Reverse Transfection of siRNA in E-Plates®

Reverse Transfection

1. Introduction

This protocol is for a basic assay using an xCELLigence® RTCA instrument to monitor the effects of siRNA knockdown. Though this protocol has been optimized using the A549 human non-small cell lung cancer cell line, assay conditions may require additional optimization if different cell lines are used.

2. Reagents, Materials, & Equipment

Reagents & Materials

Item	Manufacturer	Cat. # or Model #
A549 Cells	ATCC®	CCL-185™
Opti-MEM® I Reduced Serum Medium	Gibco by Life Technology	31985-062
Lipofectamine™ RNAiMax	Invitrogen by Life Technology	13778-075
E-Plate® 16 or E-Plate® 96	ACEA Biosciences	05469830001/05232368001

Equipment

Item	Cat. # or Model #
xCELLigence RTCA DP – Bundle (complete system)	
RTCA Analyzer	
RTCA DP Station	
RTCA Control Unit	

3. Protocol Overview

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

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 E-Plate
5. Equilibration
6. Incubation and overnight monitoring of cell attachment and proliferation

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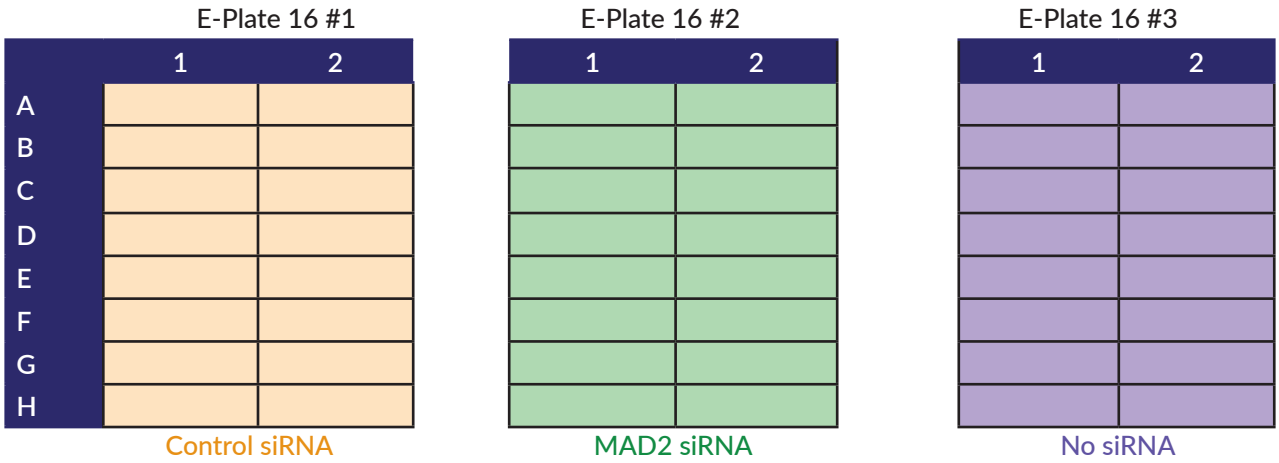
Day 2

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

Plate Layout

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



Software Setup

Step	Sweeps	Intervals	Unit	Comments
1	1	1	minute	background reading
2	100	15	minute	overnight monitoring
3	300	15	minute	compound response

4. Detailed Protocol

Day 1

1. **Warm up growth media and trypsin in a 37°C water bath 30 minutes prior to 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 entire volumes of mixture A and mixture B. Mix gently and incubate for 20 min. 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 RTCA station inside an incubator. Open the RTCA software and initiate Step 1 (1 minute and 1 sweep) to perform background measurement. Remove the E-Plate 16 from the RTCA 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 reverse transfection assay using an xCELLigence RTCA instrument depends greatly on the quality of the cells and how they are handled. It is imperative to follow the steps described here in order to ensure 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 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 1-5 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 30-60 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 for the following reason: 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** (16-24 hours)
   a. Transfer the E-Plate 16 to the RTCA plate station 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:** 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).
      » **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 effect 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:** The final concentration of solvents such as DMSO should be kept to a minimum (ideally, below 0.1%) to avoid cellular responses to the vehicle itself. When evaluating serial dilutions of a compound, it is important to maintain the same vehicle concentration in each dose.
2. **Compound addition** (10 minutes)
   a. To minimize the artifacts seen upon 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, and then pause the experiment. (This first reading in Step 3 will be used as the normalization time point.)
   d. Remove the E-Plate 16 from the RTCA station.
   e. Add 13.3 µL of the pre-warmed compound dilutions to each well of the E-Plate, and quickly return the E-plate to the RTCA station.
3. **Response monitoring** (72 hours)
   a. Resume Step 3 of the program, monitoring every 15 min. for 72 hours.

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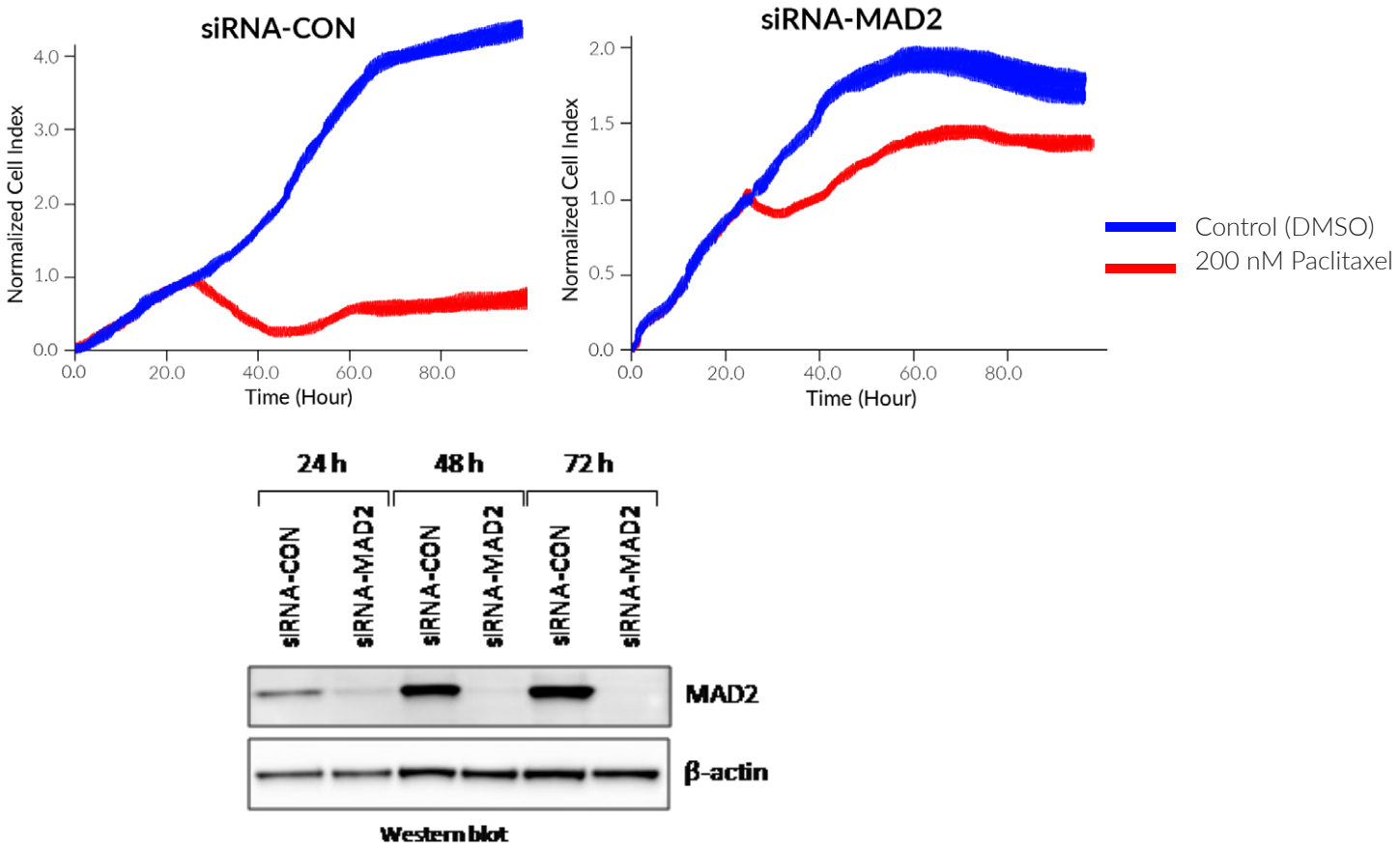
Sample Results



MAD2 knockdown mitigates the effects of Paclitaxel. The Western blot in the lower panel clearly 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 the cell index. This indicates that cell number/proliferation, cell size/shape, and/or 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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