APPENDIX

Component	Vendor	Catalog Number	Notes
Trehalose	Sigma	T9449	
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.
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 before adding to buffers

CCCP 10 mM in DMSO.

FCCP 10 mM in DMSO

Oligomycin 20 mg/mL in DMSO

Digitonin Up to 50 mg/mL in 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
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

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

DTEB is an older buffer as are T-EB or Newmeyer buffer. These buffer contain trehalose which is very expensive. Further testing showed mannitol to be a suitable replacement, and MEB is now the standard BH3 profiling buffer.

DTEB	MW	[Stock] M	Final Conc (M)	Final Vol (L)	Mass (g)
135 mM Trehalose	378.33		0.135	0.5	25.537
10 mM HEPES-KOH pH 7.5	238.3		0.01	0.5	1.192
50 mM KCl	74.55		0.05	0.5	1.864
0.02 mM EGTA	380.35	0.5	$2x10^{-5}$	0.5	20 uL
0.02 mM EDTA	292.24	0.5	$2x10^{-5}$	0.5	20 uL
0.1 % BSA	66463		0.1 %	0.5	0.500
5 mM Succinate	118.09		0.005	0.5	0.295
MEB (DTEB replacement)	**MW**	**[Stock] M**	**Final Conc**	**Final Vol (L)**	**Mass (g)**
150 mM Mannitol	182		0.15	0.5	13.650
10 mM HEPES-KOH pH 7.5	238.3		0.01	0.5	1.192
50 mM KCl	74.55		0.05	0.5	1.864
0.02 mM EGTA	380.35	0.5	$2x10^{-5}$	0.5	20.000
0.02 mM EDTA	292.24	0.5	$2x10^{-5}$	0.5	20.000
0.1 % BSA	66463	100	0.1	0.5	0.500
5 mM Succinate	118.09		0.005	0.5	0.295

DTEB / MEB Assembly:

Add solids to beaker: Trehalose / 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

Three 10X staining buffers are described below. Saponin and 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.

10X Intracellular Staining Buffer (for Cytochrome C antibody staining)
1% Saponin, 10% BSA, 20% FBS, 0.02% Sodium Azide, PBS

Per 50 mL combine:
10 mL FBS
500 mg saponin
5 g BSA
Dissolve in 50 mL PBS
Add 100uL 10% Sodium Azide (Final concentration 3mM. Saponin can contain bacterial spores so a preservative should be added)
Sterile filter. Store at 4°C.

This buffer is based off the BD 10X Perm/Wash buffer. Filtration of the saponin mixture is necessary to remove insoluble matter, and this can take several filters. Color will vary by saponin lot. If you don't want to worry about the variability of saponin preps, you can use BD PermWash (BD Biosciences Cat# 554723).

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

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	Extinction Coeff. 280 nm
hBIM	Ac-MRPEIWIAQELRRIGDEFNA-NH2	5500 cm^{-1} M^{-1}
hBID-Y	Ac-EDIIRNIARHLAQVGDSMDRY-NH2	1490 cm^{-1} M^{-1}
mBAD	Ac-LWAAQRYGRELRRMSDEFEGSFKGL-NH2	6990 cm^{-1} M^{-1}
mNoxaA	Ac-AELPPEFAAQLRKIGDKVYC-NH2	1490 cm^{-1} M^{-1}
Puma	Ac-EQWAREIGAQLRRMADDLNA-NH2	5500 cm^{-1} M^{-1}
Bmf-Y	Ac-HQAEVQIARKLQLIADQFHRY-NH2	1490 cm^{-1} M^{-1}
W-Hrk	Ac-WSSAAQLTAARLKALGDELHQ-NH2	5500 cm^{-1} M^{-1}
Puma2A	Ac-EQWAREIGAQARRMAADLNA-NH2	5500 cm^{-1} M^{-1}

Ac = Acetyl. NH2= Amide, -Y and W- designate added residues for UV absorbance measurements at C or N term respectively.

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°C.
- Working stocks: Aliquot from master, about 1-2 weeks worth (100 uL or so) Store at -20. Avoid frequent freeze-thaws.