LETAI LAB

BH3 Profiling Flow Core

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A Laboratory Guide to BH3 Profiling

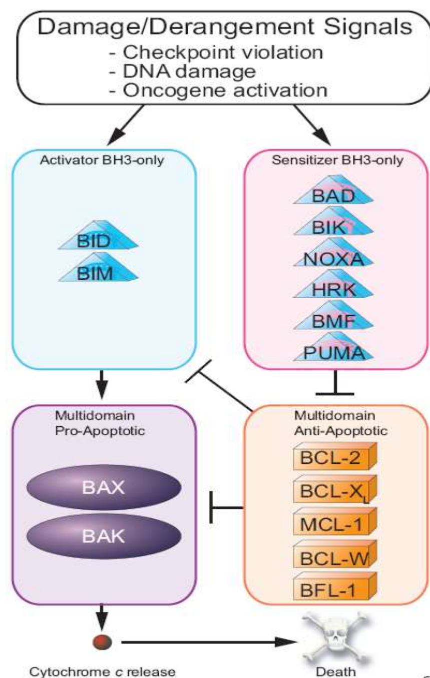


Figure 1: Classification and interactions of the BCL2 family

Why BH3 Profiling

The intrinsic pathway of apoptosis is regulated at the level of the mitochondria where more than fifteen members of the BCL-2 family of proteins interact to make this life or death decision. Many chemotherapeutic agents cause apoptosis, and the mechanism often involves changes in the levels and interactions of BCL-2 family members.

Levels of each of these proteins alone is not enough to determine how this decision will be made, and the sample size necessary to probe all possible interactions and post-translational modifications makes the task virtually impossible with cell lines and even more so with primary samples. Rather than attempt these, the Letai laboratory has developed BH3 profiling as a functional tool to determine the net response of these interactions without needing to record all of the individual interactions that determine it.

BCL-2 Family Control of Apoptosis

BH3 profiling relies on our understanding of how several broad groups of BCL2 family members interact with each other. The groups can be defined based on their structure and function as multi-domain pro-apoptotic, multi-domain anti-apoptotic, activator BH3 only, and sensitizer BH3 only. The multi-domain proteins contain BCL-2 Homology (BH) domains 1-4 in most cases while the BH3 only proteins are named because they contain only BCL2 homology domain 3, or BH3.

Functionally, the multi-domain proteins BAX and BAK are pro-apoptotic and are responsible for generating pores in the outer mitochondrial membrane when they undergo conformational changes upon activation. Mitochondrial outer membrane permeabilization (MOMP) allows for the release of cytochrome c into the cytoplasm where it can interact with pro-caspase 9 and apaf-1 to form the apoptosome, triggering downstream cleavage and activation of caspase 3 and apoptosis. Even without downstream caspase activation, MOMP will generally lead to the death of the cell even if it is no longer as efficient as with caspase activation. **All measures of response in BH3 profiling are surrogates for measuring MOMP.**

While they may not be the only proteins capable of doing so, BH3 proteins BIM and BID are capable of interacting with BAX and BAK to induce the conformational changes that leads to MOMP. This ability to activate BAX and BAK is where their designation as activator BH3s are derived. Opposing the pro-apoptotic proteins are the multi-domain anti-apoptotic proteins BCL-2, BCL-W, BCL-XL, BFL-1, and MCL-1. The proteins inhibit BIM and BID by binding them and preventing them from interacting with BAX and BAK. They can also bind the activated forms of BAX and BAK to prevent them from forming oligomers and therefore preventing MOMP. Finally, while

they cannot directly activate BAX and BAK, the sensitizer class of BH3 only proteins can bind to the anti-apoptotic proteins, acting as inhibitors of inhibitors, to release activators and activated BAX and BAK. Pre-bound sensitizers leave less available room to bind activators and activated BAX or BAK, and this makes the cell more sensitive to subsequent insults, which is where they derive their name. A summary of these interactions can be found in Figure 1. **NOTE: The BH3 domain of the BH3 only proteins is where the BH3 peptides used in profiling are derived. With few exceptions, most sequences are the native human or mouse sequence.**

The BH3 Binding Map

Not every BH3 only protein can interact with every anti-apoptotic protein, and this adds a layer of complexity that prevents a simple sum of all BH3 proteins from being used to determine the outcome of a death insult. However, the binding affinities of the BH3 domains as peptides to recombinant anti-apoptotic members is known, and this pattern also enables BH3 profiling to determine how ready a cell is to commit to apoptosis, but it also provides information regarding which anti-apoptotic proteins are being used to prevent MOMP.

	BID	BIM	BIDM	BAD	BIK	NOXA A	NOXA B	HRK	BNIP	PUMA	BMF
BCL2	66 (6)	<10	-	11 (3)	151 (2)	-	-	-	-	18 (1)	24 (1)
BCLXL	12 (9)	<10	-	<10	10 (2)	-	-	92 (11)	-	<10	<10
BCLW	<10	38 (7)	-	60 (19)	17 (12)	-	-	-	-	25 (12)	11 (3)
MCL1	<10	<10	-	-	109 (33)	19 (2)	28 (3)	-	-	<10	23 (2)
BFL1	53 (3)	73 (3)	-	-	-	-	-	-	-	59 (11)	-

Figure 2: EC50 of BH3-only peptides to N-terminal GST, C-terminal truncated anti-apoptotic proteins. Values listed are in nM. Grayed values are greater than 1 μM

The activator peptides BIM and BID can find to all anti-apoptotic proteins. PUMA and BMF, which can be called pan-sensitizers, share this behavior with BIM and BID. The remainder of the peptides have a more selective binding pattern. For example, HRK only binds to BCL-XL, and thus a response from the HRK peptide will indicate a dependency on BCL-XL. Similarly, the NOXA peptides only bind MCL-1. BAD can bind three proteins, and therefore it cannot discriminate between the three, but a response from a BAD peptide indicates that at least one of its binding partners is present and involved in the prevention of MOMP. When use in parallel with small molecules agents such as ABT-199 and WEHI-539, which bind to BCL2 and BCL-XL respectively, the identity of the anti-apoptotic protein in use can be better understood.

Three Classes of Priming

Priming can be divided into three major classes. Class A, competent but unprimed, is characterized by a response to activators BIM and BID but weak or no response to sensitizer peptides. This means that the mitochondria have functional BAX and/or BAK, but they are not sequestering any pro-death proteins that could be displaced to activate them. These cells are generally less sensitive to chemotherapy, but perturbations can convert them from Class A to Class C, primed, which will be described later.

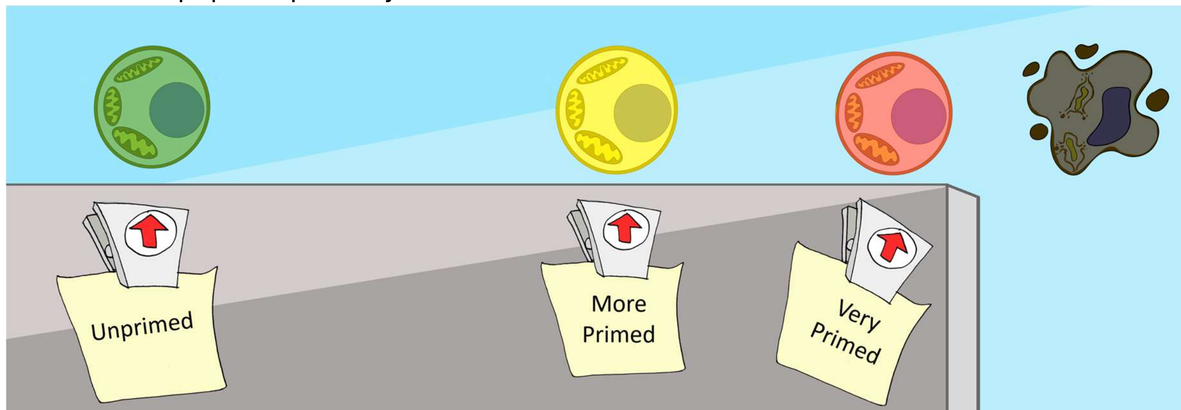
Class B cells lack functional BAX or BAK and are considered apoptotically incompetent.. This may be due to mutation, insufficient expression, or homozygous deletion of both proteins. Class B cells will not depolarize or release cytochrome c even with very high doses (100 μM) of BIM and BID over long periods because they cannot form the pores necessary to release cytochrome c. Non-specific pore forming peptides, such as Alamethicin, can be used as a control to cause MOMP in these cases to ensure that a class B profile is due to the cells and not due to an omission of a profiling component such as digitonin.

Class C primed cells will show a response to both activator and sensitizer class peptides. They have functional BAX and/or BAK to cause MOMP as well as sequestered pro-death proteins including BH3 only proteins or pre-activated BAX or BAK that have exposed their N-termini. Class C cells can be subdivided based on their responses to the sensitizer peptides as dependent on one or more anti-apoptotic protein by using the binding chart as a guide. For example, A class C MCL-1 cell would respond to BIM, BID, PUMA, BMF, NOXAA but not BAD or HRK. A class C BCL-2 would respond to BIM, BID, PUMA, BMF, and BAD but not NOXAA or HRK. It is possible, and common, for cells to depend on more than one protein, and this will cause a mixed profile. In general, class C cells are the most sensitive to chemotherapy provided that other factors, such as drug efflux pumps which are upstream of mitochondria, do not skew the response.

The Cliff Model of Priming

A simple tool to understand priming is to envision cells at different distances from a cliff, a threshold, beyond which they call in to an apoptotic death. If one cell is ten units away from the cliff and another cell is fifty units away, the one ten units away is said to be more primed. If a perturbation such as a drug is applied, and this perturbation generated twenty five units of death signal, the cell only ten units away will fly off the cliff and die. The less primed cell will move, but it will end far from the threshold of apoptosis and survive. One should note, however, that this cell is now more primed than it was when it started. This is how the class A to class C conversion can be envisioned.

A class B cell can be thought of as anchored far from the cliff where nothing can move it. Instead, one would have to collapse the cliff to kill it. Such events that can kill class B include extrinsic apoptotic signaling, necrosis, or immune-mediated killing that do not necessarily rely on the intrinsic apoptotic pathway.



iBH3 (MEB2-P25) Buffer Reagents

Potassium hydroxide solution, 45% w / v KOH
Sigma-Aldrich cat# 417661-500ML
Store at RT

Potassium chloride solution KCL 3 M
Boston Bioproducts cat# MT-253
Store at RT

EDTA solution, 0.5 M, pH 7.4,
Boston Bioproducts cat# BM-711-K
Store at 2-8°C

EGTA solution, 1 M Boston BioProducts cat# BM-723
Store at RT

HEPES buffer solution, pH 7.5, 1 M
Boston BioProducts cat# BB-103
Store at 2-8°C

Bovine serum albumin (BSA) solution, 30%, Protease Free
Gemini Bio Products cat# 700-110
Store at 2-8°C

Mannitol
Sigma Aldrich, 100 g bottle, M4125-100G
Store at RT
Dissolve entire container in 1 L water and sterile filter to get 10% mannitol
Ready made solutions of mannitol fall under the name of osmitrol, a clinical agent and thus very expensive. Making 10% mannitol is easy and much cheaper this way. Don't try to make 20%, it will start crystallizing on you later

Succinic Buffer, pH 6.5 0.2M
Boston BioProducts Cat# BB-2350
Store at RT

Pluronic F-68 10% w / v
Life Technologies, cat# 24040032
Store at 2-8°C

Preparing iBH3 Buffer

To prepare 1 liter of iBH3 buffer, combine the following in a 1 L beaker

Stock Solution (concentration)	Volume to add (mL)	Final concentration in 1000 mL iBH3 buffer
10% Mannitol	274	150 mM
200 mM Succinate Buffer	25	5 mM
BSA (30 %)	3.4	0.1 %
HEPES (1M)	10	10 mM
EGTA (1 M)	1	1 mM
EDTA (0.5 M)	2	1 mM
KCl (3 M)	50	150 mM
Pluronic F68 (10% w / v)	25	0.25% (w / v)

The pH of the buffer prepared from these reagents will still be slightly acidic. Using a pipette, add 10-20 uL of KOH solution at a time until the pH is 7.4 */- 0.1.

Sterile filter and store the buffer at 4C. The buffer is stable at least 6 months.

Assay Reagents

Digitonin

Sigma D5628
Lists as 50% by TLC, treat it like it was pure.
Prepare by dissolving 50 mg/mL to get a 5% solution.
Store at -20C to -80C

3.3M Tris Base

Research Products International T60043-4000.0.
40% Tris by weight, no pH adjustment
replaces ‘N2’ buffer in older protocols
Dilute 1:1 to get ~ 1.5 M Tris

10% Neutral Buffered Formalin

Sigma Aldrich (valid) # HT5012-1C
We use the small tubs so they can be tossed if they get contaminated with anything. They are a little easier to handle that a big jug, but a bulk container of 10% neutral buffered formalin is fine.
10% Neutral buffered formalin is roughly 3.8% Formaldehyde at neutral pH

10X Perm Wash Buffer

BD Bioscience 10X Perm/Wash buffer Cat# 554723
OR
Biolegend Intracellular Staining Permeabilization Wash Buffer (10X) Cat# 421002
Replaces staining buffer in older protocols

Cytochrome C antibody

Clone 6H2.B4
https://www.biolegend.com/en-us/search-results?Clone=6H2.B4
Biolegend offers a few formats as well as the only ASR cytochrome c that I know if in FITC
ASR antibody is used at 1:80 in 10X Perm/Wash buffer
RUO antibody is used at 1:200 in 10X perm/wash buffer because it ships more concentrated than the ASR

Additional Reagents

Surface staining antibodies

These are applied before BH3 profiling. Routine examples include things like CD45, CD3, CD33, CD34, EpCAM and many others

Live/Dead Stains

https://www.biolegend.com/en-us/live-dead
Includes Zombie stains. These need to be applied before surface antibodies and excess removed before surface staining to avoid reacting with the surface stain antibodies.

Additional Intracellular Markers

Can be added with the cytochrome c antibody in the 10x perm wash buffer. Examples include markers like COX IV and p53. NOTE: cytoplasmic markers are lost upon digitonin exposure and will not be detected

Small molecule BH3 mimetics

This includes compounds such as navitoclax, venetoclax, S63845, AZD5991, etc. These stocks are generally prepared as 25-50 mM stocks and stored the same as peptides. It is important tot note that these compounds require higher concentrations in BH3 profiling compared to viability tests. The nature of his difference is unknown, but the specificity of the compounds remains true.

Reagent Preparation

Peptide Orders

BH3 peptides are purchased as custom synthesis from peptide synthesis vendors. The Letai lab uses Biosynth https://www.biosynth.com/ (Formerly New England Peptide) for all of its peptides. This company has a great deal of experience producing these particular peptides.

Peptides are synthesized as TFA salts. We do not do salt exchange on them as the sodium salt tends to be less soluble. It is necessary to specify this when you order your peptides.

We usually order a peptide as follows:

Peptide Name, Peptide Sequence. N-terminal acetyl, C-Terminal amide, TFA Salts, 95% purity

The table below contains the name and sequence of the BH3 peptides we current use.
NOTE: mNoxaA is an older peptide replaces by MS1, but if there is a reason you can't get MS1 for yourself, you can fall back on NOXAA

We order large volumes such as 1-2 g at a time, so I have it made into 200 mg fractions so I don't have to make all the peptide at once. You can have your peptides prepped as all in one vial or split across a few. You just need to specify that when you order.

Store peptide powder stocks at -80C
Peptide solutions can be stored in glass vials for long term or plastic for short term
Fisher 0337511AA with closure 0337555B have worked well.

Peptide Name	**Sequence**	**Extinction Coeff. 280 nm**
	N-Term	**C-Term**
hBIM	Acetyl-MRPEIWIAQELRRIGDEFNA-Amide	5500 cm^{-1} M^{-1}
hBID-Y	Acetyl -EDIIRNIARHLAQVGDSMDRY- Amide	1490 cm^{-1} M^{-1}
mBAD	Acetyl -LWAAQRYGRELRRMSDEFEGSFKGL- Amide	6990 cm^{-1} M^{-1}
mNoxaA	Acetyl -AELPPEFAAQLRKIGDKVYC- Amide	1490 cm^{-1} M^{-1}
Puma	Acetyl -EQWAREIGAQLRRMADDLNA- Amide	5500 cm^{-1} M^{-1}
Hrk-y	Acetyl -SSAAQLTAARLKALGDELHQY- Amide	1490 cm^{-1} M^{-1}
MS1	Acetyl-RPEIWMTQGLRRLGDEINAYYAR-Amide	8480 cm^{-1} M^{-1}
FS1	Acetyl-QWVREIAAGLRLAADNVNAQLER-Amide	5500 cm^{-1} M^{-1}

-Y and W- designate added residues for UV absorbance measurements at C or N term respectively.

MS1 is a non-natural MCL1-specific peptide: ACS Chem Biol. 2014 Sep 19;9(9):1962-8. doi: 10.1021/cb500340w.
FS1 is a non-natural BFL1 / BCL2A1-specific peptide: Elife. 2017 Jun 8;6. pii: e25541. doi: 10.7554/eLife.25541.

Peptide Preparation

Peptides are dissolved in DMSO and stored at -20C for working stocks and -80C for long term stocks.

Dissolve your peptides
BIM, PUMA, BAD, NOXA, MS1, HRK are all soluble at 200 mg/mL or greater in DMSO
FS1 peptide is only soluble up to 50 mg/mL in DMSO

Solutions should be clear and free of powder or debris.

Quantify your peptides

Most of the BH3 peptides contain UV absorbing residues such as tryptophan and tyrosine. For peptides like HRK that do not, a c-terminal tyrosine was added to the peptide so they could be UV quantified. UV absorption at 280 nm can be used to determine the actual content of peptide in solution because the TFA in the salt will be variable and thus use of mass measurements is not reliable.

To quantify the peptide, first dilute it at least 1:20 with water. I usually ise a 1:50 dilution. This is done to reduce the DMSO absorption shoulder on the shorter wavelengths, and it will make the 275/280 nm peak clear. DO NOT dilute the peptide with buffers such as PBS as the high salt and high peptide concentration can result in precipitation.

Measure the absorbance at 280 nm for each peptide, and then apply Beer's Law to get your concentration

$$A_{280} = E(1/mol\ cm)*l\ (cm)*c\ (mol/L)*dil$$

A_{280} Absorbance at 280 nm
E Extinction coefficient. Use peptide table
C concentration in molar
l is pathlength in cm. On most nanodrop units, this will be 0.1 cm
Dil Dilution factor used. For example 1/50

$$\frac{A280}{(E * l * dil)} = C$$

Peptides from Biosynth will generally be in the 50-60+ mM range when prepared this way. FS1 is limited by solubility and so tends to come in around 15 mM.

Peptide Storage

Peptides can be freeze thawed several times, but it is good to keep your working stock at -20C and avoid a largenumber of freeze thaw cycles on your -80C stocks. Peptide aliquotes of 25-100 uL are usually made and stored at -80C so they can be used up in a short time once they start getting used.

Panel Design

Useful ranges and panel design

Peptide	Range (μM)	Binding partners	Class
BIM	0.001-100	All	Activator
BID	0.01-100	All	Activator
PUMA	0.1-100	All	Sensitizer
BAD	0.1-100	BCL2/W/XL	Sensitizer
	NOTE: BCLXL dependent cells can respond in low nM range		
NOXA	1-100 uM	MCL1, BFL1	Sensitizer
HRKy	1-100 uM	BCLXL	Sensitizer
MS1	0.1-10 uM	MCL1	Sensitizer
FS1	0.1-10 uM	BFL1/BCL2A1	Sensitizer
ABT199 (venetoclax)	0.1-10	BCL2	Small molecule sensitizer
WEHI-539	0.1-10	BCLXL	Small molecule sensitizer
A-1331852	0.1-10	BCLXL	Small molecule sensitizer
A-1155463	0.1-10	BCLXL	Small molecule sensitizer
S63845	1-10	MCL1	Small molecule sensitizer

Controls

FMO Control: this control lacks the cytochrome c antibody and is important to determine the minimal amount of staining that can occur taking spillover into account.

DMSO 0.1% Control: Used to establish the upper bound of the cytochrome c staining for normalization and is also an indicator of mitochondrial health. It is advisable to have at least 2 or 3 replicates of this when possible to average them together.

High BIM / Bim curve This is used to show that the mitochondria can be caused to release their cytochrome c in a BAX/BAK dependent manner. BIM curves are useful when comparing priming across samples. Even a smaller 3-4 dose curve can get a lot done.

Profile Layout

BIM is a very potent peptide that can activate BAX and BAK as well as displace other proteins from their binding sites in anti-apoptotic proteins. 3-10 uM is enough for most suspension lines to show robust cytochrome c release. For adherent lines, up to 100 uM may be used.

PUMA shares the ability to bind all anti-apoptotic proteins with BIM, but the 20-mer version we use cannot activate BAX and BAK. A curve of PUMA reveals how much pre-bound activators must be held in the anti-apoptotic proteins, and it will released these upon displacing them.

BAD binds to BCL2, BCLXL, and BCLW. It does not bind to MCL1 or BFL1. A response from this peptide indicates a dependence on one or more of BCL2, BCLXL, for BCLW. It is usually used with other peptides like HRK to determine if BCL2 is alone or if there is BCLXL present and in use.

MS1 is an engineered peptide created at MIT to bind tightly and selectively to MCL1. A response from MS1 indicates MCL1 dependence

FS1 is also an engineered peptide from MIT that binds only to BFL1. It is the only peptide available that can positively identify BFL1 dependence.

HRKy only binds to BCLXL. A response from HRK is generally accompanied by one from BAD. Seeing BAD without HRK indicates BCL2 alone. BAD and HRK responding indicates that BCLXL is there, but it does not rule out the possibility that BCL2 is present.

Small Molecules

Small molecules can be used like peptides in BH3 profiling. Because of these only bind to BCL2, they can be useful to help determine if BCL2 dependence is also occurring with BCLXL dependence. Most BH3 mimetics are use at 0.1-10 uM which is high above their IC50 but are necessary for reasons we do not yet know. Their binding fidelity, however, remains and this they can be useful agents in profiles.

Peptide Combinations

These are for more advanced layouts, but they can determine if co-dependence is occurring in a sample. For example, is a large open reservoir of BCL2 and a large reservoir of MCL1 are present,a broad binding peptide like PUMA will bind both pools and show a result. However, BAD and MS1, each only affecting one pool, may show no result because the displaces proteins just move to the next open pool. However, if you add MS1 and BAD to the same well, you can usually get a PUMA-like response indicating that the cells are using two non-overlapping pools of anti-apoptotic proteins.

The most common combos are BAD+MS1 and HRK+MS1.

Performing BH3 Profiling using the iBH3 method

Vol in uL	With automated pipetting	By Hand 384 well	By hand, 96 well	By Hand tubes
Plate Type	Corning 4514	Corning 3575	V or U bottom	
Peptides/Digitonin/ Buffer	5	15	30	50
Cells in Buffer	5	15	30	50
10% Formalin	3	10	20	30
1.5 M Tris	3	10	20	30
10X Perm wash/ Cytoc	3	10	20	30
Total	19	60	120	190

1. **Prepare your panel / plates / tubes**

    a. **Prepare iBH3 buffer / 0.002% digitonin.** For example, for 50 mL of iBH3/digitonin, add 20 uL of 5% digitonin to 50 mL of iBH3 buffer and mix well

    b. **Add peptides / small molecules to iBH3/digitonin buffer at 2X final concentration** For example, if your highest BIM dose is 100 uM, you need to add enough BIM peptide stock to make a 200 uM solution. This will be diluted when cells are added.

    c. **Store or use reagents**. A plate of peptides can be frozen and stored at -80C for many months at a time. This allows you to build a bank of pre-cast plates and used them. The same should work with storing aliquots in tubes if you have to stay more modular or are using facs tubes.

2. **Prepare your cells**

    a. **If you are using live/dead or zombie stains**, pellet your cells and suspend them in a buffer such as PBS to perform live dead staining. When complete, spin cells down to remove excess dye

    b. **If you need to perform cell surface marking,** add your antibodies / blocking agents at this stage. Staining is typically done in PBS or PBS+FBS. Cells can be stored at 4C while staining. Typical stain times are 30-60 minutes

    c. **Suspend cells in iBH3 buffer**. Use buffer that does NOT contain digitonin. All the digitonin you need is in your 2X mix with the peptides. Spin cells down to remove PBS and antibodies. Cells are usually suspended at 1-5 million / mL. Cell lines can often be at lower density while heterogenous samples can be loaded more heavily to find small subpopulations

3. **Expose your cells**

    a. **Add an equal volume of cell suspension in iBH3 buffer to the peptide / iBH3 buffer / digitonin** plate/tubes. For tubes, flick lightly to mix. Cells will not be permeabilized by digitonin and peptides will gain access to the mitrochondria.

    b. **Wait 30-60 minutes** for exposure to complete. Times as long as 90 min can also be used. 60 min is a good default time while extremely primed cells like some hematopoietic cells can be done in the 30-45 min window.

4. **Terminate Exposure**
    a. **Add 10% formalin** as indicated in the volume table above. Allow 10 minutes for fixation to terminate further cytochrome c release. Times up to 30 minutes can be used without detriment.

5. **Terminate fixation**
    a. **Add 1.5M tris base** This will react with any remaining formalin and prevent it from causing side reactions in the staining stage. If Tris base is used, cell washing is not needed. A volume of 1.5M Tris base equal to the 10% formalin volume used is enough to neutralize the formalin in 5 minutes

6. **Add intracellular staining reagents**

    a. **Dilute cytochrome c antibody into 10X Perm Wash**. You can also add other intracellular stains at this point, but you will need to titrate them first. The amount of cytochrome c antibody used here has already been titrated for you

        i. **For FITC ASR cytochrome c**:dilute 1:80 into perm/wash
        ii. **For other RUO cytochrome c:** Dilute 1:200 in perm wash
        iii. These volumes result in the same absolute concentration of antibody

    b. **Seal plates or tubes and mix well.** Wait at least 2 hours for cytochrome c to stain. It is highly recommended to allow samples to stain at 4C overnight for a clearer resolution of controls.

7. **Flow your samples to extract cytochrome c retention data**

    a. **Identify the target cells** by using standard singlet gating and by using your markers to identify the population you are interested in.

        i. **For cell lines** you might only use the cells alone with no other marker, or use stains like Hoechst 33342 to gate on intact nuclei as opposed to those degraded by death or damage

        ii. **For primary cells** you will likely need a few markers depending on the tissue you are using. This may be as few as 2-3 such as is the case for normal T cells or CLL cells, or it may need many more as in AML or T-ALL where 6-8 antibodies may be needed to find the target population

b. Export the cytochrome c median fluorescence intensity (MFI) of your target population

i. The median is used to avoid outliers from having a strong influence on the measurement of your cytochrome c retention.

ii. You can collect the MFI in this manner for multiple populations in a sample. Just record the MFI values of each peptide/drug treatment with the corresponding population

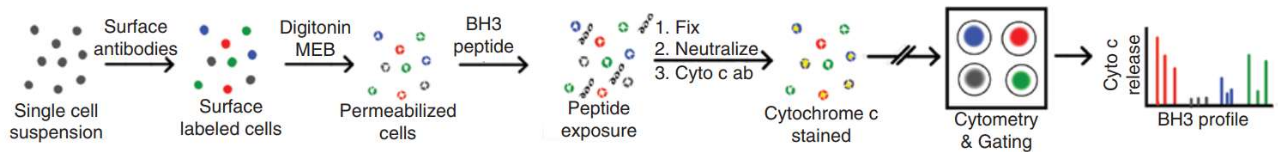


Figure 3: Schematic of the iBH3 profiling workflow

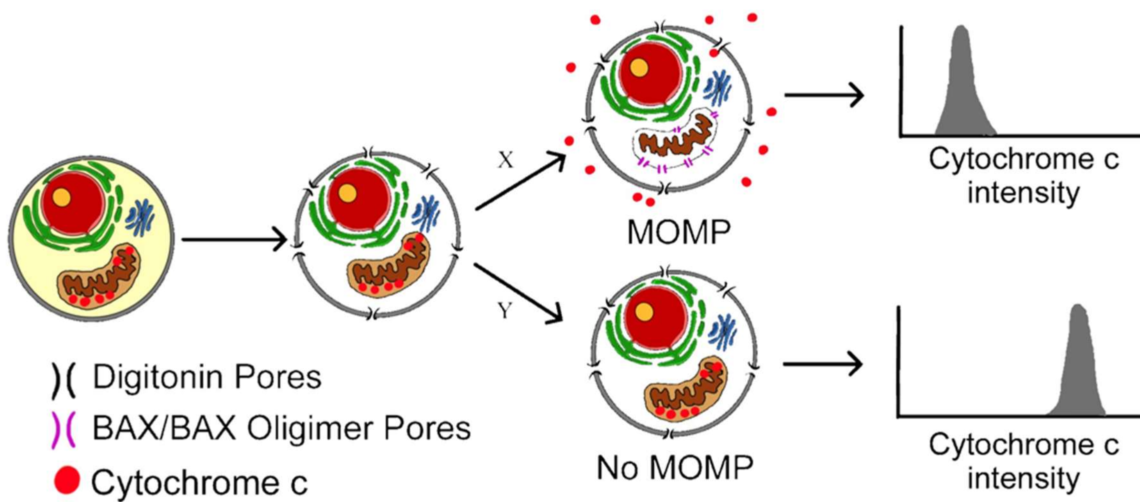


Figure 4: Correlation of cytochrome c intensiry and mitochondrial outer membrane permeabilization (MOMP) during BH3 profiling. Because the plasma membrane is permeabilized, cytochrome c leaves the cell as a whole when MOMP occurs causing a lack of staining.

Reading your BH3 profiles

1. **Normalize your MFI values**
   a. $$\% \; \boldsymbol{retained\ cytochrome\ c} = \frac{\boldsymbol{MFI_{Treatment} - MFI_{FMO}}}{\boldsymbol{MFI_{DMSO} - MFI_{FMO}}}$$
   b. **Treatments that cause MOMP will have a low retention value** because the cells will have lost their cytochrome c to the buffer. Cytochrome c will escape both the mitochondria and the cell because the plasma membrane is permeabilized by digitonin

   c. **You can invert this to get a % release value** which may be easier to read as positive responses will have larger values and inactive treatments will stay near zero. This does not change the meaning of the data but changes the presentation of it. To do this, simply use
      i. **% release = 100-%retention.**

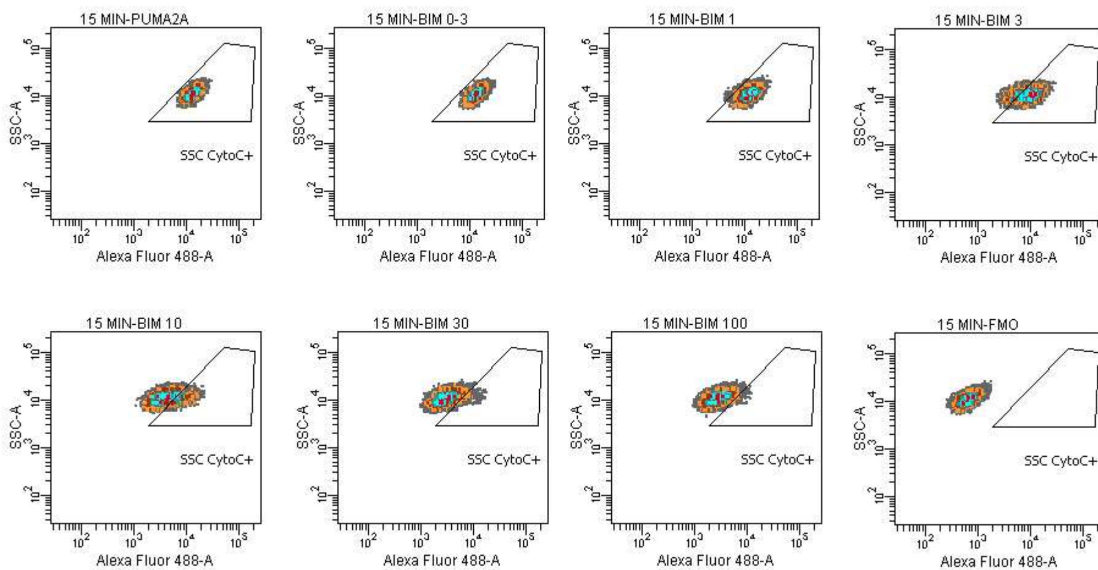


Figure 5 Loss of cytochrome c staining caused by increasing doses of the BIM peptide.

2. **Interpret your profiles**

   a. **Remember the binding chart** Keep the binding chart from the introduction handy. You will be able to use it here to determine what proteins are being used by cells for their apoptotic defense.

   b. **Start with BIM** Because BIM is an activator peptide and it binds to all anti-apoptotic proteins, it should be the most active if you used a high enough dose. **Imagine BIM as the base of a pyramid.** BIM at the same concentration compared to other peptides at the same dose is usually more potent.

i. **If BIM causes MOMP then the cells are apoptosis competent.** BIM can't release cytochrome c without BAX and/or BAK, so release of cytochrome c implies there is also enough BAX or BAK for MOMP to occur.

ii. **Cells lacking BAX or BAK wont respond to BIM**. These are fairly rare in cell lines but can happen in some normal adherent tissues. If BIM does not work, none of the other peptides should work either.

c. **Next check PUMA** If BIM caused cytochrome c release, then BAX and BAK are working. **The next question is whether the cells are pre-loaded with pro-apoptotic molecules or are the anti-apoptotic proteins vacant**

i. The Letai PUMA binds to all anti-apoptotic proteins without activation

1. **If BIM releases cytochrome c but PUMA does not**, these cells may be Class A: Unprimed. They can undergo MOMP but they don't have any pre-bound activator proteins to displace.

2. **If BIM releases cytochrome c AND PUMA releases cytochrome c** the cells are primed with some kind of pro-apoptotic molecule or protein. Because PUMA binds all apoptotic proteins, it cannot tell you which one is being used. It tells you more of a sum total of all the proteins working together.

ii. **If PUMA gives a response, you can continue to more specific peptides**

d. **Check the specific BH3 peptides to determine the apoptotic dependence more specifically**

i. **BAD is for BCL2 / BCLXL / BCLW.** The BAD peptide can bind three different proteins, so a response from BAD means you have at least one of them if not more than one present and loaded with an activator.

ii. **HRK is BCLXL Only** Response from HRK indicates a dependence on BCLXL. Because BAD binds BCLXL, an d HRK response generally appears with BAD as well. A response from BAD not not from HRK may indicate that BCL2 is being used and not BCLXL. It is not uncommon for cells to use both proteins, so molecules specific for BCL2 can be used to verify which is the case.

iii. **MS1 is for MCL1** and MCL1 is a fairly common dependency. MS1 only binds MCL1, so a response from it can be considered positive for that dependency.

iv. **FS1 is for BFL1** in a very similar way that MS1 is for MCL1. Response from FS1 is a direct indication of the cell's use of BFL1 as an anti-apoptotic.

e. **Use of small molecules as peptides**

i. **Including small molecules in the profile can be done in the same way the peptides are used.** The dependency they will indicate will depend on their specificity. For instance, ABT199 only binds BCL2, so a response from BAD and ABT199 but not HRK would indicate only BCL2 is present while a response from BAD, HRK, and ABT199 would indicate a mix of BCL2 (from Bad and ABT1 and BCLXL (from BAD and HRK)

ii. **BH3 Profiling can be combined with control cells to characterize their dependencies.** There are model cell lines that have been engineered to bear specific anti-apoptotic dependencies. Using these cell lines along with a panel of peptides to confirm their profile can be used to determine the direct mitochondrial activity of new molecules by applying a curve of the new compound and observing which of these control lines react.

1. **Cell Death Differ. 2020 Mar;27(3):999-1007.**

Useful Cell Lines

Opferman Pre-B-ALL Cell Lines

Oncotarget. 2016 Mar 8;7(10):11500-11

This is a set of 5 cell lines suitable for BH3 profiling with different dependencies on the same overall background. There are cel lines expressing human BCL2, BCLXL, MCL1, and BFL1, as well as one cell line with both BAX and BAK deleted. They are easy to grow and keep a consistent profile making them good for characterizing mitochondrial dependencies of new compounds or peptides.
BAX/BAK KO cells will not respond to any peptide, and responses of these cells to reagents can indicate that any death caused by the compound is not using intrinsic apoptosis.

MDA-MB-231

This is a human cell line that is easy to culture and can be flowed easily. While it expressed both BCLXL and MCL1, the MCL1 is usually bound by NOXA rendering unable to contribute to the binding of activator molecules that would result in MCL1 dependency. As a result, these cells are very BCLXL dependent and respond well to BAD, HRK, and small molecule BCLXL inhibitors.

DLBCL Lines

OCI-LY1

Another easily cultured BCL2 dependent line

Pfeiffer

A DLBCL line with a mostly BFL-1 dependence

Su-DHL-4 and Su-DHL-6

DHL4 is BCL2 while DHL6 is more mixed and responds to most peptides.

A number of other DLBCL lines have also been profiled: Volume 12, Issue 2, 14 August 2007, Pages 171-185

Primary Cells

Human collar PBMCs

These are usually easy to acquire. They are leftover cells from platelet donations, and they contain a good mix of normal PBMC populations. B and T cells are usually BCL2/MCL1 dependent although they do express BCLXL. Monocytes tend to be more BCLXL/MCL1 instead. These are excellent cells for practicing cell surface labeling and extracting profiles from multiple subpopulations by flow.

Chronic Lymphocytic Leukemia (CLL)

Human CLL is generally very BCL2 dependent although it can show some co-dependence on MCL1. BCL2 inhibition by small molecules causes massive apoptosis in CLL further reinforcing their BCL2 dependence. These cells do not culture without feeders so culturing can be difficult, but patients tend to have very high counts so it is not hard to bank many vials for later use.

Use CD19+ CD5+ lymphocytes as your gate to isolate these cells from mixtures.

Murine hematopoietic cells

Mouse spleen is an easy source of a large number of B-cells, and it is useful for instruction to stain B- and T-cells .

Mouse thymocytes, also easy to prep, have an excellent staining pattern of CD4 and CD8 progression with variation of profile based on single (BCL2/MCL1) or double (BCLXL) positivity for CD4 and CD8.

Example Panel Designs

General Purpose Screening

BIM 10, 3, 1, 0.3, 0.1, 0.03 uM
PUMA 100, 10, 1, 0.1 uM
BAD 100, 10, 1, 0.1 uM
MS1 10, 1 uM
FS1 10 uM
HRK 100, 10 uM
ABT199 10 uM

Combos:
MS1 10 + **BAD** 100 uM
MS1 1 + **BAD** 10 uM
MS1 10+ **HRK** 100 uM
MS1 1 + **HRk** 10 uM

FMO x2
DMSO 0.1% x4

This is a large panel, generally one of the largest I use. It covers all the single peptides and a couple of combinations as well so I will know if I have co-dependence. The panel is generally performed with technical duplicates and uses between 1 and 4 million total cells. I use it for samples I know may be diverse in their responses or when I don't know anything about an incoming sample. I routinely use this with surface markers for AML and ALL samples and any cell line that comes in.

The advantage of this panel is that you are unlikely to miss something. The downside is that it is large. This is a panel we make many plates of and freeze them so they are ready made reagents when its time to profile.

Training BIM Curve

BIM 100, 30, 10 ,3, 1, 0.3 , 0.1, 0.03, 0.01, 0.003 uM
FMO x 2
DMSO x4

This is a simple training curve that is also useful for practicing profiles, testing reagents, and building confidence. The highest doses will release cytochrome c from most cells, and the lowest doses will give little response such that when used on most cell it will produce a sigmoid dose response curve. As with drug response curves, the BIM sigmoid curves can be used to compare the EC50 of BIM from one cell type to another also though EC90 has often been more useful.

Minimal BCL2 Curve

BIM 10, 1, 0.1
PUMA 100
BAD 100, 10,1, 0.1
MS1 10
HRK 100
FMO
DMSO

This small panel can fit in one row of a 96 well plate and makes it easy to use a 12 channel pipette to makes replicates across the place for multiple samples or just for technical replicates. BIM and PUMA are important controls to prove that 1. BAX and BAK are sufficient for MOMP and 2. That the cells are primed. BIM will cause MOMP in mitochondria with enough BAX and BAK available. PUMA will respond if the cells are primed. If BIM works and PUMA does not, the cells are not primed and they shouldn't respond to the other peptides. This way you can tell lack of response caused by being unprimed vs being non-BCL2 dependent. BAD and HRK will help confirm your BCL2 dependence, and MS1 can flag MCL1 co-dependence or, if BCL2 antagonism isn't killing a sample, it can indicate if one of the primary resistance factors to BCL2 antagonists, MCL1, is present and active.