A Prototypical Cytotoxic / Viability Assay: Neutral Red Dye

Neutral Red Dye (NRD) is used to detect invitro survivability / viability after cell exposure to a potentially toxic agent. NRD is a weakly cationic chemical used as a supravital dye that readily penetrates live cells by non-ionic diffusion and accumulates intracellularly in lysosomes. Dead or dying cells, with damaged lysosomes, fail to bind NRD. Cytotoxicity is indicated by a concentration dependent quantifiable reduction of dye uptake detectable by light absorption. Failure of live cells to increase NRD uptake during a typical cell-cycle / reproductive interval is indicative of growth inhibition caused by the exogenous agent being tested. In theory, after an exposure to a Test Agent (TA) in cell culture, NRD uptake, can identify live cells, dead cells, and injured cells.

Assay Procedure:

Cell Culture Using Sterile Solutions and Aseptic Techniques

1) For each "new" Drug (Testing Agent) trial, thaw mycoplasma-free cells from a seed stock vial stored in the vapor-phase of a Liquid Nitrogen freezer. Cells should be thawed at 37 degrees C. and DMSO should be removed by washing the cells in "Complete Media" (CM) containing Fetal Bovine Serum (FBS). Cell purchased from a supplier such as ATCC should be grown in the recommended culture media containing the suggested % of Fetal Bovine serum (FBS) in a humidified, 5% CO2 supplemented, incubator at 37 degrees C. *After preparation, Complete Media should be stored at 4 degrees C. and used for no more than 2 weeks. (label Media bottles with a preparation date).
2) When growing cells are more that 80% confluent in their culture vessel, they can be sub-cultured (divided and seeded into new culture vessels with fresh CM). If the cells are growing in attached monolayers, they must be separated from the culture flask's surface. Trypsin is typically employed for this task however, this enzyme indiscriminately destroys cell surface proteins, many of which will require 24 hours or more to re-generate *At least once for each monolayer cell line, an attempt should be made to detach the cells with a less-traumatic non-enzymatic alternative as some weakly adherent cell lines will detach after 4-5 minutes in warm (37 degrees) sterile EDTA (NA-4)-Phosphate Buffered Saline (PBS), *0.2gm/L. A Corning T-75 flask has a working volume of 20ml of CM.
3) If trypsin-mediated cell detachment of a monolayer is needed, rinse the cells three times with pre-warmed Phosphate buffered- saline solution (without calcium or magnesium). After removing all traces of Fetal Bovine Serum (FBS), depending upon the culture vessel size, add 2-5 ml of pre-warmed Trypsin (0.05% / EDTA) solution rocking gently for about 30 seconds. Decant the trypsin solution and continue to the incubate the cells at 37 degrees for 3-7 minutes, frequently tapping gently and observing for cell detachment by phase contrast inversion microscopy. *The optimal incubation time for cell exposure to trypsin will vary by cell line. When most cells have detached, add 5-10 ml of Trypsin

Neutralizing Solution or 10-15ml of complete media (FBS contains natural alpha 1-antitrypsin) and rock the flask gently for 15 seconds. Remove cells after by pipetting up and down gently and then centrifuge the suspended cells gently in a 15ml conical tube (200 x G for 5 minutes). Decant the supernatant and resuspend the cell pellet again by gentle trituration with warmed CM. Count viable cells using a hemacytometer with 0.1ml of Trypan blue dye (0.4%) added to a 0.1ml cell sample *Since the cell sample is diluted 50%, the cell count determined by hemocytometer should be multiplied by 2 Count cells two minutes after mixing with Trypan blue *Exposure for longer than 5 minutes can damage cells and lead to dye uptake, causing an underestimation of the live cell count.

<u>Assessment of the "typical" Growth Characteristics (doubling time) under normal culture conditions with and without the addition of the solvent / vehicle to be used</u>

1) Before using any cell line in a TA-dilution / cytotoxicity experiment, establish a historical baseline evaluating the cell line's typical growth pattern and viability in complete media by NRD uptake (as per described protocol). This should be done in four separate 96-well plates incubating cells initially seeded at 2.5 x10e3 cells in 250ul of complete media. *Each plate should be sequentially removed from incubation and evaluated for NRD uptake after 1, 2, 3 and 4 days of culture.

<u>Preparation of Experimental Testing Plates</u>

2) A starting cell concentration of 1 x 10e4 / ml, will distribute 2.5 x 10e3 cells in 250ul aliquots into a 96-well plate using a multichannel pipette. *Using Phosphate Buffered Saline (PBS) in the top and bottom peripheral edge wells of the culture plate may to help compensate for evaporation during prolonged cultures.
3) During TA experimental runs, row 2 and 11 should be reserved for untreated cells to assess for systemic cell seeding errors. *A test run meets acceptance criteria for minimal seeding errors **if** the mean absorbance of retained NRD in rows 2 and 11 are within a **15% variance** from the *combined* mean of all untreated wells. Visually monitoring the wells daily is also an important way to detect any obvious seeding errors and to determine the important growth point of <u>*40-50% cell confluence*</u> (the optimal time for the addition of TA-dilutions to the experimental plate rows 3-10)
4) If a solvent, other than complete media is being used for the TA-dilution preparations, a parallel evaluation should be done for cells cultured 1-4 days in 96 well plates using the planned final concentration of the solvent *CM and solvent but without the TA added. This is done to exclude any adverse impact the vehicle alone may exert on cell growth or viability.

If a Preliminary Range-finding Experiment is Needed (follow: Cell Culture TA Exposure Protocol)

1) If a safe (non-toxic) invitro concentration starting point cannot be reasonably estimated, a range finding study should be performed. On the planned day of the study, prepare 50ml of a 1% (w/v or v/v) **TA stock solution** with the chosen solvent (hereafter referred to as the 1:1 dilution). Using additional solvent, perform six serial 10-fold dilutions of the 1:1 TA (1:10, 1;100, 1:1000 etc.). Before adding TA-solvent to the culture, further dilute each ten-fold TA-dilutions with complete culture media 2:100 (v/v) *1ml of the TA-solvent diluted in 49ml of complete media. *When 125ul of the TA-dilution with culture media is added to the 125ul of CM volume already in the plate, the final solvent concentration is 1% of the total volume and the final concentration of the 1% (1:1) Dilution in the cell well is a 0.01% solution.
2) During the pre-determined phase of exponential cell growth, 125ul of each TA-dilution should be added to the cells. After exposure to the TA for the chosen time interval (24-48 hrs.), cellular uptake of the NRD can then be determined (as per protocol). After the range finding experiment, an initial IC-50 concentration should be estimated. Smaller incremental dilutions (1:2, 1:3, 1:5 etc.) around this first estimated IC-50 concentration can then be made for subsequent experimental runs. When the dilution range is optimized, several different TA-dilution exposures should yield > 50% viability and several should yield < 50% viability, thus allowing for more accurate determination of the mid-point inhibitory concentration (IC-50 mM/L vs. LD-50 mM/Kg).

The Cell Culture TA Exposure Protocol: Adding the Testing Agent (TA) or Vehicle alone to Wells

When cells seeded in a 96-well plate at 2.5 x 10e3 per well, reach 40-50% confluence (24-72 hrs.), remove media by plate inversion over a sterile media disposal container and then gently blot the inverted plate on a sterile stack of paper towel. Immediately add 125ul of fresh complete Media (With FBS) and add 125ul of the final TA-dilutions (also made with complete media). For the **negative control** row, add complete culture media with 1% vehicle (No TA). The peripheral rows of wells act as blank wells (without cells) *Add 250ul of complete media during the TA test run exposure period and add NR-media and NR desorb during the plate processing. Since evaporation over a 5 day culture from a 96-well plate is estimated to be < 2%, using rows 1 and 12 for 2 concentrations of the positive control is typical SOP.

Positive Controls

At least once for each cell line, prepare a second parallel plate for a **positive control** trial using multiple concentrations of the toxic chemical sodium lauryl sulfate, Make Five 2-fold dilutions of SLS beginning with 20 mg/ml in complete media to determine each cell line's approximate IC-50

for SLS. After a historical IC-50 for SLS has been established, this dose of SLS can be used as a positive control on future TA trials.

Preparation of the Testing Agent (TA)

1) If the TA is fully soluble in serum-free Culture Media (s-fCM), this is the solvent of choice. Alternatives include: ETOH, DMSO, sterile water, sterile PBS. *Adjust pH to 7.
2) Optimally, before beginning invitro testing, the **chemical stability of the TA** during the planned time-in-culture (1-2 days), while mixed in the chosen solvent, diluted with complete media, and while exposed to 37 degrees C. heat should be confirmed by Liquid Chromatography-Mass spectrometry.
3) Fresh 100x concentrated TA-solution should be made for all desired TA final concentrations. These 100x solution are then diluted 2:100 with complete culture media to ensure that the solvent concentration does not exceed 1% (v/v) in the vehicle control (VC) wells and that the same vehicle concentration is in all TA test wells after dilution with complete culture media. *Prepare TA- dilutions under a red light if photodegradation is either known, or likely to occur.*

NRD preparation

1) Preparation of the **NRD Stock Solution**: 0.4gm NRD in 100ml water. Prepare fresh monthly. *Store in the dark at room temperature
2) Preparation of **NRD-Culture Media**: 1 ml NRD stock soln. + 79 ml of complete culture media containing Fetal Bovine Serum 5-10%. Filter sterilize NRD-Media with a 0.22um Millipore filter (will also remove large crystals). *Before using, Incubate over night at 37 degrees C. and centrifuge at 600 x G for 10 minutes to remove any crystalline material.

The measurement of NRD Uptake (Based on E. Borenfreund et. al. 1985)

1) After the chosen time of exposure of cells to the TA is complete, Remove the 250ul of culture media by plate inversion and blotting and add 250ul of **NRD-Culture Media** to each well.
2) Incubate the plate at 37 degrees in the Tissue culture incubator for 3 hours.
3) Decant wells again by plate inversion /wrist snap and blotting on paper towel and add 250ul of **Wash / Fix solution** (0.5% formaldehyde (v/v), in 1% calcium chloride made in water)
4) After 2 minutes, decant and add 100ul of **Neutral Red Desorb solution** to all wells including the blank wells. (Desorb solution: 1ml of Glacial acetic acid, 50ml water, 49 ml Ethanol) ***Prepared no longer than 1 hour prior to use**.
5) Shake on Microplate shaker for 20 minutes at room temp.
6) Using a microplate Colorimetric reader, evaluate NRD absorptions at 540-550 nm wavelength

***Other viability or cytotoxicity indicators tests are built on the design of the NRD protocol**