Method Sheet 15

Screening for inhibitors of the enzyme β-galactosidase

Overview

This method sheet explains how to screen a natural extract or compound library for inhibitors of the enzyme β-galactosidase. This enzyme hydrolyses the disaccharide sugar lactose into the monosaccharide sugars galactose and glucose. β-galactosidase activity can be measured by incubating the enzyme with the artificial substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG). Cleavage of ONPG releases ortho-nitrophenol, a yellow compound that can be quantified by measuring absorbance at 420 nm. Performing the reaction in 96-well plate format permits the screening of natural compound libraries for discovery of inhibitors of the enzyme, in a manner very similar to that used in the process of drug discovery for other enzyme targets.

Reagents

- Recombinant beta-galactosidase enzyme (1 mg/ml, frozen aliquot)
- Ortho-nitrophenyl-β-D-galactopyranoside (ONPG, 60 mg, dry powder)
- Galactose (1 M stock in water, dissolve 18 g galactose into 100 ml H_2O)
- Phosphate buffer (50 mM, pH 6.8, see notes section below for how to prepare)

Equipment

- Clear plastic 96-well microplate(s) with lids (do not need to be sterile)
- Pipette tips (compatible with each pipette)
- Plastic reservoir(s) to dispense ONPG substrate
- Multichannel pipette (8- or 12-channel) capable of dispensing 1 - 10 µl
- Multichannel pipette (8- or 12-channel) capable of dispensing 100 - 200 µl
- Suitable waste stream for disposal of spent plates and reactions
- Microplate reader capable of measuring absorbance of 96-well plates at 420 nm (450 nm is also suitable)

Method: Part 1 - Setting up the plates for assay

1) The volumes given below are sufficient to set up 5 assay plates - multiply accordingly if performing assays with more than 5 plates.
2) Prepare 200 ml of phosphate buffer according to the notes section, below.
3) Aliquot 60 ml of phosphate buffer into a beaker or bottle, and add 60 mg of substrate (ONPG compound).
4) Mix well until fully dissolved, then pour the solution into a plastic reservoir.
5) Use a multi-channel pipette to aliquot 99 µl of the substrate solution into each well of 5x 96-well plates.
6) You can now proceed directly to adding plant extracts from the library plates.

Method: Part 2 - Challenging the plates with *Phytotitre* plant extracts

1) Fully defrost a *Phytotitre* or *Puretitre* plate in a 37°C incubator (follow Method Sheet 01).
2) Carefully remove the cap mat from the library plate and place with the round domes facing upwards (do not discard the cap mat).
3) Column 1 of the library plate is left empty to allow space for negative controls - you can make use of this by pipetting 100 µl of sterile DMSO into each well of this column in the library plate to use as a vehicle only control.
4) Place both library and assay plates side by side and ensure both are in the correct orientation (i.e. check that well A1 is in the top left position).
5) Set a small volume multichannel pipette to dispense 1 µl.
6) Attach tips to the pipette and ensure all are firmly seated as in the previous method.
7) Work from left to right, starting at column 1 and moving across, one pipetting cycle at a time, to column 11.
8) It can be difficult to keep track of which column is being pipetted from and to when challenging plates for screening, so you can cover the wells that have already been completed with the lid from a 96-well plate to help keep your place as you move along the plate from column to column.
9) Push the plunger down to the first stop (not all the way to the second stop).
10) Place the tips below the surface of the liquid in each well of the respective column of the library plate (containing stock extracts).
11) Slowly allow the plunger to return to the top position.
12) Look carefully across all the tips to ensure the level of liquid in each tip is the same, if not, dispense the suspension back into the correct wells of the library plate and try again.
13) Likewise, if there are any large air bubbles in any of the tips, dispense and try again.
14) Move the pipette to the assay plate (i.e. the plate containing the substrate solution) and dispense all the liquid into the respective wells, being careful to pipette into the liquid and not onto the side of the well.
15) When dispensing, push the plunger past the first stop all the way to the second stop.
16) Discard the tips after pipetting into each column (this is essential to prevent cross-contamination of the library contents).
17) Place fresh tips on the pipette before moving on to the next column.
18) Move the plate lid marker one column to the right to make it easier to see which column is next to work from.
19) Continue until every well of the plate between columns 1 and 11 have been challenged (see plate layout, below).
20) Use a single channel pipette to add 1 µl of 1 M Galactose into each well of column 12 (this compound will be the positive control for enzyme inhibition).
21) Move the assay plate backwards and forwards, then side to side, gently several times to mix the compound into the substrate solution.
22) Avoid jarring movements that may splash liquid between wells and cause cross-contamination.

23) Reseal the *Phytotitre* library plate with the cap mat in the correct orientation and return it to the freezer for storage.

24) Repeat the process with any other *Phytotitre* library plates you may be screening on the same day.

Method: Part 3 - Adding enzyme to start the reaction

1) Aliquot 60 ml of phosphate buffer into a clean beaker or bottle, and add 12 µl of 1 mg/ml recombinant β-galactosidase enzyme (final concentration 200 ng/ml, ~0.1 U/ml).

2) Mix well, then pour the enzyme solution into a plastic reservoir.

3) Use a multi-channel pipette to aliquot 100 µl of the enzyme solution into each well of the first of the five pre-prepared assay plates already containing substrate.

4) Do this step one plate at a time.

5) As soon as pipetting of one plate is complete, use a microplate reader to measure the absorbance of the plate at 420 nm (or 450 nm if that wavelength is not available) to obtain the baseline (time = 0 h) measurements.

6) **NB**: Do not wait to take the baseline absorbance measurement until completion of pipetting into the other plates in the list - this measurement must be taken as soon as possible after supplementing the plate with enzyme.

7) Take a note of the time, then place the assay plate in a static 37°C incubator to allow the reaction to progress (does not need to be a shaking incubator).

8) Incubate the plate at 37°C for 30 minutes.

9) Use a microplate reader to measure the absorbance of the plate at 420 nm (or 450 nm if not available) a second time.

10) After placing the first plate in the incubator, you can begin pipetting enzyme solution onto the next plate in the list, and immediately afterwards, measure absorbance of that plate.

11) Continue in this way until every plate has received substrate, plant extracts and enzyme.

12) Every plate should be read at least twice - once as soon as possible after adding the enzyme, and then again after the 30 minute incubation step.

13) *Optional*: If you would like to gather more data on reaction rate from the same experiment, it is possible to read absorbance of each plate multiple times at 10 minute intervals.

Example plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	01	09	17	25	33	41	49	57	65	73	Gal
B	DMSO	02	10	18	26	34	42	50	58	66	74	Gal
C	DMSO	03	11	19	27	35	43	51	59	67	75	Gal
D	DMSO	04	12	20	28	36	44	52	60	68	76	Gal
E	DMSO	05	13	21	29	37	45	53	61	69	77	Gal
F	DMSO	06	14	22	30	38	46	54	62	70	78	Gal
G	DMSO	07	15	23	31	39	47	55	63	71	79	Gal
H	DMSO	08	16	24	32	40	48	56	64	72	80	Gal

1 µl DMSO (vehicle control) — 1 µl each plant extract or stock compound — 1 µl 1 M Galactose (positive control)

Notes

- Prepare phosphate buffer as follows: place ~160 ml distilled water (dH_2O) in a beaker, add 1.70 grammes anhydrous Na_2HPO_4, 0.96 grammes anhydrous NaH_2PO_4, stir until fully dissolved, pH to 6.8 using HCl or NaOH, make up to 200 ml by adding dH_2O.
- Column 1 of the library plate is left empty to allow space for negative controls - you can make use of this by pipetting 100 µl of sterile DMSO into each well of this column in the library plate to use as a vehicle only control.
- Column 12 of the library plate is also left empty to allow space for customers to insert their own positive controls of interest (we suggest filling each well with 1M galactose in water for these experiments).
- When dispensing compound, ensure the liquid goes directly into the larger culture volume, and does not touch and then adhere to the plastic on the side of the well.
- If using the *Phytotitre* library, the stock plant extracts are at 10 mg/ml, so transfering 1 µl of stock extract into 199 µl of assay solution represents a 1:200 dilution, which means the final concentration of plant extract in each well will be 50 µg/ml.
- Accordingly, the final concentration of DMSO (the solvent, also called vehicle, used to dissolve the plant extracts) will be 0.5% (wt:vol) when following this protocol.
- If using the *Puretitre* collection instead, the stock compounds are at 10 mM, which means the final concentration of each compound in each well will be 50 µM.