User Guide

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Reverse Transfection of siRNA in E-Plates

Introduction

RNAi has proven invaluable for understanding the functions of genes in biology and disease. This guide is for a basic assay using an Agilent xCELLigence RTCA instrument to monitor the effects of siRNA knockdown. It has been optimized using the A549 human nonsmall cell lung cancer cell line, but assay conditions may require further optimization if different cell lines are used.

Reagents, materials, and equipment

Reagents and materials

Item	Manufacturer	Product or Part Number
A549 Cells	ATCC	CCL-185
Opti-MEM I Reduced Serum Medium	Gibco by ThermoFisher	31985062
Lipofectamine RNAiMAX Transfection Reagent	Invitrogen by ThermoFisher	13778075
E-Plate 16	Agilent	5469830001

Equipment

Item	Part Number
xCELLigence RTCA DP bundle	380601050

Overview

Though this reverse transfection procedure only takes ~2 hours, cells are then monitored for an extended period of time (for example, 72 hours) with or without drug treatment.

Workflow summary

When 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 16 preparation and background measurement.
3. Cell preparation.
4. Cell seeding in E-Plate.
5. Equilibration.
6. Incubation and overnight monitoring of cell attachment and proliferation.

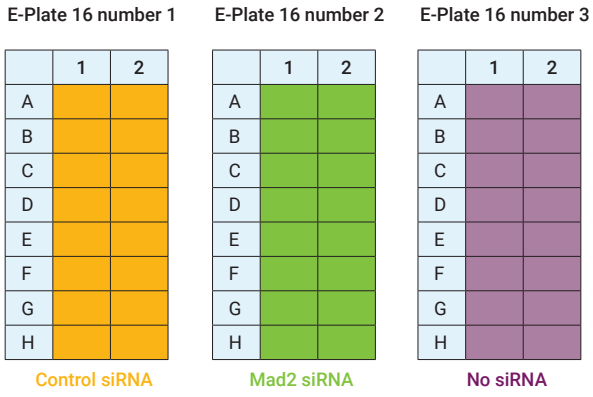
Day 2

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

Plate layout

This example uses an xCELLigence RTCA DP instrument and three separate E-Plate 16 microplates: one for each siRNA or control being analyzed. However, the process can be readily adapted for use with any of the xCELLigence instruments (RTCA eSight, S16, SP, MP, HT) and their respective E-Plates (E-Plate 96, E-Plate 384). Depending on the type/size of wells being used, volumes may need to be adjusted.

E-Plate 16 layout



Software setup

Step	Sweeps	Intervals	Unit	Comments
1	1	1	Minute	Background reading
2	100	15	Minutes	Overnight monitoring
3	300	15	Minutes	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 16 preparation and background measurement (15 minutes)
    a. Inside a tissue-culture hood, remove the E-Plate 16 from its packaging.
    b. In an Eppendorf tube, dilute 6 µL of 10 µM RNAi into 194 µL of Opti-MEM (mixture A).
    c. In another Eppendorf tube, dilute 4 µL of Lipofectmine RNAiMAX into 196 µL of Opti-MEM (mixture B).
    d. Combine the entire volumes of mixture A and mixture B. Mix gently and incubate for 20 minutes at room temperature.
    e. Add 20 µL of the A+B mixture to each well of the E-Plate 16.
    f. Place the E-Plate 16 into the Agilent xCELLigence RTCA DP instrument inside an incubator. Open the RTCA software and begin Step 1 (1 minute and one sweep) to perform a background measurement. Remove the E-Plate 16 from the RTCA DP instrument 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 reverse transfection assay (using an xCELLigence RTCA instrument) depends on the quality of the cells and how they are handled. Following the steps 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 intensity of the siRNA response can change with increasing passage number.

    a. Remove serum-containing media from the flask and gently rinse the cell monolayer once with phosphate-buffered saline (PBS).
    b. Trypsinize cells by adding 1 mL of 0.05% Trypsin/EDTA solution per T75 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 over trypsinize the cells, as this can be toxic.

    c. Stop trypsinization by adding serum-containing media at a volumetric ratio of 9:1.
    d. Count the cells under a microscope using a hemocytometer, and adjust the concentration of the cell suspension using complete growth medium without antibiotics. For the A549 cells being used in this example experiment, the concentration should be 5,000 cells/100 µL.
4. Cell addition to E-Plate 16 (10 minutes)
    a. Add 100 µL of cell suspension to each well of the E-Plate 16; these wells should already contain 20 µL of the A+B mixture.

5. E-Plate equilibration at room temperature (30 minutes)
    a. Leave the E-Plate 16 in the hood at room temperature for 30 to 60 minutes after cell addition 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, and 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 16 to the RTCA DP instrument inside a 37 °C incubator.
    b. Start Step 2 of the RTCA program, monitoring impedance overnight with readings every 15 minutes. This step monitors cell adhesion and proliferation.

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

       Critical: It is important to set the reading time for longer than the expected experimental time so that if any delays occur, no time points will be missed.

Day 2

1. Preparation of compound dilutions (30 minutes)
    a. This step is only applicable if, in addition to the siRNAs added on day 1, the effects of drugs are going to be evaluated. 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 a fresh stock for the assay.
    b. Make appropriate dilutions of compounds to be tested.

       Critical: To avoid cellular responses to vehicles such as DMSO, the vehicle concentration should be kept to a minimum (ideally, below 0.1% for DMSO). It is important to maintain equal concentrations of the vehicle for every sample, even when evaluating serial dilutions of a compound.
2. Compound addition (10 minutes)
    a. To minimize the artifacts seen during compound addition, the diluted compounds (prepared in the previous step) should be incubated in the 37 °C CO_2 incubator for 30 minutes to facilitate temperature and CO_2 equilibration.
    b. Stop Step 2 of the RTCA program.
    c. Start Step 3, take one reading, then pause the experiment (this first reading will be used as the normalization time point).
    d. Remove the E-Plate 16 from the RTCA DP instrument.
    e. Add 13.3 µL of the prewarmed compound dilutions to each well of the E-Plate, and quickly return the E-Plate to the RTCA DP instrument.

3. Response monitoring (72 hours)
    a. Resume Step 3 of the program, monitoring every 15 minutes for 72 hours.

Sample results

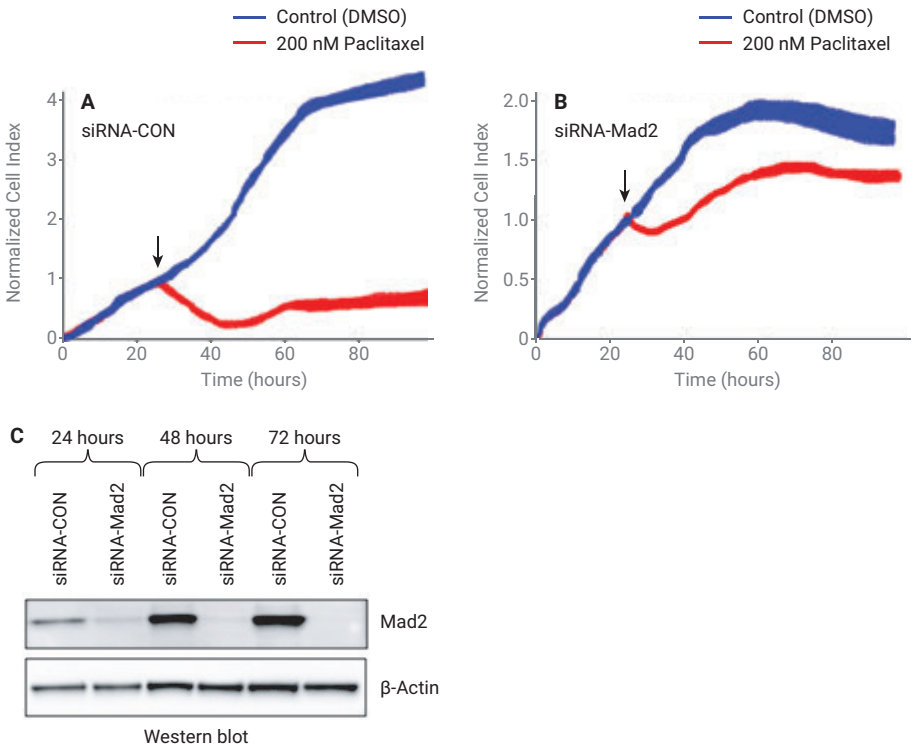


Figure 1. Mad2 knockdown mitigates the effects of Paclitaxel. The Western blot in the lower panel demonstrates efficient siRNA-mediated knockdown of Mad2 at 24, 48, and 72 hours post transfection. In the upper panels showing RTCA impedance traces, knockdown of Mad2 reduces the impact that Paclitaxel has on Cell Index. This indicates that cell number/proliferation, cell size/shape, and cell-substrate attachment quality are reduced by Paclitaxel most efficiently when Mad2 is present. The black arrows denote the time of Paclitaxel addition; Cell Index has been normalized to this time point. CON = negative control.

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