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SOP –UNITS-2.0

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RESAZURIN VIABILITY ASSAY TO DETERMINE THE HALF MAXIMAL INHIBITORY CONCENTRATION (IC_{50}) OF CANCER CELLS

Purpose

The purpose of UniTs SOP 2.0 is to describe how procedures for the measurement of cell viability by using resazurin protocol. Dosage dependent drug response can be used to determine the half maximal inhibitory concentration (IC50) of cancer cells in response to pharmacological treatment.

Scope

This SOP will be applied to cancer cell lines for measuring cell viability and testing for cytotoxic effects of drug treatments.

1. Cell culture media, reagents and solutions

- H1299 cell lines (ATCC)
- RPMI 1640 Medium (Euroclone cat.no. ECB2000)
- Penicillin/Streptomycin 100X (Euroclone cat. no. ECB3001D)
- L-glutamine 100X (Euroclone cat. no. ECB3004D)
- Fetal bovine serum (FBS) (Opticlone cat.no. ECS0183L)
- PBS-1x (Life Technologies, cat. no. 14190-094)
- Resazurin sodium salt (Sigma-Aldrich cat. no. R7017)

2. Equipment

- Cell culture incubator set at 5% CO_2, 37 °C
- Pipette aid, serological pipettes (Euroclone, cat. no. EPS05N; EPS10N)
- 48 well plate (Euroclone cat.no. 3048)
- 96 well plate (Euroclone cat.no.3096)
- Spectraplate 384 TC+LID (Perkinelmer cat. no.6007650)
- 50µl core tip stacked sterile (Hamilton cat.no. 235947)
- Standard Tube Dispensing Cassette, Identical (Steinle ST-0015)
- Synergy H1 hybrid Reader Biotek
- Multidrop™ Combi Reagent Dispenser (Thermo scientific)
- ELx405 Select Deep Well Washer (BioTek Instruments) – aspirator vacuum pump
- Multipette® plus (eppendorf)
- Software for analysis GraphPad Prism 8

3. Reagent setup

- Dissolve high purity resazurin in 1x PBS (pH 7.4) to a final concentration of 35 mg/ml (stock solution: 3.5 g for 100 ml). Filter the resazurin solution through a 0.2 µm pore-size filter into a sterile falcon tube. Store light protected at -20°C.
- Prepare the resazurin working solution by diluting the stock solution in 1x PBS (pH 7.4) to a final concentration of 0.35 mg/ml (1:100 of stock solution)
- Store the resazurin working solution protected from light at 4°C for short term use or at -20°C for long term storage.
- Prepare complete medium: RPMI 1640 medium, FBS 10%, Penicillin/Streptomycin 1x, L-glutamine 1x
- Prepare stock solution for drugs to be tested. Typically, the concentrations range between 10mM and 50mM.

4. Procedure

4.1 Preparation of reagents for IC50 Test

- Thaw resazurin working solution (if kept frozen) and warm it to 37°C to ensure that Resazurin completely dissolves. Keep at 4°C until use.
- Generate serial dilution of drugs in DMSO to be tested. The final concentration of DMSO in cultivation medium should not exceed 0,2%. To perform an IC_{50} curve use at least 8 different concentrations of the drug of interest, prepared as serial dilutions. Table 1 indicates the dilution range of drugs used in H1299 IC50 measurements.

Table 1. Range of drug concentration for different therapeutic compounds used to determine IC50 values of H1299 cells.

Name of drug	Diluent	Concentration range	IC50 of H1299 cells
VE821	DMSO, final concentration 0,2%	0.78-100 µM	35 µM
VE822	DMSO, final concentration 0,2%	0.78-100 µM	6.2 µM
Ceralasertib	DMSO, final concentration 0,2%	0.78-100 µM	7.4 µM
Doxorubicin	DMSO, final concentration 0,2%	7-10000 µM	0.25 nM
Pidnarulex	DMSO, final concentration 0,2%	0.1-4000 nM	2.4 µM
Piridostatin	DMSO, final concentration 0,2%	3.2-1000 µM	200 µM
Captothecin	DMSO, final concentration 0,2%	0.01-20 µM	1.8 µM

4.2 IC50 test in 48 well plate

- Plate 9 x 10^3 H1299 cells in in 200 µl of complete medium per well of a 48 well plate. Cell numbers indicated in SOP-UNITS-1 refer to adherent H1299 cell. For any other cell type, cell number titration is recommended to determine the optimal cell seeding density.
- Incubate the cells overnight at 37°C.
- Remove the medium from the cells using a p200 pipette and add 200 ml of serial dilution of the drug of interest. Make sure all the wells contain the same volume of medium. For each concentration a technical triplicate should be performed. At least 3 wells should be reserved as blank-controls (medium without cells) in the downstream resazurin assay.
- Grow cells for 72 hrs in a cell culture incubator.
- Prewarm resazurin working solution (0,35mg/ml) at 37°C and dilute 1:10 in prewarmed complete medium to obtain a final concentration of 0,035mg/ml.

- Carefully remove the medium from cells using a p200 pipette and add 100ml resazurin solution on top of the cells.
- For the blank controls, remove medium and add resazurin solution.
- Incubate the plate with H1299 cells for 3 hours in a standard cell culture incubator. For any other cell type incubation time needs to be optimized and can be in the range of 1-6 hours
- Measure the relative fluorescent units (RFU) using a plate reader. Excitation wavelength = 560 nm, Emission wavelength = 590 nm.

4.3 IC50 test in 96 well plates

- Plate 3 x 10^3 H1299 cells in 100 µl of complete medium per well of a 96 well plate. Cell numbers indicated in SOP-UNITS-1 refer to adherent H1299 cell. For any other cell type, cell number titration is recommended to determine the optimal cell seeding density.
- Incubate the cells overnight at 37°C.
- Remove the medium from the cells using a p200 pipette and add 100µl of serial dilution of the drug of interest. Make sure all the wells contain the same volume of medium. For each concentration a technical triplicate should be performed. At least 3 wells should be reserved as blank-controls (medium without cells) in the downstream resazurin assay. Grow cells for 72 hrs in a cell culture incubator.
- Prewarm resazurin working solution (0,35mg/ml) at 37°C and dilute 1:10 in prewarmed complete medium to obtain a final concentration 0,035mg/ml (resazurin solution)
- Carefully remove the medium from cells using a p200 pipette and add 100ul resazurin solution on top of the cells.
- For the blank controls, remove medium and add resazurin solution.
- Incubate the plate with H1299 cells for 3 hours in standard cell culture incubator. For any other cell type incubation time needs to be optimized and can be in the range of 1-6 hours
- Measure the relative fluorescent units (RFU) using a plate reader. Exitation wavelenght = 560 nm, Emission wavelength = 590 nm.

4.4 Procedure for 384 well plate

- Plate 500 H1299 cells in 40 µl of complete medium per well of a 384 well plate using a Multidrop™ Combi Reagent Dispenser. Cell numbers indicated in SOP-UNITS-1 refer to adherent H1299 cell. For any other cell type, cell number titration is recommended to determine the optimal cell seeding density.
- Incubate the cells overnight at 37°C.
- Prepare serial drug dilution. To perform an IC_{50} curve use at least 8 different concentrations of the drug need to be included into the assay. Stock solutions of used drugs will be diluted in complete medium containing 1% DMSO. For IC_{50} curve measurements in 384 well plates, 10 µl of diluted drugs will be directly added to the 40 µl cell culture medium of cultivated cells. Thus, the serial dilutions of drugs need to prepared at 5x concentration. Adding the 5x concentrated drug to the cell culture medium will result a 1x final concentration (see below) in complete medium containing 0,2% DMSO.
- Use a Multipette® plus pipetting aid to carefully add 10µl of the interested drug (5x concentrated) to the cultivated cells (in 40 µl complete medium), resulting a final volume of 50 µl.
- H1299 cells will be treated for desired period of time (72 hr) in a cell culture incubator.
- Cell culture medium is removed using an aspirator vacuum pump using ELx405 Select Deep Well Washer. In this step 10 µl of RPMI complete medium should remain in wells to avoid a loss of H1299 cells during this process. This remaining amount of complete medium should be considered when preparing the resazurin solution used for the viability readout.
- Prewarm resazurin working solution (0,35mg/ml) at 37°C and dilute 1:8 in prewarmed complete medium to obtain a final concentration Of 0,042mg/ml (resazurin solution).
- Incubate H1299 cells for 3 hours in a standard cell culture incubator. For any other cell type incubation time needs to be optimized and can be in the range of 1-6 hours.
- Measure the relative fluorescent units (RFU) using a plate reader Envision 2104 Multilabel Reader (Perkin Elmer). Exitation wavelenght = 560 nm, Emission wavelength = 590 nm.

5. Data analysis using the Graph Pad Prism 8 software

To obtain IC_{50} curves the Graph Pad Prism 8 software is used (See *Figure 1*). First, the average value of all technical replicates will be determined. Subsequently, the average of biological replicates will be determined.

Obtained values of blank samples will be subtracted from values obtained from wells containing treated or untreated cells. To obtain an IC_{50} curve from obtained data the following steps need to be performed. From the Welcome dialog in Graph Pad Prism 8, choose the XY tab, drop the list of sample data sets and choose "RIA or ELISA".

Note that the X values are logarithms of concentration. Prism offers built-in equations designed to handle X values as either concentration or log(concentration). Be sure you select the correct equation when performing nonlinear regression. Drug concentration values can be converted to log(concentration) values using Prism's Transform analysis.

Also note that this sample data set includes unknown values. These are Y values without corresponding X values. Prism can interpolate these X values. Click Analyze and then Nonlinear regression. On the Nonlinear regression dialog, open the "Dose-Response -- Inhibition" family of equations, and choose "log(inhibitor) vs. response -- Variable slope (four parameters)". Click OK and view the results.

Drug range for each drug used

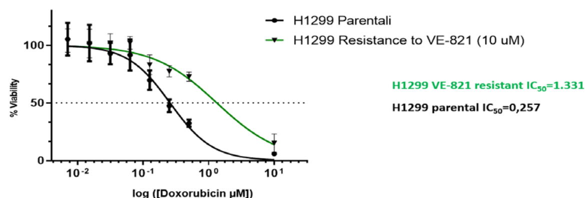

Figure 1. IC_{50} curves for Doxorubicin treated parental H1299 cells and H1299 resistant to the ATR inhibitor VE821. The percentage of viability is measured by Resazurin assay to a DMSO control.

n = 3 biological replicates per concentration; for each biological replicate 3 technical replicates were obtained; error bars indicate standard deviation.

6. Applicable references

Friedman B et al. Adenosine A2A receptor signalling promotes FoxO associated autophagy in chondrocytes. Sci Rep 11:968 (2021).

NOTES