SRB viability assay for *Acanthamoeba castellanii*

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1 Works for me Share dx.doi.org/10.17504/protocols.io.bvpen5je

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DISCLAIMER

ABSTRACT

Viability assay for *Acanthamoeba castellanii* trophozoites and cysts, which detects 1,000-100,000 cells.

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DISCLAIMER:

Reagent preparation

1 PYG medium
Protocol for preparation of PYG medium

2 EMb medium
Protocol for preparation of EMb medium

Only needed if working with cysts

3 PBS supplemented with $MgCl_2$ and $CaCl_2$ (PBS-MC)

3.1 Make Solution A (20x, 500 mL):
23.43 g Na_2HPO_4 (final concentration of **348 Milimolar (mM)**)
4.83 g NaH_2PO_4 (final concentration of **70 Milimolar (mM)**)
dH_2O to **500 mL**

3.2 Make Solution B (20x, 500 mL):
1.3 g $CaCl_2$ (final concentration of **18 Milimolar (mM)**)
2.6 g KCl (final concentration of **70 Milimolar (mM)**)
1.8 g $MgCl_2$ (final concentration of **18 Milimolar (mM)**)
80 g NaCl (final concentration of **2740 Milimolar (mM)**)
dH_2O to **500 mL**

3.3 Autoclave or filter sterilize solutions A and B. Store at **Room temperature** .

3.4 Add **50 mL** each of solutions A and B to **900 mL** autoclaved dH_2O for a final concentration of 1x PBS-MC. Store at **Room temperature** .

4 Trichloroacetic acid (TCA): make 100% (w/v) TCA by adding **227 mL** dH_2O to **500 g** bottle of TCA. Dilute to

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[M]**10 % (v/v)** in 1x PBS-MC. Store in glass container at **4 °C** .

5 Prepare [M]**1 % (v/v)** acetic acid in dH_2O in 10 or 20 L plastic carboy and store at **Room temperature** .

6 Prepare [M]**10 Milimolar (mM)** Tris-HCl pH**8** and store at **Room temperature** .

Cell preparation

7

Grow *Acanthamoeba castellanii* trophozoites in **40 mL PYG media** in vented tissue culture-treated 75 cm^2 cell culture flasks at **25 °C** without shaking. Passage 1:80 every 3-4 days.

8 Remove spent PYG media from a monolayer of trophozoites. Wash cells with **10-20 mL PYG media** , adding liquid to surface of flask opposite the cell layer to prevent dislodging adherent amoebas.

9 Add **20 mL PYG media** to flasks and close lids tightly. Gently tap all surfaces of flasks on benchtop to dislodge trophozoites. Confirm complete detachment with light microscope at 100x. Move on to next step immediately, as trophozoites begin to reattach quickly (within 5 minutes).

10 Transfer media containing detached amoebas to a sterile 50-mL conical. Measure the volume using a sterile serological pipette and record (for cell count calculation).

11 Briefly vortex conical to ensure even suspension of cells and transfer **100 µl** to a 1.5-mL microcentrifuge tube containing **100 µl trypan blue dye** . Mix by briefly vortexing and transfer **10 µl** to hemocytometer.

12 Count live cells and calculate total number of harvested amoebas. Determine total number of amoebas needed for experiment and calculate volume of cell suspension required.

13 20m

Briefly vortex conical of trophozoites to dislodge attached cells. Transfer appropriate volume of cell suspension containing desired number of trophozoites to a new conical. Pellet cells by centrifuging at **1000 x g, Room temperature** **00:15:00** (in 50-mL conical) or **00:05:00** (in 15-mL conical).

14 Gently decant media into waste container and resuspend cell pellet by briefly vortexting. Adjust volume with fresh PYG to a final cell concentration of 10^6 per mL.

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15 Briefly vortex cells to dislodge attached trophozoites and ensure even suspension, then transfer to sterile liquid trough. Add **100 µl cells** per well to sterile 96-well plates (clear, flat bottom, with lid).

Plates must be tissue culture-treated for experiments on cysts.

16 2h 5m

Allow plates to sit at **Room temperature** for approximately **02:00:00** before further processing to ensure cells have attached to plates. Before removing media for additional steps, spin plates **200 x g, Room temperature** for **00:05:00** .

Trophozoites are now ready for compound testing. To test compounds on cysts, proceed to steps 17 and 18.

17 To induce encystment, remove media from wells and discard. Add **100 µl EMb encystment media** per well. Media must be added gently to walls of wells to prevent a jet of liquid from dislodging attached cells. Trophozoites can reattach, but cysts cannot.

(Do only if testing cysts)

18 2d 0h 5m

Replace plate lid with breathable plate seal. Incubate at **25 °C** without shaking for **48:00:00** . At end of encystment, spin plates **200 x g, Room temperature** for **00:05:00** .

(Do only if testing cysts)

Compound preparation

19 Dissolve compounds in appropriate vehicle to desired concentration. DMSO, methanol, and ethanol are nontoxic to *A. castellanii* to a maximum of 1% final volume.

20 Add dissolved compounds to media. For cysts, use EMb. For trophozoites, use LB or PYG. Trophozoites will continue to divide in PYG but not in LB (nor will they encyst).

Compound testing

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21 Remove media from plates and discard. Add **100 µl media +/- test compounds** per well, pipetting media gently to walls of wells to prevent a jet of liquid from dislodging attached cells.

22 

Replace plate lid with breathable plate seal. Incubate at **25 °C** without shaking for desired time.

Fixing plates 1h

23 At end of incubation, aspirate media and discard.

Alternatively, media can be removed by flicking off into waste container. Plates can also be washed with PBS-MC (either by pipetting or by submerging) before fixing, though this is not required for most test compounds. Plates can be fixed by submerging in TCA for faster processing.

If removing the media before fixing by flicking off into waste container, appropriate safety precautions must be taken as media may contain live amoebas—we suggest dumping plates into a wide basin containing bleach in a deep sink while wearing safety goggles, face shield, lab coat, and extended cuff gloves.

24 Add **125 µl 10% TCA in PBS-MC** per well. Add TCA gently to walls of wells to prevent dislodging attached cells.

25  1h

Incubate plates for a minimum of **01:00:00** at **4 °C** . Plates can be stored at **4 °C** for several weeks.

26 

Remove TCA by washing plates by submerging in plastic trays of tap water 4 times.

TCA is caustic. Appropriate PPE must be worn to prevent contact with skin or eyes.

Do not wash by running stream of tap water into plate wells. This results in uneven washing and can dislodge attached cells.

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27

Gently tap dry on paper towels and let air dry. Dried plates can be stored at **Room temperature** indefinitely.

Stain cells with SRB dye 15m

28 Dissolve SRB dye in **1 % (v/v) acetic acid** to a final concentration of 4% (w/v). For each 96-well plate, use **0.2 g SRB dye** in **5 mL** **1 % (v/v) acetic acid** .

SRB dye is light sensitive and should be stored in the dark at **Room temperature** . We make it fresh for each experiment.

29 15m

Add **50 µl SRB dye solution** per well and incubate plates at **Room temperature in the dark** for **00:15:00** .

SRB dye solution has high surface tension. Make sure it has covered entire well bottom before incubating. Gently shaking the plate can ensure the liquid has covered the well bottom.

30

Remove excess SRB dye by washing plates by submerging in plastic trays of **1 % (v/v) acetic acid** 3 times. Gently shaking plates during submersion prevents air bubbles from excluding liquid, which can result in overstained wells. Contact time in acetic acid should be minimized to prevent overbleaching. Even washing of wells within and between experiments is critical for reproducible results. See video for example of technique.

We recommend changing first tray of acetic acid for each plate washed, and the second and third trays every other plate. This is because the majority of excess dye is removed with the first wash.

1% acetic acid is an irritant. Appropriate PPE must be worn to prevent contact with skin or eyes.

31 Gently tap dry on paper towels and let air dry.

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Measure absorbance 5m

32 Solubilize SRB dye by adding **150 µl** per well of **10 Milimolar (mM) Tris-HCl** pH **8** .

Precise pipetting is critical, as a change in volume will lead to a change in the concentration of solubilized SRB dye and the subsequent absorbance measurement.

33 5m

Incubate plate on orbital rocking platform for **00:05:00** at **Room temperature** .

34 Using a Kimwipe, remove any condensation from bottom of plate, top of lid, and inside lid. Measure the absorbance at 565 nm in a plate reader.

We use a Tecan Infinite M200 Pro plate reader with the following program: linear shake (1 mm amplitude) 30 seconds, wait 5 seconds, measure absorbance at 565 nm (25 flashes per read).

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