User Guide

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Using the xCELLigence RTCA SP Instrument to Perform GPCR Assays

GPCR assay instructions

Overview

The Agilent xCELLigence real-time cell analysis (RTCA) single plate (SP) system enables label-free, noninvasive, real-time monitoring of cell proliferation, cell size/morphology, and cell-substrate attachment quality. The use of a single 96-well plate differentiates the SP from other models.

The SP instrument (plate station) is placed in a standard CO_2 incubator. It is powered and controlled using a cable connected to an analyzer and a control unit (laptop), which are housed outside the incubator (Figure 1). The user-friendly RTCA software enables a real-time interface with the instrument, and includes instant data display and analysis functions. The SP instrument can use electronic microplates (Agilent E-Plates) with either glass or PET (polyethylene terephthalate) well bottoms in 96-well formats. Both E-Plates (PET and glass) are also available in a format where electrodes are absent from a small region in the center of the wells (Agilent E-Plate View 96), facilitating visual inspection under a microscope (bright-field, fluorescence). All E-Plates described here 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), the analyzer, and the laptop control unit (both housed outside the incubator).

Introduction

These instructions are for a basic GPCR assay using the xCELLigence RTCA SP instrument to monitor cellular responses to receptor stimulation. The process has been optimized using the HeLa human cervical cancer cell line, and assay conditions may require further optimization if different cell lines or GPCR agonists are used.

Reagents, materials, and equipment

Reagents and materials

Item	Manufacturer	Description	Product or Part Number
HeLa Cells	ATCC	Human cervical adenocarcinoma	CCL-2
PBS	Hyclone	1x DPBS (-Ca, -Mg, -Phenol Red)	SH30028.02
Trypsin	Gibco	0.05% Trypsin-Edta (1X), Phenol Red	25300
Pen-Strep	Cellgro Mediatech	10,000 IU penicillin, 10,000 mg/mL streptomycin	30-002-CI
FBS	Hyclone	Fetal bovine serum characterized	SH30071.03
EMEM	ATCC	Eagle's Minimum Essential Medium	30-2003
Filter Unit	Nalgene	Pore size: 0.20 mm; PES membrane: 90 mm	569-0020
Histamine	Sigma-Aldrich	Histamine dihydrochloride	53300
Compound Plate	Greiner	96-well polypropylene plate, V bottom	651201
E-Plate 96	Agilent Technologies	96-well electronic microplate	5469830001

Equipment

Item	Part Number
Agilent xCELLigence RTCA SP – bundle (complete system)	380601030
Separate Components	
xCELLigence RTCA analyzer	5228972001
xCELLigence RTCA SP station	5229057001
xCELLigence RTCA control unit	5454417001

Process overview

This two-day assay has been optimized for monitoring the rapid cellular response to the receptor stimulating compound histamine.

Workflow summary

At the time that cells are collected for seeding into the E-Plate, they should be ~80% confluent; passage them accordingly.

Day 1

1. Warm up reagents.
2. E-Plate 96 preparation and background measurement.
3. Cell preparation.
4. Cell seeding in the 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 looking at histamine stimulation, only 32 wells of the 96-well plate are used.

	1	2	3	4	5	6	7	8	9	10	11	12
A								Histamine 100 µM				
B								Histamine 10 µM				
C								Histamine 1 µM				
D								Histamine 100 nM				
E								Histamine 10 nM				
F								Histamine 1 nM				
G								Histamine 0.1 nM				
H								No compound control				

Software setup

Step	Sweeps	Intervals	Unit	Comments
1	1	1	Minute	Background reading
2	31	1	Hour	Overnight monitoring
3	237	15	Seconds	Compound response

Detailed instructions

Day 1

1. Warm 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. The reverse pipetting technique is effective for preventing bubble formation and ensuring consistent volume dispensation.
    c. Place the E-Plate 96 into the RTCA plate station. Open the RTCA software, and enter the experiment and plate layout information. Start Step 1 (one sweep) to perform background measurement. Remove the E-Plate 96 from the station and place the plate back in a tissue culture hood for cell seeding.

3. Cell preparation (5 minutes).

   Critical: Like any other cell-based assay, the ultimate success of this GPCR assay using the xCELLigence system depends on cell quality and how cells are handled. Following the steps described here is imperative for ensuring reliable and reproducible results. Noting the passage number of the cells is also important because, for some cell types, the intensity of the GPCR response 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 cell monolayer once with PBS.

   c. Trypsinize cells by adding 3 mL of 0.05% Trypsin/EDTA solution per T25 flask and leave the flask at room temperature or in a 37 °C incubator for 1 to 5 minutes.

      Critical: It is important to observe the cells under a microscope intermittently during trypsinization to check when they become detached. Do not overtrypsinize the cells, as this can be toxic.

   d. Stop trypsinization by adding serum-containing media at a volumetric ratio of 9:1 (27 mL).

   e. Count the cells, and adjust the concentration of the cell suspension. For the HeLa cells being used in this example experiment, the concentration should be 12,000 cells/100 µL.

4. Cell addition to E-Plate 96 (10 minutes).

   Add 100 µL of cell suspension to each well of the E-Plate 96.

5. E-Plate equilibration at room temperature (30 minutes).

   Leave the E-Plate 96 in the hood at room temperature for 30 to 60 minutes after cell addition. Allow the cells to settle to the bottom of the well evenly.

   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 plate 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 perimeter of the plate).

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

      Critical: It is important to set the reading time longer than the expected experiment time so that, if any delays occur, 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. In this process, stock solutions are produced 10x and dispensed 17 µL to 150 µL. Therefore, in the final volume (167 µL), compound concentration will be very close to 1x.

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

    b. Make appropriate dilutions of compounds to be tested.

       Critical: To avoid impedance changes caused by a vehicle such as DMSO, its concentration should be kept to a minimum. When evaluating serial dilutions of a compound, it is important to maintain the same dilutions in the control samples containing vehicle only.

2. Compound addition (10 minutes).
    a. Pause Step 2 and abort the remaining sweeps.
    b. Start Step 3, take one reading, and 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 station.
    d. Working inside the hood, add 17 µL of the prepared compound dilutions to the E-Plate wells, and return the plate to the RTCA station.

3. Response monitoring (1 to 48 hours).
    a. Resume Step 3 of the program, monitoring every 15 seconds for 10 to 60 minutes.
    b. **Optional:** Step 4 can be added to the schedule with the "auto" box checked. Cells can be monitored every 15 minutes for 24 hours or longer if needed.

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