Jun 10, 2021

Bioluminescence-based 24 well plate assay for screening fungi for activity against *Mycobacterium marinum*

Siouxsie Wiles[1], Alex Grey[1]

[1]University of Auckland

dx.doi.org/10.17504/protocols.io.bvnbn5an

Bioluminescent Superbugs Lab
Tech. support email: s.wiles@auckland.ac.nz

Siouxsie Wiles
University of Auckland

ABSTRACT

There is a real and urgent need for new antibiotics which are able to kill Mycobacteria. This group of bacteria includes *Mycobacterium tuberculosis*, a deadly human pathogen that latently infects a third of the world's population. Multi-drug resistant (MDR) and totally-drug resistant (TDR) isolates have also now evolved. Due to the difficulties and dangers involved in culturing *M. tuberculosis*, an airborne Biosafety Level 3 pathogen, faster-growing and less pathogenic mycobacterial species are routinely exploited for TB research and anti-mycobacterial drug discovery. *M. marinum* shares conserved virulence determinants with *M. tuberculosis* and is a pathogen of ectotherms (fish, amphibians and reptiles), as well as causing granulomatous skin infections in humans.

Because of the slow growth of many mycobacterial species, we routinely use luciferase-tagged strains[1] for our assays. As bacteria only produce light when alive, bioluminescence is an excellent non-destructive real-time reporter to assay for anti-mycobacterial activity in microtitre plate formats using a luminometer[2-4]or *in vivo* using sensitive imaging equipment.[5]

In this protocol, we describe a luminescence-based 24-well plate assay for medium throughput screening of fungal isolates grown on a variety of media for anti-mycobacterial activity against *M. marinum*.

References:

1. Andreu N, Zelmer A, Fletcher T, Elkington PT, Ward TH, Ripoll J, Parish T, Bancroft GJ, Schaible UE, Robertson BD, Wiles S (2010). Optimisation of bioluminescent reporters for use with Mycobacteria. PLOS One. 5(5):e10777 (doi:10.1371/journal.pone.0010777).
2. Andreu N, Fletcher T, Krishnan N, Wiles S, Robertson BD (2012). Rapid measurement of antituberculosis drug activity in vitro and in macrophages using bioluminescence. Journal of Antimicrobial Chemotherapy. 67(2): 404-14 (doi: 10.1093/jac/dkr472).
3. Dalton JP, Uy B, Phummarin N, Copp BR, Denny WA, Crosier PS, Swift S, Wiles S (2016). Effect of common and experimental anti-tuberculosis treatments on *Mycobacterium tuberculosis* growing as biofilms. PeerJ. 4:e2717 (doi: 10.7717/peerj.2717).
4. Dalton JP, Uy B, Okuda K, Hall CJ, Denny WA, Crosier PS, Swift S, Wiles S (2017).Screening of anti-mycobacterial compounds in a naturally infected zebrafish embryo model. Journal of Antimicrobial Chemotherapy 72(2):421-427 (doi: 10.1093/jac/dkw421).
5. Andreu N, Zelmer A, Sampson SL, Ikeh M, Bancroft GJ, Schaible UE, Wiles S, Robertson BD (2013). Rapid in vivo assessment of drug efficacy against *Mycobacterium tuberculosis* using an improved firefly luciferase. Journal of Antimicrobial Chemotherapy. 68(9):2118-27 (doi: 10.1093/jac/dkt155).

DOI

dx.doi.org/10.17504/protocols.io.bvnbn5an

PROTOCOL CITATION

Siouxsie Wiles, Alex Grey 2021. Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bvnbn5an

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

KEYWORDS

Mycobacterium marinum, Mycobacteria, Screening, Bioluminescence, lux, fungi, 24 well, antibacterial

CREATED

Jun 09, 2021

LAST MODIFIED

Jun 10, 2021

PROTOCOL INTEGER ID

50595

GUIDELINES

If possible, carry out steps as indicated in a biological safety cabinet to prevent contamination.

MATERIALS TEXT

Microorganism: *M. marinum* BSG101

Growth media and chemicals:

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

Description	Catalogue number	Supplier
Difco Dehydrated Culture *Media* Middlebrook 7H9 Broth	271310	Fort Richard, New Zealand
Difco Dehydrated Culture Media Middlebrook 7H11 Agar	283810	Fort Richard, New Zealand
BD BBL Middlebrook OADC Enrichment	211886 or 212240	Fort Richard, New Zealand
BD BBL Middlebrook ADC Enrichment	211887 or 212352	Fort Richard, New Zealand
Glycerol ACS reagent, ≥99.5%	G7893	Sigma-Aldrich, New Zealand
Tyloxapol non-ionic surfactant	T8761	Sigma-Aldrich, New Zealand
Difco Potato Dextrose Broth	254920	Fort Richard, New Zealand
Difco Oatmeal Agar	255210	Fort Richard, New Zealand
Difco Malt Extract Agar	211220	Fort Richard, New Zealand
Difco Czapek Solution Agar	233910	Fort Richard, New Zealand
BBL Rice Extract Agar	211567	Fort Richard, New Zealand
Bacto Yeast Extract	212750	Fort Richard, New Zealand
Bacto Tryptone	211725	Fort Richard, New Zealand
Agar, Granulated	214530	Fort Richard, New Zealand
Phosphate Buffered Saline tablets	P4417	Sigma-Aldrich, New Zealand

Plasticware:

Description	Catalogue number	Supplier
4titude Black 24 Well assay plates	4TI-0262	Millennium Science, New Zealand
4titude 24 Well Microplate Lids	4TI-0284	Millennium Science, New Zealand
Falcon 50mL Conical Centrifuge Tube	BDAA352070	In vitro technologies
Technoplast 5 mL flat bottom yellow screw cap tube	S5016SU	Mediray, New Zealand
BRAND Semi micro cuvette	BR759015	Sigma-Aldrich, New Zealand
Biotix Pipetting reservoirs 50mL	BTSR-0050-5SC	Mediray, New Zealand
Pipette tips		

Equipment:

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

- Pipettes – various sizes
- 6mm Biopsy punch (we use Paramount)
- Spectrophotometer (to measure optical density of bacterial culture)
- Luminometer (we use a Perkin Elmer Victor X)
- Sterile scalpel handle and blade
- Germinator
- Biological Safety cabinet (Herasafe KS12)

BEFORE STARTING

1. To carry out this protocol, you will need a suitably grown bacterial culture. We use an isolate of *M. marinum* BAA-535 (type M) that carries a chromosomal copy of the lux operon (*M. marinum* BSG101).[4] Constructs to make this strain are available from Addgene (https://www.addgene.org/26161/ or https://www.addgene.org/26159/). We grow *M. marinum* in Middlebrook 7H9 broth supplemented with 0.4% glycerol, 0.05% tyloxapol, and 10% Middlebrook ADC growth supplement. Cultures are grown at 28 °C with shaking at 200 revolutions per minute (RPM) until they reach stationary phase, which takes roughly 7-10 days.
2. Prepare the 7H9 agar by adding 0.5% v/v glycerol before autoclaving. This can be left to set and be re-melted in a microwave before each fungi screening experiment. The OADC should only be added to aliquots of agar before inoculating it with bacteria to prevent denaturing the proteins within the supplement.
3. Prepare the various growth media as per the manufacturer's instructions. These can be left to set and be re-melted in a microwave before each fungi screening experiment. Ensure they remain sterile.

Initial culturing of fungi

1 Sub-culture your fungus of choice onto a Potato Dextrose Agar (PDA) plate from either an existing agar culture or from frozen stocks.

2 Seal the plate with parafilm and store it in a plastic box at room temperature. Allow the culture to reach at least 50% growth for sub-culturing on to well plates for screening. Fungi can take days to months to reach maximal growth.

Preparation of 24 well plates

3 Sterilise your 24-well plates and lids using UV irradiation in a biological safety cabinet for at least an hour.

4 Dispense 0.5 mL of each agar type into the three wells each of a 24-well plate using a serological pipette. Our plate layout is shown in Figure 1.

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

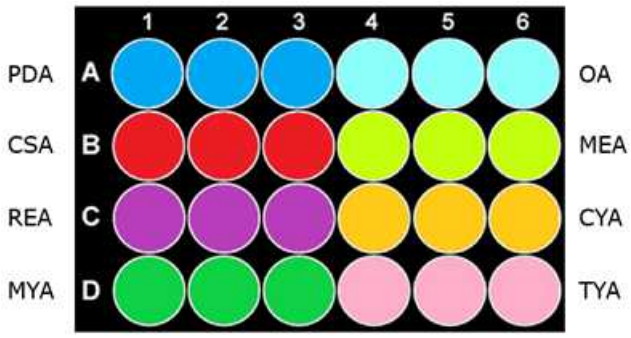

Figure 1. 24 well plate growth medium layout.

Key: PDA, Potato Dextrose Agar; CSA, Czapek Solution Agar; REA, Rice Extract Agar; MYA, Malt Yeast Extract Agar; OA, Oatmeal Agar; MEA, Malt Extract Agar; CYA, Czapek Yeast Extract Agar; TYA, Tryptone Yeast Extract Agar.

5 Allow the agar to set.

6 Store the plates at 4 °C.

Culturing fungi - carry this out in a biological safety cabinet if possible to prevent contamination.

7 Set the Germinator to 250 °C or higher. Leave the germinator on for at least 30 minutes to ensure that it reaches the appropriate temperature.

8 Attach the scalpel blade to the handle.

9 Sterilise the blade using the Germinator.

10 Using the sterile scalpel blade, section the agar culture using the scalpel blade into cubes ≤ 5 mm in diameter.

11 Use the scalpel blade to transfer the cubes of fungus into each agar-filled well of a 24-well plate. Ensure that each cube is touching the agar in the well and is inoculated fungus-side down.

12 Replace the lid and parafilm the edge of the plate.

13 Repeat for each fungal isolate. When inoculating more than one isolate, work with one isolate at a time, and sterilise the hood with 10% Trigene when switching between each to prevent cross-contamination.

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

14 Attach masking tape to the lid of each sterile 24-well plate and label it with your name, the date, and the fungus you inoculated the well plate with.

15 Store plates in a labelled plastic box. Store them agar-side down initially to ensure that the cubes don't fall off the agar but flip them over once the fungi have shown visible growth to reduce the chances of contamination.

16 Check your cultures at least twice a week for growth and contamination. If thegrowth rate differs between wells with different culture media, use the growth in PDA as a reference. We aim to screen each fungus when it reaches 200% growth: Record the age at which it either covers the entire well or stops visibly growing and screen it at double that age.

If a well is contaminated, you don't need to throw the whole plate away. Instead, use a sterile spatula, pipette tip, or similar instrument to scoop out the agar containing the contaminated culture. Discard the contaminated agar and dispose of using the appropriate procedure. Make sure you record which well(s) you have removed.

Inoculation of 24 well screening plates with *Mycobacterium marinum*

17 Record the date, the fungal isolates you are screening, and the age of these cultures. If any wells were removed, make sure you record the location of these wells.

18 Melt 0.8% 7H9 agar in the microwave and cool it back down to 50 °C in a water bath.

19 Transfer 2.7 mL aliquots of 0.8% 7H9 agar into your 5 mL tubes. You will later supplement these with 300 µL OADC to make up a final volume of 3 mL. This is enough to comfortably fill all two 24 well plates.

20 In a biological safety cabinet, use a biopsy punch to remove the centre of each well culture. Repeat for each fungus you want to test, as well as an extra uninoculated well plate, which will be used as a control. Discard these agar plugs in an empty Petri dish.

21 Measure the optical density of the bacterial culture at 600nm (OD_{600}). To do this we generally dilute the bacterial culture 1:10 in a 1.5 mL cuvette with MHB (720 µL broth + 80 µL bacteria).

22 Dilute the bacterial culture with supplemented 7H9 broth to give a final OD_{600} of 1.0 which is the equivalent of ~10^9bacteria per mL.

23 Dilute your readjusted culture of *M. marinum* 1:10 into the 7H9 agar. We usually add 300 µL of bacteria into 3 mL of agar per 5 mL tube. Vortex the mixture for 10 seconds. Use a pipette to dispense 50 µL of the agar mixture into the cylindrical holes left after the fungal plugs were removed.

24 Repeat for all well plate cultures and allow the pipetted agar to set.

Checking the bacterial inoculum

25 Add 90 µL of MHB or Phosphate Buffer Solution (PBS) to each of the wells in a single column of a 96 well plate. Mix in 10 µL of bacteria inoculum to the top well, mix and discard the tip. Using a clean tip, remove 10 µL, add it to the next

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an

well in the column and mix. Repeat to perform a 10-fold serial dilution down the column, using a clean tip each time.

26 Plate three technical replicates of each dilution onto a Middlebrook 7H11 agar plate. Incubate agar plates upside down at 28 °C until visible colonies appear. For *M. marinum* this usually takes approximately 7 – 10 days.

27 Count visible colonies to ensure inoculum was correct at approximately 10^8CFU/mL.

Measuring bacterial bioluminescence

28 We use a Perkin Elmer Victor X plate luminometer set to read 24 well plates with an integration time of 1 second per well. Measure all 24 wells of each plate, even if some have been removed and aren't inoculated with bacteria.

29 We take measurements immediately after setting up the plate (T_0) and then at 24 (T_{24}), 48 (T_{48}), and 72 (T_{72}) hours.

30 Between measurements, place lids on the plates, put in a plastic box lined with damp paper towels, and incubate at 28 °C without shaking.

Citation: Siouxsie Wiles, Alex Grey (06/10/2021). Bioluminescence-based 24 well plate assay for screening fungi for activity against Mycobacterium marinum. https://dx.doi.org/10.17504/protocols.io.bvnbn5an