40) is usually the non-ionic detergent of choice and may be used with or without sodium deoxycholate. The infected cell pellet or purified virus is resuspended in NP-40 at a final concentration of 1%, sodium deoxycholate being the same concentration. The suspension is left at 4°C for 20 min and a further 20 min at 25°C before centrifuging at 38,000 rpm in an ultracentrifuge. Depending

on the size of virus and other characteristics, the detergent treated suspension may be centrifuged in an 'Eppendorf' microfuge. The supernatant is retained as the detergent extract.

Excreted protein fraction

Many viruses selectively release specific proteins into the extracellular medium and this may be a useful means of making preparations of antigens of restricted specificity (Randall et al, 1980). Extracellular medium from infected cell monolayers is centrifuged to remove debris – if possible at 16,000 rpm for 1.5 h. The supernatant is concentrated, initially by polyethylene glycol dialysis (see chapter 4), followed by either vacuum dialysis or ultrafiltration (e.g. Amicon concentration). The concentrated suspension is ultracentrifuged to remove virion particles and dialyzed against either PBS or 0.05 M Tris/HCl buffer pH 7.0, resulting in an infected cell released protein fraction (Randall et al, 1980).

High-salt extraction

A high-salt extract of antigens from infected cells often forms the starting point for a series of further fractionation steps. An example is the purification of virus-specific DNA binding proteins which, following high salt extraction, are exposed to DEAE cellulose, phosphocellulose or DNA cellulose chromatography (Alberts et al 1968; Purifoy and Powell 1976; Powell and Purifoy 1977).

Washed and pelletted infected cells are resuspended in buffer containing 20 mM Tris HCl pH 7.5, 2 mM β-mercaptoethanol, and bovine serum albumin (4000 $\mu g\ ml^{-1}$). Following ultrasonic cellular disruption, the cells are suspended in an equal volume of high-salt buffer containing 5 mM EDTA and 1.7 M KCl and incubated at 0°C for 60′. Insoluble protein and free DNA are removed by centrifugaton at 16,000 rpm for 1 h and the supernatant dialyzed against several changes of low-salt buffer containing 50 mM KCl, 20 mM Tris HCl pH 7.5, 1 mM EDTA, 2 mM β-mercaptoethanol, and 10% glycerol. The dialysate is centrifuged at 20,000 rpm for 32 h to yield a high-salt extract.

Partial purification by adsorption/elution techniques

Adsorption and elution from erythrocytes

This simple technique is based on the hemagglutinability of certain viruses. Viruses which agglutinate red blood cells can be partially purified by adsorbing them to the appropriate erythrocytes at low temperatures, thoroughly washing the cells to remove all non-virion and non-HA antigen components, and then eluting the virus into a simple buffer at higher temperatures. The method has been used effectively for partial purification of intact, infectious influenza, parainfluenza, mumps, measles, and coronaviruses, for removing hemagglutinins from CF antigens, and for obtaining reagents enriched in hemagglutinins (Chappell et al 1984; Hierholzer and Dowdle 1970; Hierholzer et al 1972; Schmidt and Emmons 1989). Because of the number of washes the cells must undergo, the success of the procedure depends on being able to use one of the 'tougher' species of erythrocytes, such as human, monkey, or chicken, rather than rat, mouse, or guinea pig red blood cells which tend to disintegrate from the physical trauma incurred by the procedure.

1. Begin with a large-volume harvest of virus from cell culture, 20% suckling mouse brain suspension in PBS, amniotic or allantoic fluids from embryonated eggs, or any other system. Multiple freeze–thaw cycles may be indicated to release maximum virus from the cells, if the virus is stable under this treatment. Clarify the harvest at abut 5000 *g* for 20–40 min at 4°C to remove gross cellular debris. At this point, the supernatant will contain maximum amounts of whole virus and soluble antigens (if produced).
2. If a hemagglutinin preparation free of infectious virus is desired, treat the supernatant with SLS in distilled water to a final concentration of up to 5% for 1 h at ambient temperature, or with some other ionic or nonionic detergent, followed by dialysis against PBS to remove the detergent.
3. Select erythrocyte species that gives high HA titers with virus being purified, choosing a more durable cell if possible. Obtain sufficient volume of red blood cells to carry out the procedure; the blood must be obtained in Alsever's solution [20.5 g glucose + 8.0 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) + 0.55 g citric acid ($C_6H_8O_7.H_2O$) + 4.2 g NaCl + 1000 ml distilled water; final pH 6.1; filter-sterilized] as gently as possible, and used fresh to minimize trauma to the cells.
4. Gently wash the cells three times with a large excess of cold PBS, being sure to aspirate off the leukocytes which layer on top of the erythrocytes. Use a suitably large centrifuge bottle so that the reaction to follow can be carried out in the bottle. Do all wash steps at 4°C.
5. Placing the bottle with packed, washed cells in an ice bath, slowly add ice-cold virus supernatant (from

steps 1 or 2) to the cells at the final ratio of 60 ml of virus to 40 ml of packed erythrocytes. [**Note:** For influenza and mumps viruses grown in embryonated eggs, 95 ml of cold fluid harvest per 10 ml of packed chicken red cells is generally sufficient.] Mix gently and frequently over a 2 h adsorption period.

6. Pack the red cells by centrifugation at 1000 *g* for 15 min at 0°C. If removing hemagglutinins from a CF, EIA, or other antigen preparation, *save* the supernatant and stop here. If preparing an HA antigen, *discard* the supernatant and proceed as follows.
7. Wash the cells three times under the same centrifugation conditions, always discarding the supernatant. It is critical to re-suspend the cells as gently as possible at each wash step, preferably using a wooden applicator stick.
8. Elute the virus or hemagglutinins from the red cells by two successive incubations, each with 10 ml of PBS for 40–60 min at 38°C, with frequent gentle agitation, followed by centrifugation as above but at ambient temperature. Collect the supernatant, now containing the virus or hemagglutinins.
9. Pool the two eluates and test for virus or antigen titer, as appropriate. Thimerosal may be added to 0.01% final concentration as a preservative. Assuming a starting volume of 60 ml of virus, this yields a 3× concentration of virus that is free of all cell and medium components and all non-intact virus, non-HA antigen viral components. Some erythrocyte membranes and proteins, however, may be picked up in the elutions by disintegration of some red blood cells.

Adsorption and elution from calcium phosphate gel

The freshly-prepared brushite form of calcium phosphate ($CaHPO_4.2H_2O$) is one of the oldest but still most useful ways to purify viruses, proteins, and antigens (Taverne et al 1958). In hydroxyapatite form [$Ca_{10}(PO_4)_6(OH)_2$] calcium phosphate is used to separate the various forms of DNA and RNA, nucleohistones, and polynucleotides (Alberts et al 1989; Bernardi 1971). The brushite procedure, with little modification, has been used to partially purify many viruses and antigens, including adenovirus capsid components, influenza virus and hemagglutinins; Group A arboviruses belonging to Togaviridae and Group B arboviruses belonging to Flaviviridae, along with their CF and HA antigens; vaccinia virus; coronaviruses; and enteroviruses (Dowdle et al 1971; Hierholzer 1976; Hierholzer and Dowdle 1970; Hierholzer et al 1972; Simon 1962; Smith and Holt 1961; Taverne et al 1958). The method works best for virus cultures grown under maintenance medium without calf serum or other added proteins; and the final culture must be clarified by low-speed centrifugation and dialyzed thoroughly against 0.001 M phosphate buffer, pH 7.3, for 24 h at 4°C.

1. Prepare the brushite form of $CaHPO_4$ by adding equal volumes of 0.5 M $CaCl_2.2H_2O$ and 0.5 M $Na_2HPO_4.2H_2O$, both in distilled water, dropwise into a beaker, with constant mixing at ambient temperature, at a drop rate of about 60 drops min^{-1} for each salt. The precipitate $CaHPO_4.2H_2O$ + 2NaCl is formed.
2. Allow the precipitate to settle for about 1 h, decant or aspirate off the supernatant, and wash the precipitate six times in distilled water by

low-speed centrifugation, such as 700 *g* for 5–10 min. The final wash may be tested for chloride content, but this is usually not necessary.

3. Resuspend the final packed gel in 0.001 M phosphate buffer, pH 7.3, at which point the gel may be stored in the refrigerator for up to two weeks. Degas the gel under vacuum before using for column chromatography.
4. To continue with column chromatography, fill a chromatographic column with 0.001 M phosphate buffer, begin dripping out the buffer, and pour in the gel slurry (slowly to avoid bubbles and channels) until the column has the desired height of gel. The column should have a length : diameter ratio of 5:1 to 10:1, and must have a series of mesh filters at the bottom of the column to prevent blockage by the fine gel particles. The top of the gel bed should be covered with a nylon or filter paper 'float' to effect even loading of the surface. The virus sample can then be loaded, the column rinsed with one void volume of distilled water, and the desired components eluted by a gradient of increasing molarities of phosphate buffer at pH 7.3.
5. To continue with batch chromatography, which is preferable for $CaHPO_4$ work, transfer the gel slurry to a 50 ml conical centrifuge tube to obtain about 15 ml of packed gel. Centrifuge and discard the supernatant buffer. Add about 30 ml of clarified virus supernatant that has already been dialyzed against the 0.001 M phosphate buffer.
6. Gently but constantly mix the gel (now at about 33% v/v) with the virus for 2 h at ambient temperature. (This step may be done in an ice bath to protect a labile virus. Likewise, mixing may be accomplished by periodically inverting the tube rather than using a magnetic mixer, to prevent physical destruction of the virus.)
7. Centrifuge the mixture at 4°C; discard the supernatant.
8. Wash the gel once with distilled water and once with 0.001 M buffer to remove nonadsorbed components.
9. Elute with 0.005, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, and 0.5 M phosphate buffers, all at pH 7.3, in stepwise fashion to release different proteins and whole virus from the gel. (Whole virus usually elutes between 0.2 and 0.4 M.) Each elution step is done with 10–40 ml of buffer, depending on whether any concentration is desired, by incubating the buffer–gel mixture for 30–60 min in a 30–37°C water bath (as dictated by experience) with occasional mixing, and then packing the gel by centrifugation as above. All supernatants should be tested for the desired components or whole virus, as appropriate, before discarding them.

Partial purification/ purification by chromatographic procedures

Chromatography is considered by many to be the most effective means of producing purified preparations of virus proteins, this being achieved by gel filtration, ion exchange or affinity chromatography. Antigens are separated by the process of selective retention, when a 'mobile' phase (liquid) moves through a liquid or solid 'stationary' phase, the retention being based on the size and chemical or immunological attributes of the individual antigens. Gel filtration separates molecules according to their size and the choice of pore size selected in the stationery gel. Small molecules enter the pores and are retained, whereas larger molecules elute through the gel very quickly thus giving rise to a differential elution of antigens, collected in small volume fractions from the gel column. Ion exchange chromatography utilizes the intrinsic ionic charge of a molecule as the differentiating property. In this case the stationary phase of a column contains groups of chemicals with various anionic or cationic charges, these retaining ions from the mobile phase of virus antigens as they pass through the column. Ions that react strongly with the exchange are retained and eluted much later than those with weaker attachment kinetics. Elution is achieved by slowly increasing, with time, the salt concentration of the eluting buffer. More recently this technique has been refined by the introduction of high-performance liquid chromatography (Chicz and Regnier 1990).

For the purpose of antigen separation in this chapter we have chosen to describe, in detail, the method of affinity chromatography, using a monoclonal antibody as the ligand which is immobilized onto the stationary matrix.

Affinity chromatography

The reader is referred to Harlow and Lane (1988) or to Wilchek et al (1984) for excellent review manuals.

The production of columns or slurries which consist of a monoclonal antibody attached to a solid matrix allows the purification of single proteins from mixtures of antigens which are passed through the column, the selected protein being specifically retained by the antibody for later elution. The procedure is more efficient if the starting mixture of viral antigens is in a 'semi-pure' state. Purification of single virus proteins of 1000–10,000 fold are common practice with such procedures. The efficiency of the procedure depends on three criteria: (a) the purity of the starting antigen, (b) the affinity of the antibody for the antigen, and (c) the relative ease with which the bond can be split and the protein eluted.

Preparation of the antibody–matrix

This first step in the procedure requires the attachment of antibody to the solid matrix. A number of matrices exist, e.g. agarose beads, cross-linked agarose beads, polyacrylamide beads, copolymer of polyacrylamide and agarose, and

polyacrylic beads. The authors commonly use Sepharose (Pharmacia). There are also a number of procedures for achieving attachment of antibody. Essentially, however, there are three methods of coupling antibodies: (a) directly to Protein A beads; (b) to chemically activated beads; or (c) by activating the antibody before coupling. Matrices modified so as to contain secondary reagents are the most commonly used.

Preparation of a Protein A Bead–Antibody column

The method described is suitable for binding mouse monoclonal antibodies of sub class IgG2a, IgG2b and IgG3.

1. 2 mg purified antibodies/ml of wet beads are mixed in a slurry by incubating at room temperature for 60 min with gentle rocking.
2. The beads are washed twice with 10 volumes of 0.2 M sodium borate (pH 9.0), being centrifuged at 3000 *g* for 5 min or 10,000 *g* for 30 s.
3. Resuspend the beads in 10 volumes of 0.2 M sodium borate buffer (pH 9.0) and add dimethylpimelimidate (solid) to bring to a final concentration of 20 mM.
4. Mix for 30 min at room temperature with shaking.
5. Stop the reaction by washing the beads in 0.2 M ethanolamine (pH 8.0) and incubating for 2 h at room temperature in 0.2 M ethanolamine with gentle mixing.
6. After the final wash, the beads are resuspended in PBS with 0.01% merthiolate.

The beads at this stage are ready for binding to antigen.

Preparation of chemically activated beads

This procedure requires the matrix to be activated using one of a number of chemical reagents prior to interaction with antibody. The chemicals most widely used include cyanogen bromide, carbonyldiimidazole, gluteraldehyde, hydroxysuccinimide, and tosyl chloride. Procedures for activating the beads with either of these are well documented in Harlow and Lane (1988).

Many laboratories, however, purchase pre-activated beads for use in antibody binding from commercial companies. These include Reacti-Gel (Pierce), CNBr-activated Sepharose (Pharmacia), Act-Ultragel ACA 22(IBF), Affigel 10 (Bio Rad), and Activated Microspheres (KPL). The authors have used Affigel 10 (Bio Rad) with success. This comes as an immunoaffinity kit.

Cyanogen-bromide activated beads

This procedure has been selected for description as it is the most commonly used. It is suitable for agarose, cross-linked agarose, and polyacrylic beads. The method is based on that of Harlow and Lane (1988), Axen et al (1967), March et al (1974), and Kohn and Wilchek (1984).

1. Transfer 10 ml of wet beads to a sintered glass filter. Wash with distilled water. Wash with 1 M sodium carbonate buffer (pH 11.0).
2. Add 10 ml of 1 M sodium bicarbonate (pH 11.0) and transfer the beads to a suitable beaker.
3. Move to a fume hood. Weigh out 1 g of cyanogen bromide (CNBr) and dissolve in 1 ml of acetonitrile. Add the CNBr to the beads. Cyanogen bromide is extremely toxic; use only in a fume hood.

4. Incubate at room temperature for 10 min with constant agitation. Monitor the pH. Adjust as necessary to keep between 10.5 and 11.0 by adding 4 N NaOH.
5. Transfer the beads to a sintered glass filter. Use suction to draw the CNBr buffer into a vacuum flask containing 100 mM ferrous sulfate to inactivate the CNBr.
6. Sequentially wash the beads with water, several milliliters of 95% acetone, and several changes of 100 mM sodium phosphate (pH 7.5) (or an alternative binding buffer).

The beads are ready for coupling to antibody.

Coupling of antibody to activated beads
(Harlow and Lane 1988)

1. The antibody preparations must not contain extraneous compounds with amino groups, and, if these compounds have been used during the purification, the antibody preparation should be extensively dialyzed against the binding buffer of 0.5 M sodium phosphate (pH 7.5).
2. Prepare a solution of antibody at the desired concentration in 0.5 M sodium phosphate (pH 7.5). 5–10 mg of antibody per milliliter of beads should yield a high capacity column.
3. Add the activated beads and mix gently overnight at room temperature on a rocker.
4. Wash the beads twice with 0.5 M sodium phosphate (pH 7.5) and once with 1 M NaCl/0.05 M sodium phosphate (pH 7.5).
5. Add 10 volumes of 100 mM ethanolamine (pH 7.5). Incubate at room temperature for 4 h to overnight, with gentle mixing.
6. Wash twice with PBS. Add merthiolate to 0.01%. The beads can be stored at 4°C where they should be stable for several months.

The beads are now ready for binding of the antigen.

Note

A check for binding can be made by testing the protein concentration in a sample taken from step 2 and comparing it to the wash in step 4. Should antibody activity be drastically reduced through coupling, a different approach should be tried (see Harlow and Lane 1988).

Binding of antigens to antibody
(Harlow and Lane 1988)

Antigen can be bound to antibody-coated beads either by mixing in a slurry or by passing the antigen down a column of antibody bound beads.

Binding in suspension

1. Mix the antigen solution with the antibody beads.
2. Rock for 1 h to overnight at 4°C, ensuring that beads are maintained in suspension.
3. Stop agitation, transfer the beads to a suitable column and wash with 20 bed volumes of binding buffer.

The antigen is now ready for elution.

Binding in a column

Antigen samples added to columns should be free of particulate matter and need centrifugation at 100,000 *g* for 30 min prior to use.

1. Transfer antibody beads to a suitable column and wash with 20 bed volumes of PBS.
2. Apply the antigen solution to the column and allow to pass through by

gravity (three times) or with a fixed flow rate of approximately 2 ml h^{-1}.
3. Wash the column with 20 bed volumes of binding buffer.

Elution of antigens from immunoaffinity columns
(Harlow and Lane 1988)

Elution of antigen from columns requires a series of optimal conditions to be determined in order that the process is quick and efficient. Conditions vary from harsh to mild, depending on the antibody used; strategies for testing elution conditions are described in Harlow and Lane (1988). In essence, the procedure is as outlined below.

1. Use a pre-elution buffer and pass 10 bed volumes through the column.
2. Using a stepwise elution, sequentially pass samples of elution buffer (0.5 bed volumes/step) through the column. Collect each fraction in separate tubes. If either high or low pH is used to elute the column, the collection tubes should contain a neutralizing buffer. When using a gradient elution, pass the gradient of increasingly harsher elution buffer through the column.
3. Check each tube for the presence of the antigen. Combine tubes with high concentrations.
4. Return the column to the starting buffer by passing 20 column volumes through the matrix. Add 0.01% merthiolate for long-term storage (4°C).

Note

After the elution of the antigen, the eluant will often need to be changed by dialysis or by chromatography on a desalting column.

The above procedures have been used successfully in many laboratories to produce batches of purified viral antigens. It should be noted, however, that they are generalized procedures and may need specific refinements for the antibody and antigen of choice. Commercially available purification 'kits' come with excellent step-by-step protocols. The reader is also referred to Deutscher (1990) for a guide to purifying antigens.

Preparation of antigens for immunization

Formol saline inactivation

Many antigen preparations, particularly those containing virions, need inactivating before immunization protocols can commence; for most preparations this can be achieved by incubation with 0.015% formaldehyde.

Production of solid matrix antigen/antibody complexes

Binding antigens to antibodies is thought to induce a much better immune response than antigen alone (Harlow and Lane 1988). The immunogenicity can be enhanced further by binding the complex to a solid matrix such as *Staphylococcus aureus* (Randall and Souberbielk 1990). The procedure below describes the preparation of *S. aureus* (Staph A Protein), and attachment to a monoclonal antibody and an antigen resulting in a solid matrix antigen antibody (SMAA)

Preparation of formalin fixed *S. aureus*

1. Grow *S. aureus* Cowan I strain in tryptone soya broth for approximately 8 h. Centrifuge 9000 rpm for 10 min.
2. Resuspend in PBS (pH 6.95) containing 0.1% (w/v) sodium azide (PBS/azide) to give a 10% (w/v) cell suspension.
3. Wash cells twice in PBS/azide and resuspend in 10% (w/v) PBS/azide containing 1.5% (v/v) formalin. Leave stirring overnight at room temperature.
4. Wash cells in PBS/azide to remove formalin and transfer to a large flask such that the depth of liquid is $\leq$ 1–2 cm.
5. Heat at 80°C for 5 min and cool rapidly in ice bath.
6. Wash cells twice in PBS/azide, resuspend in 10% w/v PBS/azide and store in aliquots at −70°C.

Preparation of a SMAA complex

The procedure is essentially that of Randall & Young (1988) and Randall et al (1988).

1. A 10% (w/v) suspension of formalin fixed *S. aureus* is saturated with antibody by mixing with an excess of monoclonal antibody (concentrated tissue culture supernatant or ascitic fluid) at 4°C for 4 h.
2. Centrifuge at 13,000 rpm for 1–1.5 min in an Eppendorf microcentrifuge and wash twice in PBS. Resuspend in PBS to give a 10% (w/v) suspension of *S. aureus*.
3. The antibody-bound *S. aureus* is shaken overnight at 4°C with an excess of solubilized virus antigen prepared by any of the procedures outlined above.
4. The SMAA complex produced is washed three times in ice cold RIPA buffer (20 mM Tris-HCl pH 7.2, 5 mM EDTA, 0.5% (v/v) NP40, 0.1% (w/v) SDS, 0.65 M NaCl and 1 mM PMSF) followed by three washes with PBS. The complex is resuspended at 10% (w/v) or 0.5 (w/v) in PBS. The 0.5% suspension is used for immunization purposes.

Expression of antigens *in vitro*

The advent of advanced techniques in molecular biology and biotechnology has lent itself to the production of more refined systems for both the *in vitro* expression and purification of viral antigens. These may comprise several amino acids or a larger sequence complete with the post-translational modifications of the authentic protein. The nature of the desired end-product determines the system of choice (i.e. prokaryotic or eukaryotic). Whilst prokaryotic systems are convenient, with the availability of inexpensive scale-up reagents, there are distinct disadvantages in their use for protein expression. This is due to differences in processing pathways which lead to the absence of both amino terminal modifications and di-sulphide bond formation, a lack of glycosylation, and an inability to facilitate the proteolytic cleavage of signal sequence from mature polypeptide chains. In addition, because vertebrate viruses replicate in cells of eukaryotic origin, in some instances it is more feasible to produce the relevant antigen in one of the many eukaryotic systems available where any post-translational modifications can be ensured.

Ultimately, the choice of the expression system will be dependent on the role the antigen is to play. Thus it may be required for either diagnostic or therapeutic purposes. Diagnostic tests often involve the detection of anti-viral antibodies in body fluids such as serum, saliva, cerebrospinal fluid or synovial fluid which indicate prior exposure to an agent. The diagnostic tests which are used extensively include the ELISA, Western blotting, radioimmunoprecipitation, indirect immunofluorescence, single radial haemolysis-in-gel, and radio-immunoassay. For each of these systems, a different form of the antigen may be required. For therapeutic purposes, the antigen may be required as a subunit vaccine and extensive purification steps may have to be carried out before the antigen can be of use.

The initial steps in obtaining antigen expression, including the isolation of the gene or a small nucleotide sequence and its cloning into a suitable expression plasmid, are common to most systems. These and further steps are summarized below.

(i) The selection of a suitable expression plasmid;
(ii) The isolation of the whole of the gene of interest or a truncated form of it;
(iii) The cloning of the gene into the expression plasmid;
(iv) the expression and detection of the protein product.

Eukaryotic gene expression

Many references are available which describe the successful production of viral antigens in eukaryotic systems. Several types of virus have been engineered as vectors for this purpose, including adenoviruses, herpesviruses, poxviruses, baculoviruses and adeno-associated viruses. The baculovirus and poxvirus systems are here described in detail.

Baculovirus expression systems

The prototype baculovirus *Autographa california* nuclear polyhedrosis virus (Ac*M*NPV) is commonly used for the production of recombinant baculoviruses which are engineered for high-level protein expression. The production of the recombinant first involves the cloning of the gene into one of the many baculovirus

expression vectors available. The most commonly used vectors employ one of two very late baculovirus gene promoters, although studies have been carried out to stably transfect insect cells with plasmids containing genes under the control of one of the immediate early promoters (Jarvis 1991). The former include the polyhedrin or the p10 promoters. The polyhedrin gene encodes the polyhedrin protein which surrounds newly synthesized virus particles in the nucleus of the cell. The p10 promoter is thought to be involved in the assembly of occlusion bodies. Such promoters are flanked by sequences which allow recombination into the baculovirus genome to occur, and unique restriction endonuclease sites are incorporated downstream of them for the insertion of coding sequences. Further modifications to baculovirus vectors include the insertion of the beta-galactosidase gene which is of use for the screening of recombinant viruses by blue colour selection (Vialard et al 1990) or the addition of a histidine tag (i.e. pBlue Bac His ABC) which can be used for the purification of the expressed protein on nickel columns. Vectors are also commercially available which allow for the insertion of multiple coding sequences (i.e. p2Bac).

Careful consideration must be given when selecting the vector and designing the insert. The vector may be such that the insert is fused to the polyhedrin gene, in which case the translation initiation codon of the novel gene is not required but subsequent amino acids are inserted in-frame (i.e. pAC360). In non-fusion vectors (i.e. pVL1392 and pVL1393) there is a mutation (ATG to ATT) in the initiation codon of the polyhedrin gene, resulting in a requirement for an ATG codon in the inserted gene. The baculovirus transfer vector pVT-bac (Tessier et al 1991) contains the honeybee mellitin signal sequence under the control of the polyhedrin promoter; this construct has been used successfully by Sisk et al (1994) to substitute for the signal sequence of HSV-1 gD, enabling up to 25 mg of protein to be purified from one litre of culture medium.

Procedure

The propagation of baculoviruses and tissue culture maintenance is carried out at 27°C. Baculoviruses are routinely stored at 4°C.

Types of insect cell in which baculoviruses can be propagated

A number of cell lines of insect origin have been used in the propagation of baculoviruses and are commercially available. These include Sf9 cells, Sf21 cells, High 5 cells and MG1 cells. Sf9 cells are derived from *Spodoptera frugiperda* ovarian cells. Sf21 cells are of a similar origin but are larger in size and allow for elevated levels of protein production. High 5 cells are adherent cells and are derived from *Trichoplusia ni* egg cell homogenates. These cells also give 25 fold higher levels of protein expression than Sf9 cells. Cells are routinely cultured at 27°C in commercially available 'Grace's' insect medium which is supplemented with foetal calf serum. Serum-free preparations of insect medium are available for the production of recombinant proteins free from serum contaminants.

Plasmid preparation

1. 1 μg of 100 μg ml^{-1} of the baculovirus expression plasmid is added to 100 μl of competent cells which are left on ice for 30 min.
2. Cells are heat shocked at 42°C for 45 s.
3. 400 μl SOC medium is added.
4. Cells are shaken at 37°C for 1 h.
5. Cells are plated out on LB agar plates containing antibiotics (ampicillin at 100 μg ml^{-1}).
6. Plates are inverted and incubated at 37°C overnight.
7. 250 ml LB broth containing ampicillin at 100 μg ml^{-1} is inoculated with a

single colony from the plate and shaken overnight.
8. Plasmid DNA is purified from the bacteria using caesium chloride gradient centrifugation or one of the commercially available DNA purification kits.
9. The plasmid is digested with a suitable restriction enzyme, treated with calf intestinal, alkaline phosphatase and purified following agarose gel electrophoresis.
10. The gene of interest is either excised from an existing plasmid or amplified from viral nucleic acid by the polymerase chain reaction (PCR). Suitable restriction endonuclease sites are incorporated into synthetic oligonucleotide primers for cloning purposes.
11. The PCR product is phenol/chloroform extracted.
12. 100 µl chloroform is added, the sample vortexed and centrifuged at 14,000 rpm.
13. The upper phase is removed and 100 µl Tris-saturated phenol plus 100 µl chloroform is added. The upper phase is again removed and the DNA is precipitated with 1/10 volume 3 M sodium acetate pH 7.0 and 2.5 volumes ethanol at −70°C for 1 h.
14. The sample is centrifuged at 14,000 rpm, dried and digested with a compatible restriction enzyme.
15. The DNA is purified by PAGE and electroelution.
16. The DNA is quantitated and ligated into the baculovirus vector.
17. Competent cells are transformed as described above and the baculovirus expression plasmid and insert purified. This material is then co-transfected with linear Ac*M*NPV DNA into Sf9 insect cells.

Production of the recombinant virus

1. Sf9 cells are seeded into 60 mm tissue culture dishes at a density of 2×10^6 and allowed to adhere for 30 min.
2. 1 µg Ac*M*NPV DNA is mixed with 2 µg circularized plasmid DNA containing the gene of interest in 1 ml of Grace's medium containing no supplements.
3. 20 µl cationic liposome solution is added and the solution vortexed.
4. The solution is incubated at room temperature for 15 min.
5. The medium is aspirated from the Sf9 cells and replaced with 2 ml Grace's medium without supplements and cells are allowed to remain in this medium for 10 min.
6. The medium is removed from the plates; the transfection mix is then added dropwise and the plates are placed on a rocking platform for 4 h. An additional 1 ml of complete medium is then added to the plates and the plates incubated at 27°C for 48 h. The medium is harvested at 48 h and stored until required.
7. The cells are refed and observed for signs of infection for a further two days.
8. The supernatant containing the recombinant virus harvested on day 2 is titrated under an agar overlay and a plaque assay performed. If the beta-galactosidase gene has been incorporated, any recombinant virus can be selected by blue/white screening if X-gal is incorporated into the overlay. Several rounds of plaque purification are required and recombinant viruses are selected on the basis of their occlusion body-negative phenotype.

The analysis of protein expression

Protein expression can be analysed in a number of ways. These include PAGE

followed by Coomassie blue staining, indirect immunofluorescence, Western blotting and radio-immunoprecipitation. It is imperative that adequate controls are included at all times when performing these assays. For instance, if the levels of recombinant protein expression are monitored at various time points after infection of cells, lysates from cells infected with a wild-type baculovirus or cells alone must be included in any analysis being performed.

Poxvirus expression systems

The expression of antigens in poxvirus was originally described by Mackett et al (1982). Concern over the use of vaccinia virus in humans which has surfaced in recent years has resulted in efforts to overcome this, such as the production of the non-replicating canarypox virus to express both the fusion and HA proteins of measles virus (Taylor et al 1992) and an attenuated form of vaccinia virus, NYVAC (Tartaglia et al 1992; Cox et al 1993). Further, raccoon poxvirus has been used to express the rabies virus glycoprotein which has been used to vaccinate sheep (DeMartini et al 1993). Cytotoxic T-cells have been induced by inoculation with such viruses expressing the HIV-1 envelope glycoprotein (Cox et al 1993). Pox viruses have the advantage of having a large genome which can accommodate additional genes, high levels of expression, and a cytoplasmic site of transcription. For their construction, the gene of interest is cloned into a non-essential gene locus such as thymidine kinase. Recombinants are selected on the basis of their growth in TK-cells in the presence of the toxic analogue BUdR. Vaccinia virus recombinants have been used extensively for the study of both cellular and humoral immune responses to virus infection. For a review on the use of vaccinia virus on T-cell studies see Bennink and Yewdell (1990). The vaccinia T7 system has been used extensively to transcribe and translate proteins that are encoded under the control of the T7 RNA polymerase promoter. Cells are infected with the vaccinia virus recombinant expressing the T7 enzyme which are then transfected with the plasmid containing the gene under the control of T7. Expression of the protein can be detected using conventional techniques.

Procedure
(Smith 1993)

1. The gene of interest is cloned into a poxvirus expression plasmid under the control of a vaccinia virus promoter (i.e. p11 or p7.5).
2. CV-1 cells are infected with wild-type vaccinia virus at moi of 0.5 and the flask is incubated at 37°C for 2 h.
3. Plasmid DNA is precipitated by the dropwise addition of 6.5 µl 2 M $CaCl_2$ to a 1 ml solution of 1 µg plasmid DNA which has been added to 19 µl carrier DNA while vortexing.
4. The virus is removed and the precipitated DNA is added thus allowing homologous recombination to occur.
5. The flask is incubated at 37°C for 30 min, medium added and further incubated for 3–4 h.
6. The cells are refed and after a further 48 h the cells are harvested.
7. The virus is plaque purified.
8. Cells are freeze–thawed three times and titrated on TK-143 cells by the addition of 0.5 ml inoculum followed by rocking for 2 h.
9. Cells are overlayed with 4 ml medium containing 2.5% FCS, 1% low temperature gelling agarose, and 25 µg BUdR. Plaques are harvested after 48 h and further rounds of plaque purification are carried out. Purified virus is analyzed for the production of the relevant protein.

The transient expression of viral antigens

The transient expression of viral antigens in mammalian expression systems has a number of uses. The location of the protein can be determined, i.e. whether it remains in the Golgi or endoplasmic reticulum or whether it is transported to the surface of the cell. Protein interactions can be studied by co-transfection techniques, and whether the transport of a protein to a different compartment of a cell is dependent on a second transfected protein can be assessed. Eukaryotic expression vectors are available which can be used for these purposes. The gene of interest is usually inserted under the control of a strong eukaryotic promoter such as the major immediate early gene promoter of human cytomegalovirus or the RSV LTR promoter. The SV40 origin of replication is often included to ensure the replication of the plasmid in COS-7 cells. The co-transfection of such a plasmid with a plasmid containing a selectable marker will allow for the production of stably expressing cell lines.

Procedure

DEAE dextran transfection

1. The gene of interest is cloned into the vector of choice (as described previously).
2. 60 mm tissue culture dishes are seeded with COS-7 cells at a concentration of 5×10^5.
3. The next day, the monolayer is washed three times in PBS.
4. 1 μg DNA + 500 μl PBS + 5 μl DEAE dextran are mixed together and added to the cells.
5. The plates are incubated at 37°C for 30 min and rocked gently.
6. 5 ml growth medium containing chloroquin is added and the plate is incubated at 37°C for a further 3–5 hours.
7. The plate is washed and refed with growth medium and incubated for a further 48–72 hours.

Detection of proteins

Antibodies raised against the expressed proteins can be used for detection purposes.

Surface expression

1. Cells are fixed for 5 min with a solution of 2% isotonic paraformaldehyde.
2. The slides are washed three times in PBS.
3. The primary antibody is added and the slides incubated at 37°C for 1 h.
4. The slides are washed three times in PBS and the secondary antibody added and the slide is incubated as before.
5. Slides are washed and mounted in 90% glycerol/10% PBS.
6. Slides are examined using a microscope equipped with a mercury vapour lamp. FITC is excited by blue light at 490 nm and emits a green light at 520 nm. TRITC is excited by green light at 540 nm and emits a red light at 625 nm. The size of the protein can be determined by RIPA or Western blotting.

   For the detection of the protein internally, cells are fixed with a 50:50 solution of methanol and acetone for 5 min and stained as described above.

In vitro transcription/ translation systems

It is possible to use a cell-free transcription/ translation system to determine the size of processed and unprocessed forms of viral antigens. The gene encoding the protein of interest is cloned into a plasmid such as pBluescript II KS+ which contains the T3 and

T7 RNA polymerase promoter or psp72 which contains SP6 and T7 promoters. The circular plasmid is transcribed and translated in a rabbit reticulocyte lysate in the presence of the relevant polymerase enzyme. The addition of canine pancreatic microsomal membranes enables the processing of the protein to occur.

Prokaryotic gene expression

The use of bacteria for the expression of either full length or truncated forms of proteins has been described extensively. The carboxyl terminal sequence of the inserted sequence may be modified to contain additional amino acids that can be used for purification purposes such as a histidine tag (His_6) which can be used to bind the protein to a nickel column. One particular system that has been used widely for this purpose is the pGEX system (Smith and Johnson 1988). Here, the sequence of interest is inserted into a plasmid fused with the C-terminus of Sj26, a 26 kDa Glutathione S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum*. The protein can then be purified by its affinity with a glutathione-Sepharose column and eluted using reduced glutathione. The pGEX vectors are designed so that GST can be cleaved by site specific proteases such as thrombin or blood coagulation factor Xa, after which excess GST or uncleaved protein can be absorbed on glutathione agarose.

Virus polypeptides may also be expressed as β-galactosidase fusions, and others may be fused with staphylococcal protein A to allow for their purification on IgG-Sepharose. The insolubility of some of these proteins may be overcome by the use of sarkosyl buffer (Grieco et al 1992). pGEX vectors are available for the insertion of the gene or part of the gene in one of three open reading frames.

Other recently described fusion systems include the CHO recognition domain (CRD) of the galactose-specific rat hepatic lectin, which is used to create fusions with eukaryotic proteins using galactose-Sepharose for purification (Taylor and Drickamer 1991). Similarly, the pBR322 derivative pVB2 has the mg1B gene which encodes the galactose binding protein of *E. coli*. An EcoRI restriction endonuclease site allows for in-frame fusions. GBP is taken to the periplasmic space of a bacterial cell and the recombinant protein can be isolated from the periplasm by osmotic shock (Müller et al 1989).

Procedure

1. The gene is cloned into the pGEX plasmid of choice using the techniques outlined above.
2. The plasmid is used to transform JM105 cells.
3. An overnight culture of bacteria is diluted 1/50 with LB broth containing 100 μg ml^{-1} ampicillin.
4. The cells are grown to mid log phase (A600 = 0.6–1.0) and the expression of the fusion protein is induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1–10 mM.
5. The cells are grown for 3–5 h at 37°C.
6. 1 ml of culture is centrifuged at 14,000 rpm for 2 min and 100 μl PBS added. 10 μl of this is analyzed by PAGE followed by Coomassie blue staining.
7. Insolubility of the protein may be overcome by the addition of Triton X-100 to a final concentration of 1%, shaking for 30 min and analysis of the protein from the supernatant.

The purified proteins may then be inoculated into animals to raise monospecific antisera. Further techniques include fusion to the heat shock protein.

Purification of proteins to which His6 tag has been attached by affinity chromatography
(Soumounou and Laliberte 1994).

1. Bacteria are resuspended in buffer (500 mM NaCl, 160 mM Tris-HCl, pH 8.0) and lysozyme is added to a final concentration of 2 mg ml^{-1} and incubated at room temperature for 20 min.
2. Cells are freeze–thawed several times and sonicated.
3. The lysate is centrifuged at 15,000 *g* and the pellet resuspended in 20 ml of the buffer described in 1, containing 6 M guanidine-HCl, 5 mM 2-mercaptoethanol and 0.1% Tween 20 and incubated at room temperature for 30 min with vortexing.
4. Solubilized proteins are recovered by ultracentrifugation (15,000 *g* for 40 min at 4°C) and incubated with 2.5 ml Ni-NTA Agarose (Qiagen).
5. The resin is washed with buffer described in step 1, containing guanidine, and bound proteins are eluted with this buffer containing 250 nM imidazole.
6. The purified proteins are dialyzed for 24 h at 4°C against 20 mM NaCl, 10 mM Tris HCl, pH 8.0.

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