METHOD 1: JC-1 PLATE-BASED BH3 PROFILING

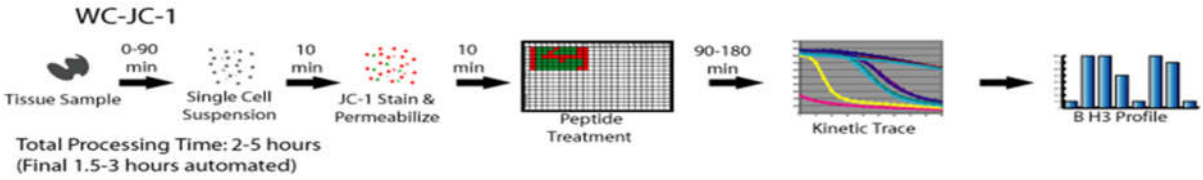

Figure 3: Overview of JC-1 Plate Procedures: Tissue samples or cell line samples are dissociated into a single cell suspension. Cells are then added to a plate containing profiling components including JC-1, digitonin, peptides, and buffer. Fluorescence at 590 nM with 545 nM excitation (JC-1 red fluorescence only) is monitored over time to produce a kinetic trace. The area under each curve is normalized to produce the final profile.

Required Materials for JC-1 Plate BH3 Profiling

1. Plate reader configured for JC-1 red fluorescence (Ex 545 +/- 10 nM,, Em 590 +/- 10 nM) Temperature control in the range of 28-32 ^{o}C is also necessary.
2. MEB Buffer (see appendix)
3. 100X Treatment Stocks (see peptide preparation in appendix) in DMSO
4. Black 384 untreated polystyrene plates
5. 50 mg/mL digitonin, 20 mg/mL oligomycin, 5M 2-mercaptoethanol in water, 100 μM JC-1 in DMSO

Preparation of Equipment

JC-1 BH-3 profiling is best suited to cell lines or homogenous samples because it provides a bulk response and not a single cell measurement. It is one of the easiest methods to use, but it requires that buffers and equipment be prepared and configured correctly.

On the equipment side, it is important to only excite the red fluorescence as excitation of the monomer can obscure the measurement. This is accomplished by exciting at 545-560 nM which is beyond the excitation and emission of the monomer. JC-1 red emission is measured at 590 +/- 10 nM. This should be easy to configure on monochrometer based units, and filter based systems should use caution in their filter selection to get the correct filter pair.

Order of Events

1. Prepare the staining solution
2. Prepare 2X stocks of treatments in staining solution
3. Array stocks in 384 well plate
    a. (optional) Freeze replicate plates for later use
4. Suspend cells in MEB
5. Add cells to 384 well plate
6. Measure JC-1 at 5 min intervals for 180 min
7. Determine and normalize area under each curve to produce final profile

Profiling Procedure

1. Calculate the amount of solution needed. Well will need 15 μL of staining solution each, and it is advisable to make 10% more per treatment than will be needed to account for any pipetting error

2. For 10 mL of staining solution, add the following in together. It is important to add the MEB last as the first three components are hydrophobic and may precipitate if added directly to aqueous media.
    a. 10 μL 20 mg/mL oligomycin
    b. 10 μL 50 mg/mL digitonin
    c. 200 μL 100 μM JC-1
    d. 20 μL 5M 2-mercaptoethanol
    e. 9760 μL MEB

3. Allow the staining solution to sit while treatments tubes are prepared. To prepare treatment tubes, add 2 μL of the 100X treatment stock for every 100 μL of 2X treatment required. Dilute to 2X using staining solution
    a. Controls

        i. Controls for Charge:

            1. 1% DMSO is the carrier for the peptide solutions and can be used as a full charge control
            2. Puma2A at the same concentration as the highest concentration of peptide used elsewhere in the profile. This often performs better than DMSO in generally having a curve more like the non-responsive peptides.

        ii. Controls for Depolarization

            1. Chemical depolarizers CCCP or FCCP: These compounds can shuttle protons through the mitochondrial membrane and cause rapid dissipation of the trans-membrane potential. This effect does not damage the membrane or cause cytochrome c release. It is possible for the electron transport chain to partially compensate resulting in a slow creep up in charge after the initial depolarization

            2. Alamethicin at 25 μM: This is a pore forming peptide that can make holes in both the plasma and mitochondrial membranes. Use of Alamethicin results in BAX/BAK independent MOMP and release of cytochrome c followed by depolarization. Because this damages the mitochondria, it is irreversible and tends to provide a more stable baseline for full depolarization. It can be used in the same manner as a peptide stock when arrayed on the plate.

4. Add 15 μL per well of each treatment to the 384 well plate. Droplets may stick to the sides of the wells, but they can be gently tapped down after all treatments have been arrayed
    a. (Optional) JC-1 plates can be sealed with a removable plate seal film and stored at -80 for later use. Plates have been stable for more than a month under these conditions.

b. (optional)To use a pre-frozen plate, thaw the plate fully at 20-25 °C, remove the sealing film, and proceed to step 5. Take care not to leave glue residue on the top of the plate.

5. Suspends cells in MEB by spinning down to remove media and resuspending at 0.67-3.33 x 10^6 cells / mL in MEB. The lower range results in 10000 cells / well and is most suitable for large adherent cells while the upper range is 50000 cells / well that can be used for very small suspension cells. A value of 1.67 x 10^6 / mL is most common and suitable for almost all samples.

6. Add 15 μL of cells in MEB to each treatment well and place in the plate reader

7. Let the plate reader perform reads every 5-10 minutes for 90-180 minutes. **IMPORTANT: Set temperature to 28-32 ºC** Failure to elevate and control the temperature can result in a lack of charging (temperature too low) to rapid depolarization and instability (temperature too high)

8. If possible, have the machine calculate the area under each curve for you. If this is not possible, it can be calculated in excel or Graphpad

9. Data Processing

   a. $Depolarization = 1 \quad \frac{AUC_{Sample} - AUC_{FCCP\ or\ Alamethicin}}{AUC_{\ DMSO\ or\ Puma2A} - AUC_{FCCP\ or\ Alamethicin}}$

   where sample is the AUC of a given treatment, Depol is the depolarized control (10 μM FCCP or 25 μM Alamethicin) and Charged is the value of the fully charged control (DMSO or PUMA2A)

   b. The AUC of the PUMA2A or DMSO wells should be 2-3X or greater than the AUC for FCCP or alamethicin. 8-10X separations are not uncommon.

   c. Graph treatment vs. depolarization to produce the final BH3 profile

   d. Hyperpolarization, where sample > charged, can result in negative values. For small hyperpolarizations, they can be considered a null response. Very large hyperpolarizations, especially when only one of several replicates of a treatment does so, could come from overloading of the well with cells or staining solution.

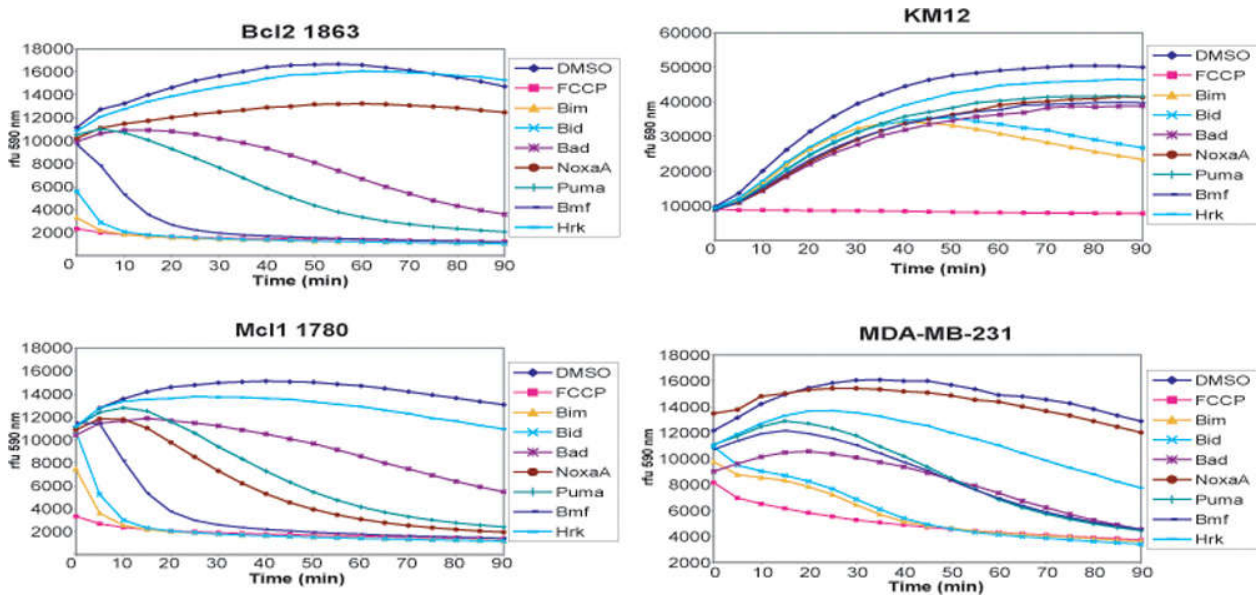

Figure 4: JC-1 Profiling Examples: From left to right and top to bottom a Class C: BCL-2 dependent line, a class A profile, A class C MCL-1 profile, and a Class C BCL-XL profile. If there were a class B profile present, it would look very much like KM12 with no lines coming away from the control curves except the control FCCP.