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.

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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.

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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.

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