APPENDIX

Component	Vendor	Catalog Number	Notes
Mannitol	Sigma	M9647	
EDTA	Sigma	E6758	Comercial solutions can be used.
EGTA	Sigma	E3889	Comercial solutions can be used.
Succinic Acid	Sigma	S3674	
BSA (IgG Free)	VWR	100182-742	(or Gemini Protease free 700-101P)
HEPES Free acid	Sigma	H4034	
Potassium Chloride	Sigma	P9541	
Potassium Hydroxide	Sigma	P5958	
Tris base	Fisher	BP152-1	
Glycine	Fisher	BP381	
Digitonin	Sigma	D5628	Its says ~50% pure. Treat it as if all pure.
FCCP	Sigma	C2920	1-10 uM final in assays
CCCP	Sigma	C2759	Can substitute for FCCP, 1-10 uM
Alamethicin	Enzo	BML-A150-0005	Control for iBH3 and JC-1
Oligomycin	Sigma	O4876	10 ug/mL final in assays.
JC1 (Enzo 52304)	VWR	89166-014	Hydrophobic. Dissolve in DMSO. Use 100 uM JC1 working stocks in DMSO, then dilute to 1uM final to prevent precipitation.
Polaxamer 188	Fisher	MT61161RM	Also known as Pluronic F68
Glass Vials	Fisher	0337511AA	
Caps for Vial	Fisher	0337555B	

Stocks Preparation for Oligomycin, JC-1, CCCP/FCCP, 2-mercaptoethanol and Digitonin

All of these stocks are made in DMSO and stored at -20°C to -80°C.

JC-1 5 mM master stock solution. Dilute to 100 µM working solution in DMSO before adding to buffers or it may form an insoluble precipitate.

CCCP 10 mM in DMSO. FCCP and CCCP are interchangeable in this assay.

FCCP 10 mM in DMSO

Oligomycin 20 mg/mL in DMSO

Digitonin Up to 50 mg/mL in DMSO

Alamethicin Dissolve in DMSO at 5 mg/mL to make a 100X stock

Regardless of what manufacturer sheets may say about the above compounds, do not attempt to dissolve them in aqueous or ethanol solutions. Most are highly insoluble in these but will dissolve neatly into fresh DMSO.

2-mercaptoethanol (BME) Dilute to 5M in water.

Multi-well plates for plate readers and HTS loaders

The following plates have been tested as suitable in iBH3 and JC-1 assays

Plate	Catalog Number		Application
Corning Flat bottom 96 clear NBS	3641	Corning	iBH3
Corning Black 384 NBS	3575	Corning	iBH3
Corning Black Low Vol 384 NBS	4514	Corning	iBH3*
Greiner 384 well Black (Fluotrac 200)	781076	Greiner	JC-1
Corning 384 well Black	3573	Corning	JC-1
Nunc 384 well Black	12-568-54	Fisher	JC-1
Eppendorf V bottom 384 well	951040481	Eppendorf	JC-1

*Low volume U-bottom plates can only be used on cytometers with a plate loader calibrated to use them. Do not use on a standard BD HTS unit. This plate has been tested to work with the Intellicyt iQue Screener Plus to provide excellent yield with that system

NOTE REGARDING PLATE SURFACES AND COMPOSITION

The NBS coatings are used for iBH3 because they yield 2-3 times as many cells during FACS over standard polystyrene or polypropylene. Polystyrene and polypropylene generally lead to cell loss to the plate walls due to hydrophobic interaction. Non-binding surfaces (NBS) are recommended for iBH3 unless the samples are not limiting. Uncoated plates are then suitable.

NBS plates should not be used in JC-1 assays. Interference with the fluorescence has been noted, but the exact cause is not yet known. Standard polystyrene plates are recommended for JC-1 plate reader applications.

Mitochondrial Buffers

Below are the current BH3 profiling buffers. MEB is relatively gentle and works best for JC-1 assays which use long exposure times and kinetic measurements. MEB2 is best for iBH3 as it provides better cell recovery, less clumping, and a faster response to peptides for the sheerer 45-60 minute incubations used in typical iBH3 assays.

MEB2-P25 includes the addition of polaxamer 188 (Also known as Pluronic F68) which can help reduce doublets and increase the yield of cells from multiwall plates, particularly for suspension lines.

MEB For JC-1 Assays	**MW**	**[Stock] M**	**Final Conc**	**Final Vol (L)**	**Mass (g)**
150 mM Mannitol	182		0.15	0.5	13.65
10 mM HEPES-KOH pH 7.5	238.3		0.01	0.5	1.19
50 mM KCl	74.55		0.05	0.5	1.86
0.02 mM EGTA	380.35	0.5	$2x10^{-5}$	0.5	20.0 uL
0.02 mM EDTA	292.24	0.5	$2x10^{-5}$	0.5	20.0 uL
0.1 % BSA	66463	100%	0.1	0.5	0.500
5 mM Succinate	118.09		0.005	0.5	0.295
MEB2 for iBH3	**MW**	**[Stock] M**	**Final Conc**	**Final Vol (L)**	**Mass (g)**
150 mM Mannitol	182		0.15	0.5	13.65
10 mM HEPES-KOH pH 7.5	238.3		0.01	0.5	1.19
150 mM KCl	74.55		0.15	0.5	5.59
1 mM EGTA	380.35	0.5	$1x10^{-3}$	0.5	1 mL
1 mM EDTA	292.24	0.5	$1x10^{-3}$	0.5	1 mL
0.1 % BSA	66463	100%	0.1	0.5	0.500
5 mM Succinate	118.09		0.005	0.5	0.295
MEB2-P25 for iBH3	**MW**	**[Stock] M**	**Final Conc**	**Final Vol (L)**	**Mass (g)**
150 mM Mannitol	182		0.15	0.5	13.65
10 mM HEPES-KOH pH 7.5	238.3		0.01	0.5	1.19
150 mM KCl	74.55		0.15	0.5	5.59
1 mM EGTA	380.35	0.5	$1x10^{-3}$	0.5	1 mL
1 mM EDTA	292.24	0.5	$1x10^{-3}$	0.5	1 mL
0.1 % BSA	66463	100%	0.1	0.5	0.500
5 mM Succinate	118.09		0.005	0.5	0.295
Polaxamer 188	Avg 8400		2.5 g/L	0.5	1.25

MEB Assembly:
Add solids to beaker: Mannitol, HEPES, Succinic acid, BSA, KCl and allow to dissolve
Add EDTA and EGTA to solution
Adjust pH to 7.5 +/- 0.1 with KOH
Add water to final volume
Filter through 0.22 micron filter and store at 4°C. MEB and DTEB are stabile for 6 months if kept clean and stored at 4°C.

Neutralizing Buffer 'N2'
1.7 M Tris base, 1.25 M Glycine, pH 9.1

Per 100 mL:
20.59 g TRIS base (M.W. 121.11)
9.38 g Glycine (M.W. 75.07)
Add water to 90 mL, dissolve and adjust pH to 9.1
Dilute to 100 mL and sterile filter to remove trace particulates. Store at RT

NOTE ON POSITIVE CONTROLS

Alamethicin is a peptide antibiotic that can permeabilize mitochondria independent of BAX and BAK. This serves as a positive control for cytochrome C release in iBH3 and can provide a better baseline depolarization in JC-1 applications because it is irreversible unlike CCCP depolarization. 15-25 micro molar concentrations have been sufficient to induce full Cytochrome C / potential loss in all applications to date.

Intracellular Staining Buffers
10X staining buffers are described below. Tween20 can be used without washing out the detergent while Triton-X100 will require a wash. The Tween20 based buffer is the current preferred non-commercial buffer for reasons of its ease to use and ease of preparation.

A saponin based commercial concentrate, BD Perm/Wash, can be used interchangeably with the tween20 buffer.

10X Tween20 Intracellular Staining Buffer (For Cytochrome C antibody staining) CURRENT METHOD

This buffer is easy to make and filters well. It produces a staining pattern similar to saponin based buffers, and it does not need to be washed out prior to FACS analysis.

Per 50 mL combine:
1 mL Tween20
5 g BSA
Add PBS to 50 mL and dissolve completely.
Sterile filter. Store at 4°C.

10X Triton-X100 Intracellular Staining Buffer (For Cytochrome C antibody staining)
If you are working with stains or antibodies that require harsh conditions such as 0.1% Triton X100, the cytochrome c antibody can be used, but the cells must be spun down after staining to remove Triton-X100 as it will negatively impact the staining intensity of fluorophores during analysis.

Per 50 mL combine:
0.5 mL Triton-X100
5 g BSA
Add PBS to 50 mL and dissolve completely.
Sterile filter. Store at 4°C.

Peptide Stocks

Peptide Name	Sequence N-Term	Extinction Coeff. 280 nm 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}
Bmf-Y	Acetyl -HQAEVQIARKLQLIADQFHRY- Amide	1490 cm^{-1} M^{-1}
Hrk-y	Acetyl -SSAAQLTAARLKALGDELHQY- Amide	1490 cm^{-1} M^{-1}
Puma2A	Acetyl -EQWAREIGAQARRMAADLNA- Amide	5500 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.

Sequence change: wHRK has been replaced by HRKy to reduce off target effects

Suggested doses for profiling

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)	0.1-1	BCL2	Small molecule sensitizer
WEHI-539	0.1-1	BCLXL	Small molecule sensitizer
A-1331852	0.1-1	BCLXL	Small molecule sensitizer
A-1155463	0.1-1	BCLXL	Small molecule sensitizer
S63845	1-10	MCL1	Small molecule sensitizer

- A-133 and A-115 are more potent than WEHI-539. In our hands, A-133 is more potent at killing than A-115 or WEHI-539
- Do not use MS1 at concentrations greater than 10 μM or you may lose specificity. Doses higher than suggested begin to behave like PUMA
- Most peptides can handle freeze/thaw 5 times, but many of the small molecules including ABT263 have lost potency after multiple freeze thaws. Try to aliquot working stocks into single use volumes when possible.

Peptides Source and Purity

- Peptides should be 95% pure or greater. It is not generally worth the cost to get 99% pure or greater while at low purities (50-70%) you cannot be sure what the impurities are or what they may interact with
- We typically purchase our peptides from **New England Peptide**, but other vendors are just as suitable. Make sure you get an HPLC trace and mass spec for proof of purity. These documents are usually standard with peptide synthesis orders.

Peptide Stock Preparation

- Peptides should be made as TFA salts. Counter-ion exchange for sodium will result in peptides of lower solubility.
- Dissolve in DMSO. Peptides are about 20-30% salt by mass, so calculate volume accordingly.
    - Dissolving lyophilized peptide powder at 50 mg/mL will generally produce peptide stocks of 10-15 mM.
- Always verify peptide concentration by UV absorbance at 280 nm.
- Master stocks: Store at -80^{o}C.
- Working stocks: Aliquot from master, about 1-2 weeks worth (100 uL or so) Store at -20. Avoid frequent freeze-thaws.

Small Molecule Inhibitors

A number of validated small molecule inhibitors are now available from several companies. We have bought compounds from the following:

- MedChem Express
- ApexBio
- Selleckchem

The publicly available compounds we often use include: (* currently favored for use in iBH3)

ABT-737	*ABT-263	*ABT-199	
WEHI-553	A-1155463	*A-1331852	*S63845