User Guide

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Using the xCELLigence RTCA SP Instrument to Perform Cytotoxicity Assays

Cytotoxicity assay instructions

Overview

The Agilent xCELLigence real-time cell analysis (RTCA) single plate (SP) instrument enables label-free, noninvasive, real-time monitoring of cell proliferation, cell size/morphology, and cell-substrate attachment quality. What differentiates the SP model from others is its use of a single 96-well plate. The SP plate station is placed in a standard CO_2 incubator. It is powered and controlled using a cable connected to an analyzer and control unit (laptop), which are housed outside the incubator (Figure 1).

The user-friendly RTCA software allows for real-time data display and analysis. The SP instrument uses electronic microplates (Agilent E-Plates) in a 96-well format that has a glass or PET (polyethylene teraphthalate) well bottom. The PET plates are also available in a format (Agilent E-Plate VIEW 96) with electrodes absent from a small center region of the well, facilitating visual inspection under a microscope. All of these E-Plates can be used with E-Plate Inserts, which enable coculture experiments.

Figure 1. Agilent xCELLigence RTCA SP instrument. This consists of the plate station (housed inside a tissue culture incubator), analyzer, and laptop control unit (both housed outside the incubator).

Introduction

These instructions are for a basic cytotoxicity assay using an xCELLigence RTCA SP instrument to monitor the kinetics of long-term cellular responses. The process has been optimized for the A549 human nonsmall cell lung cancer cell line. Assay conditions may require further optimization if different cell lines or cytotoxic compounds are used.

Reagents, materials, and equipment

Reagents and materials

Item	Manufacturer	Description
A549 Cells	ATCC	Human lung epithelioid carcinoma
PBS	Hyclone	1x DPBS (-Ca, -Mg, -Phenol Red)
Trypsin	Gibco	0.05% Trypsin-EDTA (1x), Phenol Red
Pen-Strep	Cellgro Mediatech	10,000 IU penicillin, 10,000 µg/mL streptomycin
FBS	Hyclone	Fetal bovine serum characterized
F-12K	ATCC	Kaighn's modification of Ham's F-12 medium
Filter Unit	Nalgene	Pore size: 0.20 µm; PES membrane: 90 mm
Paclitaxel	Tocris	Taxol
Hydroxyurea	Sigma	Hydroxyurea
Compound Plate	Greiner Bio-One	96-well polypropylene plate, V bottom
E-Plate 96	Agilent Technologies	96-well electronic microplate

Equipment

Item	Part Number
xCELLigence RTCA SP – bundle (complete system)	380601030
xCELLigence RTCA analyzer	5228972001
xCELLigence RTCA SP station	5229057001
xCELLigence RTCA control unit (laptop with pre-installed software)	5454417001

Process overview

A basic cytotoxicity assay may take 4 to 5 days to complete. Cells are seeded on day 1, challenged with cytotoxic agents on day 2, and their response is continuously monitored for the next 72 to 96 hours.

Workflow summary

Cells should be ~80% confluent at the time for seeding, so passage them accordingly.

Day 1

1. Reagent warm up.
2. E-Plate 96 preparation and background measurement.
3. Cell preparation.
4. Cell seeding in E-Plate 96.
5. Equilibration.
6. Incubation and overnight monitoring of cell attachment and proliferation.

Day 2

1. Compound preparation.
2. Compound addition.
3. Continuous monitoring of cellular response.

Plate layout

In this example process that tests paclitaxel, hydroxyurea, and a negative control, only 32 wells of 96-well plate are used. However, media or PBS (~50 µL) should be added to all other wells on this plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A			Paclitaxel 1000 nM									
B			Paclitaxel 100 nM									
C			Paclitaxel 10 nM									
D			Paclitaxel 1 nM									
E			Paclitaxel 0.1 nM									
F			Hydroxyurea 2 mM									
G			Hydroxyurea 0.4 mM									
H			No compound control									

Software setup

Step	Sweeps	Intervals	Unit	Comments
1	1	1	Minute	Background reading
2	31	1	Hour	Overnight monitoring
3	72	1	Hour	Compound response

Detailed instructions

Day 1

1. Warm up growth media and trypsin in a 37 °C water bath 30 minutes before the start of the experiment (30 minutes).
2. E-Plate 96 preparation and background measurement (15 minutes).
   a. Inside a tissue-culture hood, remove the E-Plate 96 from its packaging.
   b. Using a pipette, carefully transfer 50 µL of prewarmed media to each well.
   c. Place the E-Plate 96 into the RTCA SP station. Open the RTCA software and start Step 1 (1 minute and one sweep) to perform background measurement. Remove the E-Plate 96 from the station and take it back to the tissue culture hood for cell seeding.
3. Cell preparation (5 minutes).

   Critical: Like any other cell-based assay, the ultimate success of this cytotoxicity assay using an xCELLigence system depends on the cell quality and how cells are handled. Following the steps described here is imperative for ensuring reliable and reproducible results. It is also important to note the passage number of the cells because, for some cell types, the degree of cytotoxicity can change with increasing passage number.

   a. Cells should be passaged the day before the experiment so that they are 60 to 80% confluent.
   b. Remove serum-containing media from the flask and gently rinse the cell monolayer once with PBS.
   c. Trypsinize cells by adding 3 mL of 0.05% Trypsin/EDTA solution per T225 flask and leave the flask at room temperature or in a 37 °C incubator for 1 to 5 minutes.

      Critical: Observe the cells under a microscope intermittently during trypsinization to check when they become detached. Do not over trypsinize the cells as it can be toxic.

   d. Stop trypsinization by adding serum-containing media at a volumetric ratio of 9:1.
   e. Count the cells under a microscope using a hemocytometer, and adjust the concentration of the cell suspension. For the A549 cells used in this experiment, the concentration should be 5,000 cells/100 µL.
4. Cell addition to E-Plate 96 (10 minutes).
   a. Add 100 µL of cell suspension to each 32-set of wells in the E-Plate 96.
5. E-Plate 96 equilibration at room temperature (30 minutes).
   a. Leave the E-Plate 96 in the hood at room temperature for 30 to 60 minutes to allow the cells to settle to the bottom of the well.

      Critical: Failure to perform this step can result in large well-to-well variation. This is because immediate warming to 37 °C can cause convection currents to form within the well. These currents can push cells to the well perimeter, resulting in an uneven distribution of cells on the impedance electrodes.

6. E-Plate incubation at 37 °C in a CO_2 incubator (16 to 24 hours).
    a. Transfer the E-Plate 96 to the RTCA SP station inside a 37 °C incubator, and incubate for 16 to 24 hours to allow cell attachment and proliferation.

       Critical: It is important to use an incubator with high humidity (preferably >90%) to minimize evaporation of media, especially for the wells along the plate perimeter.

    b. Start Step 2 of the RTCA program, monitoring impedance for 30 hours with readings taken every hour.

       Critical: We recommend setting the reading time for longer than the expected experiment time so that, if there is a delay, no time points will be missed.

Day 2

1. Preparation of compound dilutions (30 minutes).
    a. Compound stocks should be freshly prepared in an appropriate solvent. A 10 mM stock concentration is generally recommended. Aliquots should be made and stored per manufacturer recommendations. Alternatively, thaw previously made compound stocks in a tissue culture hood or in a 37 °C water bath.

       Critical: For compounds that are not stable, it is important to make fresh stocks for the assay.

    b. Make appropriate dilutions of compounds. The final concentration on each dilution should be four times the concentration intended for the assay. For example, the concentrations for paclitaxel on this experiment should be 4000, 400, 40, 4, and 0.4 nM.

       Critical: The final concentration of solvents such as DMSO should be kept to a minimum (ideally, below 0.5%) to avoid undesirable cellular responses. When evaluating serial dilutions of a compound, it is important to maintain the same solvent concentration in each dose.

2. Compound addition (10 minutes).
    a. Stop Step 2 of the RTCA program.
    b. Start Step 3, take one reading, then pause the experiment (this first reading in Step 3 will be used as the normalization time point).
    c. Remove the E-Plate 96 from the RTCA SP station.
    d. Add 50 µL of the prepared compound dilutions to the E-Plate wells for a total of 200 µL, and return the plate to the RTCA SP station.
3. Response monitoring (48 to 72 hours).
    a. Resume Step 3 of the RTCA program, monitoring every hour for 72 hours.

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