Practical and low cost whole-organism motility assay: a step-by-step protocol

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ABSTRACT

Here, we provide a step-by-step protocol for a practical and low cost whole-organism assay for the screening of chemical compounds for activity against parasitic worms. This assay has considerable advantages over conventional methods, mainly in relation to ease of use, throughput, time and cost. It is readily suited to the screening of hundreds to thousands of compounds for subsequent hit-to-lead optimisation, and should be applicable to many different parasites and other organisms commensurate with the size of wells in the microtiter plates used for phenotypic screening.

*Keywords:*  
Drug screening  
Motility assay  
Step-by-step protocol  
Nematode  
Parasite
1. Introduction

Anthelmintic drugs are widely used to treat and/or control parasitic worms that infect humans and livestock. Owing to a heavy reliance on anthelmintics in the livestock industry, drug resistance in parasites usually develops rapidly (Waller 2006; Kaplan and Vidyashankar 2012). Therefore, there is a constant need to monitor and manage such resistance (Kaplan and Vidyashankar 2012; Leathwick et al., 2015), and to search for novel compounds (Epe and Kaminsky 2013). To identify new compounds with anthelmintic activity, researchers primarily rely on two in vitro screening approaches: target-based screening or whole-organism (phenotypic) screening.

Target-based or mechanism-based screening usually involves testing compounds against recombinant forms of the molecular target from the pathogen (e.g., ion channels or/and enzymes) to detect a disruption of a response or reaction (Woods et al., 2007; Kotze et al., 2012; Rohwer et al., 2012). Although few target-based screens have been successful in identifying new anthelmintics (cf. Swinney and Anthony 2011), the use of a recombinant redox cascade assay was successful in detecting anti-schistosomal activity of oxadiazoles (Sayed et al., 2008; Simeonov et al., 2008). Major advantages of target-based screens are that the drug target of the compound is already defined and that the assays are often amendable to high throughput formats (384- or 1536-well microtiter plates). However, a major limitation of target-based screens is the uncertainty of whether a compound, which is very active against the isolated target, will have the same or similar response in an intact whole-organism, such as a parasitic worm (Rohwer et al., 2012).

Whole-organism (phenotypic) screening methods are often used to assess the activity or toxicity of compounds on one or more developmental stages. For parasites, the toxicity of a compound is usually evaluated by measuring defects in development, feeding and/or motility (Kotze et al., 2012). Whilst these screens provide some evidence of compound permeability or bioactivity, many of these defects are complex and difficult for machine-learning algorithms to quantify, to allow for automation and the adaptation of an assay to a high-throughput format. Nonetheless, recent developments in image-based analysis tools, such as object tracking and classification through mixture models (i.e. Bayesian and Gaussian), have allowed for the establishment of medium to high throughput assays, which can automatically measure defects in motility and changes in phenotypes following treatment with chemicals (Mitchell et al., 2010; Paveley et al., 2012). In spite of this advance, such algorithms rely on the ability to identify and distinguish objects of interest in the foreground from those in the background, a process called segmentation (e.g., Buckingham et al., 2014). This process can be difficult for algorithms to reliably capture if images contain numerous objects (such as worms) that overlap or rapidly translocates the field of view (Paveley and Bickle 2013; Buckingham et al., 2014). Recent publications have reported assays that can automatically and indirectly detect motility without the need for segmentation by calculating changes in image pixel intensity (Hurst et al., 2014; Storey et al., 2014; Preston et al., 2015). These studies show that dose-response curves can be reliably produced, demonstrating the ability of the assay to detect very subtle changes in motility in response to serial dilutions of chemicals with motility inhibitory properties (Hurst et al., 2014; Storey et al., 2014; Preston et al., 2015) and the ability to screen small to medium-sized compound libraries (n = hundreds to thousands of chemicals) to identify hit compounds for subsequent lead optimisation and anthelmintic discovery. Here, extending recent work (Preston et al., 2015), we provide a step-by-step protocol of a whole-organism screening assay that we now routinely use to test chemicals against parasitic roundworms (nematodes); in this assay, we employ an automated image analysis algorithm to detect motility inhibition without segmentation (see Fig. 1). Although applied to parasitic nematodes, this assay should be useful for the phenotypic screening of a wide range of small organisms, and for numerous applications beyond drug screening.
2. Step-by-step protocol

2.1. Parasites and reagents

Infective, third-stage larvae (L3s) of parasitic nematodes (order Strongylida), such as *Haemonchus contortus* (see Veglia, 1915; Preston et al., 2015);
Reverse-osmosis deionised water;
Tryptone (cat no. LP0042; Oxoid England);
Yeast extract (cat no. LP0042; Oxoid England);
Sodium chloride (cat no. K43208004210; Merck, Denmark);
Antibiotics-antimycotic 100X (cat no. 15240-062; Gibco; contains 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Fungizone®, Life technologies, USA);
Sterile 96-well flat bottom microplates (cat no. 3596; Corning, Life Sciences, USA);
Disposal transfer pipettes (cat no. PTPO1B, Livingstone, Australia);
50 ml tubes (cat no. 352070, Fisher Scientific, USA);
Monepantel (52mM Zolvix®, Novartis Animal Health, Switzerland);
Moxidectin (1.56mM Cydectin®, Virbac, France);
0.4% v/v sodium hypochlorite (Bleach, Woolworths, Australia);
Dimethyl sulfoxide (DMSO; cat no. 2225; Ajax Finechem, Australia);
Plastic Petri dish (100x200mm; Greiner, Sigma, USA);
175 cm tissue culture flask (Corning, Life Sciences, USA);
100 ml reservoir base and disposal reservoirs (cat no. 4321 and 4322, INTEGRA Biosciences, Switzerland);
100 ml sterile glass beaker.

2.2. Equipment needed

Autoclave (model no. 2707.00 Getinge, Australia);
Laminar flow cabinet (model no. PCR60; Clyde-Apac air filtration, Australia);
Water bath (model no. JSWB-O6T; jSR, Laftech, Australia);
Centrifuge (model no. 5810; Eppendorf, Germany);
Sterilised Air pump (Airpump-S100; Aquatrade, Australia) with aerator adaptor (cat no. 159025, Vacusafe, Biotools, Australia);
ViaFlo Assist micro-plate dispenser (model no. 15040466; INTEGRA Biosciences, Switzerland);
ViaFlo Assist II electronic pipette, 12 channels, 50-1250 µl (cat no. 4634, INTEGRA Biosciences, Switzerland);
Standard pipettes (200-1000 µl, 20-200 µl and 0.5-20 µl);
Water-jacketed CO2 incubator (temperature range: 20-50 °C; model no. 2406; Shel Lab, USA);
Orbital shaker (model EOM5; Ratek, Australia).
Worm Imaging system:
Stereo dissecting microscope (head: SZ61, base: SZ-ST, Olympus, Japan);
Biopoint 2 Inverted stage, stepper Motor 4.75”x4” Travel Res 0.025 µm Rep 2 µm (LUDL Electronic products, USA);
Inverted Slide holder Insert-well Plate (LUDL Electronic products, USA);
Controller, MAC6000, Biopoint, xy, plus joystick (LUDL Electronic products, USA);
2.3. Reagent setup

**Luria Bertani Broth (LB):** 10 g tryptone + 5 g yeast extract + 5 g sodium chloride dissolved in 1 l of reverse-osmosis deionised water and autoclaved;
**LB** made by diluting (1/100) Antibiotics-Antimycotic (100X) in LB;
**20 µM monepantel** made by diluting (1/2600) Zolvix in LB*;
**20 µM moxidectin** made by diluting (1/78) Cydectin in LB*;
**Physiological saline** made by dissolving 9 g in 1000 ml of reverse-osmosis deionised water and autoclaved;
**LB** made by diluting (1/100) of 100 % DMSO stock in LB*;
**20 µM of each chemical compound in 1 % DMSO** made by diluting with LB*.

2.4. Detailed procedure (cf. Fig. 1)

**Compound dilution and screening plate setup (timing ~3 h for 384 compounds = 40 x 96-well plates)**

*Critical: perform under sterile conditions in a laminar flow hood.*

1. If compounds are frozen, thaw compounds in a water bath at 37 °C and dilute compounds to 20 µM and 1 % DMSO using LB*.
2. If compounds are in powder form, dissolve compounds in 100 % DMSO and dilute compounds to 20 µM and 1 % DMSO using LB*.
3. Using the Viaflo Assist micro-plate dispenser:
   a. Add 50 µl of the negative-control (LB* + 1 % DMSO) into 6 wells (B2-F2) of the 96-well plate;
   b. Add 50 µl of the DMSO control (LB*) into 6 wells (B8-F8) of the 96-well plate;
   c. Add 50 µl of each positive-control (monepantel or moxidectin) in triplicate into the wells B12-F12 of the 96-well plate;
   d. Add 50 µl of the 20 µM compounds into the 96-well plate in triplicate (columns 2-7, rows B-G);
   e. Add 200 µl of the sterile reverse-osmosis deionised water wells into row A (1-12), row G (1-12) and columns 1 and 11 (B-G).

**Parasite preparation (timing ~ 1 h for 40 plates)**

4. Place two-times the required number of L3s into a 50 ml tube for exsheathment (require 300 xL3/well).
5. Add 0.15 % sodium hypochlorite to the 50 ml tubes containing L3s, and incubate tubes in an incubator at 37 °C for 20 min.

*Critical: the following steps to be performed under sterile conditions in a laminar flow hood.*

6. Wash L3s by filling the 50 ml tube containing L3s with sterile physiological saline and centrifuge at 500 xg, room temperature (22-24 °C), de-acceleration level 2. Resuspend the L3 pellet in 50 ml of the saline. Repeat this step five times.

**If screening compounds on fourth-stage larvae (L4s), continue steps; if screening on xL3 continue at step 8.**
7. After the last centrifuge spin, resuspend the pellet of exsheathed L3s (xL3s) in 50 ml of LB* and transfer to 175 cm tissue culture flask. Place the flask into the incubator set to 38 °C and 20% CO₂. Ensure that the incubator tray is filled with water to create a humid environment (100 %). After seven days in culture, centrifuge and resuspend using a transfer pipette in 5 ml of LB*. Place the tubing of the air pump into the suspension to prevent L4s from settling at the bottom. (Go to step 9)

8. After the last centrifuge spin, resuspend the pellet of xL3s in 5 ml of LB* using a transfer pipette and place the tubing of the air pump into the suspension to prevent xL3s from settling at the bottom.

9. Determine the concentration of larvae and percentage of xL3s or L4s. To determine the density of larvae, aliquot 3 x 25 µl of solution on to a Petri dish and count the number of worms per 25 µl using a light microscope at x 10 magnification. For screening with xL3s: To determine the exsheathment rate, examine the same worms at x 20 magnification and record the number of worms which have shed their L2-cuticle (= sheath) to become xL3s (Fig. 1). Determine the percentage. The exsheathment rate should be between 90-100 %. For screening with L4s: examine worms at x 20 magnification, and record the number of worms which have developed a pharynx (Veglia, 1915). CRITICAL STEP: Do not begin the compound screen on L4s unless more than 80 % of worms have developed to the L4 stage.

10. Dilute the larval (either xL3s or L4s) suspension to 300 larvae per 50 µl using LB* and transfer the suspension to a 100 ml disposal reservoir tray. Connect the 8-channel adaptor aerator to the air pump and place it into the reservoir tray containing the larval suspension. Adjust the density of larvae to 300 larvae per 50 µl, as described in step 12. Dilute or concentrate suspension with using LB* as required.

11. Using the Viaflo Assist micro-plate dispenser, transfer 50 µl the homogenous larval suspension into the wells of individual 96-well plates containing the compounds and all necessary controls. To avoid potential cross-contamination among wells during pipetting, place plates into the dispenser upside down. CRITICAL STEP: Ensure that the xL3s/L4s are always resuspended, so that they are evenly dispensed into the wells.

12. Check plates under light microscope to ensure that all wells contain larvae and that the larval density is the same among wells.

13. Place the plates into the incubator pre-set at 38 °C and 10% CO₂. Place a tray filled with sterile water at the bottom of the incubator to ensure a humidity of 100 % inside the incubator.

Motility analysis (timing ~9 h for 40 x 96-well plates)

14. Following an incubation period of 72 h, place the plate on an orbital shaker rotating at speed 7 (126 revolutions per min) for 20 min at 38 °C to agitate the larvae.

15. Turn on the motorised stage controller and open the software program Image Scope.

16. Remove a plate from the orbital shaker and insert it into the XY motorised stage. Set parameters to take a 5 sec video recording of each well containing larvae. To do this, from under the ‘Acquire’ drop-down menu, open the stage controller. Select the ‘Acquire’ tab and click the button ‘Preview’. Adjust the magnification to capture the entire well in the field of view. Next, select the ‘Sample Pattern’ tab and highlight the wells to be imaged. For screening against the xL3s, half of the plate is imaged, the plate is re-agitated using the orbital shaker, and then the rest of the plate is imaged. For screening against the L4s, individual plates are imaged in groups of 3 columns (i.e. columns 2, 3, 4 B-G), as the motility of the L4s is more temperature dependent than for xL3s. Use the joystick to move the plate to well position A1 and click the button ‘Set Plate of Origin’. Go back to the ‘Acquire’ tab and tick the box next to ‘Macro’ and open the ‘Macro’ button. In the drop-down
menu, select the custom-made macro, ‘PostwellSnap’ and click the button ‘OK’. Tick the box called ‘use Sample Pattern’ and then click the ‘Acquire’ button to start imaging.

17. Convert the video recordings from Tiff files to AVI files using the video converter software.
18. Open the program Image J and the macro ‘wiggle index-batch analysis’.
19. Select the folder containing the AVI files of the videos for subsequent analysis.
20. Select the parameters shown in Table 1 and click OK. This step is performed using a ‘non-batch’ analysis version of the program (wiggle index analysis) to optimise any one of the five parameters for individual videos. Parameters can be adjusted to suit individual requirements.

Table 1
The parameters used to calculate larval motility (Preston et al., 2015).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image scale adjustment</td>
<td>1</td>
</tr>
<tr>
<td>Image blur (Gaussian)</td>
<td>2</td>
</tr>
<tr>
<td>Frames to average</td>
<td>25</td>
</tr>
<tr>
<td>Projection output</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Threshold</td>
<td>20</td>
</tr>
</tbody>
</table>

**Image scale adjustment**: This parameter allows you to scale the image down, to reduce the processing time; but, adjusting the scale of the image can reduce the sensitivity of the algorithm.

**Image blur (Gaussian)**: Increasing the value of the Gaussian blur will reduce noise, but you may not detect subtle movement.

**Frames to average**: This parameter allows you to change the number of frames averaged. Increasing the number of frames to average will reduce the detection of artefactual movement, but may inhibit the detection of limited movement.

**Projection output**: You may select the motility output to be either standard deviation, or maximum or average change in light intensity.

**Threshold**: With this function you can change the background threshold of the algorithm. Adjusting this parameter can correct for fluctuations in lighting (which may erroneously be recorded as movement).

21. Open the output folder of the file analysed and select the excel spread sheet called wiggle index. Transfer the motility index scores calculated for each well into GraphPad prism.
22. For each plate, normalise the mean motility index to the positive and negative controls. Display values using a scatter plot.
23. Select compounds which have reduced motility by $\geq 70\%$ for subsequent, secondary or tertiary screening (in the same manner as for primary screening).
3. Concluding remarks

This whole-organism screening system is able to rapidly and effectively identify compounds with properties that inhibit motility. Currently, compounds are tested against the parasitic larval stages of nematodes (order Strongylida) in 96-well plates. At three distinct time points, a five-second video is captured of each well of each plate. Video files are then processed through an algorithm, which calculates a motility score from the changes in pixel intensity over time (from frame to frame). Using the 96-well microplates, imaging larval motility at 72 h and employing defined motility algorithm parameters, the expected mean and standard error of the mean (SEM) of the motility index for the negative control is $1.7 \pm 0.04$ (Preston et al., 2015). In this assay, motility indices for test compounds are compared with well-defined positive and negative controls. For the positive control compounds, moxidectin and monepantel, the expected motility indices are $0.6 \pm 0.03$ and $0.4 \pm 0.11$, respectively (Preston et al., 2015), although some inter-laboratory variation in values would be expected. Test compounds that reduce larval motility by $\geq 70\%$ are re-screened. Compounds that reproducibly and repeatedly reduce motility are then assessed in dose-response experiments, to determine their half maximum inhibitory concentration ($IC_{50}$), and then tested for their capacity to inhibit nematode development.

Overall, the main advantages of this assay include:

1. The use of parasitic stages to screen compounds for anthelmintic properties;
2. The assay is label-free, reducing the cost of reagents and multiple time points of the same compound-treated parasites can be imaged;
3. The imaging algorithm is not based on image segmentation;
4. The assay indirectly measures motility through changes in pixel intensity;
5. This assay or a modification thereof is applicable to various species of worms, and should be adaptable to other organisms.

Acknowledgements

This work was supported by the Australian Research Council, the National Health and Medical Research Council of Australia and the Victorian Life Sciences Computation Initiative (VLSCI) grant number VR0007 on its Peak Computing Facility at the University of Melbourne, an initiative of the Victorian Government.
Fig. 1. Workflow schematic of the compound screen against nematode larvae (e.g., *Haemonchus contortus*). **Step 1:** Incubate ensheathed third stage larvae (L3) with sodium hypochlorite to produce exsheathed L3s (xL3s). **Step 2:** Prepare screening plate by adding test compounds using the Viaflo Assist micro-plate dispenser. Next, add 300 parasitic larvae to individual wells of 96-well microtitre plates. **Step 3:** Place plates in the incubator at 38 °C and 10% CO₂ for 72 h. **Step 4:** Agitate each plate for 20 min on an orbital shaker at 38 °C. **Step 5:** Transfer individual plates to the imaging system, and record a five sec video of each well. **Step 6:** Analyse video recordings for motility using the motility index algorithm. **Step 7:** Compare motility indices for individual test compounds with motilities recorded in the positive and negative control wells. Identify compounds that have resulted in a ≥ 70% reduction in motility.
References


Kotze AC. Target-based and whole-worm screening approaches to anthelmintic discovery. Vet Parasitol 2012;186:118-23.


