Method Sheet 26

Disk diffusion assays for inhibitors of bacterial growth

Overview

This method sheet explains how to perform a disk diffusion assay to explore whether natural compounds or extracts of interest have antibacterial activity. Also called the Kirby-Bauer method, this assay makes use of the fact that as compounds impregnated onto paper disks diffuse outwards into the solid medium, their concentration falls with distance from the paper disk. Bacteria inoculated near a disk containing an anti-bacterial agent will be unable to grow within a certain distance of the disk, yielding a clear zone of no growth around the disk called the zone of inhibition, or zone of clearance. Measuring the diameter of this zone gives an indication of the antibiotic potential of a new extract or compound. The experiments described below test whether the extract alone has antibiotic potential, and also if it is capable of enhancing the activity of an existing antibiotic.

Reagents and consumables

- LB agar plates without antibiotic
- Sterile 6 mm paper discs
- Sterile cotton bud applicators
- Stock antibiotic solution at 10 mg/ml (e.g. ampicillin or tetracycline)
- Stock herb extract at 10 mg/ml (or stock natural compound at 10 mM) in DMSO

Equipment

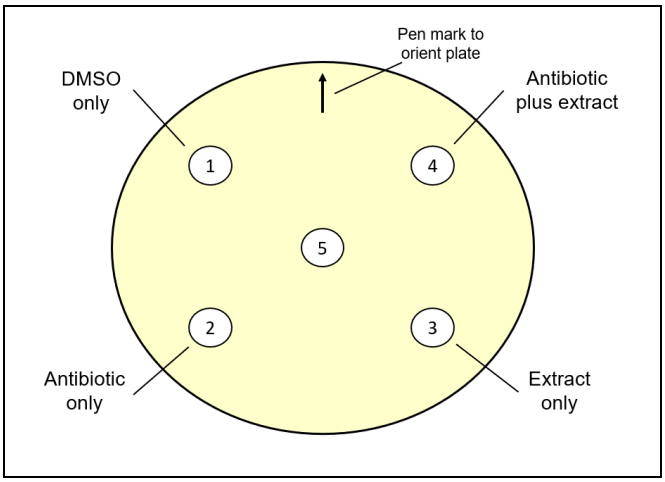
- Metal tweezers and a 37^{o}C microbiology incubator

Preparation of disks and plates (any time before the experiment)

1) The day before setting up plates, cut some paper disks from laboratory filter paper using a standard stationary hole punch to create many circular pieces of paper.
2) Collect the paper disks into a glass container with a lid and sterilise by autoclaving.
3) If you do not have any LB agar plates available, this is a useful opportunity to autoclave some LB agar at the same time.
4) After autoclaving, leave the sterile disks in a warm place with the lid slightly ajar to allow them to dry before use.
5) Once dried, close the lid firmly, and store at room temperature.
6) These disks can last indefinitely, so you will not have to repeat this preparation again during your project.
7) Pour the LB agar in standard 90 - 100 mm petri dishes (you can typically prepare 8-10 plates from 200 ml of molten agar).
8) Once cool, the LB agar plates can be stored in the fridge for several weeks.
9) Set up an overnight culture of your bacterial strain of interest the day before conducting the experiment.

Setting up plates for disk diffusion assay (Day 1)

1) Use a marker pen to draw an arrow on the bottom (not the lid) of an LB agar plate that contains no antibiotics.
2) Suspend 1 µl of overnight culture of your bacterial strain of interest (e.g. *Escherichia coli* DH5α or *Micrococcus luteus*) in 1 ml of LB in a sterile 1.5 ml microtube.
3) Pipette the bacterial suspension gently up and down several times to mix.
4) Insert a cotton bud into the culture for several seconds to coat the surface with bacteria.
5) Remove the cotton bud from the microtube and use it to streak the culture over the entire agar plate.
6) Continue streaking in many directions to ensure every part of the plate has been streaked with the inoculated cotton bud, then allow the plate to dry for 5 minutes.
7) Use 70% ethanol or a Bunsen burner to sterilise the tips of a pair of metal tweezers.
8) Remove the lid and rotate the plate so the arrow is at the top as you look at it.
9) Use the sterile tweezers to place 5 sterile paper discs onto the agar plate in the locations shown in the plate map shown below.
10) Pipette 2 µl of DMSO alone (no antibiotic or extract) onto disk 1.
11) Pipette 2 µl of antibiotic alone onto disc 2 (the positive control).
12) Pipette 2 µl of herb extract onto disc 3.
13) Pipette 2 µl of herb extract AND 2 µl of antibiotic onto disc 4.
14) Pipette nothing onto the central disk 5 (i.e. leave it untreated).
15) Take a note of which disk received which treatment in your lab book.



16) Incubate the plate at 37°C without shaking overnight.

Measurement of zones of clearance (Day 2)

1) The next day, remove the plate from the incubator and use a ruler to measure the zones of clearance around each disk in millimetres.
2) If there is no zone of clearance around a disk, in other words the bacterial growth occurs right up to the edge of the disk, record this as zero (0).
3) For any disks where there is an obvious ring of no bacterial growth around the disk, measure across the whole diameter of this outer circle, from one edge of bacterial growth across to the other.
4) Note these measurements in your laboratory notebook in a table with the names of each treatment.
5) You should conduct this experiment at least 3 times independently to be able to conduct statistical analysis of the data.

Notes

- If disk number 4 spills liquid onto the neighbouring agar, try pipetting the first 2 µl and allowing it to dry for several minutes, before pipetting the next 2 µl.
- If the zone of clearance is not circular, measure across both axes then take the average.
- In this experiment, disks will receive 20 µg of antibiotic and/or 20 µg of extract in total.
- If you would like to expand on these experiments to create additional charts, a simple variation is to repeat the same experiment using different antibiotics.
- If there is little impact of the herb of interest, larger quantities can be tested using the well diffusion method, which simply replaces paper disks with holes cut into the agar.
- Before inoculating the plate with bacteria, use a drinking straw to cut circular holes in the agar in the same locations as the paper disks shown in the plate map above, remove and discard the resulting agar plugs leaving empty circular wells.
- Pipette up to 20 µl of extract or antibiotic into each well, then continue as per the method above.