Jun 09, 2021

Bioluminescence-based Minimum Inhibitory Concentration (MIC) testing of fungal extracts against Mycobacterium marinum

Forked from Bioluminescence-based Minimum Inhibitory Concentration (MIC) testing of pure compounds isolated from fungi against Mycobacterium marinum

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 dx.doi.org/10.17504/protocols.io.bvnan5ae

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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). This protocol is modified from the method described by Dalton et al (4).

References:

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DOI

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PROTOCOL CITATION

Siouxsie Wiles, Alex Grey 2021. Bioluminescence-based Minimum Inhibitory Concentration (MIC) testing of fungal extracts against Mycobacterium marinum. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bvnan5ae

FORK NOTE

FORK FROM

Forked from Bioluminescence-based Minimum Inhibitory Concentration (MIC) testing of pure compounds isolated from fungi against Mycobacterium marinum, Shara Van De Pas

KEYWORDS

Minimum inhibitory concentration, MIC, Mycobacteria, Mycobacterium marinum, sensitivity testing, anti-mycobacterial activity, antibiotic activity

CREATED

Jun 09, 2021

LAST MODIFIED

Jun 09, 2021

PROTOCOL INTEGER ID

50594

MATERIALS TEXT

Microorganism: Mycobacterium marinum BSG101 (4)

Growth media and chemicals:

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A	B	C
Description	**Catalogue number**	**Supplier**
BD BBL Dehydrated Culture Media Mueller Hinton II Broth Cation-Adjusted	212322	Fort Richard, New Zealand
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 ADC Enrichment	211887 or 212352	Fort Richard, New Zealand
BD BBL Middlebrook OADC Enrichment	211886 or 212240	Fort Richard, New Zealand
Glycerol ACS reagent, ≥99.5%	G7893	Sigma-Aldrich, New Zealand
Tyloxapolnon-ionic surfactant	T8761	Sigma-Aldrich, New Zealand
Methanol EMSURE® ACS	106009	Merck, New Zealand
Dimethyl sulfoxide (DMSO), reagent grade (99.5%)	D5879	Sigma-Aldrich, New Zealand
Rifampicin, ≥97% (HPLC)*	R3501	Sigma-Aldrich, New Zealand
Phosphate Buffered Saline tablets	P4417	Sigma-Aldrich, New Zealand

Growth media and chemicals required
*Make up a stock solution of 100mg/mL in methanol then dilute to 1mg/mL in DMSO

Plasticware:

- Nunc F96 MicroWell Black Polystyrene Plates, Catalogue number NUN137101, Thermo Scientific (we use black plates to prevent light spill-over between wells)
- Pipette tips
- Biotix Pipetting reservoirs 50mL, Catalogue number BTSR-0050-5SC, Mediray, New Zealand

Equipment:

- Pipettes – various sizes
- Spectrophotometer (to measure optical density of bacterial culture)
- Luminometer (we use a Perkin Elmer Victor X)

SAFETY WARNINGS

Mycotoxins produced by fungi can be highly toxic and carcinogenic, so make sure you wear gloves when handling fungal extracts.

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. Dissolve the extracts in dimethyl sulfoxide (DMSO) to reach a final concentration of 50 mg/mL. Dissolve the extract completely so that no particles remain. Use the vortex and centrifuge to achieve this. We use the following equation to work out how much DMSO to add: volume (mL) = mass (mg)/concentration (mg/mL).
3. Make up sufficient sterile Muller Hinton Broth (MHB) as directed by manufacturer. **Important: supplement with 10% ADC and 0.05% tyloxapol.**

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Preparing 96 well plates

1 We test doubling dilutions of each extract fraction in duplicate with a maximum concentration of 1000 µg/mL (Fig. 1A). Each round of screening also requires control wells containing a series of dilutions of the solvent used (we use DMSO), an antibiotic(we use rifampicin at a maximum concentration of 10 µg/mL when testing against *M. marinum*), and a broth control (Fig. 1B). Using the plate layout described in Figure 1, each 96 well plate can contain either 1 complete set of a crude extract and its 5 fractions or the appropriate controls for a testing round on a separate plate. Record the code of each extract that you are testing in your lab book.

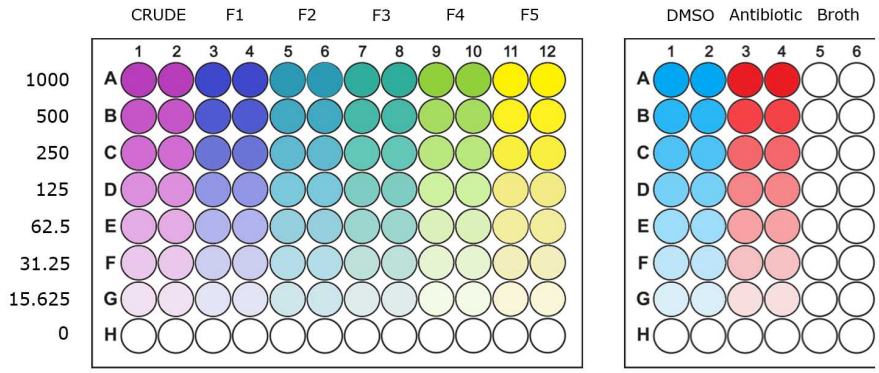

Figure 1: Example 96-well plate layout.

2 Set up your plate according to Figure 1. Use masking tape to make a front label on the lid including your name or initials, the date, the details of the bacteria being tested, and the extract being tested.

3 The final volume you will be using in each well is 100 µL. Add 50 µL of supplemented Mueller Hinton Broth (MHB) to all the wells on the first plate except the top row (A).

4 Add all extracts at double their required concentration to wells in the top row of the plate. If the extracts were dissolved at 50 mg/mL, add 96 µL MHB to wells in the top row and 4 µL of each extract fraction. Mix well by gently aspirating up and down using a pipette and make sure to change pipette tips between extract fractions. For the controls, add 96 µL supplemented MHB to wells in the top row and 4 µL of DMSO to the "DMSO" column, and 2 µL of rifampicin to the "Antibiotic" column. Add 100 µl supplemented MHB to the "Broth" column.

5 Using a multichannel pipette, gently aspirate repeatedly to homogenise the wells of each row. Then transfer 50 µL from the first row to the second row and aspirate to mix. Discard tips and repeat the doubling dilution down the plate, changing tips between rows, until you reach row G. Do not continue the dilution into row H.

6 Aspirate 50 µL from the wells in row G and discard the solution. This will leave row H as a growth control containing no extract/DMSO/antibiotic.

Adding the bacteria

7 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).

8 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). Dilute the bacterial culture with supplemented MHB to give a final $OD_{600}$of 0.001 which is the equivalent of ~10^6 bacteria per mL. We do this by diluting

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the bacterial culture to an OD_{600} of 0.01 and then diluting that 1 in 10 to give 0.001.

9 Calculate the volume of bacteria you will need for all the plates you have set up. You will need 50 µL in all wells to get to 100 µL total volume. Calculate the number of total wells and multiply by 50 µL, for example, 128 wells x 50 µL = 6400 µL or 6.4 mL (V2). Add some extra to account for any pipetting errors and to check the actual inoculum by plating out for colony forming units (CFU).

10 We usually make up our bacterial inoculum in a 50 mL Falcon tube using a serological pipette to add the appropriate volume of supplemented MHB.

11 Tip the bacterial inoculum into a pipetting reservoir and use a multichannel pipette to add 50 µL to all the wells excluding the MHB sterile control column.

Checking the bacterial inoculum

12 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 bacterial inoculum to the top well, mix and discard the tip. Using a clean tip, remove 10 µL, add it to the next well in the column and mix. Repeat to perform a 10-fold serial dilution down the column, using a clean tip each time.

13 Count visible colonies to ensure inoculum was correct at approximately 10^6CFU/mL.

14 Plate three technical replicates of each dilution onto a Middlebrook 7H11 agar plate supplemented with 0.4% glycerol and 10% Middlebrook OADC growth supplement. Incubate agar plates upside down at 28 °C until visible colonies appear. For *M. marinum* this usually takes approximately 7 – 10 days.

Measuring bacterial bioluminescence

15 We use a Perkin Elmer Victor X plate luminometer set to read 96 well plates with an integration time of 1 second per well. If we haven't filled the entire plate, we change the settings so that the machine doesn't measure the empty wells.

16 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.

17 Between measurements, place lids on the plates, put in a plastic box lined with damp paper towels and incubate at 28 °C with shaking at 100 RPM.

18 After the final time-point, if the light has reduced to background levels in any wells (for our machine this is < 10 relative light units [RLU]) we plate 3 x 10 µL aliquots from each well onto fresh Middlebrook 7H11 agar plates (supplemented with 0.4% glycerol and 10% Middlebrook OADC growth supplement) to check for bacterial viability. We define the minimum bactericidal concentration (MBC) as the lowest concentration at which no colonies appear after 10 days of incubation at 28 °C.

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