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Bioluminescence-based Minimum Inhibitory Concentration (MIC) testing of fungal extracts against Escherichia coli

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ABSTRACT

In this protocol, we describe how to obtain the minimum inhibitory concentration (MIC) of fungal extracts using a bioluminescent derivative of *Escherichia coli* ATCC 25922.

MATERIALS

Microorganism: *Escherichia coli* 25922 lux

Plasticware

	Description	Catalogue number	Supplier
	90mm Petri Dishes	LAB-021MR	Medi'Ray
	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
	Nunc F96 MicroWell Black Polystyrene Plates	NUN137101	Thermo Scientific
	Biotix Pipetting reservoirs 50mL	BTSR-0050-5SC	Mediray, New Zealand
	Pipette tips		

Growth media and chemicals

	Description	Catalogue number	Supplier
	Mueller Hinton II Broth Cation Adjusted	212322	Fort Richard, New Zealand
	Agar, Granulated	214530	Fort Richard, New Zealand
	Dimethyl sulfoxide (DMSO), reagent grade (99.5%)	D5879	Sigma-Aldrich, New Zealand
	Phosphate Buffered Saline tablets	P4417	Sigma-Aldrich, 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 compounds isolated from fungi.

BEFORE START INSTRUCTIONS

Prepare media. You will also need an overnight culture of E. coli to test against. The day before, Inoculate 10 mL of MHB in a 50 mL tube with *E. coli* 25922 lux and incubate overnight at 37 degrees C with shaking at 200 rpm.

Preparing 96-well plates

1 We test doubling dilutions of each extract fraction in duplicate with a maximum concentration of [M] 1 mg/mL. Each round of screening also requires a control plate containing the solvent the extract was dissolved in (e.g. DMSO), an antibiotic (to be used as a positive control, e.g. erythromycin), and broth (negative control to test the growth of the testing organism). Using the plate layout described in Figure 1, each plate can contain either one complete set of a crude extract and five fractions of decreasing polarity, or the appropriate controls for the testing round.

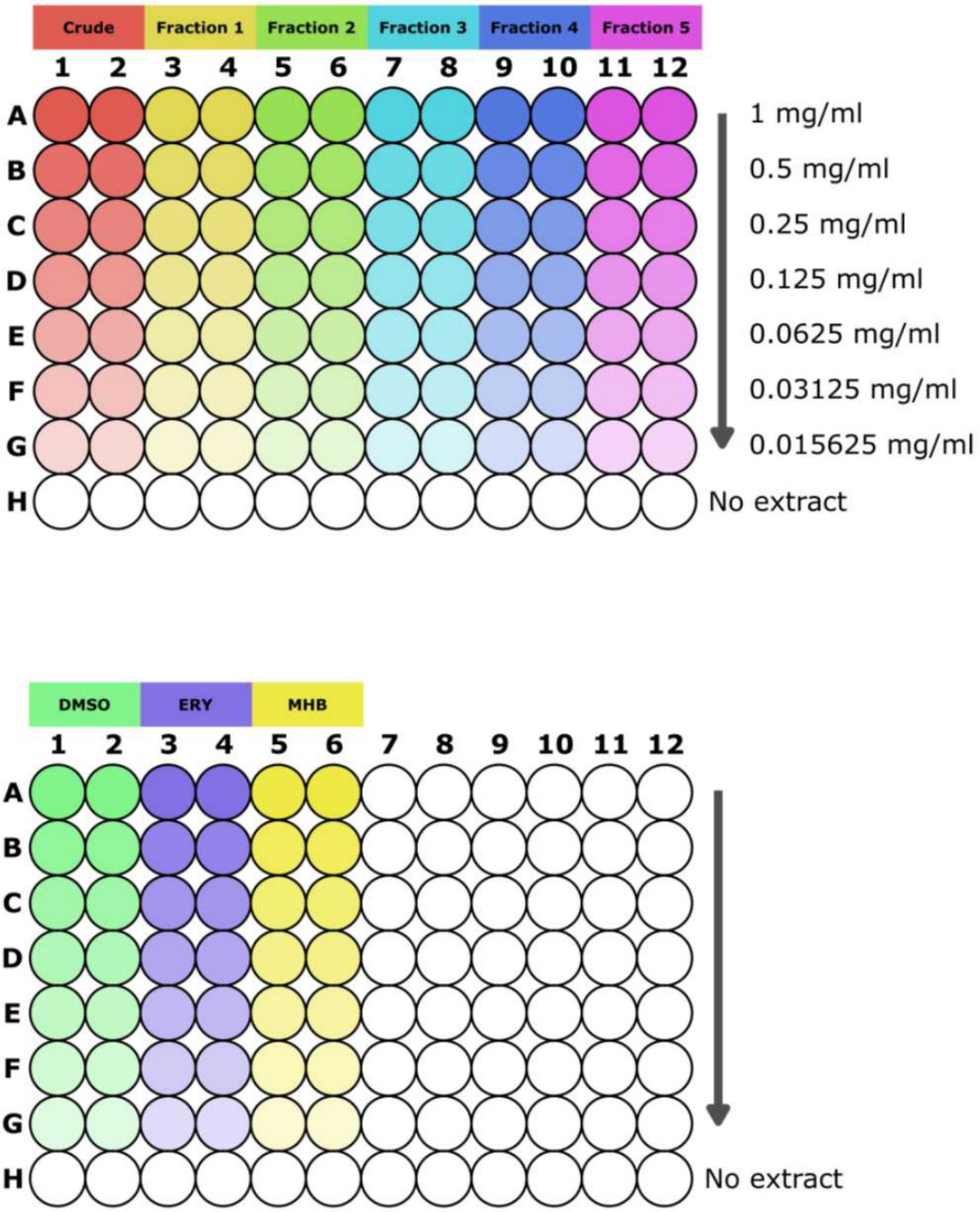

Figure 1: Plate layout for MIC testing of fungal extracts from active ICMP isolates.

The top plate shows the layout of the crude extract and the five fractions from each fraction. The bottom shows the control plate setup.
Control plate: 4% DMSO (Row A, Col 1-2), 0.25 mg/mL Erythromycin (Row A, Col 3-4), sterile MHB (Row A, Col 5-6).

Set up your plate according to Figure 1. Use masking tape to make a front label on the lid

2 including your name or initials, the date, the name of the bacteria you are testing against, and the name of the extract being tested.

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

4 Add all the extracts at double their required concentration to row A of the plate. If the extracts were dissolved at [M] 50 mg/mL , add 96 µL MHB to row A and 4 µL of each extract fraction. For the controls add 96 µL MHB to row A in the "DMSO" columns and 4 µL DMSO to these columns. Add 1 µL of [M] 50 mg/mL Erythromycin to the "ERY columns" and 99 µL MHB. Lastly, add 100 µL sterile MHB to the "Broth columns."

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.

Preparing bacterial inoculum

7 **This step needs to be done the day before.** Inoculate 10 mL of MHB in a 50 mL tube with E. coli 25922 lux and incubate overnight at 37 °C with shaking at 200 rpm.

8 Measure the optical density of the overnight culture of *E. coli* at 600nm (OD_{600}). We do this by diluting a sample of the overnight culture 1:10 in a 1.5 mL cuvette with MHB (720 µL broth + 80 µL bacteria).

Dilute the bacterial culture with MHB to give a final OD_{600} of 0.001 which is the equivalent of ~5 x 10^5 bacteria per mL. We do this by diluting the bacterial culture to an OD_{600} of 1 and then diluting that 1 in 1000 to give 0.001.

9 Make up the bacterial inoculum in a 50ml Falcon tube using a serological pipette to add the appropriate volume of MHB. Tip the inoculum into a pipetting reservoir and use a multichannel pipette to add 50 µL to all the wells excluding the sterile MHB control columns.

Checking inoculum concentration

10 Add 90 µL of MHB or Phosphate Buffer Solution (PBS) to each of the wells in a single column of a clear 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.

11 Plate three technical replicates of each dilution onto a MHA plate. Incubate agar plates upside down at 37 °C overnight. Count the colonies the following day.

12 Count visible colonies to ensure theinoculum was correct. It should be approximately ~5 x 10^5 CFU/mL

Measuring bioluminescence

13 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.

14 We take measurements immediately after setting up the plate (t=0) and then at 2, 4, 6, and 24 hours.

15 Between measurements, place lids on the plates, put them in a plastic box lined with damp paper towels, and incubate at 37 °C with shaking at 100 RPM.

16 After the final timepoint, if the light has reduced to background levels in any of the wells (for our machine this is < 10 relative light units [RLU]) plate 3 x 10 µL aliquots from each "dark" well onto fresh MHA to check for bacterial viability. We define the minimum bactericidal concentration (MBC) as the lowest concentration at which no colonies appear after overnight incubation at 37 °C.