## Method Sheet 11

# Preparation of dose dilution series from individual extracts

## Overview

This method sheet explains how to prepare a dose response curve from an individual plant extract using the serial dilution method. This is commonly done in the replication phase after hit discovery, to seek better evidence that the hit extract has genuine biological activity against the target of interest. The results of these experiments are also helpful in estimating the likely potency of the active compounds present in the plant extracts. This method assumes that the serial dilutions will be used in a bioassay-based experiment in 96-well format with 100 µl of target cell culture or reagent per well. The method shown here is applicable to projects employing bacterial growth assays, mammalian cell viability assays and enzyme inhibition assays - follow the same protocol but use the most appropriate medium for your experiments as the diluent.

## Reagents

- Plant extract at 10 mg/ml in DMSO
- For bacterial growth assays - Luria Broth (LB) as diluent, Ampicillin at 100 mg/ml in $H_2O$ (standard concentration for molecular biology) as positive control
- For mammalian cell growth assays - cell culture medium as diluent, SDS at 10% in $H_2O$ as positive control
- For β-galactosidase enzyme activity assays - enzyme assay buffer as diluent, 1 M galactose in $H_2O$ as positive control
- DMSO alone (for equivalent vehicle control experiment)

## Equipment

- P20, P200 and P1000 pipettes
- Sterile pipette tips, 96-well microplates and microtubes of appropriate sizes
- Appropriate culture incubator
- Appropriate microplate reader

## Method

1) Prepare a 96-well assay plate containing, for example, mammalian cells, bacterial cultures, or enzyme suspensions of interest, at a volume of 100 µl per well (see previous Method Sheets for how to prepare these).
2) In this example, we will prepare a dose curve from 128 µg/ml to 0.5 µg/ml using the doubling dilution method.
3) Defrost a vial or tube containing the plant extract at 10 mg/ml in DMSO by placing in an incubator, dry block or water bath at 37°C.
4) While the tube is defrosting, use a pen to label 10x sterile 1.5 ml microtubes with 256, 128, 64, 32, 16, 8, 4, 2, 0.5 and 0 on the lid or side of the tube and line them up in a rack.

5) Aliquot 12 ml of culture medium, or enzyme buffer (these are the diluents), from your stock medium bottle into a sterile tube.
6) Pipette 974 µl of this medium into the first tube (labelled 256), and pipette 500 µl of this medium into the next 9 tubes.
7) Close the cap now on tube number 10 (labelled '0') to prevent accidental contamination with extract.
8) Add 25.6 µl of extract into the first tube (labelled 256), and either vortex briefly, or mix up and down gently with a larger pipette tip with the pipette set to 500 µl.
9) To begin the serial dilution step, remove 500 µl of the suspension from the first tube, and add it to the second tube (labelled 128) in the row.
10) Mix up and down gently with the same tip, leave all the 1 ml of suspension within the tube and discard the empty tip.
11) Replace the tip with a fresh one, and repeat the process, taking 500 µl of suspension from the tube containing 1 ml, and placing it on top of the existing 500 µl of medium in the next tube, then mixing gently.
12) Repeat until all tubes have been treated in this way, except for the tenth (last) tube in the row - this tube must receive no extract to serve as a negative control.
13) Once all serial dilutions are complete, rotate the rack containing the 10 tubes a half turn so that the tube containing no extract (labelled 0) is at the left-hand side, and the tube containing the highest concentration of extract (256) is at the right-hand side.
14) Pipette 100 µl of the solution from the first tube (labelled 0) onto each of 4 wells in the first column of the plate intended for use as shown in the plate map below, without discarding the existing medium from the wells.
15) Note that this step results in a halving of the concentration of extract (100 µl of extract solution onto 100 µl of medium already in the wells = 1:2 dilution), so the final concentration of extract in the well will be half of what it was in the microtube.
16) Place a fresh tip on the pipette and repeat with the next dilution in the row, working from left to right, for all other relevant wells according to the plate maps shown below.
17) Now challenge the other wells as shown in the plate map below with the relevant positive control compounds, which will differ depending on the type of assay and experiment you are performing.
18) For mammalian cell growth / viability assays, add 10 µl of 10% SDS to 490 µl of medium, mix and then add 100 µl directly onto each of the bottom right four wells of the plate (a positive control for maximum cell killing).
19) For bacterial culture growth / viability assays, add 10 µl of 100 mg/ml ampicillin to 490 µl of medium, mix and then add 100 µl directly onto each of the bottom right four wells of the plate (a positive control for maximum cell killing).
20) For β-galactosidase enzyme activity assays, add 10 µl of 10 mM PETG to 490 µl of medium, mix and then add 100 µl directly onto each of the bottom right four wells of the plate (a positive control for maximum enzyme inhibition).
21) Return the plate to the incubator for an appropriate period of time (e.g. 18 - 24 h for bacterial or mammalian cell growth assays, 1 hour for enzyme activity assays).
22) After the incubation period, remove the plate from the incubator and measure cell number / culture density / enzyme activity using the relevant method.

23) Repeat the same experiment (or set up at the same time in a separate plate) using the same volumes of DMSO alone instead of extract (this is a vehicle control experiment, see notes below).

## Plate layout

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | - | - | - | - | - | - | - | - | - | - | - | - |
| B | - | 0 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | - |
| C | - | 0 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | - |
| D | - | 0 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | - |
| E | - | 0 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | - |
| F | - | - | - | - | - | - | - | - | - | - | - | - |
| G | - | - | - | - | - | - | - | - | - | - | - | - |
| H | - | - | - | - | - | - | - | - | Pos | Pos | Pos | Pos |

Negative control wells (medium alone)

Serial dilutions of extract (concentrations in µg/ml)

Positive control wells

## Notes

- Always change your tip in between using different solutions, to avoid cross-contaminating the stock reagents.
- Avoid pipetting air into the samples while performing the doubling dilution, as bubbles interefere with the assay and give poor results.
- If you do find bubbles in your plate, you can prick them with a sharp piece of tissue after rolling it between finger and thumb.
- Keep all extract stock solutions at -20$^{o}$C, and return them to the freezer promptly after use.
- For mammalian cell assays, more reproducible results are frequently obtained by avoiding use of the outer wells of the plate (except for the SDS wells, which we can use here because this reagent kills all the cells).
- Please note that although you are challenging 4 wells with the same concentration of extract, these are what we term internal “technical replicates”, which is not the same as performing 4 separate experiments.
- The challenged plate represents only one experiment - you would have to repeat the whole experiment with three further plates in the same way to have completed four independent experiments.
- Because multiple doses of extract are assayed in this experiment, a single concentration of DMSO for vehicle control is not sufficient.
- Instead, for a suitable vehicle control test, it will be necessary to repeat the whole experiment using the same volumes of DMSO alone instead of stock extract - this is necessary to establish whether the solvent alone (DMSO) has an effect on the phenotype of interest.
- Feel free to modify the protocol to match the concentrations and plate layouts you prefer for your own experimental needs.