Molecular Parasitology Lab Manual

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Molecular Parasitology Lab Manual

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1. Preparation of Somatic Antigens

A very wide variety of procedures have been used to obtain soluble somatic fractions from nematode parasites and methods commonly used for one species or group may not be appropriate for another. Amongst filarial nematodes, adult worm extracts have been made by freeze-thawing [1] or lyophilisation [2] of worms before being pulverised manually or in a French pressure cell [3]. Microfilariae are considerably harder to disrupt, and require sonication to release somatic proteins. Homogenised or sonicated parasites are then usually incubated for some time in PBS or other buffer, to yield a "Crude Somatic Extract" (CSE [3]). Protease inhibitors (**Method 31**) must be included during this process or breakdown of protein antigens will occur. At the conclusion of the incubation, soluble antigens are recovered by high-speed centrifugation. One of the many possible approaches to somatic extract preparation is outlined in **Method 1.1** A further important question is whether to include detergents in the extraction buffer, in order to solubilise hydrophobic (generally membrane-band) proteins, as discussed in **Method 1.2.** In either case, the term 'antigen' should not be applied to a heterogeneous somatic extract, but only to individual molecular components for which immunogenicity has been established. The protocol outlined below is one of many possible approaches to somatic antigen extraction.

1.1 Somatic Extract Preparation

Procedure:

1. Take a recorded number of parasites up in PBS (**Appendix 1**), containing protease inhibitors (**Method 31**). Break up adult worms or infective larvae by repeated strokes of the homogeniser. Sonicate microfilariae for 3 minutes at 6 μm on ice. A useful homogeniser for small quantities is Jencons H103/32/324 with a ground glass barrel. Keep on ice at all times.
2. Allow suspension to stand on ice for 60 mins, agitating occasionally.
3. Centrifuge disrupted parasite suspension 10000 **g**, 30 mins, 4°C and pass supernatant through a Millex 0.2 μm filter. Measure protein concentration (**Method 5**) aliquot and store at -70° C.

Notes:

1. The pellet may be further extracted by repeating the disruptive procedure in detergent (**Method 1.2**)
2. Fractionation of crude somatic extracts may be attempted by a number of techniques such as gel filtration (**Method 22**), lectin affinity chromatography (**Method 26**) or ion exchange chromatography.
3. Even on ice, homogenisation may activate enzymes which alter critical antigens. For example, disrupting *Trypanosoma brucei* bloodstream forms allows a phospholipase to cleave a lipid anchor from the major surface (VSG) antigen [4].
4. For some purposes, such as coating an ELISA plate (**Method 17**), whole suspension of somatic extract may be used without centrifugation to remove insoluble material.

1.2 Detergent Solubilisation

No single detergent exists which is ideal for all purposes, and selection of a detergent will depend on a number of properties [5]. For example the strong alkyl ionic detergents SDS (sodium dodecyl sulphate) and CTAB (cetyl trimethylammonium bromide) tend to denature proteins, although 1% CTAB was reported to be the most effective detergent for solubilisation of adult *B. malayi* proteins [6]. The nonionic detergents such as Triton X-100 and Tween-20 do not denature or dissociate inter-protein links, but form large micelles and cannot be removed by dialysis. The bile salt, deoxycholate (DOC), has a high critical micelle concentration (CMC) and therefore retains its monomeric form even at high concentration. DOC is therefore dialysable [7] but can only be used in low ionic strength (10 mM) buffers at pH 8 or above. Perhaps the optimum detergent available is the synthetic glycoside, n-octyl glucoside (nOG), which has a high CMC, is dialysable and is operative in a broad range of buffers [8]; however its cost often precludes use on a large scale. Finally, a detergent of a different nature is Triton X-114 [9] which forms a homogenous solution in water at room temperature but separates at 37°C into detergent (hydrophobic) and aqueous (hydrophilic) phases. The character of individual proteins can therefore be studied according to their differential distribution in the two phases following TX-114 solubilisation.

Procedure

1. Make homogenate or sonicate as in **Method 1.1** using PBS, protease inhibitors and 1.5% n-octyl glycoside (Sigma O-8001)
2. Centrifuge and recover supernatant
3. If supernatant is required for coating of ELISA plates, remove detergent by exhaustive dialysis [7]

1.3 References

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2. Parasite Culture and Excretory-Secretory (ES) Antigens

In vitro- released, or Excretory-Secretory (ES), culture fluids have been collected from a number of helminth species, and this literature has recently been reviewed [1,2]. The protocol below is adapted from that used for the nonfilarial nematode *Toxocara canis* [3,4] unusual among these parasites for its longevity *in vitro* (see also **Method 37**).

In Vitro *Culture of Parasites*

Reagents and Materials:

1. RPMI-1640 medium supplemented with additional glucose (to 1%), HEPES (25 mM), penicillin (100 U/ml) and steptomycin (100 µg/ml). Gibco Cat No 041-2400 is supplied with HEPES. Add fresh glutamine (2 mM) to liquid which has been stored. Prepare sterile 10% glucose in RPMI for feeding cultures.
2. Parasites harvested in sterile medium or washed x5 in medium with 10x antibiotics.
3. Millex 0.22 µm disposable filters (Millipore SLGV 025 BS) and Amicon YM-10 membranes (cut-off 10,000 molecular weight) to fit suitable ultrafiltration cell (e.g. 8010 or 8050)
4. For Bradford protein determination (see **Method 5**): Bio-Rad protein assay kit (500-0001),or Coomassie Brilliant Blue G250 (Sigma B-5133)

Procedure:

1. Incubate parasites in serum-free culture medium in an atmosphere of 5% CO_2. Avoid conical tubes in which parasites would pellet: flat bottom sterile bijoux (Sterilin 129A) or round bottom tissue culture tubes (Falcon) are often ideal.
2. Change medium at regular intervals. *T.canis* are routinely cultivated with weekly changes [3,4]. *Brugia* adults require daily changes, or daily addition of sterile glucose to 1% and adjustment of pH with $NaHCO_3$. Aim for a density of 2 adult female or 10 male *Brugia* worms per ml and 20,000 microfilariae per ml. For microfilariae, include 2% FCS in the culture medium.
3. Filter supernatant prior to storage at -70°C. Discard medium if significant proportion of parasites are dead. Pool and concentrate supernatants by diafiltration in Amicon cell. Aim for 100x concentration and three washes with PBS. Take samples for protein determination and freeze remainder in small aliquots at -70°C.
4. Determine protein concentration by the Bradford procedure, as in **Method 5**

Notes:

1. Day 0-1 ES may be significantly different from later days.
2. Use gentamycin (10 µg/ml) in place of penicillin-streptomycin if bacterial contamination is a problem.
3. For some purposes (eg collection of secreted enzymes) Amicon diafiltration should be performed at 4°C or in an ice bath, avoiding prolonged handling.

References

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3. Metabolic Labelling of Filariae In Vitro

A wide variety of radioactive precursors, amino acids, sugars and fatty acids may be used to biosynthetically label products of living parasites. Of these, ^{35}S-methionine is most widely used for nematode parasites [1,2] as this isotope has a higher energy than ^{14}C or ^{3}H and is more readily detected. The conditions described below may be adopted for other precursors with appropriate modification of the culture medium.

Reagents:

1. ^{35}S-methionine (Amersham SJ. 204, 1000 Ci/mMol). Store in small aliquots at -70°C until required.
2. Methionine-free MEM medium (Flow 16-222-49) or similar, to which should be added Non-Essential Amino Acids (Flow 100x solution, Cat No 16-810-49), HEPES (10 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (10 µg/ml) and additional glucose (to 1%).
3. Foetal calf serum in cases where parasites cannot survive in serum-free medium
4. Parasites in sterile medium; where sterility is not assured, wash x5 in medium containing 10X antibiotics.
5. Millex 0.22 µm disposable filters, SLGV 025 BS (low protein binding).

Procedure:

1a. Incubate parasites in methionine-free medium. Add isotope diluted in medium through a disposable 0.22 µm filter. Place culture in 37°C incubator, 5% CO_2, for 16-60 hrs. Check viability at intervals with an inverted microscope.

1b. For filarial ES, incubate 10 females or 20 male parasites or 50,000 microfilariae in 2 ml of methionine free medium containing 200-500 µCi of isotopes. Add glucose (to 1%) and 20 µl of $NaHCO_3$ (or more to neutralise the culture medium) daily. Continuous culture are kept up to 3 days for adult ES and 2 days for microfilarial ES (2% FCS is included in microfilarial culture).

2. At conclusion of culture, recover supernatant and Millex filter. Isolate labelled secreted macromolecules by dialysis, TCA (**Method 6**) or ethanol precipitation, or desalting on G25 or similar column.

3. For filarial surface antigens, wash parasites 3x in PBS containing protease inhibitors (**Method 31**), then incubate for 60 mins in 1.5% n-octylglucoside on ice, agitating regularly. Filter and centrifuge the supernatant for 30 mins, 10000 **g**, 4°C.

4. For biosynthetically labelled somatic antigens, homogenise or sonicate the parasites as detailed in **Method 1,** followed by harsh extraction if required.

Notes:

1. ^{35}Sulphur is a beta emitter and therefore can only be detected in contact with scintillants. Good general purpose scintillation cocktails which take up aqueous samples include Aquasol (NEN) and PCS II (Amersham). Details of impregnating polyacrylamide gels for fluorography are given in **Method 10.**
2. Parasites with high viability *in vitro* may be preincubated in met-free medium to deplete their endogenous methionine pool.
3. Non-Essential Amino Acids (Ala, Asn, Asp, Gly, Glu, Pro and Ser) significantly enhance incorporation of label by filarial parasites [3].
4. The BSA in FCS interacts with labelled methionine to produce a spurious band in culture [4,5]. Therefore FCS should be added only when essential to parasite survival.
5. Selected inhibitors may be included in the culture medium, e.g. Tunicamycin or 2-deoxyglucose which inhibit N-linked (via Asn) glycosylation.

References

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4. Labelling of Surface Antigens

Four techniques have been used for extrinsically labelling exposed antigens on the surface of nematodes and other parasites. The chloramine T procedure [1,2] has now been superceded by Iodogen [3-5] which introduces radio-iodine onto tyrosine residues without invoking a damaging redox reaction. The third technique utilises the Bolton-Hunter reagent [6,7], an iodinated succinimide which conjugates to amino groups, predominantly labelling lysine positions. Both Iodogen and Bolton-Hunter procedures, described here for nematode worms, consist of exposing living parasites to radiolabel, separating iodinated organisms from free label by centrifugation or sedimentation, and solubilising cuticular molecule in detergents. Other surface-labelling approaches use lactoperoxidase [5,8], which is highly vectorial due to the exclusion of enzyme by most biological surfaces, and iodosulphanilic acid [9,10]. The use tritiated borohydride labelling of carbohydrates [11] has so far only been reported on trematodes [12].

4.1 Iodogen Labelling

Iodogen is insoluble in aqueous media and, coated on a tube, acts as a solid phase mediator of iodination [4].

Reagents:

1. Iodogen (1,3,4,6-tetrachloro-3α,6α,-diphenylglycouril: Pierce Chemical Company 28600) made up at 1mg/ml in methylene chloride. Add 50-200 µl into polypropylene tubes (1.5 ml Eppendorf tubes are convenient). Higher concentrations of Iodogen may prove toxic to filarial worms [5]. Allow solvent to evaporate and store sealed at room temperature.
2. Sodium ^{125} Iodide (Amersham IMS.30, 100 mCi/ml or equivalent). Standard quantities used are 250–500 µCi.
3. A suspension of parasites in PBS. Minimum quantities which have proved practical are: 5000 microfilariae, 500 infective larvae, 1 adult.
4. Saturated solution of tyrosine in PBS.
5. A solution of PBS containing protease inhibitors (see **Method 31**):

0.5 ml	200 X	Protease inhibitors EDTA, EGTA, TLCK, NEM in water
0.5 ml	200 X	Protease inhibitors TPCK, PMSF, pepstatin in ethanol
~ 0.25 ml	1 M	NaOH to pH 8.3 per 100 ml.

Procedure:

1. Transfer parasites to dried Iodogen tube. The tube may be pre-rinsed with PBS to remove flakes of reagent. Excess buffer used in transfer (particularly with adult worms) may be removed.
2. In a well-vented fume cupboard, add radio-iodine. Seal vessel and agitate regularly. The reaction may be carried out on ice or at room temperature. Continue agitation for 10 mins.
3. Add 10% by volume saturated tyrosine solution.
4. Transfer contents of labelling tube to 10 ml centrifuge tube and add cold PBS containing protease inhibitors. Separate parasites as follows:

Microfilariae	400 *g*	5 mins
Infective larvae	100 *g*	3 mins
Adults	1 *g*	as required

5. Measure activity in parasite pellet, and wash 3 x in PBS. Solubilise pellet as in section **4.3** below).

4.2 Bolton-Hunter Labelling

The Bolton-Hunter reagent, N-succinimidyl 3-(4-hydroxy,5-[^{125} I] iodophenyl) proprionate, is an iodinated ester which hydrolyses to conjugate to amino groups [6]. It is highly unstable in aqueous solution, and must be kept moisture-free at 4°C until use in labelling reaction.

Reagents:

1. Bolton-Hunter Reagent (Amersham IM. 5861, 5 mCi/ml in benzene). An aliquot containing 200-500 µCi of ^{125} I is placed in a glass tube and dried under a gentle stream of dry nitrogen.
2. 0.1 M borate buffer, pH 8.5.
3. 2 M glycine solution.
4. Buffer containing protease inhibitors as in **4.1**
5. Parasites, washed in 0.1 M borate buffer, pH 8.5 before labelling.

Procedure:

1. Place parasite suspension in 0.1 M borate buffer into tube in which Bolton-Hunter reagent has just been dried down. Agitate on ice for 10 mins: the ester half-life at 0°C is 9 mins.
2. Add 10% by volume of 2 M glycine and continue agitation for further 5 minutes.
3. Transfer parasites to 10 ml centrifuge tube and wash with cold protease-inhibitor buffer as in **4.1**
4. Measure activity in pellets and take for solubilisation as in **4.3** below.

4.3 Solubilisation of Surface Labelled Molecules

Reagents:

1. 15% n-octylglucoside (Sigma O-8001) solution, as stock solution kept at -20°C.
2. PBS containing protease inhibitors as above.
3. 2-Mercaptoethanol, kept sealed at 4°C if not a new bottle.
4. 'Harsh' extraction buffer:

10 ml	0.1 M phosphate pH 7.4	(Final concentration 10 mM)
4.8 g	Urea	(8M)
5.0 g	SDS	(5%)
50 µl/ml	2-Mercaptoethanol freshly added	(5%)

Procedure:

1. Add nOG to a final concentration of 1.5%.
2. For "detergent-released" antigens incubate with occasional agitation for 1-16 hours on ice [13,14].
3. To disrupt filarial parasites:

Microfilariae	Sonicate, 10 x 1 min at 8 µm wavelength
Infective Larvae	Homogenise, or sonicate 5 x 1 minute
Adult worms	Homogenise (see **Method 1**)

4. Centrifuge homogenates, suspensions, etc: 30 mins, 4°C, 10000 *g*.
5. Supernatants are taken as Surface Antigen preparations. Test TCA precipitability as in **Method 6.2**
6. Vortex pellet and add detergent and buffer as before plus 5% fresh 2-mercaptoethanol. Incubate 37°C for 1 hr with occasional reagitation [13,15]. Centrifuge as above and recover supernatant; dialyse for antigenic studies.
7. Resuspend pellet in 'harsh' extraction buffer and incubate 100°C, 30 mins or 65° C for 2 hours, then centrifuge as before. Save supernatant and discard pellet.

Alternative Procedure:

This procedure employs 2-mercaptoethanol to dissolve the disulphide linked framework of the cuticle and releases all surface-labelled antigens from adult *Brugia malayi* [16]

1. Incubate labelled parasites in PBS-protease inhibitors. Add fresh 2-mercaptoethanol to final concentration of 5%. Incubate for 30 mins at 37°C and recover supernatant.
2. Centrifuge supernatant 10,000 g for 30 mins at 4°C. Dialyse against PBS containing protease inhibitors if required for immunological reactions. Homogenise pellet from incubation as for (3) above.

4.4 References

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5. Protein Detection

Two general techniques are given here for the determination of concentration of a known protein by UV absorbance (**Method 5.1**) or unknown proteins or mixtures by dye-binding (**Method 5.2**)

5.1 Ultra-Violet Absorbence

Nearly all proteins characteristically absorb UV light with a peak at 278-280 nm, due to the presence of aromatic rings in tryptophan and tyrosine residues. The absorbance of an individual protein is therefore a function of the relative proportion of these residues. If this absorbance value is known, this is the most accurate way of determining concentration. Measurement of absorbance (optical density, OD_{280}) requires quartz cuvettes as glass absorbs light at this wavelength.

Extinction co-efficients give the absorbance at 280 nm of a 1 cm pathlength of a 1% (10mg/ml) solution of a given protein. These can vary widely and it is not valid to extrapolate from BSA or any other standard protein. Some examples are:

BSA	6.6
Immunoglobulin	14.0
Toxocara canis ES	10.0

5.2 Protein Determination by the Bradford Method

The determination of protein concentrations by the dye-binding [1,2] is generally preferable to Lowry [3] on account of speed, convenience and stability of reagents. The basis of the assay is the shift in peak absorption by Coomassie Brilliant Blue from 465 nm to 595 nm when the dye is bound by protein. Few substances interfere with this reaction, except detergents which will also bind dye and give spurious positive results.

Reagents:

1. Bradford Reagent, either purchased from Pierce (Cat. No. 23-200) or BioRad (Cat. No. 500-0006, or part of kit 500-0001). The stock reagent may be kept indefinitely at 4°C. The BioRad stock reagent must be diluted 1/5 in deionised water prior to use and store no longer than 2 weeks at room temperature. The Pierce reagent is ready to use.

Procedure 1 (Cuvette method):

1. To 100 µl of protein sample (containing 1-100 µg of protein), add 0.9 ml (for 1-20 µg)or 5ml (for 20-100 µg) of Reagent. Mix well by vortex or inversion. Include a full range of known concentration reference protein (e.g BSA) to construct a standard line.
2. Read after two minutes and before 60 mins at 595 nm, measuring difference between sample and blank. Draw graph of protein standards and read off unknown concentrations by OD value.

Procedure 2 (Microtitre method):

1. For samples containing 150-1500 µg/ml of protein add 5 µl of test sample (diluted or undiluted) to a well in an ELISA microtitre plate and add 200 µl of Bradford reagent. Mix by pipetting, and read as above with BSA standards. For samples containing 1-25 µg of protein add 50 µl of test sample (diluted or undiluted) per well and add 150 µl of Bradford reagent.

Notes:

1. Bradford Reagent can be made up as follows:

100mg Coomassie Brilliant Blue (Sigma B5133)	(Final concentration 0.01%)
dissolved in 50 ml 95% ethanol	(4.7%)
100 ml 85% Phosphoric acid	(8.5%)
Make up 1 L with distilled water.	

   Alternative recipes for the reagent [2] substitute 3% perchloric acid for 8.5% phosphoric.
2. Protein-dye complex binds to quartz cuvettes but not to glass or plastic.
3. Bound dye has a broad absorption peak: readings may also be taken at 620 nm [2]
4. Reference protein concentrations should be determined by OD at 280 nm. For BSA the extinction coefficient for a 1 mg/ml solution is 0.66.

5.3 References

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6: Iodination of Soluble Proteins

Of the many techniques available for radiolabelling soluble proteins, the Iodogen method [1] is most widely used. This labels at tyrosine residues, and avoids the damaging effect of the original Chloramine T procedure [2] without introducing the elaborate procedure of lactoperoxidase catalysis [3]. The Bolton-Hunter reagent [4], although more expensive, may be more appropriate for proteins with a high lysine/low tyrosine composition (see Note 3).

6.1 Iodogen Labelling

Reagents:

1. Iodogen, prepared as in **Method 4.1**
2. Protein in PBS, ideally 1 mg/ml
3. Saturated tyrosine solution. Add ~100 mgs tyrosine to 20 ml water. Store away from light.
4. Sodium ^{125} Iodide (Amersham IMS. 30)
5. Sephadex G-25 column, 2.5 ml in plastic syringe, equilibrated with PBS or PBS-0.5% Triton X-100. G-25 beads should be swollen overnight in buffer and may then be stored indefinitely at 4° C in the presence of 0.1% sodium azide. Support the beads in the column by inserting a filter paper circle at the base of the syringe. Suitable filter circles may be mass produced with a cork borer or similar punch.

Procedure:

1. Rinse Iodogen coated tube twice with PBS. Add 10 µg of protein to vial containing 100 µg of Iodogen.
2. Add PBS to take volume up to 50 µl, allowing for volume of isotope to be added.
3. In a well-ventilated fume cupboard add 250-500 µCi of Na ^{125}I
4. Agitate frequently, keeping vial on ice.
5. After 10 mins, add 5 µl of saturated tyrosine solution.
6. Load onto G-25 column, collecting 250 µl fractions.
7. Test each fraction for total radioactivity and TCA precipitability (**Method 6.2**)
8. Retain first peak which should have high TCA precipitability (>80%) and discard remainder. Aliquot and store at -20° C.

Notes:

1. Addition of detergent (e.g 0.5% Triton X-100) reduces loss of labelled protein on Sephadex column.
2. Dialysis may be substituted for desalting by gel filtration.
3. For Bolton-Hunter labelling, follow **Method 4.2** and add 10 µg protein in borate buffer in place of parasites, then follow from Procedure 4 above.

6.2 TCA Precipitation

Reagents:

1. 10% Trichloracetic acid (can be diluted from 100% stock)
2. Normal serum, e.g. NRS

Procedure

1. Add to duplicate tubes (e.g. LP3 or Eppendorf) 5 µl of normal serum. Vortex.
2. Add 1-5 µl of radiolabelled antigen. Vortex and count.
3. While vortexing the tube, fill slowly with cold 10% TCA
4. Allow to stand on ice for up to 60 minutes.
5. Centrifuge 10 minutes, 4°C, top speed bench centrifuge
6. Discard supernatant, count pellet and calculate % precipitated.

6.3 References

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7. SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis of proteins in polyacrylamide gels in the presence of SDS is a primary analytical method in immunochemistry. The addition of SDS serves both to denature multimeric proteins into subunit components and to saturate sample molecules with negative charges so that gel migration is generally proportional to molecular weight [1,2]. From the original tube gel technique [1] has developed slab gels which may be used for simultaneous analysis of multiple samples, for two-dimensional analysis of single samples (**Method 8**), or for immunoblotting procedures (**Method 15**).

The percentage acrylamide in slab gels may be varied according to the molecular weight range in which resolution is required; gradient gels (**Method 7.2**) offer separation over a particularly wide range, and such gels are well worth the more elaborate preparation procedure (see Figure 1 below). There is further choice in the level of cross-linker used to polymerise acrylamide, *N,N'*-methylene-bis-acrylamide ("bis"). Gels of all types include a short stacking gel, designed to concentrate samples at the interface between stack (pH 6.8) and main (pH 8.8) gel sections. Finally, conditions of electrophoresis vary according to the type of gel, the power unit used and whether cooling is available. In the recipes below, procedures are given which have been proved applicable to the analysis of surface antigens of nematode parasites.

Although most proteins migrate in true proportion to their molecular weight, anomalous results have been reported with extensively glycosylated [3] or collagenous molecules [4]. In one instance a single amino acid change causes a shift in mobility [5], and migration often depends critically on whether samples have been reduced or not (for example [6]). Consequently, SDS-PAGE estimates provide an apparent molecular weight which may be at variance with the true value but is nevertheless an invaluable aid in identifying and characterising parasite molecules.

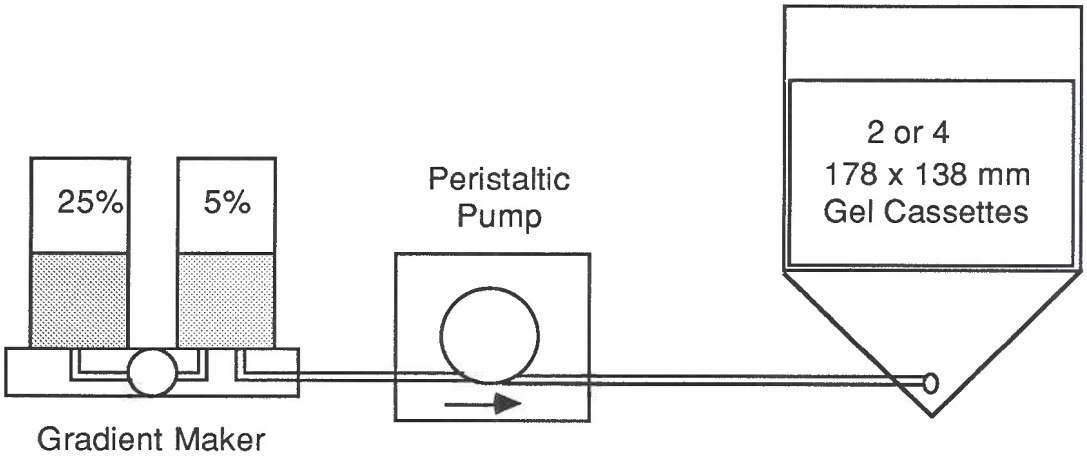

Figure 1 : Preparing Gradient PAGE gels for the Pharmacia system (**Method 7.2**)

7.1 General Procedure

Reagents and Equipment:

1. Gel electrophoresis tank, homemade or obtained from LKB (2001), Pharmacia (GE2/4LS), BioRad (Protean) or other manufacturers. Gel pourer, glass plates, spacers, sealing tape, sample combs as appropriate. For Pharmacia use 139 x 178 x 2 or 3 mm glass plates, 0.7mm spacers (19-4917-01), sample combs (19-4920-01) and sealing tape (DRL 1607). Pharmacia GE2/4 LS gel caster requires disperser nets (19-4781-01).
2. Power supply delivering up to 200 V, 200 mA, such as LKB 2197.
3. Acrylamide Grade 1 (BDH 44313), bis-acrylamide, ammonium persulphate (Sigma A-6761), TEMED (*N, N, N′, N′*,-tetramethylethlene diamine, Sigma T-8133), Trizma base (Tris, Sigma T-1503), HCl, sodium dodecyl sulphate (SDS, specially purified: BDH 10807), Glycine (BDH: 28458).
4. Sample buffer: see **Method 7.4**
5. Materials for gel drying and staining or autoradiography: see **Methods 7.5, 9 and 10**
6. Molecular weight marker proteins: see **Method 7.6**

Stock Solutions:

1. Solution A (Acrylamide): 250 g Acrylamide (Final concentration 40%)
   3.375 g Bis (0.54%)
   Make up to 625 ml with water, and filter through Whatman No. 1.
2. Solution M (Main gel): 480 ml 1M HCl
   366 g Tris base (Final concentration 3M Tris-HCl)
   8 g SDS (0.8%)
   2.3 ml TEMED (0.23%)
   Adjust pH to 8.9, make up to 1000 ml with water. Store at ambient temperature for up to 12 months.
3. Solution S (Stack gel): 121.1 g Tris base then HCl to pH 6.8 (1 M Tris-HCl)
   8 g SDS (0.8%)
   4 ml TEMED (0.4%)
   Make up to 1000 ml with water. Store at ambient temperatures.
4. Ammonium persulphate solution: 10% (100 mg/ml) in water, keeps 24 hours : 3-5 ml will usually suffice.
5. Electrophoresis gel tank buffer (made up to 5x):
   1440 g Glycine (When diluted, 0.38 M)
   300 g Tris Base (0.05 M)
   50 g SDS (0.1%)
   Adjust pH to 8.3 make up to 10 L and dilute 1/5 when required. Store at ambient temperature.

Procedure:

1. Clean gel plates with detergent, water and 96% alcohol. Assemble cassettes, insert combs and place in caster.
2. Make up gels according to either homogeneous or gradient recipes (see below), pouring gel to 1 cm below the sample comb. Gently overlay with water saturated–butanol to ensure level surface. Allow to set for 60 mins.
3. Make up stack mixture in following proportions:

12.5 %	Solution A	Usually: 5 ml
12.5 %	Solution S	5 ml
1.0 %	Ammonium persulphate	0.4 ml
74.0 %	Water	30 ml

   Remove overlay from gels and pour stack solution over top of combs. Allow 30 mins to set, then remove comb, trim and insert in gel electrophoresis tank.
4. Fill tank with buffer and commence cooling, if available, to 4°C.
5. Add samples (see **Method 7.4**) to each well using a Gilson Pipetman or similar micropipette and begin electrophoresis at 25 mA per thin (0.7 mm) slab gel, constant current, for 4-6 hrs, or 6mA per thin gel overnight. Thick (2.7 mm) gels require double current.
6. When blue dye reaches end of gel, dismantle cassette and process gel for staining, blotting or autoradiography.

Notes:

1. Determine volumes of gel solutions required for each apparatus. Pharmacia 178 x 137 x 0.7 mm gels in the GE2/4LS gel pourer require 50 ml of main gel mixture, and 5 ml of stack solution, per slab [7].
2. Cool tank by connecting coil to refrigerated recirculating pump (e.g LKB Multitemp 2219).
3. Unpolymerised acrylamide is a neurotoxin and should not be inhaled or touch unprotected skin.
4. Rapid polymerisation may take place at warm temperatures. Slow polymerisation may result from an old (> 6 months) stock bottle of ammonium persulphate.
5. Storage of Solution A on 1 g Amberlite mixed bed resin (MB3, BDH 55008) may prolong shelf life.

7.2 Gradient (5-25%) Gels

Reagents and Equipment

1. All materials described in **Method 7.1**
2. Gradient maker (Pharmacia GM-1 or similar) and Peristaltic pump (Gilson Minipuls or similar) and tubing (Anachem 116-0532-200), arranged to feed gel caster as in Figure 1 above.

Procedure

1. Make up separately 5 and 25% solutions, omitting ammonium persulphate until ready to start pumping. Use the following volumes per 100 ml:

	5%	25%
Solution A	12.5	50.0
Solution M	12.5	12.5
Water	74.6	37.3
Amm persulphate	0.4	0.2

2. Degas each solution separately, load into gradient maker (5% solution in front chamber when pumping into bottom of gels) and pump at 10 ml/min (top speed on Gilson Minipuls using 116-05320-200 tubing). Check carefully for leaks in tubing before pumping.
3. When pumping from beneath, use 50% glycerol to push all the acrylamide into cassettes. Overlay with water saturated n-butanol and allow to stand for one hour. Once set pour off overlay, rinse well, and add stack (Procedure 3 above).
4. Run under the conditions in Procedure 5 above.

Notes:

1. To avoid polymerisation before the gradient is cast, catalyst and initiator concentrations have been minimised. Colder acrylamide mixtures also polymerise more slowly.
2. Routinely, to make 4 5-25% gels on a Pharmacia GSC-2 apparatus, use:

	For thin (0.7 x 138 x 178 mm) gels		For thick (2.7 x 138 x 178 mm) gels	
	5%	25%	5%	25%
Solution **A**	6 ml	30 ml	16 ml	80 ml
Solution **M**	6 ml	6 ml	16 ml	16 ml
Water	36 ml	12 ml	96 ml	32 ml
Amm persulphate	180 µl	100 µl	450 µl	250 µl

Do **not** exceed these volumes or gel will not clear disperser nets which must then be thrown away.

7.3 Homogenous Preparative Rod (10%) Gels

1. Take glass tubes
2. Clean 60 mins conc HCl then rinse well in dist water and 96% EtOH. Dry.
3. Siliconise by immersion in dimethyldichlorosilane solution (BDH 33164). Recycle solution. Dry.
4. Rinse in distilled water and 96% EtOH. Dry
5. Seal base with parafilm.
6. Each gel volume is less than 3 ml
7. For 10 gels, make 30 mls as follows:
   7.5 ml A (40% Acrylamide)
   3.75 ml M
   0.3 ml AP
   18.45 ml Water
   Fill each tube up to the mark at 10 cms and overlay with n-butanol. When set, wash out overlay and add stack. Make 10 mls as follows:
   1.25 ml A
   1.25 ml S
   0.1 ml AP
   7.4 ml Water
   Run 1.5 mA per gel for 1 hour, and then 2.5 mA per gel for three hours or until dye reaches end of gel.
8. For homogenous 10% slab gels, makeup gel mixture in following proportions: for 4 thin gels use;
   25 % Solution A
   12.5 % Solution M
   0.7 % Ammonium Persulphate
   62 % Dist-di water
   Degas mixture and pour into gel cassettes up to 1 cm below the sample comb.
9. For 2 homogenous 18% slab gels, use:
   27 ml Solution A
   7.5 ml Solution M
   25.25 ml Water
   0.25 ml Ammonium persulphate

7.4 PAGE Sample Buffer

Reagents:

1. SDS and Tris as in **Method 7.2**
2. Glycerol (BDH 10118)
3. Bromophenol Blue (BDH 44305) 0.2%
4. Mercaptoethanol (Sigma M-6250) and Iodoacetamide (Sigma I-6125)

Procedure:

1. Make up the following Studier buffer [8]

12.5 ml	1M Tris pH 6.8	0.125 M
40 ml	SDS	10%
20 ml	Glycerol	20%
2 ml	0.2% Bromophenol Blue	0.042%

   Add water to final volume of 100 ml. Store at room temperature.
2. Where reducing conditions are required, add 5% fresh mercaptoethanol (= 0.713 M) immediately before use.
3. Where nonreducing conditions are required, add 1 mg/ml iodoacetamide.
4. Add sample to be electrophoresed to this buffer, incubate for 5 mins at 100° C, and load onto gels. Where samples are to be stored, subject to boiling immediately after adding SDS-containing buffer.

Notes:

1. Dithiothreitol (DTT, or Cleland's Reagent: Sigma D-0632) offers a more stable reducing agent as an alternative to mercaptoethanol.

7.5 Fixing and Drying Gels

Reagents and Equipment:

1. Gel drier, such as BioRad 1125 B, and vacuum source of at least 29 mm Hg
2. Gel fixer (25 % methanol, 7.5% acetic acid)

Procedure:

1. Immerse gel following electrophoresis in gel fixer for at least 20 minutes. Fixed gels may be kept for 24 hrs or more in this solution.
2. Transfer gel to filter paper (Whatman No. 1) of suitable size, wetting the filter paper to permit repositioning of the gel to ensure flatness.
3. Place gel on gel drier and turn on heat and vacuum. At 80° C expect a 0.7 mm gel to dry in 60 mins, a 2.7 mm gel in 2 hrs; at 60°C allow twice as long.

Notes:

1. This procedure is suitable for ^{125}I-labelled samples for autoradiography. Gels for fluorography (**Method 10.2**) are immersed in scintillant after step 1, and various staining protocols (**Method 9**) require different fixing or handling steps.
2. Substituting AcrylAide (Miles 49.543.1) for bis in gel solutions, at equivalent concentrations, circumvents the need for vacuum drying, but requires GelBond PAG film (Miles) in place of filter paper for good results.
3. When fixing gels containing high levels of free counts (e.g. culture supernatants containing labelled amino acids), the fixer should be changed three or four times and discarded. This removes any free label and prevents high background.

7.6 Molecular Weight Markers

Reagents:

1. One of the following kits of marker proteins of known molecular weights:
   Pharmacia 17-0551-01 (Mol Wt Range 14,400 - 94,000)
   Boehringer 750115 (20,000-170,000)
   BDH Electran 44264 (12,300-78,000)

Procedure:

1. Place marker sample, appropriately labelled if necessary in one or more wells of a slab gel.
2. On staining or autoradiography, measure the migration of each marker protein and plot mobility (linear) against log of molecular weight. Make a separate plot for each gel.
3. Measure mobility of parasite antigens and read apparent molecular weight on the corresponding plot.

Notes:

1. Disparate estimates of molecular weight from gels of different percentage acrylamide are indicative of heavy (over 10%) glycosylation [3]: the lower the acrylamide concentration the higher the molecular weight will anomalously appear to be.

7.7 References

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8. Two-Dimensional Electrophoresis of Proteins

Two-dimensional electrophoresis is one of the most powerful tools in the analysis of complex mixtures of proteins, separating firstly by charge (isoelectric focussing: IEF [1] or nonequilibrium pH gradient electrophoresis: NEPHGE [2]) and then by weight by PAGE [1,3]. The first dimension may be carried out under non-denaturing [1] or denaturing [3] conditions: the second (PAGE) dimension is always performed in the presence of SDS.

IEF Gels: proteins are loaded on the basic side of the 1st dimension rod gel – acidic proteins therefore enter the gel first. Generally IEF gels do not resolve proteins with a pI greater than 7 even when basic ampholines are used, but the resolution of acidic proteins is superior to those obtained by the NEPHGE method.

NEPHGE Gels: proteins are loaded on the acidic side of the rod – basic proteins enter the gel first but electrophoresis is stopped before proteins reach the isoelectric point or run off the gel. This method resolves proteins over a broad range of pI from approximately pH 4 to pH 10.

Reagents and Equipment:

1. Gel tanks for IEF tubes and PAGE slabs, or combination apparatus such as Pharmacia GE-2/4LS.
2. Power pack delivering up to 500 V.
3. Gel cassettes (**Method 7**) and 140 mm x 2.7 mm internal diameter glass gel tubes (Pharmacia 19-4793-01).
4. PAGE chemicals and ultrapure Urea (BDH 45204), Nonidet-P40 (NP40: Sigma N-6507), sodium hydroxide (BDH 45212) and orthophosphoric acid (BDH 45010).
5. q Ampholines from LKB (pH range 3.5-10, No.1809-001 and pH range 5-7, No.1809-021).
6. Agarose (BDH 44250).

Stock Solutions:

1. Acrylamide stock solution: 28.38% (w/v) acrylamide (BDH 443136C) and 1.62% (w/v) bisacrylamide (BDH 443003N) in nanopure water. Store in the dark for up to 2 weeks at 4°C.
2. **IEF** Gel mixture:

5.5 g	Urea	(Final concentration 9.2 M)
2.0 ml	Nanopure water	
1.33 ml	Acrylamide stock	(4%)
0.1 ml	Ampholines pH 3.5-10	
0.4 ml	Ampholines pH 5-7	(2% total)
2.0 ml	10% NP40	(2%)

Dissolve the urea at this point using a 37°C water bath if necessary. Then, immediately before pouring, add:

7 µl	TEMED
10 µl	10% (w/v) Ammonium persulphate (freshly made).

3. **NEPHGE** Gel mixture:

5.5 g	Urea	(Final concentration 9.2 M)
2.0 ml	Nanopure water	
1.33 ml	Acrylamide stock	(4%)
0.5 ml	Ampholines pH 3.5-10	(2%)
2.0 ml	10% NP40	(2%)

Dissolve the urea at this point using the 37°C water bath if necessary. Then, immediately before pouring, add:

14 µl	TEMED
20 µl	10% (w/v) Ammonium persulphate (freshly made).

4. Stock RNase and DNase: Make up 50mg/ml RNase and 1mg/ml DNase in 0.01M Tris-HCl pH 7.4 with 0.005 M $MgCl_2$ and store at -70°C.
5. Lysis buffer:

2.75 g	Urea	(9.5 M)
1 ml	10% NP40	(2%)
250 µl	3-10 Ampholines	(2%)
40 mg	Dithiothreitol (DTT, BDH 44149)	(0.78%)
4 µl	RNase Stock	
4 µl	DNase Stock	

Add nanopure water to 5 ml. Aliquot and store at -70°C for up to 2 months. Do not refreeze aliquots.

6. 4X Lysis buffer:

180 µl	NP40	(18%)
41 µl	3.5-10 Ampholines	(41% total)
55 mg	DTT	(17%)
4 µl	RNase Stock	
4 µl	DNase Stock	

Add nanopure water to 1 ml. Store in 50 µl aliquots at -70°C.

7.Immunoprecipitate elution solution:

250 µl	10% SDS solution	(0.5%)
40 mg	DTT	(0.78%)

Nanopure water to 5 ml.

8. Overlay mixture: 1% ampholines in 8 M urea (4.8 g in 6 ml).
9. IEF electrophoresis buffer

Upper (–, cathodic)	0.05 M NaOH (2 g/litre)
Lower (+, anodic)	0.023 M H_3PO_4 (1.44 ml/litre)

10. NEPHGE electrophoresis buffer

Upper (+ ve, anodic)	0.01 M H_3PO_4 (576 µl/litre)
Lower (–ve, cathodic)	0.02 M NaOH (800 mg/litre)

Make up 4 litres of lower buffer And 1 litre of upper buffer. Degas both for 10-15 minutes before use.

11. Equilibration buffer:

10 ml	Glycerol	(10%)
50 ml	10% SDS	(5%)
6.25 ml	1 M (121 mg/ml) Tris pH 6.8	(62.5 mM)
1 ml	2% Bromophenol blue	
27.75 ml	Deionised water	

Add conc HCl to pH 6.8. Add 5% 2ME before use.

12. Sealing agarose:

1 g	Agarose
6.25 ml	1 M Tris pH 6.8
20 ml	10% SDS
73.75 ml	Deionised water

Aliquot and store at 4°C.

13. Ethanol – KOH bath solution: 10 g KOH in 500 ml 95% ethanol or methanol.
14. Chromic bath solution: conc sulphuric acid, saturated with potassium dichromate.
CAUTION: THESE SOLUTIONS ARE **EXTREMELY** CAUSTIC. DO **NOT** MIX THE SOLUTIONS – EVEN IN SMALL QUANTITIES.

Sample Preparation:

To x µl sample add x µl lysis buffer and x mg urea.
For larger volumes: to x µl of sample add x/4 µl of 4x lysis buffer plus x mg urea.
For immunoprecipitates: boil precipitates in 50 µl of the immunoprecipitate elution solution. Cool, then add 12.5 µl of 4x lysis buffer and 12.5 mg urea. after loading overlay with 10µl 10% NP-4O.

Procedure:

1. Wash glass rods in distilled water immediately after use. Immerse in chromic acid overnight, then wash in distilled water before transferring to EtOH-KOH bath. Leave for at least 1 hour. Rinse in distilled water then absolute alcohol just before pouring gels.
2. Seal end rods with two layers of nescofilm or parafilm. Place them around a 500 ml bottle and fix in place with a rubber band. Mark off the desired length of gel on every rod (usually 10-12 cm).
3. Pour the gels using a glass Pasteur pipette. Squirt the gel in vigorously initially to prevent air bubbles forming at the bottom of the rod. Tap rods on the bench to remove any small bubbles. The gels will polymerise in 15-30 minutes. Overlay the gel mixture with 20 µl water. After 1-2 hours the overlay is removed and replaced with 20 µl lysis buffer. The gels should be left for a further two hours before use. Do not leave for longer than 24 hours.
4. Samples can now be loaded onto NEPHGE gels but IEF gels need pre-running at the following voltages:
   200V for 15 minutes
   300V for 30 minutes
   400V for 30 minutes or longer.

5. Gently remove all the liquid above the gels and load the samples against the wall of the rod with a Hamilton syringe (Supelchem). IEF gels can be loaded with up to 20 µl sample while NEPHGE gels can be loaded with as much as 200 µl; however small sample volumes give better results.
6. Overlay sample with 20 µl overlay solution and overlay this with upper electrophoresis buffer to the top of the rod.
7. Electrophorese for the folowing period: IEF gels 7,500-9,500 volt-hours (400 or 500 volts for 16 hrs). NEPHGE gels – 2,000-2,5000 volt-hours (500 volts for 4-5 hrs). REMEMBER THE ELECTRODES ARE REVERSED FOR NEPHGE GELS.
8. After electrophoresis the gels are removed by connecting each rod to a 2 ml syringe filled with equilibration buffer (without 2ME) via a length of tubing and applying light pressure. Difficult gels can be removed by running a long narrow needle (A R Horwell) connected to a syringe containing equilibration buffer down between the gel and the rod. When the needle is near the bottom of the rod it is slowly removed while injecting the buffer. The gel should fall from the rod when the needle is removed.
9. The gels should be extruded into 5 ml equilibration buffer. 5% 2ME can then be added and the tube sealed. Equilibration times can be varied from 15 mins to 1 hour. At this point the gels can be loaded onto the 2nd dimension or frozen in equilibration buffer at -70°C.
10. The 2nd dimension gels are similar to normal slab gels made with a flat stack. To ensure good contact between rod and slab gel, the top of the stack must not be wet.
11. Once the rod gel holder is attached to the slab gel molten sealing agar is run along the length of the holder. Ensure that no bubbles are trapped in the agar and that the surface is level.
12. The rod is then poured onto a rectangular piece of teflon and the excess equilibration buffer removed. The rod can then be rolled onto the holder and sealed in pace with more molten agar.
13. Electrophorese at 12 mA/gel overnight or 35 mA/ gel during the day.

Notes:

1. When labelled proteins are analysed unlabelled molecular weight markers can be added to the sample. These proteins can be used to approximate molecular weight and the pI of the labelled proteins if the 2nd dimension gel is stained and orientated with the autoradiogram. 10 µg of markers can be visualised by staining with Coomassie blue. Alternatively molecular weight markers can be incorporated in the sealing agar [4] producing a series of horizontal lines across the whole slab gel giving a more accurate indication of molecular weight.
2. Proteins solubilised in low concentration (<2%) of SDS can also be analysed. SDS will dissociate from proteins in the IEF gel forming a bulge at the acidic end of the gel. Proteins bound to SDS will migrate away from NEPHGE gels due to their negative charge. SDS can be competed out by overlaying the sample with 10 µl 10% NP40, however significant protein loss still occurs. Thus SDS in samples should be avoided if possible for NEPHGE gels and is reported to have detrimental effects on protein patterns in IEF [5].
3. An alternative method of solubilising proteins has been described [6] using 9.5M urea and 5mM K_2CO_3
4. Proteins must not be heated above 37°C when urea is present.

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9. Staining Polyacrylamide Gels for Proteins and Carbohydrates

The original standard stain for PAGE gels, Coomassie Brilliant Blue [1] is a straightforward technique although wanting in sensitivity (**Method 9.1**). More recently a range of silver staining techniques have been developed [2-5] which offer 100-fold higher sensitivities. Of these, the simplest and most economical procedure is that of Merrill et al [5] which is described in **Method 9.2**. Both these techniques are directed at polypeptides, and for staining carbohydrate components, the Periodic Acid-Schiff's (PAS) stain is available. **Method 9.3** is a modification [6] of the original techniques [7,8].

9.1 Coomassie Blue Staining

Reagents:

1. Coomassie Brilliant Blue R-250 (Sigma B-0630)
2. Methanol (BDH)
3. Glacial acetic acid (BDH)

Procedure:

1. Make up stain solution: 5 g Coomassie Blue in 2.5 litres of 25% methanol/ 7.5% acetic acid and filter.
2. Make up destain: 25% methanol, 7.5% acetic acid (same as gel fixer in **Method 7.5**)
3. Immerse gel immediately after electrophoresis in stain for between 5 and 120 mins, then transfer to destain. Agitate continuously, changing destain when necessary. A block of sponge (4 x 8 cm) will absorb stain and speed the desatining process.

Notes:

1. For longer term storage, keep gels in acovered tray of 5% methanol, 7.5% acetic acid, or dry gel onto filter paper (**Method 7,.5**).

9.2 Silver Staining

Reagents:

1. BioRad Silver Stain Kit 161-0443, containing oxidiser, silver reagent and developer. Dilute oxidiser and silver reagent as required; make up stock bottle of developer (115g makes 3.6 L) and store at roomtemperature.
2. Methanol, ethanol and acetic acid.

Procedure:

1. Fix the gel immediately after electrophoresis in 400 ml 40% methanol-10% acetic acid for 30 mins.
2. Immerse in 200 ml 10% ethanol-5% acetic acid for 15 mins twice, to remove excess SDS.
3. Immerse in 200 ml 10% oxidiser for 5 mins (Note 1).
4. Immerse in 400 ml deionized water for 5 mins, twice (Note 6).
5. Immerse in 200 ml 10% silver reagent for 20 mins (Note 2).
6. Immerse in 400 ml deionized water for 1 minute.
7. Immerse in 200 ml developer, 3 times, 30 secs, 5 mins and up to 5 mins respectively
8. Stop development in 400 ml 5% acetic acid for 15 mins and transfer to water.
9. Dry down gel if required.

Notes:

1. Oxidiser is [2] 3.4 mM potassium dichromate (1 g/L) in 3.2 mM nitric acid (215 µl/L)
2. Silver Reagent is [2] 12 mM silver nitrate (2.05 g/L). Some protocols illuminate for first 5 mins on light box.
3. Developer is [2] 280 mM sodium carbonate (29.7 g/L) in 0.5% formalin (0.5 ml/L of commercial formalin = 37% w/v formaldehyde). It takes about 20 minutes to dissolve completely at room temperature. Avoid breathing fumes.
4. Water quality is essential for successful staining. If conductivity is over 1 µmho, deionise water by mixing for 15 mins with Amberlite MB-1 or MB-3 mixed bed resin (1g/100 ml) and filter
5. Wear gloves throughout or fingerprints will develop!
6. Washing in deionised water removes any traces of acetic acid which would stop the developing reaction.
7. Times given are for thin gels (0.5-1.0 mm), thicker gels require approximately twice as long.
8. If bands do not appear after the initial silver staining soak the gel in water for 30 mins and the repeat the process from Step 5. The developing time can be adjusted to prevent overdevelopment and high background.
9. Additional washes at steps 2, 4, 6 may be beneficial.

9.3 Periodic Acid - Schiff Staining

Reagents:

1. Isopropanol (25%)-acetic acid (10%) fixer and acetic acid (7.5%) solution
2. Periodic acid (H_5I_6, Sigma P7875), 0.2% solution.
3. Schiff's Reagent (Sigma S-5133)
4. Sodium metabisulphate ($Na_2S_2O_5$: Sigma S-9000), 0.5% solution

Procedure:

1. Fix slab gel overnight in isopropanol-acetic acid
2. Soak for 30 mins in 7.5% acetic acid
3. Immerse 60 mins in 0.2 % periodic acid at 4°C
4. Immerse immediately, without washing, in neat Schiff's Reagent, 60 mins at 4°C in dark (cover with foil)
5. Fix in three changes of 0.5% metabisulphate, 10 mins each, in dark
6. Rinse and store in 7.5% acetic acid, photographing if desired

Notes:

1. Gels cannot be dried on filter paper as residual stain colours paper
2. Fuschin-sulphate solution may be substituted for Schiff's Reagent: Dissolve 16 g potassium metabisulphate and 60 g sodium thiosulphate in 2 litres of water; add 21 ml of concentrated HCl and 8 g basic fuschin (Pararosanaline, Sigma P-7632). Stir 4 hrs, room temperature. Add decolourising charcoal and filter 15 mins later. Store at 4°C.
3. Potassium metabisulphate may be substituted for the sodium salt in Step 5.
4. A combined periodic acid-silver stain has been described [6] in which periodate-oxidised carbohydrates react with an ammoniacal silver solution used in some protein-silver stains [3].

9.4 References

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10. Autoradiography and Fluorography

Direct autoradiography records photographically the presence of radioisotopes such as ^{125}I and ^{32}P. A number of modifications to this technique have been developed which permit both more efficient detection of these isotopes by indirect autoradiography of dried PAGE slab gels (**Method 10.1** [1,2]) and the detection of low energy beta emitters such as ^{3}H, ^{14}C, and ^{35}S by fluorography (**Method 10.2** [1, 3, 4]).

In indirect autoradiography, intensifying screens are placed behind the autoradiographic film to capture high energy beta (^{32}P) or gamma (^{125}I) rays which would otherwise not be recorded [1,2]. In fluorography, the gels are impregnated prior to drying with a scintillant to detect beta particles of too low an energy to reach the film [1,3].

In both indirect autoradiography and fluorography, sensitivity is greatly enhanced by using film which has been briefly pre-exposed, and by exposing plates at -70°C [1, 4].

10.1 Autoradiography

Reagents and Equipment:

1. X-ray film (Fuji RX or Kodak X-omat) and cassettes, 18 x 24 cm.
2. Intensifying screens (eg Ilford Fast Tungstate or DuPont Lightning Plus)
3. General purpose developer and fixer (eg Ilford PQ and Hypam)
4. Low-intensity flash gun, with filters (eg Kodak Wratten Nos 21 or 22)

Procedure:

1. Flash X-ray film to increase sensitivity [1]. Aim to fog film to 0.1 above base density with shielded flash gun: at this density, the outline of a coin or key laid on the film can barely be made out.
2. Place film on intensifying screen mounted in cassette, with flashed side face down on the screen. Place the dried gel (**Methods 7.5**) or NC blot (**Method 15**) on the upper (unflashed) side of the film and close the cassettes.
3. Incubate the cassette for 1-3 days at -70°C.
4. Recover the film and develop (5 mins in Universal Developer at 1/4 dilution at 20°C), briefly immerse in stop solution (1.25% acetic acid), fix (5 mins Fixer at 1/10), wash well in distilled water and allow to dry.
5. Re-expose gel or blot as indicated by the result of the autoradiograph.

Notes:

1. Indirect autoradiography with intensifying screens reduces exposure time by about tenfold for ^{125}I, but introduces some loss of resolution. Exposures without screens may be used for samples with sufficient radioactivity, in which case unsensitised film is used and autoradiographs may be incubated at room temperature.
2. Safelights for X-Ray film should be Ilford NX914 or Kodak 6B.
3. Automatic processors (eg Gevamatic 2200) are available for X-ray films.

10.2 Fluorography

Reagents and Equipment:

1. X-Ray film, cassettes and chemicals as in **Method 10.1**
2. One of the following scintillants for impregnating gels:
   (a) Amplify (Amersham NAMP. 100)
   (b) EnHance (New England Nuclear) or
   (c) A cocktail of DMSO (dimethylsulphoxide) and PPO (2, 5-diphenyloxazole)
   This protocol is based on the Amplify reagent which is faster and safer than the alternatives.

Procedure:

1. Run PAGE slab gel as in **Method 7** or **8** and fix for 30 mins or overnight, as convenient. Change and discard fixer once and remove any free counts and so prevent high background.
2. Immerse gel in Amplify for 30 mins. Re-use reagent.
3. Recover gel and dry immediately at 60°C.
4. Expose impregnated gel to autoradiography as in **10.1**, omitting intensifying screens, and placing flashed side of film next to gel.

Notes:

1. EnHance is used for 60 mins on fixed gels, and requires a 60 min rehydration step in water prior to drying. Use in a fume cupboard.
2. The cocktail procedure entails two 30 min incubations in DMSO followed by 3 hours in 20% PPO in DMSO and 60 mins in water prior to drying. Perform in a fume cupboard.
3. A spray form of EnHance is available to impregnate nitrocellulose paper with scintillant (NEN Cat No. NEF-970)

10.3 References

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11. Protein Cleavage and Peptide Maps

The cleavage of proteins at defined sites to produce derivative peptides may be useful for both analytical and preparative purposes. In this section, emphasis is given on the analytical approach (**Method 11.2**) in which the pattern of partial digests with one protease (*Staphylococcus aureus* V8 protease) is generated as a "fingerprint" of an individual protein [1]. However, the same or different reagents may also be used to prepare peptides for immunological or chemical studies.

11.1 Agents Cleaving Proteins at Defined Sites

(see also **Method 30**)

Agent/Enzyme	Cleavage Site	Notes	Reference
BPNS-skatole (Pierce)	Trp-X	Often low yield	[2]
Cyanogen Bromide	Met-X	Some bonds resistant; Met. converted to homoserine	[3,4]
Trypsin	Arg-X, Lys-X		[5]
V8 Protease	Asp-X, Glu-X		[6]
Hydroxylamine	Asn-Gly		[7]

Note that CNBr is **extremely** hazardous on inhalation.

11.2 One-Dimensional Peptide Map

A "fingerprint" of proteins may be produced by partial digestion with a number of proteases as even a small number of possible cleavage sites will generate a large number of peptides before digestion is allowed to go to completion. These peptides can readily be analysed by SDS-PAGE (**Method 7**), and side-by-side comparisons of different proteins will quickly reveal any close structural relationship. The method below is applicable to a range of enzymes [1,5,8], but is given for *S. aureus* V8 protease, an SDS-resistant, stable enzyme which cleaves on the C-terminal side of Asp and Glu residues [6].

Reagents:

1. *S. aureus* V8 protease (V8P) from Boehringer Mannheim.
2. Radiolabelled proteins to be digested.

Procedure:

1. Set up 6 aliquots of labelled protein in eppendorf tubes and heat all samples, 100°C, 3 mins.
2. Leaving one tube as control, add V8P to final concentrations of 10, 32, 100, 320 and 1000 µg/ml. Incubate all tubes for 30 mins at 37°C
3. Heat all samples, 100°C, 3 mins, add SDS-sample buffer (containing 2ME), heat for a further 3 mins
4. Run on SDS-PAGE 5-25% gel (**Method 7.2**) or 18% homogeneous gel (**Method 7.3**).

11.3 References

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12. Covalent Coupling of Enzymes, Haptens etc. to Proteins

12.1 Conjugation of Enzymes to Antibodies

As an alternative to radiometric assays, an enzymatic label may be attached to antibody probes so that a powerful range of techniques such as ELISA (**Method 17**) become available. Although widely used reagents such as enzyme-linked anti-immunoglobulins are commercially available, for many research purposes antibodies need to be conjugated in the laboratory. This method, the two-step glutaraldehyde conjugation [1], proved most successful in our laboratory for the conjugation of peroxidase to a monoclonal IgM antibody. This method exploits the fact that glutaraldehyde will not cross-link peroxidase which can therefore be prepared in an "activated" state before addition of immunoglobulin molecules [1].

Reagents:

1. Horseradish Peroxidase Type VI (Sigma P-8375), 2000 Units
2. 0.1 M phosphate buffer pH 6.8
3. Glutaraldehyde (25%) (BDH 36080 2F)
4. Sepharose G-25 (Pharmacia) 5 ml column in saline; prepacked PD-10 columns (Pharmacia) are suitable.
5. 1 M carbonate buffer pH 9.5
6. 0.2 M lysine (36.5 mg/ml)
7. Saturated ammonium sulphate

Procedure:

1. Weigh out contents of peroxidase vial (approx. 75 mgs) and dissolve in 200 µl 0.1 M phosphate buffer containing 10 µl of 25% glutaraldehyde. Stand overnight at room temperature.
2. Desalt on a G25 column in saline, recovering exclusion volume which will be brown in colour.
3. Add 5 mg of antibody in 1 ml saline together with 100 µl 1 M carbonate buffer, pH 9.5., for each ml of peroxidase. Incubate for 24 hours at 4°C.
4. Add 100 µl of 0.2 M lysine, leave for 2 hrs at room temperature
5. Dialyse against three changes PBS, or Amicon diafiltrate with a PM100 membrane
6. Precipitate at 4°C with an equal volume of saturated ammonium sulphate; stand for 20 minutes
7. Wash precipitate twice in 50% saturated ammonium sulphate
8. Amicon diafiltrate against three changes of PBS (PM100 membrane)
9. Concentrate to desired final volume and add 0.1% BSA to stock. Aliquot and freeze

Notes:

1. Peroxidase has a molecular weight of 40,000 and a characteristic absorbance peak at 403 nm.
2. A range of other enzymes may be coupled to antibodies by various techniques [2]. Alkaline phosphatase can be conjugated to immunoglobulin by co-incubation of 15 mgs and 5 mgs respectively in 0.2% glutaraldehyde for 2 hours [3] and this method is widely applicable to many protein-ligand pairs.

12.2 Conjugation of Phosphorylcholine to Proteins

Phosphorylcholine (PC) is present in a wide range of nematode species [4,5] as well as many other organisms. A conjugate of PC to a carrier protein such as bovine serum albumin is useful to measure anti-PC antibodies by ELISA or other techniques. This method, adapted from that published by Péry [6] is based on an older protocol for coupling nucleotides to proteins [7].

Reagents:

1. Cytidine-5'-diphosphocholine (CDPC, Sigma C-0256), MW 488
2. Sodium periodate ($NaIO_4$), 0.1M in water (214 mgs/10 ml)
3. Ethanediol, 1 M (558 µl/10 mls)
4. Protein to be conjugated. BSA is obtained from Sigma (A-7906)
5. 10 mM Na_2CO_3-$NaHCO_3$ buffer pH 9.6 (0.3 mls 1 M $NaHCO_3$ + 0.7 ml 1 M Na_2CO_3 in 100 mls H_2O)
6. 5% (0.47 M) Na_2CO_3
7. Sodium borohydride (Na BH_4), fresh 0.2 M solution (757 mgs/100 ml)
8. 1 M formic acid (1.89 mls/50 ml)
9. 1 M NH_4OH (6.65%)
10. Sigma Antifoam (Sigma A-5632)

Procedure:

1. Dissolve 50 mgs of CDPC (approx 100 µMoles) and dissolve in 2.5 ml of 0.1 M $NaI0_4$.
2. Incubate 20 minutes at room temperature, in sealable flask, stirring , with pH probe inserted.
3. Add 150 ml of 1 M ethane diol.
4. Dissolve approx 2 µMoles of protein (for BSA=140 mgs) in 5 mls of 10 mM carbonate buffer pH 9.6
5. Slowly add CDPC to the protein solution together with 5% Na_2CO_3 to maintain pH at 9.6
6. Proceed for 1 hour, maintaining pH 9.6
7. Add 5 mls of 0.2 M $NaBH_4$ and 1-2 µl of Sigma Antifoam (pH will rise to 10.5).
8. Stand overnight at room temperature.
9. Add 2.5 mls 1 M formic acid. The pH will drop to 4.0 and both effervescence and precipitation may occur.
10. Stand for 1 hour.
11. Neutralise with approx. 0.9 ml 1 M NH_4OH.
12. Centrifuge to remove unresolubilised precipitate.
13. Dialyse or diafiltrate against PBS to remove all unwanted salts and reagents.

12.3 References

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13. Polyclonal Antibody Production

The huge variety of customised protocols for generating for generating polyclonal antibodies probably reflects the ability of the immune system to mount a response under nearly any circumstances. To optimise this response in the form of a high serum concentration of specific antibody, the general rules are to use a moderate antigen dose (10-100 µg for a mouse, 100-500 µg for a rabbit), administered with adjuvant (eg Freund's) at least for the primary immunization, and to allow a 1-6 month resting period to generate long-lived immunological memory. Freund's adjuvant is mineral oil combined with ("Complete") or without ("Incomplete") killed mycobacteria. An emulsion of soluble protein in the oil provides a depot of antigen at the injection site, while the mycobacteria enhance the nonspecific activation of macrophages partcipating in the response.

Reagents:

1. Complete Freund's Adjuvant (Sigma F-4258)
2. Incomplete Freund's Adjuvant (Sigma F-5506)
3. Syringes 2ml
4. 3-way Leur fitting stopcocks, Pharmaseal K 69B (American Hopsital Supplies, Didcot)

13.1 Mouse Polyclonal Antiserum

Procedures:

1. Make an emulsion of equal volumes of antigen solution (typically 1 mg/ml) and CFA
2. Inject 0.2 ml of antigen(100 µg) in CFA, ip into each of 4-5 mice.
3. After 4 weeks inject 0.2 ml in IFA
4. Bleed 4-5 days after challenge. Boost at fortnightly intervals if required

13.2 Rabbit Antiserum: Fast Protocol

Procedures:

1. Make an emulsion in CFA as above, mixing 1 ml CFA with 1 ml of antigen at 200 µg/ml
2. Inject 1 ml (100 µg) intramuscularly in the upper leg
3. One week later, inject another 1 ml in CFA in the other leg
4. After 6 weeks inject emulsion in IFA into 3-5 subcutaneous sites
5. Bleed 7 days later
6. Repeat bleeds, boosting if titre drops

13.3 Rabbit Antiserum: Slow Protocol

Procedures:

1. Inject emulsions in CFA at 0 and 1 weeks as in (1) above
2. Rest animal for 3 months
3. Challenge with 100 µg soluble antigen i.v.
4. Bleed 7 days later
5. Repeat bleeds, boosting if titre drops

Notes:

1. If quantities of antigen are limiting, direct injection of 10 µg of protein in CFA into the popliteal lymph node of a rabbit may efficiently prime the immune response [1]. Challenge with subcutaneous injection as in **Method 13.2**
2. Antibodies may be raised to insoluble proteins, particles or resuspended nitrocellulose blots mixed with adjuvant. A method for dissolving nitrocellulose bound with blotted proteins in DMSO prior to immunisation has been described [2].

13.4 References

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14. Monoclonal Antibody Production

The generation of monoclonal antibodies [1-3] involves a sequence of different techniques, many of which vary somewhat between laboratories. The procedure as a whole has been subdivided here into the following sections:

14.1 Immunisation Protocols
14.2 Media for Cell Culture
14.3 Sterility
14.4 Myeloma Fusion Partners
14.5 Feeder Cells
14.6 Hybridisation by Cell Fusion
14.7 Culture and Feeding Hybrid Cells
14.8 Screening
14.9 Cloning
14.10 Ascitic Fluid Production
14.11 Cryopreservation
14.12 Isotype Determination
14.13 Specificity Analysis

A large literature exists on these techniques, many of which are brought together in Methods in Enzymology, volume 121 (1986).

14.1 Immunisation Protocols

The standard mouse strain for monoclonal immunisation is the BALB/c as this is the genotype of the available myeloma cell fusion partners [3]. Hybrids between genetically different cells grow equally well *in vitro* but growth and ascitic fluid production *in vivo* requires histocompatible hybrid (F1) mice.

Immunisation protocols vary infinitely, but a standard regimen is to inject 10-100 µg of antigen i.p. in Freund's Complete Adjuvant (Sigma F-4258), followed at days 28, 29 and 30 by 1 µg soluble antigen i.v. Spleen cells are taken for fusion 2 days after the final injection.

Faster, and more economical, immunisation procedures are to inject small quantities (20 µg) of antigen directly into the spleen 4 days pre-fusion [4], or to sensitize normal lymphocytes *in vitro*, a procedure which may be more effective for poorly or non-immunogenic targets [5].

14.2 Media for Cell Culture

Three related culture media are involved in monoclonal production, centering around the ability of aminopterin to block major pathways of nucleic acid synthesis. Normal cells possess HGPRT (hypoxanthine guanine phosphoribosyl transferase) and thymidine kinase which provide bypass pathways for normal growth, but as the myeloma cell partner has been selected as HGPRT –ve, it is killed in the presence of aminopterin. Myeloma cells are therefore grown in basic tissue culture medium. Immediately after fusion, unfused myeloma cells are killed by culture in medium containing hypoxanthine, aminopterin and thymidine (HAT). Cells are grown in HAT for two weeks, followed by two weeks in HT (hypoxanthine, thymidine without aminopterin) to ensure no residual poisoning of hybrid cells occurs.

Tissue Culture Medium (TCM):

RPMI 1640 with HEPES	(Gibco 041-2400-M)	500	ml
Glutamine 200 mM	(Gibco 043-5030-H)	5	ml
Pyruvate	(Gibco 043-1360-H)	5	ml
Penicillin-Streptomycin	(Gibco 043-5140-H)	5	ml
Foetal Calf Serum, Myoclone	(Gibco 011-6180-H)	100	ml

Filter through 0.2µ filter (Nalgene 120-0020) and store at 4°C for up to 7 days.
Add additional glutamine to older media.

HAT Medium:

Tissue Culture Medium		600	ml
HAT (50 X Concentrate)	(Gibco 043-1060-H)	12	ml

HT Medium:

Tissue Culture Medium		600	ml
HT (50 X Concentrate)	(Gibco 043-1065-H)	12	ml

Notes:

1. Gentamycin (Gibco 043-5710-D) is often preferred to Penicillin-Streptomycin. Use at 10 µg/ml or 0.5 ml of 10 mg/ml per 500 ml medium.
2. Fungizone may of slight value in coping with fungal contamination.
3. Serum substitutes are available, reviewed in Reference [6]
4. Sterility test all batches of medium by incubating 10 ml in screw-top flask at 37° C for 3-4 days prior to use.
5. Sigma produces 50x HAT (H0262) and HT (H1037). Use 1 vial (10 mls/lyophilised) per 500 ml. RPMI.

14.3 Sterility

Always follow sterile procedures. Carry out all non-animal procedures in laminar flow hoods in a dedicated tissue culture room and use a separate incubator for each worker. All media should be filtered after making up even though each individual component is nominally sterile. Contaminations require drastic responses. Fumigate culture room if contamination persists:

mix - 5 g paraformaldehyde powder
5 g (or 7ml tube of crystals) potassium permanganate
7 ml water

Seal room and leave overnight or longer.

14.4 Myeloma Fusion Partners

One cell line in wide use is the non-secreting, non-heavy or light chain synthesising line Ag8, i.e. P3X63-Ag8.653 [3,7]. Others in use include NS-1, which retains light chain synthesis; fuller details are given in references [3,6]. Obtain the cell line either cryopreserved (see section **14.10**), or growing in a flask when they will be tolerant of short-term temperature drops during transfer between laboratories. Commence the cycle by adding 1 vial (10^7 cells) to 10 ml of TCM, then passaging after two days.

The growth cycle of myeloma cells *in vitro* follows a 4-5 day cycle. Seeding cells out in 30 ml at 2 x 10^4 per ml (total 6 x 10^5) will yield a flask at 8 x 10^5 per ml (total 2 - 3 x 10^7) after 5 days.
Approximately 2 x 10^7 myeloma cells will be required for a single fusion, and it is important that these cells are in logarithmic growth at the time of fusion: therefore set up 4 flasks at 2 x 10^4/ml 3 days before fusion and expect each to yield 5 x 10^6 total cells.

14.5 Feeder Cells

Single cells require feeder cells for effective growth. Therefore whenever plating out cells immediately following fusion, or when cloning by limiting dilution, place cells in wells containing feeder cells. Peritoneal macrophages are the most effective feeder cells. It is often most convenient to use BALB/c cells, but allogeneic (eg CBA) or even xenogeneic (rat) cells are equally good. For mice:

1. Kill mice by cervical dislocation and pin out. Douse in 70% ethanol. Do not take into tissue culture facility. Perform on open bench or, better, in a flow hood outside the culture room.
2. Carefully cut back abdominal skin without puncturing peritoneal membrane. Again douse in ethanol, and place ice on peritoneum.
3. Inject with 27 G (0.4 x 12 mm) needle 5 ml of ice-cold RPMI 1640 without serum but containing 50 U/ml Heparin (Evans sodium heparin injection BP, 25000 U/ml).
4. Withdraw needle and massage peritoneum manually. Use ice and alcohol liberally.
5. Insert 21 G (0.8 x 40 mm) needle and slowly withdraw fluid. Try to keep the bevel in sight through the peritoneal membrane. Take care not to block needle with peritoneal contents. Expect to recover about 4 ml fluid.
6. Pool cell suspensions, wash (centrifuge 5 mins at no more than 400 g) and resuspend in HAT or HT for fusion mixtures and clones respectively.
7. Expect to recover between 1 - 3 x 10^6 cells per mouse, and plate out at 10^4 per well in 100 µl. For each mouse, resuspend cells in 20 ml and distribute across two 96 well plates.

14.6 Hybridisation by Cell Fusion

1. Warm pre-packaged PEG 1500 (Boehringer Mannheim 779 512)
2. Set up 37°C water bath or beaker in flow hood to accomodate fusion tube.
3. Prepare spleen cell suspension from 2 immunised mice. Collect serum from the donor mice (if long-term immunized) to assess antibody levels at the time of fusion. After cervical dislocation, soak in 70% ethanol and cut abdominal skin. Douse exposed peritoneal membrane in ethanol and, using fresh instruments cut the membrane above the spleen, on the left hand side. Excise spleen and transfer to 35 mm petri dish containing 1 ml of medium. Ideally, have a 'dirty' flow hood outside the tissue culture suite for this operation.
4. In a 'clean' flow hood make a suspension with scissors and scalpel. Transfer cells to 10 ml conical, stand for 5 minutes to allow clumps to settle, and transfer the supernatant to a fresh tube. Centrifuge 5 mins at 400 g and resuspend the cells in serum-free RPMI 1640. Count an aliquot for viability with trypan blue.
3. Mix cell suspensions containing cells from one spleen and 2 x 10^7 myeloma cells. Co-pellet in a 50 ml conical tube.
4. Resuspend pellet and hold in 37°C water bath for 1 min
5. Add PEG dropwise over 2 mins, mixing continually but gently.
6. Add 2 ml of serum free medium dropwise over 2 mins in the same manner.
7. Gradually accelerate the addition of medium so that a total of 20 ml is added over 10 minutes.
8. Spin down, resuspend the pellet in medium and check for viability and fusion by trypan blue.
9. Meanwhile, remove supernatants and nonadherent cells from feeder layer paltes. Then resuspend fused cells in 200 ml HAT for distribution across 10 96-well plates (200µl per well).
10. Place plates in 37°C incubator until feeding is required (see below).

Notes:

1. If prepackaged PEG is not available, take 1 gm of PEG 1500 (BDH)and add 1 ml of RPMI 1640 and 200 µl of DMSO (Sigma D-5879). Autoclave in glass bottle and store at room temperature until needed.

14.7 Culture and Feeding Hybrid Cells

1. At day 5 remove 100 µl from each well and replace with fresh HAT medium
2. Repeat at day 10. Look for growing clones each day hereafter.
3. On day 14 remove 100 µl from each well, retaining supernatants from wells containing hybrids for screening (see below). Feed all cultures with 100 µl of HT (not HAT).
4. Continue feeding cultures with HT up to day 28, thence with Tissue Culture Medium.

14.8 Screening

A variety of techniques are available for screening hybrid cells for specific antibody production. The most important considerations are that any assay provide quick results and be able to handle hundreds of samples at any one time. For these reasons, ELISA is the screening method of choice. However, more selective screening may be achieved by immunofluorescence on intact organisms, immunoprecipitation with labelled antigens and Western blotting on prepared strips. The most practical approach to ELISA screening is to transfer 100 µl of superantants with a multichannel pipette to the corresponding wells of an antigen-coated ELISA plate.

14.9 Cloning

Positive wells in the primary screen may contain both positive cells and non-secreting cells which will outgrow the secretors in time, or independently derived cells producing an irrelevant specificity. For these reasons, cloning should be accomplished as soon as a positive result is obtained. Cloning may be undertaken either by counting cells and distributing less than 1 per well, or by serial dilution of a cell suspension. The latter is less precise, but faster and not subject to vagaries of cloning efficiency. Two rounds of cloning, the first at a higher cell density, may be employed to guarantee monoclonality.

1. Harvest cells from each positive well when nearing confluency (> 10^5 cells)with a sterile Gilson and transfer to a plastic tube
2. Count a sample
3. Make dilutions of 50, 20, 5 and 2 cells/ml each in 5 ml volumes. Transfer excess cells to wells of a 24-well plate. Distribute each dilution among 24 wells of a 96-well plate. All plates should have been seeded with feeder cells, preferably the previous day.
4. Feed wells as above, taking supernatants for testing when wells are more than half confluent.
5. Select two or three positive wells which are certain to be monoclonal by microscopic examination and dilution. Transfer these to 24-well plates, and after a few days to 25 cm^2 flasks, then 75 cm^2 flasks. Always set up duplicate cultures in case of contamination.

14.10 Ascitic Fluid Production

1. Inject 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane: Aldrich T 2,280-2) ip into BALB/c mouse or into F1 hybrids if non-BALB/c splenocytes used.
2. Transfer 1-4 x 10^6 hybridoma cells ip 7 to 21 days later. Days 10-14 are reported to be optimal [8,9]
3. From 7 days post passage, check mice daily for abdominal swelling. When swollen, tap fluid with 18 G needle, collecting into 10 ml tube containing 500 U heparin. It should not be necessary to kill the mice, from which further fluid can be collected every 2 days.
4. Centrifuge gently to pellet hybridoma cells which can be passaged to a fresh recipient or cryopreserved (See below). Note that passage of ascites cells usually produces a more rapid tumour than cells from culture. Keep a record of the passage number in case antibody secretion is lost.

Notes

1. Incomplete Freund's Adjuvant (Sigma F-5506) has been reported to be equally effective as Pristane [10].
2. Don't inject the entire supply of a cell line into a single mouse!
3. Wash hybridoma cells in serum free medium for injection to avoid raising anti-FCS antibody in ecipient mice
4. Ascites fluid may contain up to 10 mg/ml of monoclonal antibody but often includes much lipid and congulated particles. Filtration through glass wool has been reported to remove lipid aggregates. The fluid should then be centrifuged (10,000 *g*, 10 mins).
5. If solid tumours form, lavage peritoneum with PBS and collect fluid to obtain antibody [11].

14.11 Cryopreservation

1. Count cell suspension, and centrifuge.
2. Resuspend in cold neat FCS, keep on ice and when ready to freeze add an equal volume of cold 20% DMSO (D-5879) in Tissue Culture Medium.
3. Aliquot into Cryotubes (Nunc 3-68632-A) at 10^6- 10^7 viable cells per tube
4. Place in a slow freezer, either Union Carbide HandiFreeze in liquid nitrogen vapour, or in a polystyrene box within a -70°C deep freeze.
5. Thaw quickly by rapidly placing in 37°C water bath add warm medium and transfer to 10 ml conical with warm medium.

Note

1. Minimal volumes (eg 0.2 ml) are preferable as thawing will be more rapid and survival increased.

14.12 Isotype Characterization

It is important to determine the class and subclass of any monoclonal antibody, and this analysis is best performed on culture supernatants where no host immunoglobulins are present. Use a Serotec Mouse Monoclonal Typing Kit (MMT OIK).

14.13 Analysis of Monoclonal Specificities: Inhibition Assay

Like polyclonal antibodies, monoclonals (MAbs) can be used in assays such as Western blotting (**Method 15**), Immunoprecipitation (**Method 16**), and Immuno-microscopy (**Methods 20** and **21**); however additional assays can take advantage of the single specificity of a monoclonal reagent. In this inhibition assay, the ability of different antibodies (MAb vs. MAb or MAb vs Polyclonal) to inhibit each other is measured. This assay is based on that used for *Toxocara canis* and *Dirofilaria immitis* MAbs [12,13].

Procedure

1. Coat ELISA or Immunoassay plate with limiting concentration of antigen (50-1000 ng/ml). Antigen excess will reduce the sensitivity of the assay.
2. Add serial dilutions of 'blocking' antibody (MAb or PAb). Incubate for 60 mins at 37°C.
3. Add ^{125}I labelled monoclonal at a dilution pre-determined to give 50-75% of peak binding. Incubate for 60 mins at 37°C.
4. Wash and count wells. Calculate % inhibition as $\frac{\text{Control cpm - Test cpm}}{\text{Control cpm}} \times 100$

14.14 References

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15. Immunoblotting from Polyacrylamide Gels: Western Blotting

The electrophoretic transfer of antigens from PAGE slabs to paper on which they may be tested immunologically was originally prepared by Southern's DNA blotting technique. [1]. This elegant approach, which permits the immunological analysis of each polypeptide band in a complex mixture after separation on PAGE gels [2,3], has generated a range of transfer procedures, paper grades, and techniques for visualisation of bound antibodies [2-10]. Antigens insolubilised on nitrocellulose can also be used to activate T lymphocytes after presentation by suitable cells [11].

As an alternative to immunoprecipitation (**Method 16**), immuno-blotting is often preferred, but the crucial difference is that immunoprecipitation can be performed on trace of radiolabelled antigen (**Methods 3, 4, and 6**) whereas blotting requires microgram quantities of antigen for each gel on track. A further factor is that many monoclonal antibodies bind well in immunoprecipitation but fail to react on blots. This is because two types of epitopes are destroyed during electrophoresis:

[a] Sites formed by the combination of or at the interface between subunits of different molecular weight.

[b] Conformational epitopes abolished by exposure to SDS.

The procedure outlined here (**Method 15.1**) is for antigens in the most common molecular weight range (30-100 K) and employs an enzyme-linked reagent to reveal binding [12]. **Method 15.2** deals with molecules outside this range, and **Method 15.3** with the use of radiolabelled probes for antibody binding.

15.1 Enzyme-Linked Immunoblotting

Reagents and Equipment:

1. Semi-dry blotting apparatus such as LKB Novablot (2117-250) and power supply capable of producing at least 200 mA.
2. Nitrocellulose (NC) paper, from Schleicher and Schüll (BA 85) or Millipore (HAWP 304 P)
3. Whatman 3 MM filter paper.
4. Transfer Buffer:

28.83 g	Glycine	(Final concentration 192 mM)
6.05 g	Tris-base	(25 mM)
400 ml	Methanol	(20%)
2.0 g	SDS*	(0.1%)

   * optional see **Method 15.2.** Make up to 2 litres with water pH 8.3.
5. Blot Solution A: 5% Normal rabbit serum, foetal calf serum or skimmed milk powder (Blotto) [13] pH 7.4 in PBS
6. Blot Solution B:

1.00%	Normal rabbit serum or Blotto
0.10%	Triton X-100
0.05%	Tween 20 in PBS

7. Washing solution: PBS, 0.10% Triton-X100, 0.05% Tween-20 (PBS-TT).
8. Peroxidase-conjugated anti-immunoglobulin. Available from BioRad cat. nos.: mouse (172-1011), rabbit (172-1013), human 172-1001).
9. 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma D-5637)
10. Rocking table (eg Luckham RT4), shaker or rotator.
11. Incubation trays (eg. BioRad, Luckham or homemade). Alternatively a bag sealer (domestic model) and polythene bags (Jencon).
12. Amido black stain (Sigma N3005): 1% stain in 35% glacial acetic acid makes a 5x stock solution.

Procedure:

1. Run PAGE slab gel as in **Method 7**, loading 1-10 µg of protein per well, or cast gel without well-former and load protein across whole surface of gel.
2. Soak 8 pieces of 3MM paper cut to the size of the gel in transfer buffer. Place 4 pieces on the lower (+) carbon electrode taking care to eliminate bubbles, then place wet NC paper on top. Place gel on top of NC and the remaining 4 pieces of wet filter paper on top of the gel,again making sure no bubbles are trapped. Place the upper (–) carbon electrode on the sandwich, put on the lid and attach the electrodes. Transfer for 2 hours at 0.8 mA per $cm^2$of gel.
3. Recover NC paper and remove strip containing marker proteins, quench the rest of the NC in Blot Solution A for 2 hrs at room temperature, or overnight at 4°C. The marker proteins can be visualised by staining for 10 minutes in amido black, and destaining in water as required.
4. Rinse in PBS-TT, 3 washes of 10 mins each.
5. Incubate in antibody diluted in Blot solution B: 2 hrs at room temperature or overnight at 4°C, with constant agitation. A typical dilution for polyclonal antiserum would be 1:100. Either use incubation trays or place the NC in a plastic bag cut to size and sealed with a bag sealer.

6. Rinse as in step 4.
7. Incubate in peroxidase-conjugated antibody, diluterd to the manufacturers recommedation in Blot Solution B, for 60 mins, or in a radiolabelled affinity-purified antibody or protein A (note 7)
8. Rinse as in Step 4, with a final wash in PBS to remove detergent inhibiting the peroxidase enzyme.
9. Incubate in 3,3'-diaminobenzidine substrate: per 100 ml mM Tris pH 7.6 add 50 mg of DAB and 10 µl of 30% hydrogen peroxide. Agitate gently for 30 mins, or less if reaction proceeds rapidly. Rinse in water and allow the NC to air dry.

Notes:

1. Blotting times and voltages will vary for each set of antigens and should be verified in preliminary experiments: larger molecules require considerably more volt-hours (**Method 15.2**)
2. Likewise, optimal dilutions for antisera and conjugated antibodies should be determined under laboratory conditions.
3. A good alternative substrate is 4-chloronaphthol [13] (Sigma C-8890). Make a stock soluton of 30 mgs in 10 ml ethanol, and a working substrate of 100 ml PBS, 100 µl hydrogen peroxide and 2 ml 4-chloronaphthol stock solution. Note that this substrate yields a particularly light-sensitive product.
4. A method using alkaline phosphate-conjugated antibody, and B-naphthyl phosphate/Fast Blue B substrate, has been described [4]
5. Omit azide from all buffers when peroxidase reactions are to be used.
6. Adding final concentrations of 0.03% cobalt chloride and nickel ammonium sulphate has been reported to raise sensitivity significantly [5]
7. If using radiolabelled antibody (**Method 15.3**) incubate overnight at 4° C.
8. Zwittergent has been recommended in place of SDS in blot buffer (6).
9. The efficiency of binding of strongly basic proteins is relatively low; however it has been reported that replacement of the transfer buffer with 25 mM ethanolamine/ glycine pH 9.5, containing 20% methanol is beneficial.
10. Submerged or wet blotting provides an alternative to the semi-dry method described above, but is considerably less economical on transfer buffer. Briefly, blotting apparatus either LKB 2005 Transphor unit, or BioRad 170-3919 Trans-Blot system, and accompanying sponge pads and gel sandwich holder are required. After electrophoresis, the gel is placed carefully on a sheet of NC paper, eliminating air bubbles. Place three pieces of wet Whatman paper on the other side of gel and use additional sheets to ensure gel sandwich is firmly placed in holder between sponges (Bio-Rad 170-3908) and/or 'Scotch-brite' (Bio-Rad 170-3909) pads. Insert holder in blotting apparatus, making sure NC paper is on the anodic (+ red) side of the gel. It may help to always place the gel on the black half of the sandwich and insert in the tank aligned with the black (–) terminal. Fill tank with transfer buffer and commence cooling. Transfer overnight at 30V, 0.1-0.15 A or 4-5 hrs at 60V, 0.2 A.

15.2 High and Low Molecular Weight Antigens

Improving transfer of large (>100,000 kDa) antigens requires (a) raised transfer voltages and times; (b) addition of 0.1% SDS to the transfer buffer; and (c) the use of thin (<1mm) slab gels. Semi-dry blotting which includes SDS seems to transfer high molecular weights adequately. Recovery of low molecular weight antigens may be improved by using activated paper to covalently bind proteins in place of NC paper to which proteins may adsorb and desorb nonspecifically. The most widely used papers are diazobenzyloxymethyl (DBM) paper [3], diazophenylthioether (DPT) [7] and zeta bind filters [8]. A further advantage of covalently binding papers is that they can be re-used after removing the first round of antibody with 8 M urea and 0.1 M mercaptoethanol [3]. Some antigens, particularly when heavily glycosylated, do not bind directly to nitrocellulose. However, if the NC paper is precoated with specific antibody, an affinity transfer to paper can be achieved (see **Method 27**) [15].

15.3 Autoradiographic Techniques

Reagents and Equipment:

1. ^{125}I-labelled anti-immunoglobulin, Protein-A or other probe (eg lectins) iodinated as in **Method 6.**
2. X-Ray Film, cassettes and chemicals (see **Method 10.1**)
3. Add 0.1% sodium azide to buffers as preservative

Procedures:

1. Carry out electrophoresis as in **Method 15.1.**
2. Quench NC paper in Blot Solution A.
3. Rinse and treat with test antibody in Blot Solution B.
4. Rinse and incubate with 10^5-10^6 cpm/ml of radiolabelled probe in Solution B.
5. Wash in 6 changes of PBS-TT, 10 minutes each
6. Ensure paper is completely dry and set up autoradiograph for 24 hrs at -70°C; develop as in **Method 10.**

Notes:

1. The autoradiographic technique has the advantages that, although slower to yield a result, the exposure may be varied to suit the level of binding, and by using labelled antigen in one track the electrophoretic transfer may be verified.
2. Backgrounds may be minimised by using normal serum of the same species as the radiolabelled antibody in Blot Solution B
3. Inclusion of 0.02% SDS in Solution B and in rinses also reduces background levels [8]
4. Iodinated [16] or peroxidase-conjugated [10] lectin may be used as in a single step in place of steps 3 and 4. When using lectins, carrier proteins should not be glycosylated and haemoglobin should be used in place of normal serum.

15.4 References

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16. Immunoprecipitation

Immunoprecipitation of labelled parasite antigens by antibody may be used to test either the level of antibody (radioactivity precipitated) or its specificity (by analysis of precipitated bands) [1]. This protocol is an assay of the second type, and uses Staphylococcal Protein A to precipitate antigen-antibody complexes.

Reagents:

1. Antigen, labelled with ^{125}I, ^{35}S, etc
2. Test and control sera
3. PBS-0.5% Triton X-100 (Sigma T-6878)
4. *Staphylococcus aureus* formalin-fixed bacteria (Bethesda Research Laboratories: Immunoprecipitin, 9321 SA)
5. Protein-A Sepharose (Pharmacia or Sigma P3391)
6. SDS-PAGE Studier sample buffer (see **Method 7.4**)

Procedure:

1. To 50 µl of PBS-Triton in a 1.5 ml Eppendorf vial add 2.5 µl of serum or 10 µg of purified antibody. To test tissue culture fluids use 50 µl of neat supernatant in place of PBS.
2. Preclear antigen by mixing with the pellet from 50 µl of 10% *S.aureus* suspension. Incubate 15 mins at 4°C, spin and add 10^4-10^6 cpm supernatant to tubes containing test antibody; count tubes with ^{125}I-labelled antigens are used.
3. Incubate overnight at 4°C.
4. Add 2.5 µl of anti-immunoglobulin and incubate for 30 mins at 4°C (Note 2).
5. Add 20 µl of 50% suspension of Protein-A-sepharose. Incubate 60 mins at 4°C.
6. Fill tube with cold PBS-Triton and centrifuge (top speed, bench centrifuge, 4°C, 10 mins).
7. Wash at least three more. Vortex the pellets well before adding buffer. If background problems are anticipated, transfer precipitates to a fresh Eppendorf vial.
8. Vortex pellet well and add 30 µl of SDS-PAGE sample buffer containing freshly-added 5% 2-mercaptoethanol. Incubate 5 mins, 100°C without delay before centrifugation.
9. When using iodinated antigens, count pellets before loading on SDS-PAGE.
10. Spin samples (Eppendorf centrifuge) and load supernatants onto gel.

Notes:

1. To produce quantatitive results, set up triplicate tubes each with 50 µl buffer, 2.5 µl of serum and the appropriate quantity of antigen.
2. *S. aureus* Protein A does not bind all subclases of immunoglobulin (reviewed in [3]) and a small quantity of polyclonal anti-Ig is added to bridge between Protein A and nonbinding antibody classes. An excess of Anti-Ig serum may alternatively be substituted for Protein-A at step 4/5: 50 µl of a goat or rabbit anti-Ig would be typical, although longer than 60 mins is often required for full precipitation. When using anti-Ig with tissue culture supernatants, add 5 µl of normal serum as a carrier.
3. The samples may be resolubilised in SDS-PAGE loading buffer without 2-mercaptoethanol if analysis under nonreducing conditions is desired.
4. Background binding may also be reduced by using a more stringent wash buffer prepared as follows:

25 ml	1 M Tris-HCl pH 7.2
14.5 g	NaCl
25 ml	10% Triton X-100
10 ml	1 M lithium chloride
5 ml	10% SDS
435 ml	water

5. A variation of this approach is *in situ* immunoprecipitation of surface exposed antigens [4]. Intact labelled parasites are mixed with antibodies, incubated, washed extensively and then solubilised under conditions which do not disrupt antigen-antibody complexes. Complexes are then precipitated with Protein-A or anti-Ig, washed and analysed on SDS-PAGE. Precipitated antigens are presumed to have been exposed on the parasite surface.

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Method 17. ELISA

The use of enzyme-labelled antibodies in immunoassays has enabled rapid and simple protocols to be constructed which, moreover, do not depend upon expensive or sophisticated equipment [1,2]. There are now available a range of enzyme labels and substrates for each enzyme to suit the particular application in mind. This protocol combines horseradish peroxidase-coupled anti-immunoglobulin (HRP-anti-Ig) and an ABTS chromogen which provides a strong visual signal. Alternative procedures are described in **Method 17.2** and **17.3.**

17.1 Standard Procedure

Reagents and Equipment:

1. ELISA 96-well plates such as Nunc Immunoplate 96F (Gibco Cat No 4-39454)
2. Carbonate buffer for antigen attachment:

   45.3 ml 1.0 M (8.4%) $NaHCO_3$

   18.2 ml 1.0 M (10.6%) Na_2CO_3, made up to 1 litre.
3. Blocking solution: 3% FCS or 0.5% skimmed milk powder
4. PBS with 0.05% Tween-20 (Polyoxyethylenesorbitol monolaurate: Sigma P-1379)
5. HRP-conjugated anti-Ig of the appropriate species (a range is available from Sigma and BioRad)
6. ABTS substrate (2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate] from Kirkegaard and Perry, Dynatech)

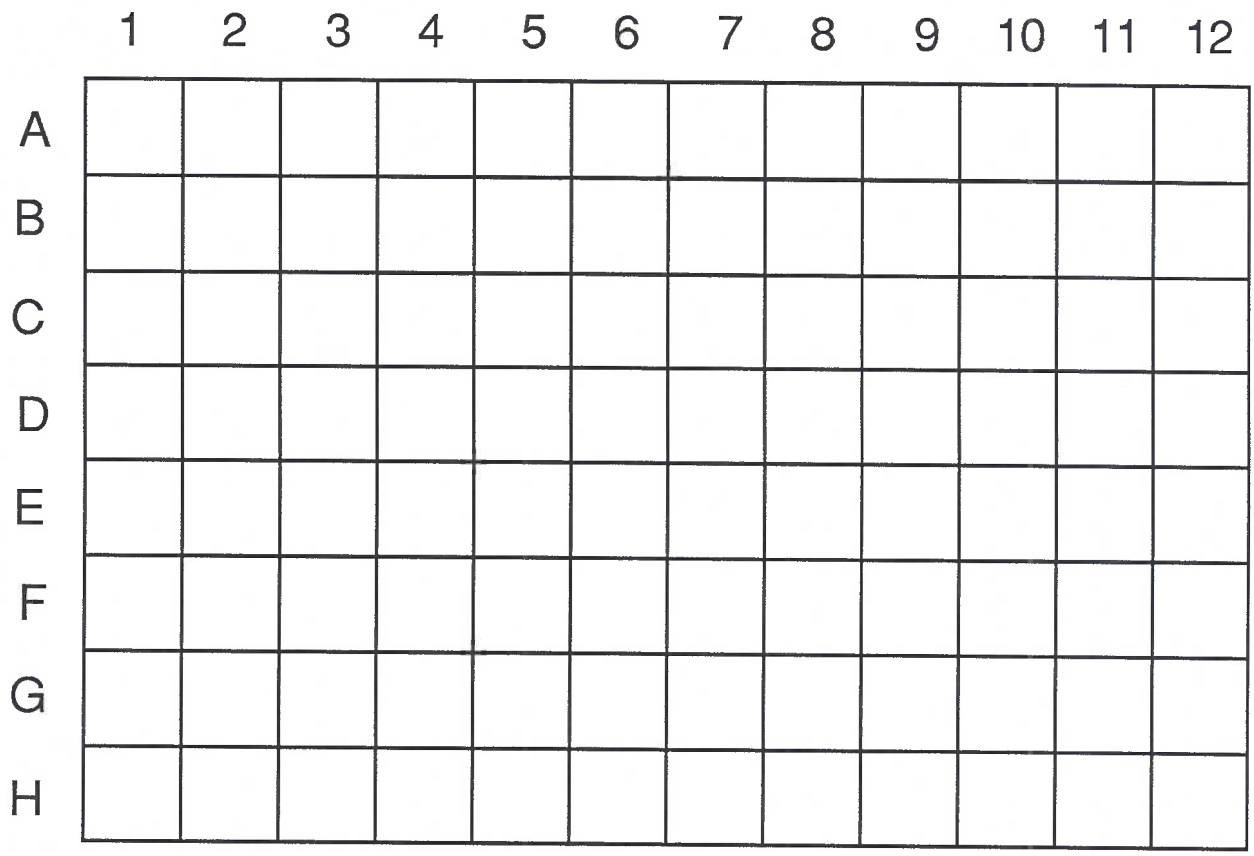
Procedure:

1. Make up antigen to optimal dilution (eg 1 µg/ml) in carbonate buffer and add 200 µl per well. Incubate 37°C 3 hrs or 4°C overnight or longer as required.
2. Remove antigen and add 200 µl per well of blocking solution. Incubate for 30 mins at 37°C
3. Wash 3 x in PBS-Tween, 3 mins per wash
4. Add serum dilutions, 200 µl/well
5. Incubate either 30 mins at 37°C or 2 hours room temperature
6. Remove serum and wash 3 x in PBS-Tween as before
7. Add 200 µl/well conjugate diluted (typically at 1/1000) in buffered PBS-Tween
8. Incubate 30 mins at 37°C or 2 hrs at room temperature
9. Make up fresh substrate solution: equal volumes of ABTS (Solution A) and buffered hydrogen peroxide (Solution B).
10. Remove conjugate, wash plate 3x in PBS/Tween and finally in water, and add substrate at 200 µl/well
11. Stand plate at room temperature for up to 30 mins and read in ELISA reader between 398 and 415 nm.

Notes:

1. Do **not** use PBS or sera containing azide as it inhibits the enzyme reaction
2. Plate-based assays such as this and **(Methods 5, 18 and 19)** are greatly facilitated by multichannel pipettes (Flow Titertek) and simple plate washers (eg Nunc Immunowash 4-48909 A or Titertek Handiwash 110. Flow Catalogue No 78-440-00)
3. A standard 96 well plate matrix and protocol sheet is set out on the following page. This can be xeroxed and used for a variety of plate-based assays and culture experiments.
4. Other enzymes most often used are alkaline phosphatase [2] and beta-galactosidase [3] and urease [4].
5. Tween-20 inhibits peroxidase, hence final rinse in distilled water.

96 Well Plate Matrix



Date _____ Expt. No_____

Plate Coated _____ Date,Time,Temperature _____

Plate Blocked _____ Date,Time,Temperature _____

1st layer _____ Date,Time,Temperature _____

2nd layer/
conjugate _____ Date, Time, Temperature _____

Substrate _____ Date, Time, Temperature _____

Notes

17.2 Alternative Substrate - OPD

Reagents:

1. Orthophenylene diamine (Sigma P-3888)
2. Methanol (BDH 45102)
3. Hydrogen peroxide (Sigma H-1009)
4. 0.1 M Acetate-citrate buffer, pH 5.0 Dissolve 8.2 g sodium acetate (BDH 10236) in 900 ml water
   Add 12 ml 1 M (19.2%) citric acid (BDH 10081) to pH 5.0.
   Make up to 1 litre and store at 4° C.
5. 4 M sulphuric acid (BDH 10276)

Procedure:

1. Follow steps 1-8 as in **Method 17.1**
2. Make up the following fresh substrate solution:

1	ml	10 mg/ml OPD in methanol (less than 7 days old)
10	µl	30% hydrogen peroxide
99	ml	Citrate buffer pH 5.0

3. Add substrate as in Step 10 in **Method 17.1**
4. Cover plate with foil at room temperature. Read at 30 mins at 405 nm.

Notes:

1. OPD solution must be stored at 4°C in the dark
2. OPD may be carcinogenic: wear gloves
3. Another, very sensitive, substrate is TMB (3,3',5,5'-tetramethylbenzidine, Miles 98-050-01), made up as follows [5]:

1	ml	10 mg/ml TMB in dimethylsulphoxide
99	ml	0.1 M sodium acetate/citric acid buffer pH 6.0. Make up as for pH 5.0 buffer but add only 1 ml of citric acid.
15	µl	30% hydrogen peroxide

   Stop the reaction after 30 mins with 2 M H_2SO_4 and read at 450 nm. This substrate is not light-sensitive and readings may be taken during the reaction at 655 nm

17.3 Other ELISA Applications

ELISA assays can be effective in other contexts, such as measurement of the affinity of monoclonal antibodies. Monoclonal antibody binding can be measured in the absence or presence of increasing concentrations (0-8M) of chaotropic agents such as urea or thiocyanate. Higher affinity antibodies require high chaotrope concentrations for inhibition of binding [6].

17.4 References

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18. Immunoradiometric Assay

An ImmunoRadioMetric Assay (IRMA) is one in which radiolabelled antibody is used to measure the presence of target ligand, and differs from RadioImmunoAssay (RIA) in which labelled antigen is used. The IRMA described here was first used with monoclonal antibodies in an assay for interferon [1] and subsequently applied to malarial sporozoites [2] and to the phosphorylcholine epitope of *Onchocerca gibsoni* [3], which is present on circulating antigen from all filarial species [4,5].

In principle, the test involves the binding of monoclonal antibody to a plastic surface, which is then saturated with a carrier protein (eg BSA). In a replica plate, test serum is incubated with the same or different monoclonal antibody, which has been labelled with ^{125}I. This mixture is the added to the monoclonal-coated plate which, after further incubation, is washed and cut up so that each well can be counted in a gamma counter. This is therefore a **two-site** assay , requiring antigen to be bound at distant sites by each monoclonal. In the case of repeating epitopes (present in both malarial sporozoite and filarial antigens [2-5]), the same monoclonal may be used for both first and final layers.

Reagents:

1. PBS and Blocking Solution (PBS, 0.5% BSA, 0.1% sodium azide)
2. Diluent for labelled antibody (PBS, 5% Normal Rabbit Serum, 0.05% Tween 20, 0.1% azide)
3. Washing solution (PBS, 0.05% Tween 20, 0.05% azide)
4. Monoclonal antibodies:
   (a) for coating plates: 1-10 µg/ml in PBS, as determined to be optimal. Prepare 5 ml per plate.
   (b) for iodinated monoclonal (see **Method 6**). Use at 2 x 10^6 cpm/ml in Diluent. Prepare 3 ml per plate
5. PVC 96-well plates (eg Flow Titertek Immunoassay Plate, Cat No 77-176-05)

Procedure:

1. Coat the plates with MAb in PBS by adding 50 µl to each well and incubate overnight at room temperature or longer at 4°C.
2. Remove MAb from plate and add 60 µl of Blocking Solution. Incubate for at least 30 mins at room temperature, and wash three times in Washing Solution.
3. In a separate, uncoated plate add 30 µl per well of diluted serum. Start with a 1:2.5 dilution in Diluent.
4. Add 30 µl of iodine-labelled MAb at 2 x 10^6 cpm/ml (60000 cpm/well)
5. Incubate at least 4 hours at 37°C, sealing plate (with eg Titertek Plate Sealers, Flow Cat. No. 77-400-05)
6. Transfer 50 µl of serum-MAb mixture from each well to the MAb-coated plate and incubate overnight, sealed, at room temperature.
7. Wash plates three times in Washing Solution, cut up plates and count individual wells in gamma counter.

Notes:

1. This test can be adapted to assay free antigen by exposing MAb-coated plates to antigen-containing solutions, washing three times, and then incubating with iodinated MAb. Incubation times should be 4 hrs to overnight for each stage.

References

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19. Two-Site ELISA Assay for Antigen Detection

This procedure is a direct adaptation of the two-site IRMA (**Method 18**) to use an enzyme conjugated second antibody in place of a radiolabelled probe. It is based on the detection of circulating filarial antigen with the monoclonal Bp-1 [1], and on the ELISA for sporozoites in infected mosquitoes [2]. A more recent application has been to detect *Toxocara canis* infection with the monoclonal Tcn-2 [3].

Reagents

1. **Blocking Solution** : PBS containing 0.5% BSA (Sigma A-9647)
2. **Diluent for conjugated antibody**: PBS, 5% Normal Rabbit Serum (Serotec WAS-04), 0.1% Tween 20 (Tween 20 = Polyoxyethylenesorbitol monolaurate: Sigma P-1379)
3. Washing solution (PBS, 0.1% Tween 20)
4. Monoclonal antibodies:
   (a) for coating plates: 1-10 µg/ml in PBS, as determined to be optimal. Prepare 10 ml for each plate to be coated. Monoclonal Bp-1 is used at 1 µg/ml [1].
   (b) for conjugated monoclonal, the dilution must be determined by titration. Current batches of horseradish peroxidase (HRP)-Bp-1 work at $^1/_{2000}$ dilution [1]. Prepare 10 ml for each plate to be assayed.
5. ELISA 96-well plates such as Nunc Immunoplate 96F (Gibco Cat No 4-39454)
6. ABTS substrate, as in **Method 18** or for greater sensitivity, TMB (see 7-11 following)
7. TMB substrate: 3, 3', 5, 5'-tetramethylbenzidine (Miles 98-050-01)
8. DMSO : dimethylsulphoxide (Sigma D-5879)
9. Hydrogen peroxide (Sigma H-1009)
10. 0.1 M Acetate-citrate buffer, pH 6.0. Dissolve 8.2 g sodium acetate (BDH 10236) in 900 ml water
    Add 1 ml 1 M (19.2%) citric acid (BDH 10081) to pH 6.0.
    Make up to 1 litre and store at 4° C.

Procedure

1. Coat the plates with MAb in 100 µl PBS per well; incubate overnight at room temperature, or longer at 4°C.
2. Remove MAb from plate and add 200 µl of Blocking Solution. Incubate for at least 30 mins at room temperature, and wash three times in Washing Solution.
3. Add dilutions of serum samples, 100 µl per well, starting from $^1/_{10}$ in PBS-Tween.
4. Incubate at least 4 hours at 37°C, sealing plate (Titertek Plate Sealers, Flow Cat. No. 77-400-05, are useful)
5. Wash plates three times in Washing Solution,
6. Add 100 µl/well conjugate diluted (typically at $^1/2000$) in 5% NRS or 0.1% BSA
7. Incubate at 37° or 4°C as before
8. Make up a fresh substrate solution of ABTS (see **Method 18**), or TMB as follows:
   1 ml 10 mg/ml TMB in DMSO
   15 µl 30% hydrogen peroxide
   99 ml Citrate buffer pH 6.0
9. Remove conjugate, wash plate 3x in PBS/Tween and finally in water, and add substrate at 200 µl/well
10. Stand the plate at room temperature until colour develops for up to 30 mins. Readings may be taken at this point at 655 nm (TMB) or 410 nm (ABTS).
11. If TMB is used, add 25 µl of 4 M H_2SO_4 (BDH 10276) per well and read plate visually or spectrophotometrically at 450 nm, or if this wavelength is unavailable, at 410 nm.

Notes

1. Do **not** use PBS or sera containing azide as it inhibits the enzyme reaction
2. Tween-20 inhibits peroxidase, hence final rinse in distilled water
3. Different antibodies can be employed as 'capture' and 'indicator' ligands [4]. Thus, a mouse monoclonal can be paired with a rabbit polyclonal serum, and binding measured with species-specific-enzyme conjugated anti-Ig reagents, circumventing preparation of individual antibody-enzyme conjugates [5].

References

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20. Immunofluorescence

The binding of antibodies to target tissues or cells can be visualised if those antibodies are directly coupled to a fluorochrome or indirectly bound by a fluorescent reagent. Fluorochromes emit visible light (of an "emission" wavelength) when exposed to light of a different ("excitation") wavelength, usually in the ultra-violet range. The most widely used fluorochrome is fluorescein, conjugated to proteins from fluorescein isothiocyanate (FITC); others include rhodamine (TRITC) and phycoerythrin. Parallel techniques may be used with fluoresceinated lectins which will directly bind specific carbohydrates [1], see also **Method 26**. This protocol describes the indirect fluorescent antibody test (IFAT) on intact parasites [2] however, similar procedures may be followed for studying parasite sections (see Note 2).

Materials and Reagents

1. Living parasites, test and control antibodies.
2. FITC-conjugated anti-immunoglobulin of the appropriate species (Sigma hold a wide range).
3. PBS, including 0.1% azide if desired (see note 1)
4. Evans Blue counterstain
5. 50% glycerol, 50% PBS, 0.1% azide for mounting; or anti-fadent mounting solution AF-1 (Citifluor, London EC1)
6. Flow Multi-test slides, Code No. 60-408-05 (8 wells/slide)

Procedure

1. Incubate washed parasites in test or control sera, generally at 1/50 and higher dilutions in PBS, for 30 mins-2 hours at 4° C.
2. Wash x 3 in PBS. Eppendorf tubes are convenient for experiments with microfilariae as a brief spin in a microcentrifuge should pellet the parasites after each wash.
3. Incubate parasites in FITC-anti-immunoglobulin (1/20 or ^{1}/40) in PBS containing Evans Blue counterstain at 1/100,000 for 30 or 60 mins on ice.
4. Wash parasites in PBS once and take up pellet into 5µl mounting buffer. Transfer to one well of a multi-test slide. Place cover slips over the whole slide. Seal with nail varnish to preserve slide.
5. Examine under fluorescence microscope. Store slides at 4°C in dark for later reference if required.

Notes

1. For *Toxocara canis* [2] and *Ancylostoma caninum* [3] all incubations must be done on ice, and 0.1% azide included in the medium [2]. For most other parasites, including filarial worms these precautions do not appear to be necessary [4], although incubations at 4°C are still advised.
2. IFAT on acetone-fixed [5] or paraformaldehyde-fixed [6] sections follows a similar timetable but all operations are carried out on sections fixed onto glass slides. Once fixed, sections can be stored at -20°C, but note that both fixation and freezing may expose additional antigens on intact worms.
3. Some parasites (eg *Brugia* microfilariae) can be stored frozen once air-dried on to slides and then fixed by a two-second immerison on acetone [7].
4. IFAT can be used for screening or characterising monoclonal antibodies by direct analysis of undiluted tissue culture supernatant at step 1 above.
5. Fluorescence rapidly quenches under UV illumination, but loss can be significantly reduced by incubation in proprietary mounting solutions and by anti-quenching buffers [8].

References

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Method 21: Immuno Electron Microscopy

The ultrastructural definition and resolution of electron microscopy can be combined with immunological probes to provide accurate localisation of antigenic molecules in parasite organisms [1,2]. The preparation of specimens for this analysis falls into the following sections: **21.1** Fixation and Dehydration; **21.2** Resin infiltration and Polymerisation; and **21.3** Sectioning and Immunostaining. Acrylic resins have been found to give the most acceptable compromise between morphology and antigenicity: both Lowicryl and L R Gold have been used and the latter has provided the best results.

21.1 Fixation and Dehydration

This is probably the most important factor in achieving good labelling. Before attempting any immunogold work find out whether the antigen(s) to be studied are sensitive to the fixatives used in E.M. These are predominantly glutaraldehyde and (to a lesser extent) paraformaldehyde. A rapid means to test this is by comparing in an IFAT (**Method 20**) methanol–fixed specimens with glutaraldehyde and glutaraldehyde-paraformaldehyde–fixed specimens. As a rough rule of thumb use 1.0 – 1.5% glutaraldehyde for polyclonal antibody staining and 0.25 – 0.5 % glutaraldehyde with 2 – 4% paraformaldehyde for monoclonal work.

Fixation times are also important and will vary with the nature of the tissue. For cell suspensions, 15 mins should be sufficient, but for more compact tissues times may be extended for up to 1 hour. When dealing with very sensitive antigens fixation times should be reduced to a minimum, though this may affect ultrastructure. A balance must therefore be determined for a particular specimen type.

Dehydration should be carried out at low temperature, using precooled methanol, to minimise antigen movement within the specimen as water molecules are extracted.

Procedure

1. Wash specimen 3 x in PBS to remove any serum components or other contaminants.
2. Fix in 0.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4, 30 mins at 4°C.
3. Wash 3 x in 0.1 M sodium cacodylate buffer. Specimens can now be dehydrated directly or may be stored in sodium cacodylate buffer at 4°C for an extended period of time.
4. Dehydrate by sequential immersion in:
   a) 35% ethanol, 15 mins, at 4°C.
   b) 50% ethanol, 30 mins, at –20°C
   c) 75% ethanol, 30 mins, at –20°C
   d) 95% ethanol, 30 mins, at –20°C
   e) Absolute alcohol, 2 changes of 30 mins. each at –20°C

21.2 Resin Infiltration and Polymerisation

LR Gold (London Resin Company) has two components: Monomer and an Activator (Benzil). Initially, the monomer must be infiltrated into the specimen, followed by a mixture of 0.1% activator in the monomer.

Procedure

1. Place specimen in a 2:1 mixture of absolute methanol and monomer for 1 hour at –20°C
2. Transfer to a 1:2 mixture of absolute methanol and monomer for 1 hour at –20°C
3. Transfer to 100% monomer for 2 hours at –20°C
4. Transfer to 100% monomer and activator for 2 hours at –20°C
5. Transfer to 100% monomer and activator and leave for up to 72 hours at –20°C. Optimal infiltration times may also vary; chitinous tissue, for example, requires 72 hours.
6. Transfer to small gelatin capsules (Agar Aids) and fill with monomer and activator. The activator is light sensitive and so resins can now be polymerised at low temperature by ultraviolet light.
7. Place capsules in an ELISA plate and place under UV source at –20°C. Continue for 72 hours –20°C.
8. Strip gelatin capsule to reveal block and section (**Method 21.3**).

21.3 Sectioning and Immuno-Staining for Electron Microscopy

Using an ultramicrotome, 90 - 120 nm sections are cut by electron microscopy methods. Sections are picked up on nickel grids (Agar Aids) coated in a Formvar plastic support film. The latter is necessary since LR Gold is less stable under the electron beam than conventional epoxy resins.

The immunolabelling is best performed on parafilm strips inside a sealable container. The basic schedule given below can be modified as appropriate (see Notes)

Procedure

1. Float each grid on a drop of 1% BSA,.01% Tween–20 at room temperature for 30 mins. Use Watchmaker's forceps to handle the grids
2. Dip wash grids 3 x in PBS–Tween
3. Make up 500 µl of antibody diluted 1:50 (see *Note 1*) in PBS-Tween, and place one drop per grid on film. Float grids at room temperature for 60 mins
4. Dip wash grids 20 x in PBS–Tween
5. Wash, 3 x 5 mins in PBS–Tween
6. Float on gold labelled probe (eg Janssen goat anti-mouse 15 nm particles), diluted in PBS–Tween (see *Notes 2 and 3*), at room temperature for 60 mins
7. Dip wash grids 20 x in PBS–Tween
8. Wash, 3 x 5 mins in PBS–Tween
9. Dip wash 50 x distilled water
10. Stain grids in uranyl acetate and Reynold's lead citrate (see *Note 4*)as in conventional EM work. Stain for 10 mins in uranyl acetate, then dip wash 10 x in large volume of water and dry on filter paper. Stain for 5 mins in Reynold's lead citrate, then 3 dip washes and dry as for uranyl acetate staining.
11. Examine under EM. See *Notes 6 and 7* for troubleshooting.

Notes

1. Initially test a range of antibody dilutions from 1/20 to 1/100 (or 1/250 in the case of monoclonals) on duplicate grids to determine the optimum primary antibody working concentration.
2. For best results use one of the Janssen Pharmaceutical range of gold conjugates, diluted 1/20 or 1/30.
3. The size of gold particles conjugated to secondary antibody range from 5 to 40 nm and should be selected in relation to the size of the specimen and the magnification to be used. Note that the larger the particle used, the lower the observed binding intensity due to steric hindrance.
4. Uranyl acetate is made up as a saturated solution (>8%) in 50% ethanol. Protect from light.
5. Reynold's Lead Citrate is made up by adding 1.33 g lead nitrate and 1.76 g sodium citrate to 30 ml boiled water. After mixing for 30 mins, add 8 ml 1 M NaOH and dilute to 50 ml. Protect from CO_2.
6. If problems occur of heavy background labelling, extend washing periods, and consider an extra blocking step (Step 1) between the two antibody incubations (Steps 3 and 6). Repeat with greater dilutions of each antibody. Antibodies stick readily to the Formvar support film so ignore high background that occurs off section.
7. If very low intensity labelling is seen with little evidence of localisation, check the quality of both primary antibody anf gold conjugate. Epitopes may have been destroyed during fixation, so shorter fixations at lower fixative concentrations should be tried.

21.4 References

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22. Gel Filtration

Gel filtration permits the preparative separation of molecules by their native molecular weight, due to the differential penetration of the gel matrix by molecules of different size. In contrast to PAGE, large molecules move more rapidly than small as they diffuse over a smaller volume while travelling through a column.

This principle is exploited in two procedures. Firstly, where proteins are to be separated from low molecular weight molecules they can be rapidly desalted by gel filtration (**Method 22.1**); this is routinely used following iodination (**Method 6**) and other trace labelling techniques. Secondly, complex mixtures of macromolecules may be separated by smaller size differences (**Method 22.2**) and their respective molecular weights estimated by the relative migration of marker proteins (**Method 22.3**). The choice of gel matrix, and conditions of column chromatography, are dictated by the molecular weight range of antigens involved [1-3].

22.1 Desalting Columns

Reagents:

1. Sephadex G-25 Medium (Pharmacia 17-0033-01) beads, or prepacked in 9 ml PD-10 columns (17-0851-01)
2. Buffer in which desalted protein is required.

Procedure:

1. If not using a prepacked column, swell beads in buffer (3 hrs, 20° C) and pour into a suitable column such as a 2 ml syringe, using glass wool or a circle of filter paper to support the beads.
2. Wash well in buffer, allow surplus to drain away without letting the bed run dry
3. Add sample without disturbing the beads
4. Allow sample to enter the column and add buffer, rinsing down the walls of the column
5. Collect fractions of 10% of the column volume and measure each fraction for protein (OD or TCA-precipitable cpm) and for free tracer (OD or TCA-soluble cpm)

Notes:

1. Some substances (eg lectins) may react with the dextran of Sephadex: BioRad P6 polyacrylamide beads are an alternative matrix for desalting.
2. Inclusion of detergent (0.5% Triton X-100) reduces loss of protein due to non-specific adherence to the column.

22.2 Fractionation of Antigens

Reagents and Equipment

1. Gel matrix selected from:

			Molecular Weight Range		
Sephadex	G25	(Pharmacia)	1,000	-	5,000
	G50		1,500	-	30,000
	G100		4,000	-	150,000
	G200		5,000	-	600,000
Sepharose	6B	(Pharmacia)	10,000	-	4,000,000
	4B		60,000	-	20,000,000
	2B		70,000	-	40,000,000
Ultrogel	AcA 34	(LKB)	20,000	-	350,000
	AcA 22		100,000	-	1,200,000
	A6		25,000	-	2,400,000
	A4		55,000	-	9,000,000
	A2		120,000	-	25,000,000

   (Many other matrices are available from these suppliers and from BioRad) BioGel P2, P4, P6 etc.
2. Fraction collector, UV monitor and recorder are available from Pharmacia/LKB and other manufacturers.

Procedures:

1. Sephadex and Bio-Gel beads require swelling, while Sepharose and Ultrogel are supplied pre-swollen.
2. Select a column with a volume approximately 100 times that of the sample to be fractionated.
3. Pour a slurry of swollen beads, equilibrated with the running buffer, into the column, connect buffer reservoir to the column and outlet to fraction collector.
4. Load sample (1% or less of column volume) collecting required volumes in each tube.
5. Measure each sample for cpm (for radiolabelled preparations), OD (for tubes which the UV monitor has shown to contain protein) and/or by any other appropriate bio-assay (eg IRMA: **Method 18**)

Notes:

1. An even, uninterrupted flow rate is essential to sharp separation of peaks in gel filtration, as is sample loading without disturbing the column bed. The rate of flow is determined by the height of the reservoir above the outlet, or may be controlled by a peristaltic pump.
2. If the sample volumes exceed 2% of the column volume, peaks will broaden and may therefore overlap.
3. Store columns when not in use in 0.1% sodium azide.

22.3 Molecular Weight Determination

Reagents:

1. Set of standard calibration proteins, eg Pharmacia 17-0445-01.

Procedure:

1. Prepare column as outlined in **Method 12.2**, and load a sample of calibration proteins. Collect fractions and determine elution volumes (Ve) of each marker protein.
2. Determine void volume (Vo) by elution volume of Blue Dextran 2000
3. Determine total column volume (Vt)
4. For each protein determine the value of Kav = (Ve-Vo)/(Vt-Vo) and plot Kav against log molecular weight.
5. With the same column, run sample of protein(s) to be analysed, measure the elution volume for each, and thence calculate Kav for each.
6. From the calibration plot, estimate the molecular weight for each peak.

Notes:

1. Elution volumes are taken as the volume at which the peak elutes
2. Occasionally proteins have Kav >1, signifying some interaction with the column matrix; Kav <0 can only result from a poorly packed column.

22.4 References

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23. Antibody Purification

Various methods are available for purification of antibodies from serum, ascites or tissue culture supernatants, depending on the class(es) of antibody, the quantity and purity required and the starting material involved. Two methods are given here: precipitation with ammonium sulphate [1] which readily provides large quantities of antibody although other components co-purify (**Method 23.1**), and affinity chromatography on anti-immunoglobulin or Protein A columns for lower yields of high purity antibodies (**Methods 23.2**). Other approaches are briefly described in **Method 23.3,** and in **Method 25** on Fast Protein Liquid Chromatography.

23.1 Ammonium Sulphate Precipitation

Reagents

1. Ammonium sulphate (BDH 10033), saturated solution, pH 7.0. Make up 550 g to 950 ml with water, warm to dissolve and filter, adjust pH with ammonium hydroxide, make up to 1 litre and refrigerate. Crystals should form confirming that the solution is saturated.
2. Saline (0.85% NaCl)
3. Dialysis membrane or Amicon diafiltration assembly. (Cell number 8010 or 8050 with XM100A membranes)

Procedure

1. **For each ml** of serum or ascites at 0°C, add 1 ml of cold saline pH 7.0
2. Add 2 ml of cold saturated ammonium sulphate dropwise while stirring
3. Stir for 30 mins on ice while a white precipitate (globulin) forms
4. Centrifuge 30 mins at top speed in refrigerated (0°C) benchtop centrifuge
5. Discard supernatant and take up precipitate in 2 ml of saline per ml of original serum
6. Repeat steps 2-5 twice more: the final supernatant should be colourless. The final precipitate should be taken up in 1 ml or less.
7. Dialyse or Amicon diafiltrate extensively against PBS to remove all ammonium sulphate.

Notes

1. Test for sulphate in dialysate or filtrate by adding first a drop of 1 M HCl and then saturated barium chloride: precipitation reveals presence of sulphate ions.
2. Sodium sulphate may be used to precipitate immunoglobulin at room temperature (ideally 25°C). Follow the same procedure, adding 36% sodium sulphate to a 50% dilution of serum in PBS. The third precipitation may be carried out in 12-14% final concentration sodium sulphate.

23.2 Protein A Affinity Chromotography

This method yields whole immunoglobulin fractions in high purity without regard to antigenic specificity. A similar approach using immobilised antigen will select only antibody specific to that antigen and is more appropriate when sufficient quantities of a well-characterised antigen are available.

Reagents

1. Protein A-Sepharose beads (Pharmacia 17-0780-02)
2. Sample and Running Buffer: 6.055 g Tris-base (Final concentration 0.05 M)
   29.2 g NaCl (0.5 M)
   Adjust pH to 8.0 with HCl and make up to 1 litre.
3. Elution Buffer: 15.14 g Glycine (0.2 M)
   29.2 g NaCl (0.5 M)
   Make up to 1L, adjusting pH to 2.8 if necessary.
4. Collection Buffer: 12.11 g Tris-base (1.0 M)
   Make up to 100 ml, adjusting pH to 8.5.

Procedures:

1. Prewash column in at least 10 volumes running buffer. Connect outflow to UV detector and record.
2. Wash column in Glycine-HCl elution buffer. After a small peak continue washing until the UV reading returns to baseline.
3. Wash again in running buffer until pH of effluent returns to 8.0
4. Mix serum or ascites containing antibody with equal volume of running buffer and load onto column.
5. Run column in a running buffer until flow-through is complete and UV returns to baseline. Expect at least 95% of OD to pass through unbound.
6. Elute bound fraction with elution buffer (pH 2.8), collecting in tubes containing sufficient collection buffer (pH 8.5) to neutralise final volume in each tube. Typically this requires 7-10% by volume of collection buffer: eluted material.
7. Wash column again in running buffer once UV returns to baseline. If column is to be stored (>1 week) include azide in buffer and keep at 4°C
8. Pool eluted peak, measure OD and calculate yield. Dialyse or Amicon Diafiltrate against PBS or other required buffers, concentrate and aliquot for storage.

Notes

1. Calculate yield of immunoglobulin from OD_{280}, using extinction coeffiecient for a 1 mg/ml solution of 1.4
2. The capacity of an immunosorbent column is determined empirically, so that on first use of a column, flow-through fractions should be rerun. Further binding reveals that the column was overloaded on the first run.
3. Ascitic fluid often needs clarification to remove debris and micro-clots. Fluid should be centrifuged at 10,000 *g* for 30 mins and passed through a 0.2 µm filter if necessary.
4. To purify Protein-A non-adherent (generally IgM) antibodies, substitute anti-immunoglobulin Sepharose beads and elute according to the same procedure

23.3 Other Methods for Antibody Purification

Other approaches for isolating immunoglobulin from serum or ascites include:

1. Ion-exchange purification of IgG subclasses on DEAE-Sephadex [2]; some enzymatic activity co-purifies [3] but ion-exchange may be combined with prior ammonium sulphate precipitation [4]
2. Sephadex G200 gel filtration, in which IgM is a dominant component of the exclusion peak (**Method 22**)
3. Ion-exchange purification on Affigel Blue [3] to which all IgG classes bind.
4. Boric acid precipitation of IgM along similar lines to ammonium sulphate precipitation. To 1 ml of ascites add dropwise 20 mls of cold 2% boric acid. Stand at 4°C, 3 hours. Dialyse supernatant against PBS or other desired buffer.

23.4 References

[1] Heide K & Schwick HG (1978)Salt Fractionation of Immunoglobulins. In Handbook of Experimental Immunology (Ed Weir DM, Blackwell, Oxford) p7.1

[2] Fahey JL & Terry EW (1978) Handbook of Experimental Immunology p8.1

[3] Bruck C, Portetelle D, Glineur C & Bollen A (1982) One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE affi-gel blue chromatography. *J Immunol Methods* **53**: 313-319.

[4] Hudson L & Hay FC (1976) Practical Immunology (Blackwell, Oxford) p152

24. Purification of Antigen by Affinity Chromotography

Affinty chromatography exploits the specific and reversible nature of biological ligands [1]. One example is **Method 23.2** in which the whole immunoglobulin fractions from serum or ascites are isolated. This section deals with purification of specific antigen by a similar procedure, using methods which can then be extended to isolate specific antibody populations.

24.1 Coupling of Protein to Solid-Phase Adsorbent

Reagents

1. CNBr-activated Sepharose 4B (Pharmacia 17-0430-01)
2. Sintered glass filter (Gallenkamp FPJ-300-050D)
3. HCl, 1 mM (83 µl concentrated HCl per litre)
4. Coupling buffer:
   8.4 g $NaHCO_3$ (Final concentration 0.1 M)
   29.22 g NaCl(0.5 M)
   Adjust pH to 8.5 and make up to 1 litre.
5. Blocking buffer:
   either ethanolamine (2-aminoethanol, Sigma A-5629) 12.1%, pH 8.0 or 0.1 M Tris-HCl, pH 8.0
   Ethanolamine pH11 requires substantial HCl to reduce pH
6. Washing buffers:
   a) 5.7 ml Glacial Acetic acid (0.1 M)
   29.22 g NaCl (0.5 M)
   Adjust pH to 4.0 and make up to 1 litre.
   b) 12.12 g Tris base (0.1M)
   29.22 g NaCl (0.5M)
   Adjust pH to 8.0 and make up to 1 litre.

Procedure

1. Dialyse protein (1-5 mg/ml) into coupling buffer at 4°C. Use 20 mgs of protein per g of dry beads. Measure OD immediately before coupling.
2. Swell CNBr-activated Sepharose in ice-cold 1 mM HCl by washing on a sintered glass funnel for 15 mins. Use 200 ml per g of beads.
3. Mix protein and beads in an end-over-end rotator for 3 hrs at room temperature.
4. Separate beads by gentle centrifugation, recover supernatant of coupling reaction, measure OD and calculate quantity bound.
5. Block remaining active groups by the addition of 2M ethanolamine (or 0.1M Tris-HCl) to the beads and continue mixing overnight at 4°C.
6. Pour beads into a column, eg a 1ml syringe
7. Wash coupled beads alternately with pH 4.0 and pH 8.0 washing buffers.
8. Store in Coupling Buffer or PBS with azide at 4°C.

Notes

1. Affigel 10 (BioRad 153-6046) and LKB activated ultrogels are alternative affinity support matrices. These are now available with a choice of coupling methods and with spacer arms to improve accessibility between immobilised and liquid-phase ligands.
2. Antibody-coupled Sepharose can also be used in a two-site assay to capture target antigen from infected serum:
   1. Incubate 50 µl of serum of beads for 8 hrs at room temperature on end-over-end rotator
   2. Spin and wash 3 times with PBS-0.5% Triton X-100
   3. Resuspend in 30 µl of PAGE loading buffer (**Method 7.4**). Boil for 5 mins, spin and load supernatant onto gel.
   4. Blot gel onto nitrocellulose (**Method 15**)
   5. Process blot using a suitable radiolabelled second antibody (10^6 cpm/ml) to visualise antigen captured by the antibody-coupled Sepharose

24.2 Purification of Target Antigen on Solid Phase Immunosorbents

Reagents

1. Immunosorbent column prepared as in **Method 24.1**
2. Running (Tris-Saline), Elution (Glycine-HCl) and Collection (Tris-base) Buffers, each as specified in **Method 23.2.**

Procedure

1. Follow the same procedure as **Method 23.2**, prewashing the column in elution and running buffers.
2. Load the preparation from which antigen is to be purified, ensuring pH is around 8.0
3. Once the flow-through is completed, elute as in **Method 23.2.** wash column and store as appropriate.
4. Check both flow-through and eluted fractions for antigenic activity by appropriate immunological assay

Notes

1. With affinity chromatography large volumes may be loaded onto columns, and antigen thereby isolated from dilute preparations.
2. Certain antigenic sites are damaged by the low pH used for elution; as an alternative, 50 mM (0.52%) diethylamine in saline (0.85% NaCl), pH 11.5 elution may be used, collecting into glycine to neutralise eluted fractions.
3. Highest affinity antibodies may fail to dissociate even these extremes of pH and are thus less suitable for affinity chromatography.

24.3 Reference

[1] Cuatrecasas P and Anfinsen CB (1971) Affinity Chromatography. *Methods Enzymol* **22** : 345-378

25. Fast Protein Liquid Chromotography (FPLC)

FPLC is a rapid automated system for the separation of molecules such as immunoglobulins and enzymes, without the loss of their bioactivity. This is achieved with low filtration pressures and the use of glass and plastic on all contact surfaces, unlike HPLC which uses high pressures and stainless steel components. A wide range of columns are available for techniques from simple gel filtration (Superose) to ion exchange and complex reverse phase (Mono series). The methods outlined below are for the use on the Pharmacia system fitted with two P-500 pumps and gradient programmer GP-250 [1], but can be adapted to any system.

General procedures

1. All buffers and eluents must be made with Millipore-Nanopure water, and then passed through a 0.22 μm filter. Samples must also be filtered or spun at 10,000*g* for 10 mins prior to application to the column. PBS is a suitable eluent for proteins, 0.1 M Tris/HCl 1mM EDTA for DNA/RNA. Samples should contain no more than 10 mg of protein at a concentration of less than 30 mg ml^{-1}.

2. Columns are stored in 20% ethanol and must be equilibrated with 50 ml eluent before use. A method can usually be programmed for this, adjusting the flow rate so the column is ready at a suitable time eg. a low flow rate overnight.

3. High back pressure, gel bed packing and loss of resolution are indicative of a dirty column. Although reversing the column and equilibriating it may cure the problem temporarily, to maintain column life and performance it must be cleaned after every 5th run. In the case of gel filtration columns, such as the Pharmacia Superose series, this is achieved by reversing it and washing with 50 ml of each of the following:

70 %	Formic acid
20 %	Ethanol
0.1 M	NaOH
	Made up to 50 ml with water

Superose columns should then be equilibriated with 50 ml of 20% ethanol. The top filter should be changed if the gel bed continues to pack. Other columns need different cleaning and storage prorcedures – check the manufacturers instructions.

4. Superose 6 separation range 5 000 – 5 000 000 M_r
Superose 12 separation range 1 000 – 300 000 M_r

5. Flow rates: for large molecules, with low coefficients of diffusion 0.1 – 0.3 ml min^{-1}; for small molecules or group separations 0.7 – 1.2 ml min^{-1}; for immunoglobulins 0.2 ml min^{-1} is ideal.

References

1. FPLC ion exchange and chromatofocusing. Principles and methods. Pharmacia Fine Chemicals (Free on request).

26. Lectin Affinity Chromotography

Lectins are carbohydrate-binding proteins which interact with specific sugar structures [1,2]. An increasingly wide range of lectins, mostly from plants, are becoming available and many may be purchased immobilised on solid-phase adsorbents for affinity chromatography. This procedure describes the use of lentil (*Lens culinaris*) lectin, a mannose- and glucose-specific ligand widely used in the characterisation of mammalian glycoproteins [3] and nematode antigens [4]. The coupling of proteins to solid-phase adsorbents is described in **Method 24.1**

Reagents

1. Lentil lectin immobilised on agarose (Sigma L-2132, or Miles 79-138-1)
2. α-Methyl mannoside (Sigma M-6882), 0.1 M (19.42 g/L)
3. 1 M $CaCl_2$ (14.7g/100 ml) and 0.1 M $MnCl_2$ (1.98 g/ 100 ml).
4. 10 mM Tris-saline pH 7.4 (8.18 g NaCl, 1.21 g Tris-base per litre: requires 3.5-4 ml 1M HCl to pH), containing 0.1 mM manganese chloride (Sigma M-3634, 20 µg/ml) and 1 mM calcium chloride (Sigma C-3881, 147 µg/ml))
5. Nonidet P-40 (Sigma N-6507) or Triton X-100 (Sigma T-6878), plus 0.1% azide

Procedure

1. Set up immobilised lectin in a small column (eg. 1 ml syrimge) and wash extensively with Tris-saline containing 0.3% NP-40 or 0.5% Triton X-100.
2. Wash with 0.1 M α-methyl mannoside in same buffer.
3. Wash again with sugar-free running buffer.
4. Load sample (eg 100 µl) let run into gel, add 500 µl buffer, let run into gel and clamp off for 30 mins and monitor effluent for cpm or other appropriate assay.
5. When effluent has returned to baseline, elute bound fraction with α-methyl mannoside.
6. Store column in buffer containing both sugar and 0.1% sodium azide.

Notes

1. Among other commonly used lectins are:

Arachis hypogaea (Peanut, PNA)	Galactose [D-gal-ß(1-3) Gal NAc]
Bandeiraea simplicifolia B4 (BSB4)	Terminal D-galactose, Blood Group B (α-D-Gal-D-GlcNAc)
Concanavilin A (Jack Bean, Con A)	Mannose, glucose
Helix pomatia (Snail, HPA)	N-acetyl galactosamine, Blood Group A- (α-D-Gal NAc)
Limulus polyphenus (Horsehoe crab)	Sialic and glucuronic acids
Lotus tetragonolobus	Fucose
Wheat Germ Agglutinin (WGA)	N-acetyl glucosamine
Ulex europeus agglutinin (UEA)	Fucose, Blood Group H [α-L-fucose $(D\text{-}GlcNAc)_2$]

   Further details of lectin specificities, molecular weights, cation dependence etc are in Ref [5].

2. Although Con A has a similar specificity to Lentil lectin, its affinity is 50 times higher [3]; consequently competitive elution with sugar is relatively ineffective and further elution 0.1M HCl is required, which may in turn affect the bioactivity of eluted molecules. Collect HCl eleuted fractions into 2 M Tris (approx 25 µl will neutralise 0.5 ml of 1 M HCl).

References

[1] Goldstein IJ & Hayes CE (1979) The lectins: Carbohydrate-binding proteins of plants and animals. *Advan Carbohydr Chem Biochem* **35**: 127-340

[2] Sharon N & Lis H (1975) Use of lectins for the study of membranes. *Methods Membrane Biol* (Ed Korn ED, Plenum Press, NY) **3**: 147-200

[3] Hayman MJ & Crumpton MJ (1972) Isolation of glycoproteins from pig lymphocyte plasmid membrane using *Lens culinaris* phytohemagglutinin. *Biochem Biophys Res Comm* **47**: 923-930

[4] Parkhouse RME, Philipp M & Ogilvie BM (1981) Characterisation of surface antigens of *Trichinella spiralis* infective larvae. *Parasite Immunol* **3**: 339-352

[5] Handbook of Biochemistry and Molecular Biology, Volume II, Proteins (Ed Fasman GD) 3rd Edition 1976, CPC Press p546

27. Agarose/Acrylamide Gels

These large-pore gel systems were designed to resolve large polymers such as proteoglycans [1] and in this method is combined with immunoblotting to detect specific high molecular weight antigens. The recipe below is for a 0.6% agarose, 1.2% acrylamide composite gel.

27.1 Gel Electrophoresis

Reagents

1. TAS (Tris-Acetate-Sulphate) buffer — 2.42 g Tris base (Final concentration 40 mM); 0.071 g Na_2SO_4 (1 mM)
   Dissolve in water and pH to 6.8 with glacial acetic acid . Make up to 500 ml.
2. 1.54 ml ß- dimethylaminopropionitrile (Sigma D1008) in 24 ml water. Freshly made. [6.4 % (v/v)]
3. 0.3 g Ammonium persulphate in 3 ml water [10% (w/v)]. Must be made up fresh
4. 2 x Loading buffer: 40 g sucrose in 1/2 dilution of TAS bromophenol blue (see Note 1)
5. Size Markers: Lambda HindIII fragments stored in loading buffer (see Note 2)
6. Agarose: BRL Ultrapure (5510UB)

Method

1. Dissolve 0.48 g agarose in 45 ml Solution A and heat to boiling. A microwave oven is particularly convenient. Cool to 52° C (hand hot).
2. Solution B: Dissolve 0.93 g acrylamide, 0.03 g bis in 23.6 mls Solution A
   Add 9.6 mls DMAPN solution
   Add 1.8 mls Ammonium persulphate solution
   Add to agarose (total volume 80 ml) and pur into 1 mid-size gel.
3. Add 1.8 ml of Solution C to the acrylamide mix, add quickly to agarose and pour gel. which will set rapidly.
4. Load samples in loading buffer and run each gel at 50-75 mA for 3-5 hrs, until bromophenol blue nears end of gel
5. Cut off track(s) containing size markers and make a diagonal cut on the main gel at the bottom of the first sample track for orientation.
6. Immerse the gel strips containing marker into 5 µg/ml Ethidium Bromide (caution: highly carcinogenic) for 10 mins, then into distilled water

Notes

1. Loading buffer may contain 4M urea to dissociate noncovalent aggregates.
2. Lambda DNA Hind III fragments are:

23130 bp	15270 K
9416 bp	6210 K
6557 bp	4330 K
4361 bp	2880 K
2322 bp	1530 K
2027 bp	1340 K
564 bp	370 K
125 bp	83 K

   Heat 5 mins, 65°C then cool rapidly before use to dissociate hybrid fragments
3. Plain agarose gels are run in Tris-Borate-EDTA (TBE):

21.6 g	Tris Base	(108 g)
11.0 g	Boric Acid	(55 g)
1.86 g	EDTA	(9.3 g)

   Make up to 1 Litre with water. Bracketed figures are for 1 litre of 5 x solution.
4. Agarose-acrylamide gels run in 1/4 dilution of Solution A: 10 mM Tris 0.2 mM Na_2SO_4 pH 6.8

27.2 Affinity Transfer Blotting

In this procedure, target antigens are captured onto nitrocellulose paper by binding to specific (usually monoclonal) antibody pre-coated onto the paper. This technique is particularly valuable for characterising molecules with little or no affinity for nitrocellulose [2].

Reagents

1. Coating Solution: Antibody in PBS, at 50 µg/ml of other concentration as experimentally determined.
2. Blot solution: 1 M NH_4Ac, 20 mM NaOH
3. Blocking solution: 5% Foetal Calf Serum in Tris-Buffered Saline (see below) without detergent. Add azide to keep.
4. Labelled antibody: ^{125}I-labelled antibody, 10^5-10^6 cpm/ml, in TBS with detergents and 2% FCS
5. Wash solution: Make 2 Litres of 20 mM Tris-Buffered Saline:

17.5 g saline	[0.875%]
40 ml of 1M Tris pH 7.5	[0.02 M]
10 ml of Triton X-100	[0.5%]
1 ml Tween 20	[0.05%]

Procedure

1. Coat NC paper by incubating in 50 µg/ml antibody in PBS, 4-6 hours at 37°C.
2. Blot agarose-acrylamide gel by laying gel onto coated nitrocellulose paper lying on 3 sheets of Whatman 3MM soaked in blot solution, themselves lying on a 5 cm thick pad of adsorbent paper towels. Cover gel with a further sheet of wet filter paper, and place a glass plate over these followed by a moderate (1 kg) weight. Leave overnight at room temperature.
3. Block the NC paper for 60 mins, room temperature, rocking
4. Incubate NC paper with labelled monoclonal antibody 1-10 x 10^5 cpm/ml, 4 hours, rocking, room temperature.
5. Wash extensively (>6 x 10 mins/wash) in TBS-Triton-Tween, dry off in oven and set up for autoradiography

Notes

1. Blotto (5% skimmed milk powder) can be used in blocking solution in place of FCS except where any phosphorylcholine specificity is involved (see **Method 15**).
2. Filtration of labelled antibody through 0.2µ filter reduces spots on autoradiograph from aggregates.

27.3 References

[1] McDevitt CA & Muir H (1971) Gel electrophoresis of proteoglycans and glycosaminoglycans on large-pore composite polyacrylamide-agarose gels. *Anal Biochem* **44**: 612-622.

[2] Handman E, Greenblatt CL & Goding JW (1984) An amphipathic sulphated glycoconjugate of *Leishmania:* characterisation with monoclonal antibodies. *EMBO J* **3**: 2301-2306.

28. Enzymatic Deglycosylation of Glycoproteins

Deglycosylation of glycoproteins is important for elucidating the structure, function and biosynthesis of molecules. For such studies it is important to maintain the integrity of the protein part of the molecule and consequently a variety of specific glycosidases which cleave carbohydrates and oligosaccharide chains have been isolated [1,2]. Glycosidases fall into two groups: **exo**glycosidases, which are active on terminal residues only and usually act very slowly; and **endo**glycosidases, which cleave oligosaccharide chains from glycoproteins and polysaccharides. Endoglycosidases can be classified into three major groups: (1) the endo-*N*-acetyl-β-D-glucosaminidases (endo D, endo F and endo H) which cleave the di-*N*-acetylchitobiose core of Asn-linked oligosaccharide chains at GlcNAc-↑-GlcNAc, individual enzyme specificity is determined by the antennary structure; (2) endo-β-D-galactosidase which hydrolyzes galactosidic bonds (Gal-↑-Glc or Gal-↑-GlcNAc) in poly(*N*-acetyl-lactosamine) type oligosaccharides; and (3) endoglycopeptidases (or peptide *N*-glycosidases: PNGases) and peptide *O*-glycosidases (endo-*N*-acetyl-α-D-galactosaminidase), which cleave between the amino acid residue and the oligosaccharide side chain in *N*-acetylglucosaminylasparagine and *N*-acetylgalactosaminylserine/threonine structures respectively.

Peptide *N*–Glycanase F [3,4] has perhaps the widest application in identifying the presence of *N*-linked oligosaccharides of glycoproteins because it readily cleaves most high-mannose oligosaccharides, hybrid oligosaccharides and biantennary, triantennary and tetraantennary complex oligosaccharides. It will not cleave oligosaccharides attached to either amino- or carboxy- terminal Asn or oligosaccharides *O*-linked to serine or threonine.

28.1 Peptide: N–Glycanase F

Procedure:

1. Make up digestion buffer:

300 µl	100 mM	1,10 phenanthroline (19.8 mg/ ml)
500 µl	7.5 %	NP40
900 µl	550 mM	Sodium phosphate pH 8.6 (Add 200 µl 1 M NaH_2PO_4 to 20 ml 1M Na_2HPO_4, and water to a final volume of 36.7 ml)

2. Take 5 µl of labelled antigen, add 5 µl of 1% SDS, 1.6% 2-mercaptoethanol and boil for 3 minutes (if using smaller volumes of antigen, add water to compensate).
3. Add 17 µl of digestion buffer. It is important to add the excess NP40 to the antigen/SDS mixture before introducing the enzyme (note 1).
4. Add either: (a) 3 µl of *N*-glycanase (note 2) (eg. at 10 U/ ml)
   or (b) 3 µl of 50% glycerol, 2.5 mM EDTA (control)
5. Incubate at 37°C for 24 hours.
6. Boil for 3 minutes and after adding SDS-PAGE loading buffer, run on gel.

Notes

1. The enzyme is inhibited by SDS in the absence of non-ionic detergents.
2. *N*-glycanase can be purchased from Genzyme or BCL (Glycopeptidase F: 903-337).

28.2 References

[1] Thotakura NR & Bahl OP (1987) Enzymatic deglycosylation of glycoproteins. Meth Enzymol **138**: 350-359.
[2] Keesey J (1987) Biochemica Information. 1st edition. Available from Boehringer Mannheim Biochemicals.
[3] Plummer TH, Elder JH, Alexander S, Phelan AW & Tarentino AL (1984) Demonstration of peptide: *N*-glycosidase F activity in endo-β-*N*-acetylglucosaminidase F preparations. J Biol Chem **259**: 10700-10704.
[4] Tarentino AL, Gomez CM & Plummer TH (1985) Deglycosylation of asparagine-linked glycans by peptide: *N*-glycosidase F. Biochemistry **24**: 4665-4671.

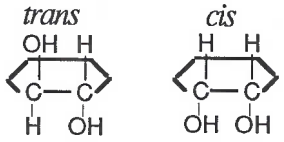
29. Chemical Cleavage of Carbohydrates

Two straightforward methods are described below for investigations of carbohydrate antigens or side-groups, while more elaborate procedures are also available. These are:

Method	Effect	Reference
Periodate	Breaks hexose ring and therefore disrupts epitope; does not break saccharide chain	**Method 29.1**; [1,2]
Alkali	Cleaves *O*-linked and some *N*-linked oligosaccharides	**Method 29.2**; [3,4]
Hydrofluoric Acid	Cleaves all oligosaccharides	[5]
TFMS	Cleaves peripheral *N*-linked sugars	[6]
Hydrazine	Cleaves GlcNAc links (including therefore *N*-links)	[7]

29.1 Periodate Oxidation

Sodium periodate ($NaIO_4$) will oxidise hexose sugars where there are adjacent hydroxyl groups on carbons 2 and 3; this reaction is much more efficient in *cis* diols than *trans* diols (see below).



Periodate will also oxidise other sites, such as histidine on the peptide backbone, and minimal conditions [2] are therefore to be preferred.

Reagents:

1. Sodium periodate (Sigma S-1878), 5 mM in 0.2 M sodium acetate buffer pH 4.7
2. Bio-Gel P6 column (Bio-Rad) in a 2 ml syringe
3. PBS containing 0.5% Triton X-100

Procedure:

1. Mix equal volumes of antigen solution (eg at 2 mg/ml) and 5 mM $NaIO_4$.
2. Incubate at 4°C in the dark for 30 mins.
3. Load onto P6 column equilibrated in PBS-0.5% Triton X-100. Wrap column in aluminium foil to exclude light.
4. Collect the exclusion peak containing glycoproteins with oxidised sugars for epitope analysis.

Notes

1. Iodine liberated during the reaction will attack tyrosine residues in the presence of light.
2. P6 is an acrylamide matrix. Do not use Sephadex as the periodate will react with the column matrix itself!
3. Oxidation with 50 mM $NaIO_4$ would be considered severe treatment.

29.2 Alkaline Degradation

O-glycosidic linkages between glycans and the β-hydroxyamino acids serine and threonine are easily split in mild alkali solutions (0.05 – 0.1 M) by beta-elimination. The same conditions will continue to cleave each reducing sugar from the oligosaccharide once released from the peptide backbone; this 'peeling' reaction can be inhibited by including 0.5 M sodium borohydride (Sigma S-9125) if the oligosaccharides need to be kept intact. *N*-linkages are generally considered to be stable in the mild conditions required to release *O*-linked chains but are cleaved in severe conditions (1.0 M). However other workers [4] have shown that even the mild conditions used to dissociate *O*-linked sugars will also liberate many *N*-linked glycans.

Reagents:

1. 0.2 M KOH
2. N_2 gas
3. 2 M acetic acid (HAc)

Procedures:

1. Mix equal volumes of sample and 0.2 M KOH to give final concentration of 0.1 M.
2. Fill vial with N_2, seal and incubate in the dark overnight at 37°C.
3. Add 2 M HAc to pH 6.5. Approximately 320 µl per ml of KOH will be required. Use a replicate tube to titre the exact volume; then add same volume to sample.
4. Dialyse extensively against PBS or other desired buffer, in the dark.

29.3 References

[1] Eylar EH & Jeanloz RW (1962) Periodate oxidation of the α_1 acid glycoprotein. *J Biol Chem* **237:** 1021-1025.

[2] Miller-Podraza H & Fishman PH (1982) Translocation of newly synthesized gangliosides to the cell surface. *Biochemistry* **21:** 3265-3270.

[3] Iyer RN & Carlson DM (1971) Alkaline borohydride degradation of blood group H substances. *Arch Biochem Biophys* **142:** 101-105.

[4] Ogata S-I & Lloyd KO (1982) Mild alkaline borohydride treatment of glycoproteins – a method for liberating both *N*- and *O*- linked carbohydrate chains. *Anal Biochem* **119:** 351-359.

[5] Mort AJ & Lamport DTA (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. *Anal Biochem* **82:** 289-309.

[6] Edge ASB, Faltynek CR, Hof L, Reichert LE Jr & Weber P (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal Biochem* **118:** 131-137.

[7] Takasaki S, Mizuochi T & Kobata A (1982) Hydrazinolysis of asparagine-linked sugar chains to produce free oligosaccharides. *Methods Enzymol* **83:** 263-268.

30. Protease Specificities

A range of proteolytic enzymes are available which will cleave proteins at specific sites or in a more general manner. Extensive details of a large number of individual proteases (or proteinases) can be found in three volumes of *Methods in Enzymology*, Volumes **19** (1970), **45** (1976) and **80** (1981). Proteases are classified into four classes according to their mechanism of action:

SERINE PROTEASES: An evolutionarily conserved group of proteases including trypsin, chymotrypsin and elastase, these share homologous sequences around active site serine and histidine residues [1,2]. Several helminth secreted proteases fall into this class [3,4]. Inhibitors include DFP (diisopropylfluorophosphate) and PMSF (**Method 31**) which covalently bind into the active site.

ASPARTYL PROTEASES. These have a critical aspartic acid residue in the active site. Also termed acid proteases, this group includes pepsin and the HIV protease. These are inhibited by pepstatin.

SULPHYDRYL PROTEASES. These have cysteine residues in the active site which must be in the reduced state to maintain free sulphydryl groups. Activity is therefore optimised in the presence of cysteine or dithiothreitol, while iodoacetamide and other SH-reactive groups are strongly inhibitory. Members of this group include bromelain and papain.

METALLOPROTEINASES. These enzymes require a metal group, such as zinc in the case of collagenase, and are reversibly inhibited by EDTA or other chelators.

Enzyme	Type	Preferred Cleavage Site	Mol Wt
Bromelain (Pineapple)	Sulphydryl	Broad Specificity	33,000
Chymotrypsin (Bovine Pancreas)	Serine	Trp, Try, Phe	25,000
Collagenase (*Clostridium*)	Metalloprotease (Zn)	Pro–X–↓–Gly–Pro only	105,000
Elastase (Porcine Pancreas)	Serine	Neutral Amino Acids	25,900
Factor Xa (Bovine Plasma)	Serine	Gly–Arg–↓–X only	55,000
Papain (Papaya)	Sulphydryl	Lys, Arg, Leu, Gly–↓	23,000
Pepsin (Porcine Gastric Mucosa)	Aspartyl (Acid)	Broad Specificity	34,500
Pronase (*S. griseus*)	Mixed	Broad Specificity	Mixed
Proteinase K (*Tritirachium album*)	Serine	Broad Specificity	18,500
Subtilisin (*B.subtilis*)	Serine	Broad Specificity	27,600
Trypsin (Bovine Pancreas)	Serine	Lys, Arg–↓ only	23,500
V8 Protease (*S. aureus*)	Serine	Asp, Glu–↓ only	27,000

Notes

1. Proteases generally are far more active against denatured than native proteins.
2. Trypsin cannot easily be purifed free of chymotrypsin and should therefore be treated with TPCK, an irreversible inhibitor of chymotrypsin.
3. V8 Protease, also termed Endoproteinase Glu-C was originally reported to cleave only at Glutamic acid residues in bicarbonate and acetate buffers but at both Glutamic and Aspartic acids in phosphate buffers [5]; subsequent reports have not confirmed this [6].

References

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31. Protease Inhibitors

Whenever protein molecules are extracted from living organisms there is always the problem that the desired material will be degraded by proteases released during the extraction process. To combat this a cocktail of protease inhibitors can be made up and added to solutions whenver this problem is likely to arise. Below are outlined the properties [1-3] of a range of protease inhibitors all easily obtainable from Sigma and a protocol for a basic cocktail. Some inhibitors are only soluble in organic solvents, therefore two stock solutions should be made, and added to solutions as required.

Substance	Sigma Cat No	Mol Wt	Working Conc	Properties
EDTA	ED-2SS	292.2	1 mM	metal chelator
EGTA	E-4378	380.3	1 mM	Ca/Mg chelator
NEM	E-3876	125.1	1 mM	protects sulphydryl groups
TPCK	T-4376	351.9	0.1 mM	chymotrypsin/ thiol protease inhibitor
PMSF	P-7626	174.2	1 mM	serine protease inhibitor
TLCK	T-7254	369.3	0.2 mM	trypsin/ thiol protease inhibitor
1,10-P	P-9375	198.2	1 mM	divalent cation chelator
pHMB	H-0642	360.0	1 mM	protects sulphydryl groups
aprotinin	A-1153	6,511.0	0.3 µM	serine protease inhibitor
leupeptin	L-2884	475.0	0.1 µM	serine & thiol protease inhibitor
pepstatin A	P-4265	685.0	0.1 µM	aspartyl protease inhibitor
α_1 antitrypsin	A-6150	54,000.0	0.1 µM	serine & thiol protease inhibitor
SBTI	T-9128	20,100.0	10 µg/ ml	serine & thiol protease inhibitor
E-64	E-3132	357.0	0.0020 mM	thiol protease inhibitor

The following protocol is for 2 stock solutions which produce the working concentrations of six inhibitors to cover the range of likely proteases.

Procedure

1. Make up each of the following stock solutions, to be stored at –20°C:

EDTA*	2.92 g/10 ml water	(10 ml of 1 M)	(Keep pH 8 to obtain complete dissolution)
EGTA*	3.80 g/10 ml water	(10 ml of 1 M)	(Keep pH 8 to obtain complete dissolution)
NEM	1.25 g/10 ml water	(10 ml of 1 M)	
TPCK	352 mg/ 10 ml ethanol	(10 ml of 0.1 M)	
PMSF	581 mg/ 10 ml ethanol	(10 ml of 0.33 M)	
Pepstatin	6.85 mg/10 ml ethanol	(10 ml of 1 mM)	

2. Mix 1 ml aliquots of the water soluble inhibitors and make up to 5 ml with water. This gives a 200x solution, designated PI-A (aqueous). Store in aliquots at –20°C:.
3. Mix 3 ml of 0.33 M PMSF with 1 ml of 0.1 M TPCK and add 1 ml of 1 mM Pepstatin. This gives a 200x solution, designated PI-B (organic). Store in aliquots at –20°C:.

Abbreviations

EDTA ethylene diamine tetracetic acid, disodium salt
EGTA ethylene glycol bis (2-amino ethyl ether) -*N,N,N',N'*-tetracetic acid
PMSF phenyl methyl sulphonyl fluoride
TLCK N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl
TPCK N-tosylamide-L-phenylalamine chloromethyl ketone
1,10-P 1,10-phenanthroline
pHMB p-hydroxymercuribenzoic acid
SBTI soybean trypsin inhibitor
E-64 *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane

Notes

1. Complete dissolution of some components is only obtained if the solution is warmed.
2. Most of these inhibitors are toxic and precautions should be observed.

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32. Detection of Proteases in Solution

A sensitive microplate assay for the detection of proteolytic enzymes using radiolabelled gelatin as substrate has been described [1]. The assay substrate is susceptible to a wide range of proteolytic enzymes [1], and the microplate format allows large numbers of samples to be assayed economically, a considerable advantage over conventional substrates such as Azocoll (Note 2). It can also be used for inhibition and pH dependence studies which allow characterisation of enzymes present [2].

Reagents:

1. A stock solution of 0.1% bacteriological gelatin (Gibco 152-0221) in distilled water, and boiled for 10 minutes to destroy endogenous proteolytic enzymes.
2. Radiolabel 10 µg of bacteriological gelatin with 400 µCi of Bolton-Hunter reagent (**Method 6**). Determine the percentage of TCA precipitable counts (**Method 4.3**) and store labelled protein at –20°C.
3. PVC microtitre 96–well plates (Flow Titertek immunoassay plate).
4. 0.06 M carbonate buffer pH 9.6 (**Method 17**) and PBS, pH 7.4 (**Appendix 1**).

Procedure:

1. Coat microtitre plates with 30,000 cpm per well of radiolabelled gelatin, diluted in 50 µl carbonate buffer, and allow the supernatant to evaporate by incubation overnight , uncovered in a dry 37°C incubator.
2. Wash wells 3 times with PBS (10 minutes each at roo temperature), then incubate for 2 hours at 37°C with PBS, and then wash a further 3 times. Plates can be stored dry at room temperature until required.
3. Add duplicate 50 µl samples of enzymes at various dilutions in PBS, to the plate and incubate for 16 hours at 37°C.
4. Aspirate the supernatants from the plate and cut up the wells. Count individual supernatants and wells in a gamma counter. The percentage counts released can then be calculated for each sample as follows:

$$\frac{cpm_{(supernatant)}}{cpm_{(supernatant)} + cpm_{(wells)}} \times 100$$

The background counts released by buffer alone should be subtracted for each sample.

Notes

1. Bolton-Hunter reagent is better than Iodogen as lysines are more abundant thsn tyrosines in a collagenous based substrate.
2. An alternative substrate is Azocoll [3] (Sigma A-8143) which when digested releases dye into the supernatant which can be quantified spectrophotometrically at 540 nm. Enzyme samples are added to eppendorfs containing 5 mg of Azocoll in 0.5 ml of PBS and incubated overnight at 37°C. Tubes are then spun in a microfuge for 5 minutes and the absorbance of the supernatant measured. Suitable controls of buffer alone should be included.
3. Protease inhibitors (**Method 31**) added individually or in combination will indicate the type of enzyme.
4. Using a range of buffered pH in place of PBS for procedure 3 will indicate the pH optimum for enzyme activity.

References

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33. Electrophoretic Separation of Proteolytic Activities

These methods allow individual proteases in an otherwise perhaps complex mixture to be visualised simultaneously with their molecular weight, by using SDS-PAGE gels which have been copolymerised with a suitable enzyme substrate. Gelatin was first used as a substrate by Heussen [1] and the method described here is that used by McKerrow [2] to detect an elastinolytic proteinase secreted by *Schistosoma mansoni* cercariae. The second method using a separate indicator gel follows that described by Hotez and Cerami [3], based on the original work of Granelli-Piperno and Reich [4].

33.1 Copolymerisation of Substrate in SDS-PAGE Gel.

Reagents:

1. SDS-PAGE 5-25% gradient gels copolymerised with 0.1% bacteriological gelatin (Gibco 152-0221). Dissolve gelatin in 24 mls heated water, allow to cool and add 12 ml to the mixtures for casting gels in place of the distilled water, a further 24 ml of water will be required for the 5% solution (**Method 7**).
2. 100 ml of 2.5% aqueous Triton X-100.
3. Incubation buffer: 200 ml 0.1M Tris or PBS, pH 7.4, and with or without 2mM Dithiothreitol (DTT) (Note 2)
4. Coomassie Brilliant Blue stain and destain (**Method 9.1**).

Procedure:

1. Load up to 10 µg of protein per track, unreduced and unboiled. (Boiling and reduction can be used as negative controls). If possible run radiolabelled proteins and markers in parallel with the samples so that after autoradiography proteolytic bands can be visualised in relation to other components.
2. Run the gels quickly (25 mA/gel) with fresh running buffer at a temperature of 4-10°C. (Note 3).
3. After electrophoresis place gel in 2.5% Triton X-100 and rock at room temperature for 30 mins to remove the SDS. Then gently agitate the gel for 60 mins in incubation buffer with one change. Replace the buffer and put the gel at 37°C overnight (Note 4).
4. Fix the gel and stain with Coomassie for 60 mins. Destain as necessary. Areas of proteolysis appear as clear bands on a blue background and are best visualised for photography on a light box.
5. Dry and autoradiograph the gel to visualise markers and labelled proteins.

33.2. Use of a separate indicator gel

Reagents:

1. Agarose gel (cast on a gel plate) containing: 4.0 ml of 2% skimmed milk powder or 0.1% bacteriological gelatin made up in PBS; 7.2 ml of 2.5% agarose; 10.4 ml of 0.1 M Tris/HCl pH 8.0
2. 100 ml of 2.5% aqueous Triton X-100
3. Prestained molecular weight markers.
4. Coomassie Brilliant Blue stain and destain (**Method 9.1**).

Procedure:

1. Run sample on a 0.7mm SDS-PAGE gel. Typically 10 µg of protein per track in loading buffer, unreduced and not boiled are loaded on a 5-25% gradient gel. Prestained molecular weight markers can also be run.
2. After electrophoresis place gel in Triton X-100 and rock at room temperature for 30 mins. Agitate for 60 mins in distilled water with one change .
3. Overlay gel onto agar plate, place in a sealed container, and incubate at 37°C in a moist environment for at least 12 hours. Bands on the agarose gel can be visualised by staining with Coomassie, and destaining as required. The PAGE gel can be Coomassie stained and/or dried and autoradiographed if radiolabelled markers have been used.

Notes:

1. A variety of other substrates can be substituted for gelatin eg. Casein (Sigma C-0376) or skimmed milk powder.
2. DDT activates thiol proteases and may enhance proteolytic activity. Replicate gels incubated in the presence and absence of DDT should be run.
3. The gels are run quickly and in the cold to limit the amount of enzyme activity during electrophoresis which produces smears.
4. Replicate gels can be incubated in buffer at a variety of pHs to determine optimum pH for enzyme activity.
5. The addition of specific protease inhibitors to the incubation buffer allow enzyme activity to be categorised.
6. To obtain optimum enzyme activity pH can be altered in the acrylamide gel by soaking it in a buffer of appropriate pH for 10 min after the final wash, or in the agarose gel by changing the pH of the buffer.

33.3 References:

[1] Heussen C and Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrate. *Anal Biochem* **102**: 196-202.

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34. Enzyme Assays

A few of the huge range of enzyme assays available are described here, particularly where they have been applied to parasite species, and where modifications have been introduced to minimise material required for analysis. The three enzymes selected are Glutathione-S-Transferase (GST :**Methods 34.1** and **34.2**), Superoxide Dismutase (SOD : **Methods 34.3** and **34.4**), both potential detoxification enzymes, and Acetylcholinesterase (AChE : **Method 34.5**), an enzyme involved in helminth neuroregulation but which is also frequently secreted.

34.1 Glutathione S-transferase (GST)

The following is an assay for enzyme activity in secretions or soluble extracts[1,2]; identification of the enzyme on PAGE gels is described in **Method 34.2**, while details of affinity purification are given in Refs [1,3].

Reagents

1.	Chloro-2,4-Dinitrobenzene		(CDNB: Sigma C6396)	Store at room temperature
2.	Glutathione		(GSH: Sigma G 4251)	Store dessicated at 4°C
3.	Make up stock solutions:	1 M	Phosphate buffer pH 6.5	(Final concentration, 0.1 M)
		25 mM	CDNB in ethanol	(0.5 M)
		10 mM	GSH in distilled water	(1 mM)

Procedure

1. For 1 ml reaction mixture in a cuvette, add sample to 100 µl phosphate buffer, 20 µl CDNB and water to a volume of 900 µl.
2. Add 100 µl of GSH then X µl of sample or enzyme.
3. Measure rate of increase of absorbance (Note 1) at 340 nm for 5 mins against test and control samples.

Notes

1. A sensitive spectrophotometer is required eg. Techtron DMS 200 or Beckman DU70).
2. 1 unit of activity is defined as the amount of enzyme which catalyses the formation of 1 µmole of product per minute ($\Delta E = 9.6\ mM^{-1}cm^{-1}$).

34.2. Visualising Glutathione S-Transferase On Polyacrylamide Gels

Materials

1.	CDNB, Glutathione and 0.1 M potassium phosphate buffer pH 6.5 as in **Method 34.1**		
2.	Phenazine methosulfate	(PMS: Sigma P 9625)	Store dessicated at 4°C
3.	Nitroblue tetrazolium chloride	(NBT: Sigma N 6876)	Store dessicated at 4°C
4.	Coomassie blue 0.1% - in methanol /acetic acid /water (50/10 /40)		
5.	Destain: 7% acetic acid		
6.	Solution A: 4.5 mM GSH 1.0 mM CDNB 1.0 mM NBT made up in phosphate buffer pH 6.5		
7.	Solution B:3.0 mM PMS in 0.1 M Tris buffer pH 9.6		
8.	Solution C: 25% isopropanol in 10 mM Tris buffer pH 7.5		

Procedure A [4]

1. Run sample on SDS-PAGE gradient gel, and then wash gel in distilled water (3x) over 1-2 hours.
2. Remove and incubate gel in 50 ml of Solution A at 37°C in incubator or water bath for 1 hour.
3. Wash gel with 3 changes of distilled water.
4. Place gel in 40 ml Solution B at room temperature. Blue formazan should appear in 3-5 min in all areas except those containing the enzyme glutathione S-transferase.
5. Wash gel with water and place in 1 M NaCl (colourless bands remain defined for 1 month).

Procedure B [4,5]

1. Prerun SDS-PAGE gradient gel, without stack, for 3 hour at 20 mA to remove ammonium persulphate.
2. Pour stack and then run sample as usual.
3. After electrophoresis, wash gel in solution C for 1 hour and then in fresh solution C for another 30 minutes.
4. Wash gel 3x 10 mins in 10 mM Tris pH 7.5.
5. Incubate gel in 50 ml of Solution A at 37°C in incubator or water bath for 1 hour.
6. Wash gel with 3 changes of distilled water.
7. Place gel in 40 ml Solution B (room temp). Blue formazan should appear in 3-5 min in all areas except those containing the enzyme glutathione S-transferase.
8. Wash gel with water and place in 1 M NaCl (colourless bands remain defined for 1 month).

34.3 Superoxide Dismutase (SOD) Enzyme Assay

Superoxide dismutase is assayed by indirect methods ie. by detecting the inhibition of reactions directed by oxygen radicals. Xanthine-xanthine oxidase is the most widely used system to generate oxygen radicals for reducing substrates such as cytochrome C [6] or nitroblue tetrazolium [7]. Other more sensitive methods include luminol [8] and chemiluminescence [9] assays, but these require special detection apparatus.

Materials

1. Cytochrome C (from horse heart Type VI: Sigma C7752)
2. Xanthine (Sigma X0626)
3. Xanthine oxidase (Sigma X4500)
4. EDTA (Sigma ED2SS)
5. Solution A: 0.5 M Potassium phosphate buffer, pH 7.8 (Final concentration, 0.05 M)
6. Solution B: 1×10^{-4} M cytochrome C (1×10^{-5} M)
   5×10^{-4} M xanthine 5×10^{-5} M)
   1×10^{-3} M EDTA in distilled water (1×10^{-4} M)

Methods

1. Add 100 µl of Solution A, 100 µl solution B, and 700 µl – X µl of distilled water to a cuvette. Enzyme reaction is started by adding 100 µl of Xanthine oxidase (1 unit) and immediately X µl of enzyme/extract/ES to the reaction mixture. (Total reaction mixture is 1 ml).
2. Enzyme reaction is measured in DMS 200 spectrophotometer at 550 nm. SOD enzyme activity is determined as the % inhibition of the Xanthine oxidase enzyme activity measured by change of OD at 550nm in the absence of SOD (1 unit of enzyme activity = 50% inhibition).

34.4 Staining SOD in Gels

This follows the procedure given in Ref [7]

Materials

1. Nitroblue tetrazolium chloride (NBT: Sigma N 6876)
2. *N,N,N',N'*-Tetramethylethylenediamine (TEMED: Sigma T 1833)
3. Riboflavin (Sigma R 4500)
4. 0.036 M potassium phosphate buffer, pH 7.8
5. 2.45×10^{-3} M nitroblue tetrazolium in phosphate buffer (solution A)
6. 0.028M TEMED and 2.8×10^{-5} M in phosphate buffer (solution B)
7. 25 % isopropanol in phosphate buffer (solution C)

Procedure

Prepare SDS-PAGE gradient gel by either method below:

1. Run SDS-PAGE gradient gel as usual.
2. After electrophoresis, wash gel indistilled water (3x) over 1-2 hours.

OR

1. Prerun SDS-PAGE gadient gel, without the stack, for 3 hour at 20 mA to remove ammonium persulphate.
2. Pour stack and then run sample as usual.
3. After electrophoresis, wash gel in solution C for 1 hr and then into fresh solution C for another 30 mins.
4. Wash gel 3x 10 minutes in phosphate buffer.

THEN PROCEED TO:

1. Incubate gel in solution A for 1 hour at room temperature.
2. Transfer gel to solution B for 30 minutes.
3. Place gel on a dry surface and illuminate for about 15 minutes. Gel becomes uniformly blue except at places containing superoxide dismutase.

34.5 Acetylcholinesterase (AChE)

Acetylcholinesterases are abundant products in many helminths and are secreted by a range of nematode species [11] and by *Schistosoma mansoni* [12]. Enzyme activity can be measured by the Ellman reaction [13], in this protocol miniaturised onto an ELISA plate format [14].

Reagents:

1. Acetylthiocholine (Sigma A 5751), 75 mM
2. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB: Sigma D 8130), 10 mM.
3. 0.1 M phosphate buffer, pH 8.0
4. 0.1 M eserine (Sigma E 8375).)

Procedure:

1. Add 260 µl 0.1 M phosphate buffer to each well
2. Add 10 µl 10 mM DTNB
3. Add 2.5 µl acetylthiocholine
4. Add 25 µl sample for enzyme assay
5. Incubate 15 mins, 37°C
6. Stop reaction with 0.1 M eserine
7. Read OD at 412 nm.

Notes

1. 1 unit of AChE is defined as 1 µmol of substrate hydrolysed per minute.

34.6 References

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35: Maintenance of Brugia malayi Filarial Nematodes

The life cycle of *Brugia malayi*, zoophilic (subperiodic) strain [1], can be maintained in jirds (*Meriones unguiculatus*) which are susceptible to subcutaneous [2,3] and intraperitoneal [4] routes of infection. In the latter case, development of infective larvae to adult stage worms, and the release of newborn microfilariae, occurs entirely within the peritoneum [4] greatly facilitating parasite recovery. *Mastomys* rats are also susceptible to long term patent infections by the subcutaneous route.

Mosquitoes, either *Aedes togoi* or *Ae. aegypti* carrying a recessive susceptibility gene [5], may be fed on anaesthetised jirds with circulating microfilariae from a subcutaneous infection, or artifically fed with peritoneal microfilariae mixed with fresh blood in a membrane feeder. Within these mosquitoes, the nematodes develop over a 10-14 day period to infective larvae. These larvae, recovered from killed, crushed mosquitoes, may then be inoculated intraperitoneally into jirds to complete the cycle [6].

B. malayi is infective to man and in some instances filarial disease may result in lymphodoema, lymphangitis, lymphadenitis, periodic fever and elephantiasis. The adult parasites do not, however, multiply in mammalian hosts but give rise to large numbers of microfilariae. Only the infective larvae are infective to man, and hence the only hazard to be contained is that of the infected mosquito; infected jirds present no danger.

Although *Aedes aegypti* and *Ae. togoi* are not found naturally in the UK, they may survive for some time in warmer weather or within laboratory facilities. Therefore infected mosquitoes must be completely contained once they have fed on microfilaraemic blood.

35.1 Infection of Jirds with Brugia parasites

(a) Infection with Infective Larvae: Take 0.2-0.5 ml of suspension of L3 in RPMI 1640 containing antibiotics (100 U/ml pencillin, 100 µg/ml streptomycin) containing between 50 and 300 L3 as required. For intraperitoneal injections use a 19G needle and stagger the points of penetration by piercing the peritoneal membrane at least 1 cm from the site of skin penetration; this should prevent any material leaking out through the wound.

(b) Transplantation of Adult Worms [7,8]: Make a small (1-2 cm) incision in the skin and pull out some of the peritoneal membrane of an anaesthetised jird. Make an incision here just large enough to admit a pasteur pipette containing the adults to be transferred; expel the contents of the pipette in one swift movement or else the adults will remain behind adhering to the glass. Press the incision closed, and suture the skin if necessary. Pasteur pipettes may be adapted to give a wider opening by cutting and rounding in a flame; it is often convenient to place the adults to be transferred in a watch glass so that transfer of counted numbers can be verified. For some purposes a syringe and 18G needle may be used; the risk of damage to the parasite is higher. In this case follow the procedure as in (a) of staggering points of entry between skin and peritoneum.

35.2 Harvesting parasites from Jirds

Peritoneal Lavage

An anaesthetised (ether or chloroform) infected jird is injected with 5ml of warm (37°C) RPMI 1640 medium containing 100 U/ml of herapin and antibiotics. After approximately 1 minute of massage of the peritoneum, excess fluid is withdrawn via a needle and syringe. Typically 3-4 ml is recovered.

If a large number of microfilariae are required, the jird may be killed, and the skin cut back to reveal the peritoneal membrane. A lavage with 5 ml of warm RPMI is carried out twice, yielding about 8 ml of fluid.

The parasites at this stage are infective only to mosquitoes.

35.3 Feeding Mosquitoes

Membrane Feeding

1. Mix microfilariae at 10-15,000/ml with fresh mammalian blood, generally from rats or guinea pigs. Blood should be drawn into a container containing anticoagulant (heparin at 100 U/ml).
2. Position glass membrane feeder on netting of mosquito cage, and connect heating chamber to supply of 37°C water.
3. Fill feeder with blood/mf mixture and agitate at frequent intervals.
4. Vacate room (human presence distracts the mosquitoes); leave a small light trained near the membrane but otherwise darken the room.
5. Allow mosquitoes to feed for 1 hour or more as required or as convenient.
6. Maintenance of mosquitoes on 5 mM PABA (para-amino benzoic acid Sigma A-9878) is beneficial.

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35.1 Infection of Jirds with Brugia parasites

(a) Infection with Infective Larvae: Take 0.2-0.5 ml of suspension of L3 in RPMI 1640 containing antibiotics (100 U/ml pencillin, 100 µg/ml streptomycin) containing between 50 and 300 L3 as required. For intraperitoneal injections use a 19G needle and stagger the points of penetration by piercing the peritoneal membrane at least 1 cm from the site of skin penetration; this should prevent any material leaking out through the wound.

(b) Transplantation of Adult Worms [7,8]: Make a small (1-2 cm) incision in the skin and pull out some of the peritoneal membrane of an anaesthetised jird. Make an incision here just large enough to admit a pasteur pipette containing the adults to be transferred; expel the contents of the pipette in one swift movement or else the adults will remain behind adhering to the glass. Press the incision closed, and suture the skin if necessary. Pasteur pipettes may be adapted to give a wider opening by cutting and rounding in a flame; it is often convenient to place the adults to be transferred in a watch glass so that transfer of counted numbers can be verified. For some purposes a syringe and 18G needle may be used; the risk of damage to the parasite is higher. In this case follow the procedure as in (a) of staggering points of entry between skin and peritoneum.

35.2 Harvesting parasites from Jirds

Peritoneal Lavage

An anaesthetised (ether or chloroform) infected jird is injected with 5ml of warm (37°C) RPMI 1640 medium containing 100 U/ml of herapin and antibiotics. After approximately 1 minute of massage of the peritoneum, excess fluid is withdrawn via a needle and syringe. Typically 3-4 ml is recovered.

If a large number of microfilariae are required, the jird may be killed, and the skin cut back to reveal the peritoneal membrane. A lavage with 5 ml of warm RPMI is carried out twice, yielding about 8 ml of fluid.

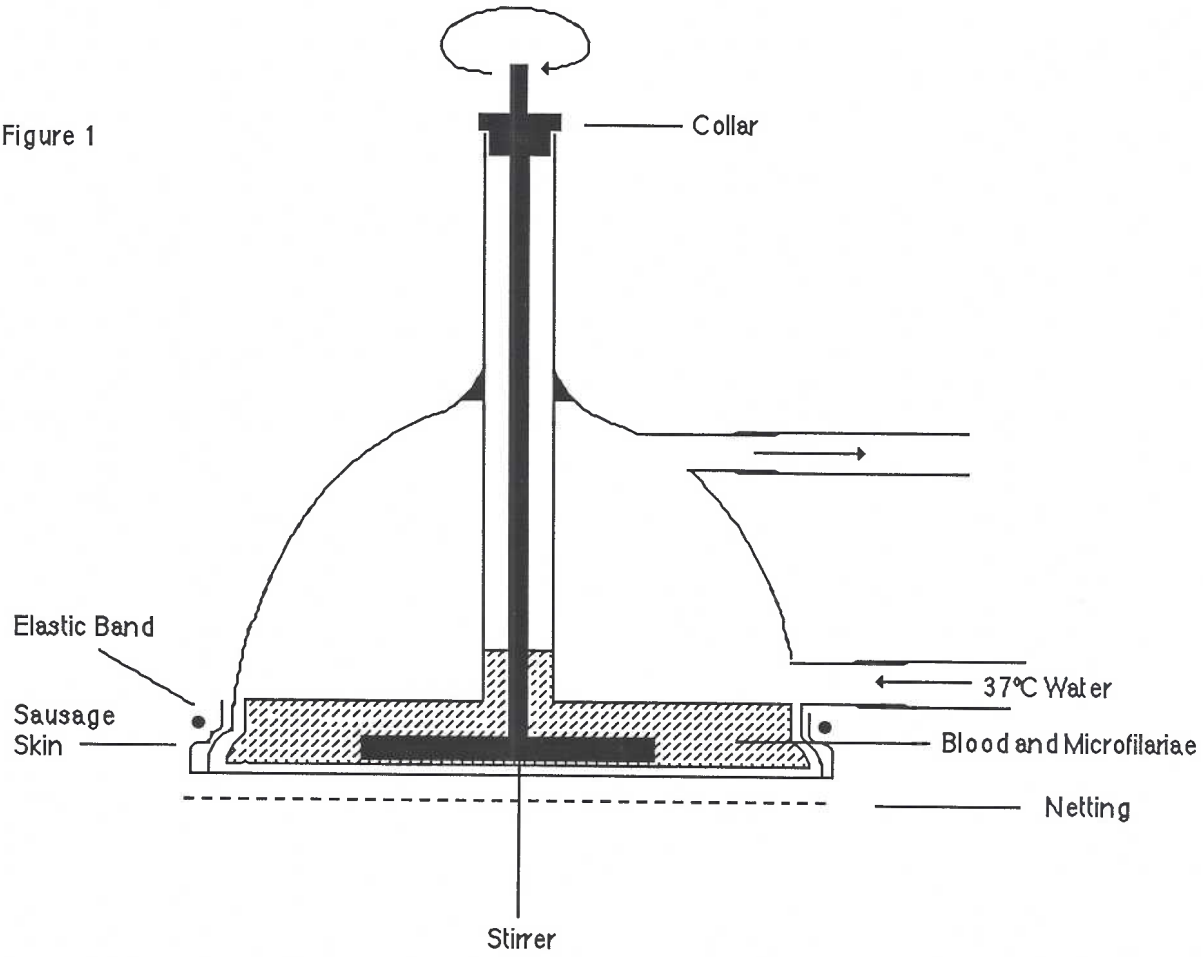
The parasites at this stage are infective only to mosquitoes.

35.3 Feeding Mosquitoes

Membrane Feeding

1. Mix microfilariae at 10-15,000/ml with fresh mammalian blood, generally from rats or guinea pigs. Blood should be drawn into a container containing anticoagulant (heparin at 100 U/ml).
2. Position glass membrane feeder on mosquito cage netting, and pump 37°C water through heating chamber.
3. Fill feeder with blood/mf mixture and agitate at frequent intervals.
4. Vacate room (human presence distracts the mosquitoes); leave a small light trained near the membrane but otherwise darken the room.
5. Allow mosquitoes to feed for 1 hour or more as required or as convenient.
6. Maintenance of mosquitoes on 5 mM PABA (para-amino benzoic acid Sigma A-9878) is beneficial.

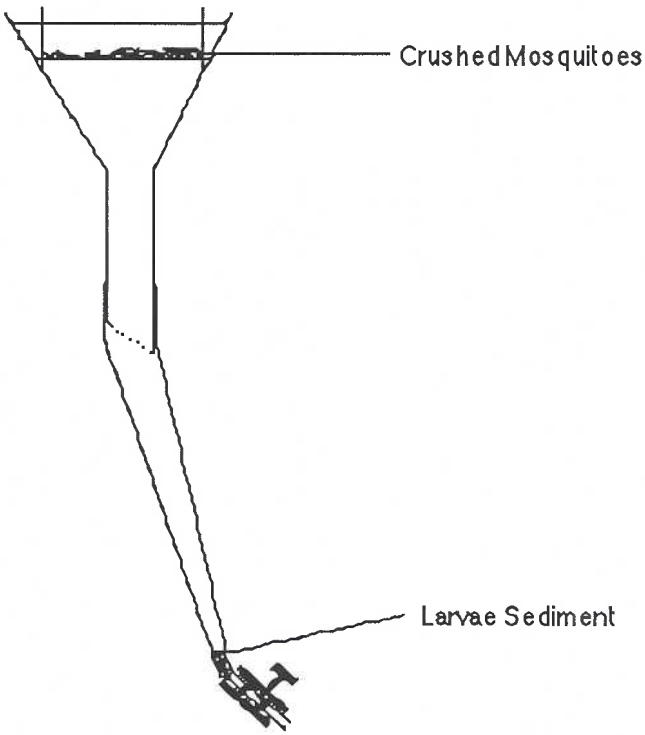
Figure 1



35.4 Harvesting Infective Larvae from Mosquitoes

1. Kill mosquitoes by placing cage(s) in CO_2 box for 10 minutes, or spray thoroughly with pyrethroid/piperonyl butoxide insecticide (eg Cooper Flykiller). This procedure must not take place in areas used for mosquito maintenance; organophosphate insecticides must not be used or the cages cannot be recycled.
2. Tap out mosquitoes onto a glass plate (75 x 100 mm or larger) and crush under a gently rolled glass test tube.
3. Transfer crushed mosquitoes into a Baermann apparatus containing an Endecotts Brass/Bronze 75 micron 100 mm diameter sieve immersed in saline at 30 ± 3°C (see Figure 2). Keep the top of the funnel covered with a glass plate or petri dish.
4. Collect infective larvae after 30-120 minutes through outlet tube by releasing clip.
5. Bleach and autoclave the empty cage.

Figure 2



35.5 Purification of Microfilariae

Two techniques produce purified microfilariae from blood or peritoneal fluid. One, originated by Taylor *et al* for *Onchocerxa volvulus* [9] employs Sephadex-G-25 columns; the other uses lymphocyte separation medium (LSM) which consist of 9.4% sodium diatrizoate and 6.2% Ficoll at a density of 1.077-1.080.

Sephadex Purification

Reagents:

1. PD10 Pharmacia prepacked Sephadex G25 columns (17-0851-01)
2. RPMI 1640 warmed to 37°C.

Procedures:

1. Equlibrate PD10 column in warm medium
2. Load microfilariae, washed in medium on PD 10 column in approx 1 ml volume
3. Collect 2 ml fractions
4. Expect cells to elute in 5-10 ml volume, microfilariae in 15-30 mls
5. Inspect each fraction and pool cell-free microfilariae. Unacceptable cell contamination can be remedied by re-running fractions on the same column.

Lymphocte Separation Medium (LSM)

LSM (Cat. No. 36427/8410-01) is supplied by Organon Teknika Corporation, Box 15969, Durham NC 27704.

Procedure

1. Centrifuge microfilaraie at 800 *g* for 10 mins at 4°C
2. If pellet is red, lyse in 3 mls distilled water
3. Resuspend in 3-5ml RPMI 1640
4. Overlay gently onto 5 mls LSM in a 15 ml conical
5. Spin for 15 mins at 800 *g* at 4°C
6. Remove supernatant, and try to take cells adhering to tube. Microfilariae are in the pellet. If red, lyse in water
7. Wash twice in RPMI 1640 before counting.

35.6 Exsheathment of Microfilariae

This method produces high (>99%) purity viable exsheathed microfilariae of *Brugia malayi*, following the high Calcium protocol of Devaney [10,11].

Reagents

1. Phosphate-Free Buffer: make up to 100 ml containing:

680 mg	NaCl
220 mg	$NaHCO_3$
1000 mg	Glucose (or 10 ml x 10%)
40 mg	KCl (or 1 ml x 4%)
1.7 mg	Phenol red (or 1 ml x 1.7 mg/ml)

2. 2 M $CaCl_2$
3. 20 mgs/ml $MgSO_4.7H_2O$

Procedure

1. Take up microfilariae in 5 ml buffer.
2. Add 50 µl 2M $CaCl_2$ (=20 mM) and 50µl 20 mg/ml $MgSO_4$ (=0.2 mg/ml)
3. Incubate at 37° C taking samples at intervals for inspection. Expect exsheathment to be complete after 20 mins but sometimes longer times (up to one hour) are necessary.
4. Gently centrifige (3 mins 1000 rpm) and purify parasites according to **Method 35.5**

Notes

1. Ca^{++} precipitates slowly in this buffer, hence it and Mg^{++} are added at the moment parasites are ready.
2. Papain also effects exsheathment [10,11], but digests all surface proteins too.
3. Exsheathed microfilariae require slightly harder centrifugation.

35.7 References

[1] Partono F & Purnomo (1987) Periodicity studies of *Brugia malayi* in Indonesia: recent findings and a modified classification of the parasite. *Trans R Soc Trop Med Hyg* **81:** 657-662

[2] Ash LR & Riley JM (1970) Development of subperiodic *Brugia malayi* in the jird, *Meriones unguiculatus* with notes on infections in other rodents. *J Parasitol* **56:** 969-973

[3] Ash LR (1973) Chronic *Brugia pahangi* and *Brugia malayi* infections in *Meriones unguiculatus*. *J. Parasitol* **59:** 442-447

[4] McCall JW, Malone JB, Ah H-S & Thompson PE (1973) Mongolian jirds (*Meriones unguiculatus*) infected with *Brugia pahangi* by the intraperitoneal route: a rich source of developing larvae, adult filariae and microfilariae. *J Parasitol* **59:** 436

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[6] Denham DA (1982) Experience with a screen for macrofilaricidal activity using transplanted adult *Brugia pahangi* in the peritoneal cavities of *Meriones unguiculatus*. **In:** Animal Models in Parasitology, Ed Owen DG, Macmillan, London. pp 93-104

[7] Suswillo RR & Denham DA (1977) A new system of testing for filaricidal activity using transplanted adult *Brugia* in the jird. *J Parasitol* **63:** 591-592

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[9] Taylor DW, Goddard JM & McMahon JE (1984) Isolation and purification of microfilariae from nodules of *Onchocerca volvulus*. *Trans R Soc Trop Med Hyg* **78:** 707-708.

[10] Devaney E & Howells RE (1979) The exsheathment of *Brugia pahangi* microfilariae under controlled conditions *in vitro*. *Ann Trop Med Parasit* **73:** 227-233.

[11] Devaney E (1985) Lectin-binding characteristics of *Brugia pahangi* microfilariae. *Trop Med Parasitol* **36:** 25-28.

36: Maintenance of Nippostrongylus brasilensis

36.1 Infection of Rats

Inject 3000-6000 infective larvae (L3) subcutaneously into an anaesthetised Sprague-Dawley (SD)rat. Use a 21 G needle, insert under the flank skin for 2-3 cms and inject smoothly causing a bleb to form under the skin. Routine maintenance is usually carried out in outbred SD (Sprague-Dawley) rats. The larvae migrate to the lungs, where they moult to L4 after 24 hours. From 48 hours L4 begin to arrive in the gut, where they moult once more to reach the adult stage by day 6 [1,2]. *N. brasiliensis* will also infect mice [3] but are expelled a little faster.

36.2 Recovery of Adult Worms

Adult worms are found loosely attached to the anterior half of the small intestine. Remove the top half of the intestine and cut open with scissors, inserting rounded end into the lumen. Place the open intestine into a muslin bag, tied with cotton, and immerse in warm (37° C) saline in a Baermann apparatus. Parasites, dull red in colour, will collect in the bottom tube within 30 minutes. Recovery is easier and cleaner if food is withheld from rats for the 24 hours before harvest.

36.3 Culture of Larvae

From day 7 post-infection, *Nippostrongylus* eggs appear in faeces of infected rats. Rats should be kept in wire-bottomed cages and faeces collected on a paper covered tray beneath. Collect faeces on days 7, 8 and 9, and each day soak in water and make a paste with an equal volume of granulated charcoal (BDH 33034). Distribute the paste onto 90mm sterile plasti petri dishes, and incubate in the dark at approximately 28°C [4].

Eggs will hatch, and larvae moult twice over the following six days. Cultures can be inspected with a dissecting microscope and top illumination. Larvae remain infective for at least four weeks, and a proportion are still infective at six weeks.

An alternative "cleaner" method of larval culture is to add the faecal-charcoal paste as a heap in the centre of the petri dish. If the filter paper remains moist, larvae will migrate to the periphery of the dish.

36.4 Collection of Larvae

An adapted Baermann apparatus is set up with a funnel and collection tube of tap water at 30-37°C. At the top of the funnel, use paper clips to fix a double muslin sheet containing a folded sheet of lens tissue under 1 cm of water. Do not let the muslin sheet overhang the funnel or water will leak out. Add the contents of 1-3 petri dish cultures and stand for 60 minutes to allow larvae to collect in the bottom tube.

Larvae can be stored in water or saline for 1-2 days at 4° C. Wear gloves when handling larvae as they may penetrate human skin although any infection would be short-lived.

36.5 References

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[3] Westcott RB and Todd AC (1966) Adaptation of *Nippostrongylus brasiliensis* to the mouse. *J. Parasit* **52**: 233-236.

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37. In vitro Culture of Toxocara canis Infective Larvae

The Ascarid nematode *Toxocara canis* is found at high prevalence in dog populations all over the world. Human infection with second-stage larvae occurs via the ingestion of embryonated eggs and leads to the conditions visceral larva migrans (VLM) and ocular toxocariasis [1]. Infective larvae are readily cultured *in vitro*, surviving for more than one year in serum-free culture, and produce large amounts of excretory-secretory antigens, which form the basis of the current diagnostic test.

Isolation of T.canis Eggs:

Adult *T.canis* are obtained from puppies aged 4 weeks to 6 months by either postmortem or anthelmintic purge. Anthelmintic treatment of dogs is by administration of Piperazine phosphate (at 500 mg/kg) in the afternoon, and the faeces collected the following morning. Adult worms are isolated and rinsed in water to remove adherent faecal material. Females are identified and the bifurcated uterus dissected into 5% formalin. Eggs are released from the teased uteri and stored in 5% formalin for at least one month to allow them to embryonate. Embryonation is complete when motile larvae can be seen inside the egg under the inverted microscope.

Hatching T.canis Larvae:

Embryonated eggs are washed thoroughly with water and a 10% suspension of eggs mixed with an equal volume of sodium hypochlorite solution (NaOCl) (10-14% w/v available chlorine) until the outer pitted coat has been dissolved away. (This usually takes 10 to 20 minutes if the NaOCl is fresh). Eggs are then washed in distilled water until no odour is detectable, alternatively either by repeated centrifugation or by filtration on a 25 mm diameter 12 µm Nucleopore membrane with 50 ml of water. Eggs are then transferred to 20 ml of sterile RPMI 1640 containing penicillin/streptomycin (at 100 U/100 µg/ml) or gentamycin (10 µg/ml), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) and 1% glucose (culture medium) [2]. This is maintained at 37°C and 5% carbon dioxide/95% nitrogen is gently bubbled through the suspension. Hatching is complete after 30-60 minutes.

Larvae are then isolated from debris and unhatched eggs by passing the larval suspension over a sterile Baermann apparatus, consisting of 6 layers of lens tissues in a 5 ml syringe, maintained at 37°C in an atmosphere of 5% carbon dioxide, 100% humidity. Most viable larvae migrate through the tissues within 60 minutes [3].

Culture of T.canis Larvae:

Larvae are dispensed into fresh culture medium (RPMI, penicillin/streptomycin, HEPES and glucose) typically sterile 7 ml Bijoux or small tissue culture flasks at concentrations of 10 000 larvae per ml. Cultures should be checked regularly under the inverted microscope for motility and those with greater than 5% mortality discarded. If fungal contamination is problematic then fungizone can be added (50 µg/ml). Medium is changed weekly by first tilting the culture vessel to allow the larvae to sediment and then aspirating off the supernatant, which is then replaced with fresh medium. The harvested supernatant is centrifuged at 10 000 x *g* (or passed through a 0.2 µm filter) to remove any accompanying larvae, and stored immediately at -80°C.

Production of ES antigen:

Culture medium is pooled and concentrated 50-100 fold on Amicon PM 10 membranes in a diafiltration unit, all procedures being carried out at 4°C. Protein determination is carried out by the Bradford method (**Method 5**) using a Biorad kit, and concentrated ES is aliquoted at 1 mg/ml and stored at -80°C.

References

[1] Gillespie SH (1988) The epidemiology of *Toxocara canis*. Parasitology Today **4**: 180-182.

[2] Maizels RM, De Savigny DH & Ogilvie BM (1984) Characterisation of surface and excretory-secretory antigens of *Toxocara canis* infective larvae. *Parasite Immunol* **6**: 23-37.

[3] De Savigny DH (1975) In vitro maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigen for use in serodiagnostic tests for visceral larva migrans. *J Parasitol* **61**: 781-782.

Appendix 1. Buffers

Phosphate-buffered saline:

To make PBS either use PBS tablets (Flow, Cat.No. 28-103-05) or make up:

8.00 g/L NaCl
1.15 g/L Na_2HPO_4
0.20 g/L KCl
0.20 g/L KH_2PO_4

Tris-buffered saline:

8.75g/L NaCl
2.42g/L Tris base
adjust pH with conc. HCl

Other Buffers

0.1 M Acetate buffer pH 4.0:	13.6g NaAc.$3H_20$ + 22 ml glacial HAc in 1 L
0.1 M Borate buffer pH 8.0:	23.33g H_3BO_3 + 4g NaOH in 1 L adjusted with saturated solution of H_3BO_3 (26.6g/L)
1.0 M Tris buffer pH 8.0:	121.1g Tris base + ~ 44.2 ml conc HCl to 1 L
0.2 M glycine-buffer pH 2.8:	5.14 g/L Glycine, adjust pH with conc. HCL
0.1 M Acetate-Citrate pH 5.0/6.0	8.2g/L sodium acetate adjust pH with 1 M (19.2%) citric acid

Saturated Solutions

Ammonium sulphate: 103.3g/100 ml H_2O at 100°C

Molarities of Common Reagents

		Molarity	To make 1 M	density (g/ml)
2-Mercaptoethanol	100%	14.26 M	70.1 ml per L	1.11
Conc. HCl	38%	12.04 M	83.0 ml per L	1.2
Glacial Acetic Acid (HAc)	99.5%	17.57 M	56.9 ml per L	1.05
Conc. H_2SO_4	96%	35.21 M	28.4 ml per L	1.84
Conc. Ammonium Hydroxide	38%	15.03 M	66.5 ml per L	0.9

Appendix 2: UK Suppliers

Company	UK Address	UK Telephone
Amersham	Lincoln Place, Green End, Aylesbury, HP20 2TP	0800 515313
Anderman/Scheicher & Schüll	145 London Rd, Kingston-on-Thames, Surrey, KT2 6NH	(01) 541 0035
BCL (Boehringer Mannheim)	Boehringer Mannheim House, Bell Lane, Lewes, E. Sussex, BN7 1LG	(0273) 480444
BDH	Freshwater Road, Dagenham, Essex, RM8 1RZ	(01) 597 7591
BioRad	Caxton Way, Watford, Herts,WD1 8RP	(0923) 240322
Du Pont/NEN	Wedgewood Way, Stevenage, Herts, SG1 4QN	(0438) 734026
Flow Laboratories	Woodcock Hill, Harefield Road, Rickmansworth, Herts, WD3 1PQ	(0923) 774666
Gallenkamp	Belton Road West, Loughborough Leics. LE11 0TR	(0509) 237371
Gibco/BRL	PO Box 35, Trident House, Renfrew Road, Paisley, Scotland. PA3 4EF	(041) 889 6100
Horwell	73 Maygrove Road, London NW6 2BP	(01) 328 1551
Jencons	Cherrycourt Way Industrial Estate, Stanbridge Road, Leighton Buzzard, Beds, LU7 8UA	(0525) 372010
Luckham	Victoria Gardens, Burgess Hill, Sussex RH15 9QN	(04446) 5348
Millipore	11-15 Peterborough Rd, Harrow HA1 2YH	(01) 864 5499
New England BioLabs	CP Labs, PO Box 22, Bishops Stortford, CM23 3DH	(0279) 758200
Northumbria Biologicals	South Nelson Inustrial Estate, Cramlington, NE23 9HL	(0670) 732992
Pharmacia/LKB	Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks, MK9 3HP	(0908) 661101
Pierce & Warriner (UK)	44 Upper Northgate Street, Chester, CH1 4EF	(0244) 382525
Sigma	Fancy Road, Poole, Dorset, BH17 7NH	0800 373731
Sterilin	Sterilin House, Clockhouse Lane, Feltham, Middx, TW14 8QS	(01) 695 1155

Appendix 3: Amino Acids and Codes

The single letter code works on the following principles: wherever possible the first letter of the amino acid is used; where more than one start with the same letter the most common residue keeps the first letter (eg. A for alanine, G for glycine, L for leucine); F for phenylalanine and R for arginine take the initial sounds. Tyrosine takes a Y, its second letter, and tryptophan is given W on account of its double ring structure. Lysine is K for no better reason than K is next to L in the alphabet.

The aspartate (D, N) and glutamate (E,Q) residues are allocated firstly on the basis that the acids are early in the alphabet and the amines later; and secondly that the aspartates are before the glutamates. An alternative rule is to remember these mis-pronunciations (!) AsparDic Acid, GluEtamic Acid, AsparagiNe and Qlutamine.

Single Letter Code	Three Letter Code	Amino Acid	Side Chain	Group
A	Ala	Alanine	CH_3	Nonpolar
C	Cys	Cysteine	CH_2–SH	Polar, uncharged
D	Asp	Aspartic Acid	CH_2–COOH	Polar, Acidic
E	Glu	Glutamic Acid	CH_2–CH_2–COOH	Polar, Acidic
F	Phe	Phenylalanine	CH_2–C_6H_6	Nonpolar,aromatic
G	Gly	Glycine	None	None
H	His	Histidine	CH_2–$C_3N_2H_3$ (imidazole ring)	Polar, Basic
I	Ile	Isoleucine	CH–(CH_3)CH_2–CH_3	Nonpolar
K	Lys	Lysine	CH_2–CH_2–CH_2–CH_2–NH_2	Polar, Basic
L	Leu	Leucine	CH_2–CH_2–(CH_3)CH_3	Nonpolar
M	Met	Methionine	CH_2–CH_2–S–CH_3	Nonpolar
N	Asn	Asparagine	CH_2–CO.NH_2	Polar, uncharged
P	Pro	Proline	N-CH_2–CH_2–CH_2–C* (imino ring)	Nonpolar
Q	Gln	Glutamine	CH_2–CH_2–CO.NH_2	Polar, uncharged
R	Arg	Arginine	CH_2–CH_2–CH_2–NH–C–(NH_2)–NH_2	Polar, Basic
S	Ser	Serine	CH_2–OH	Polar, uncharged
T	Thr	Threonine	CH–(CH_3)OH	Polar, uncharged
V	Val	Valine	CH–(CH_3)CH_3	Nonpolar
W	Trp	Tryptophan	CH_2–C_2HNH–C_6H_6	Nonpolar
Y	Tyr	Tyrosine	CH_2–C_6H_5–OH	Polar, uncharged

* **N** and **C** of the peptide backbone

Important Sequences in Proteins

Asn-X-Ser)
Asn-X-Thr) *N*-linked glycosylation sites [1]

Ser-Asp-X-Ser-Leu)
Ser-Glu-X-Ser-Leu) Phosphorylation sites

Reference

[1] Kornfield R and Kornfield S (1985) Assembly of asparagine-linked oligosaccharides. *Ann Rev Biochem* **54**: 631-664.