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We use this protocol and it's working

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Conducting Dynamic BH3 Profiling Adapted From Letai Lab

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ABSTRACT

Dynamic BH3 Profiling is used to test cellular proximity to apoptosis after treatment by exposure of in situ mitochondria with the BH3 domain of apoptotic proteins to induce mitochondrial cytochrome C release. Cytochrome C release is determined by flow cytometric measurement of cytochrome C fluorescence in cells and corresponds with the cellular proximity to mitochondrial outer membrane permeabilization, change of which is termed "priming".

MATERIALS

All material information and preparation are dutifully discussed in the Letai lab BH3 Profiling Appendix.

bh3_profiling_guide_appendix_20170814 (accessed 20220419).pdf

Before Starting

1 Allow the MEB2-P25 to equilibrate to room temperature (RT) if stored at 4 degree. Equilibration may take 1 to 2 hours. Preferably, you can pull the bottle out the night before to allow RT equilibration.

Note

Note: BH3 Profiling is temperature-sensitive. As temperature increases, cytochrome c release increases as well. Conduct experiments in a constant temperature room.

2 Prepare your peptide panel by diluting your peptides in DMSO to 100x the final concentration desired at exposure. If you do not know the desired final concentration, titrate your peptides to determine a concentration that causes around 20% cytochrome c release.

Note

Note: You can save these working dilutions in the minus 20 for at least a week. Peptides are stable and can survive a few freeze thaws but the chemical BH3 mimetics should be made fresh.

	A	B
	Peptide	Suggested Range (uM)

	A	B
	BIM	0.001-100
	BID	0.01-100
	PUMA	0.1-100
	BAD	0.1-100
	NOXA	1-100
	HRKy	1-100
	MS1	0.1-10
	FS1	0.1-10
	ABT199	0.1-1
	WEHI-539	0.1-1
	A-1331852	0.1-1
	A-1155463	0.1-1
	S63845	1-10

Adapted from "A Guide to BH3 Profiling" Appendix Revision 2017-08-14

3 Determine the minimum concentration of digitonin to permeabilize all your cells. While the Letai BH3 Profiling Guide recommends 0.001% final concentration, this may not work for every cell due to the amount of cholesterol at the cell membrane. Test final digitonin concentrations of 0.008-0.00025%.

3.1 Testing Digitonin Mediated Cell Permeabilization with Trypan Blue

1. Dilute the digitonin stock to 0.016% in MEB2-P25 and perform a 2-fold serial dilutions five times (0.008%, 0.004%, 0.002%, 0.001%, 0.0005%) to make a panel of 6 different digitonin concentrations (at 2x final) in MEB2-P25 buffer.

2. Take 400,000 cells you wish to BH3 profile and resuspend in 40 uL of MEB2-P25.

3. Combine 5 uL of cells with 5 uL of the 6 digitonin concentrations.

4. Wait 5 minutes to allow permeabilization

5. Add 10 uL of trypan blue, mix, and transfer to a hemocytometer.

6. Permeabilized cells will appear blue under the microscope.

7. Select the lowest concentration of digitonin that results in 100% permeabilization.

BH3 Peptide Exposure

4 Count your cells and take 2,000,000 cells per treatment condition in a 1.7mL tube use in the assay.

Note

Note: In this protocol, we will expose 200,000 cells to 8 different peptides. The assay tolerates cell concentrations between 0.5 million per mL to 4 million per mL at the BH3 exposure stage. In this protocol, we use 2 cells million per mL. Scale up or down as necessary.

5 Spin down the cells at 500xg for 5 minutes.

6 During the spin, prepare your 2x peptide solutions in MEB2- P25. The peptide stocks are made at 100x and should be diluted 1:50. Prepare enough for every cell line and treatment used in the assay.

Minimum Total Volume= (#cell line x #treatment condition + 1)* 25 uL

Note

Note on panel: Always include in your peptide panel DMSO only, PUMA2A, and Alamethicin to act as controls for cytochrome C.

7 Aspirate media and resuspend with 1mL PBS and spin down in cold centrifuge to wash the cells.

8 If you need to stain your cells with cell surface markers, block and stain cells now then spin down before proceeding to digitonin permeabilization.

Note

Note on staining: A fixable viability dye would be advisable if your treatment causes significant amounts of death or if your starting sample has variable initial viability. Decide now if you would like to include a fixable viability dye and proceed from step 9.

9 During this step, dilute the digitonin to 2x of the final concentration determined in step 3.1 in MEB2-P25 buffer.

10 Resuspend the cells in 250uL in the diluted digitonin/MEB2-P25 solution. This is enough for ten 25uL peptide exposures, thus preparing extra.

11 In a V-Bottom plate, place 25uL of 2x peptide at the bottom of the well.

12 Place 25 uL of cells on the wall closest to you.

Gently tap after the addition to each row.

12.1 

Mix the 25 uL of cells and 25uL of peptide by gently tapping the sides of the plate. Don't tap to hard or you may lose sample. Proper tapping technique: keep plate on benchtop while tapping.

Tapping will be necessary in future steps to best mix the solution. Alternatively, a reliable plate vortexer can be used to mix, however inappropriate settings or use may cause solution to spill out of the well.

Note

Note: The tapping technique is used to control the exposure of cells to peptide if using a single channel pipet. Exact time of exposures can be kept in combination with this tapping method and a timer. Alternatively, you may add and mix the cells with a reliable multichannel pipettor.

13 Incubate for 1 hr at room temperature. If you have highly sensitive cells, incubate for 30 minutes. Hardier cell lines may require 1.5 hr. Determine this time for your cell system.

Ceasing BH3 Exposure and Cytochrome C Staining

14 During incubation, prepare Cytochrome C Stain: 1:40 human cytochrome c (Biolegend) in 10x Cytochrome C Staining buffer, you need 10uL/well, prepare extra.

15 Fix the cells by adding 16.5uL of 8% PFA. This is a final of ~2% PFA.

Gently tap after the addition to each row.

Note

Note: Use the freshest available PFA. Inadequate fixation can lead to cytochrome c loss during staining.

16 Incubate at room temp for 20 minutes

17 Neutralize with 16.5uL N2 buffer.

Gently tap after the addition to each row.

18 Wait at least 5 min.

19 Add 10uL of 1:40 diluted Cytochrome C Staining mix to each well

Gently tap after the addition to each row.

20 Seal the plate with a foil seal or clear seal if kept in the dark.
Incubate for 4 hours at room temperature (in a drawer) or overnight (16hr minimum) at 4 degrees.

Flow Analysis

21 Add ~50uL PBS to increase volume to ~150uL

22 On the cytometer, open the BH3 Profile Template and BH3 Profiling Instrument Settings

23 Ensure that the threshold for FSC-H is excluding junk. This assay will have notable amounts of debris.

24 Run the DMSO control to set cytochrome c high population above the second log

25 Run the BIM 10uM control to check if cytochrome c low population is below the second log.

26 Acquire 125uL. Set acquisition settings to acquire 10,000 events.