H. POLYGYRUS EXCRETORY/SECRETORY PRODUCT (HES) PREPARATION

Heligmosomoides polygyrus produces a suite of immuno-modulatory molecules when maintained in *in vitro* culture, and these also include many major antigenic targets of the parasite. This protocol describes culture of *H. polygyrus* adult worms rom the point at which adult worms have been recovered from infected mice (see Life Cycle Protocol), the collection of excretory-secretory product (HES), and its concentration and quantification.

Reagents and Equipment

(a) For Adult worm culture

1. Hanks' Balanced Salt Solution (HBSS), no calcium or magnesium (Gibco Life Tech Cat.No. 14170088).
2. RPMI1640 Medium, no glutamine (Gibco Life Tech Cat.No. 31870025)
3. P/S: Penicillin (10,000 U/ml) and Streptomycin (10,000 µg/ml), Gibco, Life Tech Cat.No. 15140122. Aliquotted into 5 ml tubes and stored at -20°C.
4. L-Glut: L-Glutamine (200 mM), Gibco Life Tech, Cat.No. 25030024
5. D-Glucose, Fisher Chemical Cat.No. G/0500/53. Make 25% glucose solution (25 g of D-Glucose up to 100ml of water, mix on stirring plate until complete dissolution). Sterile filter and store 20ml aliquots at -20°C).
6. Gentamicin. 10 mg/ml (Gibco Life Tech 15710049).
7. Hpoly media – (all worm cultures are in this – NB. DON'T add FCS!!!!!!!)
   500 ml RPMI1640
   + 20 ml of 25% glucose solution (final concentration 2%) (*Note 1*)
   + 5 ml P/S (final concentrations 100 U/ml penicillin, 100 µg/ml streptomycin)
   + 5 ml L-Glut (final concentration 2 mM).
   + 5ml Gentamicin (final concentration 100 µg/ml)
8. T25 Tissue culture flasks

(b) For HES Concentration

1. Amicon 50 ml Stirred Pressure Cell (Millipore Cat.No. UFSC05001)
2. Amicon membranes: Ultracel 3kDa, Ultrafiltration Discs Chemistry Regenerated Cellulose (RC) Filter diameter 44.5mm (Millipore, Cat.No. PLBC04310) (*Note 2*).
3. Nitrogen cylinder and Nitrogen regulator (see Appendix)
4. Stirring plate in fridge
5. Dulbecco's PBS, no calcium, no magnesium (Gibco Life Tech Cat.No. 14190094).
6. Low Binding protein tubes: 0.5 ml Polypropylene Protein LoBind Microcentrifuge Tube, Eppendorf, Cat.No. 0030108094; and 1.5 ml Polypropylene Protein LoBind Microcentrifuge Tube, Eppendorf, Cat.No. 0030108116.

(c) For Protein Concentration and Storage

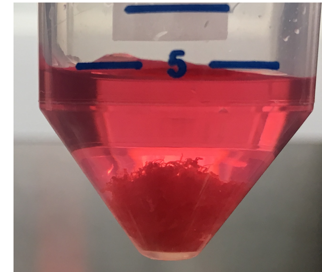
1. <u>Bradford assay</u>:

- Coomassie Plus Bradford Assay Reagent (Pierce Cat.No.23236)
- 96 Well Clear PVC Assay Microplate (Corning Cat.No. 2595)
- Bovine Serum Albumin Standard Ampules, 2 mg/ml (Pierce Cat.No. 23209)
- Plate reader (reading at 595 nm); *or*
Nanodrop spectrophotometer (reading at 280 nm)

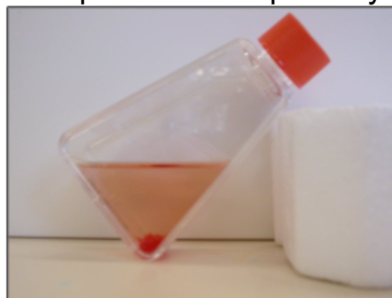
2. Low Protein Binding 0.22 µm Filter, 33 mm diameter (Millex-GV Cat.No. SLGV033RS).
3. Endotoxin (LPS) Assay :
   - Endpoint Chromogenic LAL assay (Lonza Cat.No. 50-647U) *or*
   - LAL Chromogenic Endotoxin Quantitation Kit (Pierce Cat.No. 88282)

Protocol

(a) Adult worm culture



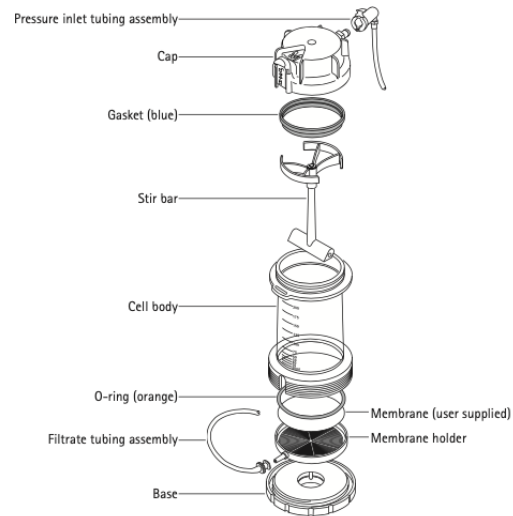
1. Collect adult worms from Baermann apparatus (see *Life Cycle Protocol*). Use a plastic pipette to put worms in a 50 ml tube and wash 6 times with HBSS (prewarmed to 37°C), leaving to settle before removing media.
2. *NOTE: worm culture must be kept sterile from this point onwards.*
3. Then move to a laminar flow hood (room B642) and wash another 6 times in HBSS (prewarmed to 37°C) supplemented with P/S (5ml P/S per 500 ml of HBSS).
4. Soak worms in 10% Gentamicin (1 ml added to about 10 ml media left in tube) for 20 min, leaving tube resting at an angle to ensure worms are fully covered.
5. Wash again 6 times with HBSS+P/S and count total number of worms.
6. Distribute into T25 flasks with approx. 1000 worms in 15 ml Hpoly media in each and place in 37°C incubator (5% CO_2) for a total of 3 weeks.
7. Collect HES-containing culture media from worm cultures (flasks) at 24 hr, and twice a week thereafter, expecting to recover ~12 ml and replenishing with an equal volume of fresh Hpoly media. Set aside the first 24 hr culture medium due to potential contamination with host proteins or LPS – can be processed separately or discarded).



8. Centrifuge HES-containing media in 15 ml or 50 ml tubes, at 400 x g for 5 min to pellet eggs released in vitro. Pass the supernatant through 0.2 µm low-protein-binding filter with a syringe into 50 ml tube, to ensure sterility and exclude any remaining eggs. Store in the -20°C freezer clearly labelled with date of worm harvest and date of HES collection.
9. At the end of 3 weeks, worms may be discarded, or frozen in RNAlater etc.

(b) HES Concentration

1. Pool 500-1,000 ml of HES supernatant (usually from frozen stock, and not including the first 24 hr collection) and concentrate over a 3,000 MWCO filter in the 50 ml Amicon concentrator under nitrogen pressure. (*Note 3*, and *Appendix*).
   NOTE: Be very careful not to let the filter run dry.
2. Add each tube of HES into the filtration device as required (typically 100-140 ml per day), until the volume is concentrated down to 2-5 ml.



3. In order to remove buffer salts and metabolites from the HES-containing culture media, add 50 ml of pyrogen-free PBS to the filtration device and then concentrate down to approximately 2 ml. Repeat this step twice (150 ml of PBS in total).
4. Transfer the concentrated HES into a 1.5 ml Low Binding Tube, filter sterilize through a 0.2 μm filter, in a laminar flow hood and measure protein concentration as below. Expect a protein concentration of 0.5-1.0 mg/ml.

(c) For Protein Concentration and Storage

To determine protein concentration, either use the Bradford assay as detailed here, or measure the OD at 280 nm and convert to protein concentration using an empirically determined extinction coefficient. In either case, measure protein concentration before and after filtration through 0.2 μm filter

Bradford Assay

1. Prepare BSA standards in PBS from 2,000 ug/ml to 1.95 ug/ml (doubling dilution with vortex and centrifugation between each dilution).
   NOTE: Can be stored at 4°C for months
2. On ice and in laminar flow hood, set aside 22 μl of concentrated HES in Low Binding tube.
3. Add 10 μl of BSA standard dilutions, PBS (blank) or sample per well of a 96 well plate (each in duplicate).
4. Add 190 μl of Coomassie Plus Bradford Assay per well and mix well (homogenous colour).
   NOTE: avoid bubbles, they interfere with reading
5. Read at OD at 595 nm with a plate reader (see Appendix for VersaMax)
6. Perform a chromogenic LAL assay according to the manufacturer's protocols on each batch of HES prior to use (see *LAL Chromogenic Endotoxin Quantification Protocol*). If LPS levels are greater than 1 U LPS per 1 μg protein, consider not using this batch for in vivo experiments or in vitro cultures.
7. Aliquot in Low binding protein tubes, label with batch number and date, and freeze at -80°C.

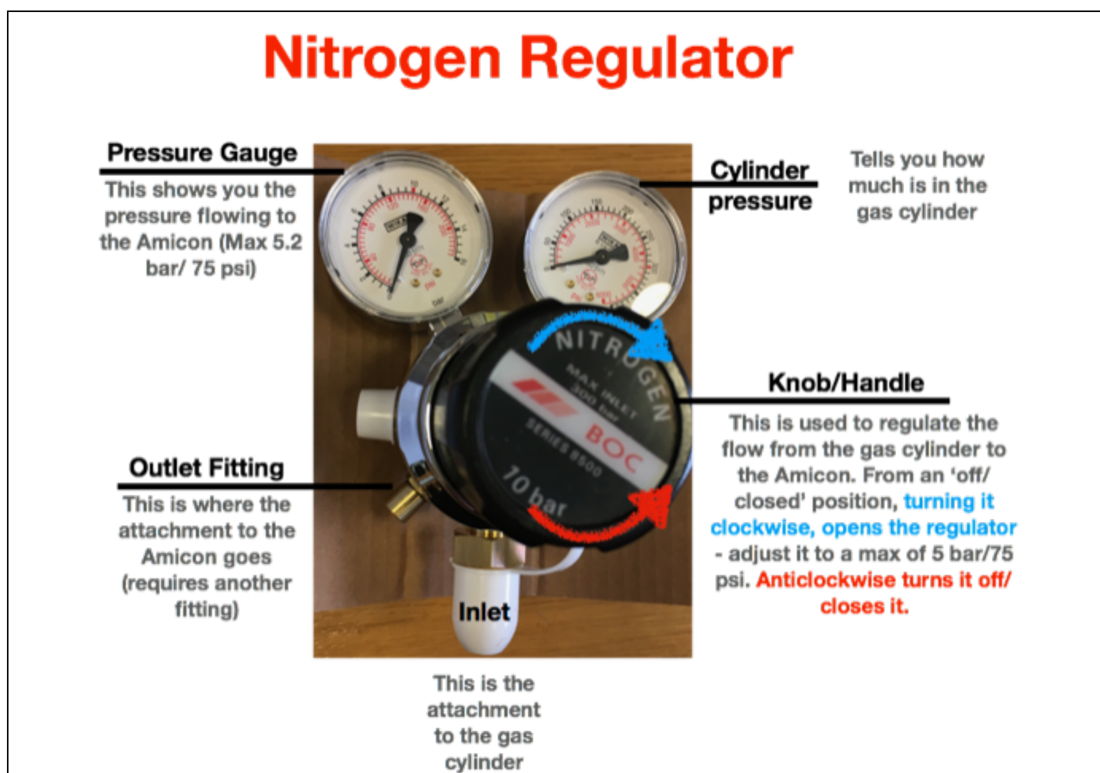
Notes

1. RPMI1640 is supplied with 0.2% glucose, so the final concentration is actually 2.2%.
2. The Amicon membrane can be stored in 20% ethanol and re-used; doing so is likely to reduce loss of proteins adhering to the membrane.
3. To set up the filter device, first wash the 3 kDa membrane shiny side down in a 1 liter beaker with distilled water for 3 x 20 min whilst stirring. Assemble Amicon as per manufacturer's instructions with filter membrane shiny side up. Place in cabinet at 4°C on a stirring plate and run through 50 ml of dH_20 first, then start adding defrosted HES.
4. Process the HES collected at 24 hr separately in the same manner; it may contain LPS and some host proteins and, while not suitable for functional experiments, it is a useful source of individual molecules that may be isolated by monoclonal antibody affinity purification.

References

[1] **Johnston, C. J. C., Robertson, E., Harcus, Y., Grainger, J. R., Coakley, G., Smyth, D. J., McSorley, H. J., Maizels, R.** 2015. Cultivation of Heligmosomoides Polygyrus: An Immunomodulatory Nematode Parasite and its Secreted Products. *J. Vis. Exp.* (**98**), e52412.

Appendix 1 : Nitrogen Regulator for Amicon Pressure Cell

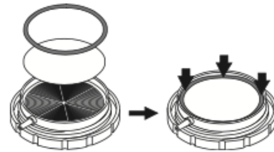


Appendix 2 Assembly of Amicon Pressure Cell

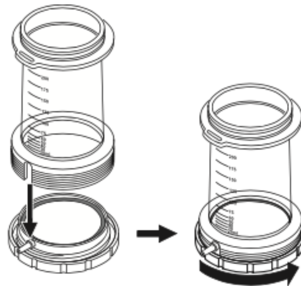
Assembly and Operation

1. Snap membrane holder onto base.

2. Place membrane into membrane holder, oriented as indicated in membrane instructions. Place O-ring on top of membrane and push down gently to seat membrane in holder.

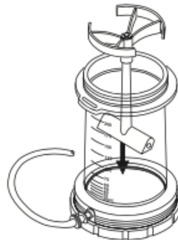


3. Align filtrate port on membrane holder with slot in bottom of cell body. Screw base into cell body.



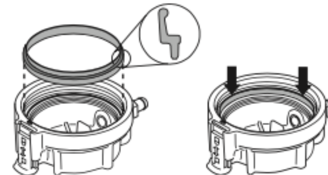
4. Attach filtrate tubing assembly to filtrate port on membrane holder.

5. Insert stir bar into cell body until support ring is seated on ridge inside the top of cell body.



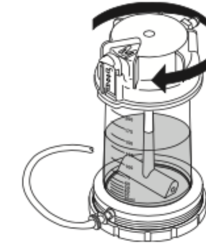
6. Pour desired sample into cell.

7. With cap oriented as shown, seat large diameter of gasket in gasket groove. Gently push the gasket down to seat it fully.

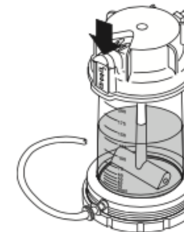


IMPORTANT! To avoid leakage between cap and cell body, make sure that the gasket is free of dirt/debris and oriented correctly in the cap.

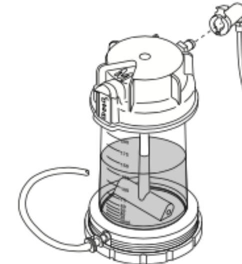
8. Screw cap onto cell body until it stops.



9. Move blue slide-lock downward to close pressure relief valve and lock cap in place.

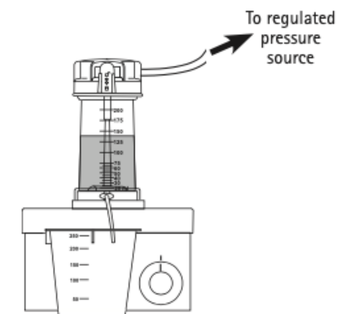


10. Attach pressure inlet tubing assembly by inserting female connector onto quick-connect fitting on cap until it clicks.



11. Place stirred cell on magnetic stirrer.
12. Insert filtrate tubing into an appropriate collection container.
13. To concentrate, connect free end of pressure inlet tubing to a pressure source.
14. Initiate stirring and pressurize stirred cell to desired pressure. Refer to membrane instructions for optimal operating pressures.

    WARNING: Do not exceed pressure limit of 5 bar (75 psi).
15. Collect filtrate until desired concentration factor is achieved.



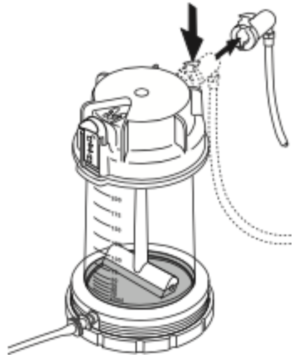
Shut down and disassembly

1. Once filtration is complete, turn off pressure at the source, then turn off magnetic stirrer.

   WARNING: Do not disconnect pressure inlet tube until stirred cell is depressurized.

2. Move blue slide-lock upward to vent residual pressure and disengage cap lock.

3. Disengage the quick-connect fitting by pressing down on the metal tab and pulling the fitting away from the cap.



4. Unscrew cap and remove from cell body.

5. Remove stir bar and recover concentrated sample. Save filtrate sample if required.

Appendix 3

Bradford Protein Determination With VersaMax plate reader:

- Connect the plate reader to the computer, start SoftMax Pro software and select "Protocol/Protein assay/Bradford".
- In Plate01, double click on Template and enter samples, standards and blank on the map. For Standard, click on Series and complete concentrations.
- Place the 96 wells plate in the plate reader and clic on "Read"

ersaMax 22.2 °C Read StakMax

Untitled

formulas by using the formula editor views, activated by double-clicking the column titles.

Exp01

Plate01 Settings Template Reduction Display

Plate01

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.011	0.001	0.021	0.035	0.043	0.134	0.274	0.348	0.582	0.730	0.937
B	-0.000	0.003	0.005	0.010	0.038	0.061	0.141	0.301	0.379	0.597	0.740	0.970
C	0.827	0.824		0.886	0.891		0.880	0.872				
D												
E												
F												
G												
H												

Endpoint
Lm1 595
Automix: Off
Calibrate: On
Column Priority

Start Read:
16:24 28/01/2020

Wavelength Combination: !Lm1
Mean Temperature: 22.1
Data Type: Absorbance
Plate Blank: Used Lm1 = 0.274
Reader: VERSAmax ROM v3.13 31Oct98

Standards

Standards (ug/ml)

Sample	Conc	BackCalcConc	Wells	Value	MeanValue	SD	CV
St01	2000.000	Range?	A12	0.937	0.954	0.024	2.5
		Range?	B12	0.970			
St02	1000.000	779.024	A11	0.730	0.735	0.007	0.9
		804.276	B11	0.740			
St03	500.000	496.353	A10	0.582	0.590	0.011	1.8
		519.194	B10	0.597			
St04	250.000	232.797	A9	0.348	0.364	0.022	5.9
		260.443	B9	0.379			
St05	125.000	170.825	A8	0.274	0.287	0.019	6.6
		192.325	B8	0.301			
St06	62.500	71.692	A7	0.134	0.137	0.005	3.4
		75.975	B7	0.141			
St07	31.250	16.237	A6	0.043	0.052	0.012	23.3
		26.258	B6	0.061			
St08	15.625	11.270	A5	0.035	0.037	0.002	6.4
		13.170	B5	0.038			
St09	7.813	3.397	A4	0.021	0.015	0.008	53.1
		-3.073	B4	0.010			
St10	3.906	-7.914	A3	0.001	0.003	0.003	100.0
		-5.638	B3	0.005			
St11	1.953	-2.233	A2	0.011	0.007	0.005	74.1
		-6.472	B2	0.003			

Smallest standard value: 0.001
Largest standard value: 0.970

Unknowns

Unknowns

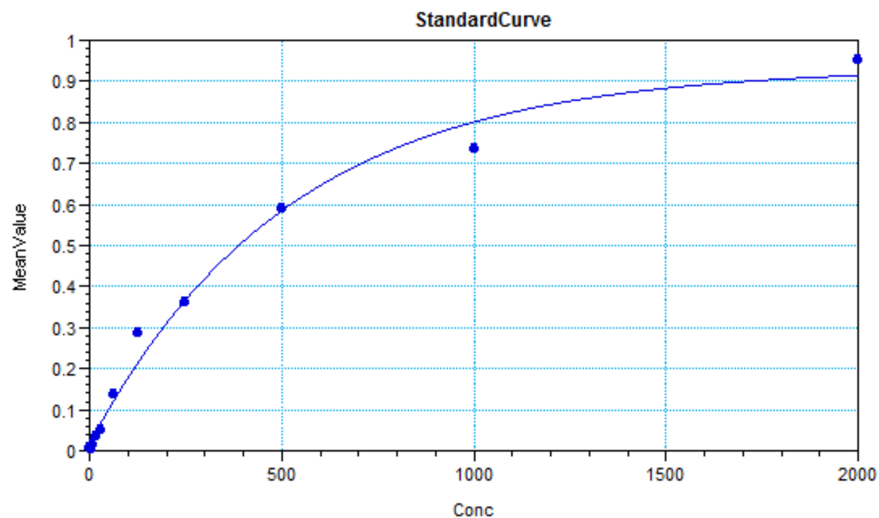
Sample	Wells	Value	R	Result	MeanResult	SD	CV
tube A	C1	0.827		1113.426	1106.483	9.819	0.9
	C2	0.824		1099.539			
tube B	C4	0.886		1526.561	1554.471	39.472	2.5
	C5	0.891		1582.382			
Tube C	C7	0.880		1471.312	1434.311	52.328	3.6
	C8	0.872		1397.310			

R - Outside standard range

Unk_Dilution

Control

StandardCurve Fit: Exponential



Exponential Fit: y = A + B * (1-exp^(-x/C)):	A	B	C	R^2
Std (Standards: Conc vs MeanValue)	0.015	0.919	517	0.989

Weighting: Fixed

- R^2 should be the closest to 1
- Standard curve is often the best with “Expontial” (select this option with button above the curve)

Updated by Claire Ciancia and Rick Maizels 22 April 2020