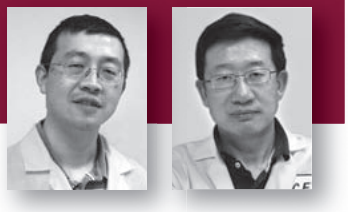
Roche

Cellular Analysis Application Note No. 4

Using Real-Time Data from the xCELLigence System to Determine Optimal Time Points for Gene and Protein Expression Analysis

Jing Zhang and Dr. Ning Ke
ACEA Biosciences



1 Introduction

We have explored the utility of the Roche xCELLigence System for assessing RNAi-mediated knockdown of gene function. The xCELLigence System of Real-time Cell Analyzer (RTCA) Instruments allows for label-free, continuous monitoring of cell phenotypic changes using electrical impedance as readout.

The cells are seeded in standard microtiter plates which are integrated with microelectronic sensor arrays. The interaction of cells with the electronic biosensors leads to the generation of a cell-electrode impedance responses which signify the status of the cells in terms of cell number, cell viability, cell morphology and cell attachment quality.

Real-time, continuous measurement ensures the documentation of cell phenotypes in the form of time-dependent cell response profiles (TCRP). The TCRP provides predictive mechanistic information regarding the modulation of targets and pathways, in addition to allowing the assessment of the optimal time for performing endpoint analyses.

To demonstrate the utility of the xCELLigence System in functional genomics using RNAi, we focused on Eg5, a kinesin involved in the mitosis pathway. Our results indicate that the xCELLigence System can provide a very specific TCRP, allowing users to determine the optimal times for endpoint analysis. The integration of these xCELLigence System-generated cell response profiles is easily extended to other targets and pathways for end-point analysis.

2 Methods and Material

siRNA transfection and cell proliferation assays

HeLa cellsValidated siRNAs targeted to Eg5 and control siRNAs were purchased from Ambion. HeLa cells were reverse transfected with the indicated siRNA using the X-tremeGENE siRNA Transfection Reagent. Briefly, the desired amount of siRNA (50 nM final concentration) was mixed with transfection reagent in 20 µl of Opti-MEM and incubated for 10 minutes in individual wells of the specialized 96-well microtiter plates with integrated gold microelectrodes at the bottom of each well (E-Plates). About 5000 cells in 100 µl media were then added to the wells containing the siRNA-transfection reagent mixture, after which the Cell Index values were recorded using the RTCA SP Instrument. Data were collected every 15 minutes for the entire duration of the experiment.

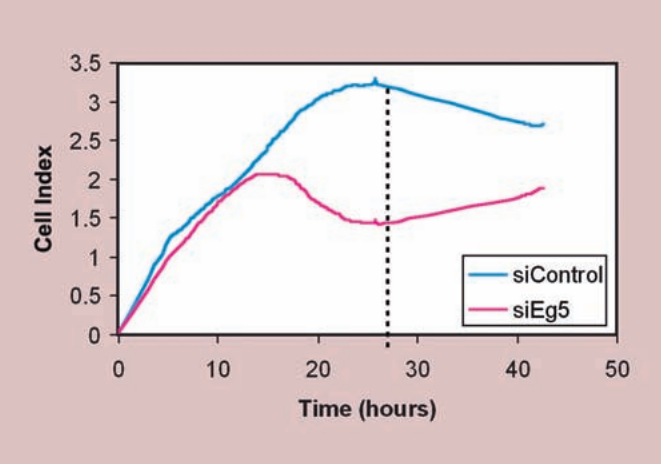


Figure 1. HeLa cells were reverse transfected in E-Plates 96 with Eg5 siRNA or Control siRNA (see text for details). Cell responses were continuously monitored using the xCELLigence System by recording Cell Index values. For RNA and protein analysis, cells were harvested from the E-Plates 96 at 27 hours post-transfection (dashed line).

Gene expression analysis

For RNA expression analysis of Eg5, HeLa cells were reverse transfected with 50 nM Eg5 siRNA and the control siRNAs in a 96-well E-Plate (5000 cells/well). At 27 hours after transfection (dashed line in Figure 1), total cellular RNA was purified using the High Pure RNA Isolation Kit.

Gene specific primers and probes were designed using the Universal ProbeLibrary Assay Design Center, and purchased from IDTDNA (primers) and Roche (probes). Real-time RT-PCR was performed using the LightCycler® 480 Instrument with the LightCycler® 480 RNA Master Hydrolysis Probes kit. GAPDH was used as the reference gene. Advanced relative quantification was used to obtain normalized changes in Eg5 mRNA levels relative to controls. Changes in the expression levels of CNTL2 siRNA transfected samples, in triplicate samples with standard deviations, were calculated (see Figure 2).

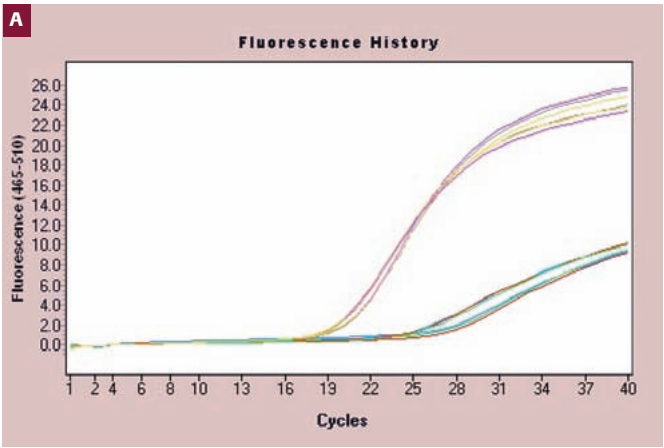


Figure 2. Total cellular RNA was isolated from siControl or siEg5 transfected cells grown in xCELLigence System Instrument E-Plates, using the High Pure RNA Isolation kit from Roche Applied Science. (A) Changes in Eg5 RNA expression were determined using the LightCycler 480 Real-Time PCR Instrument; (B) Approximately 65% knockdown of Eg5 mRNA expression was observed in cells compared to siControl cells.

Protein Expression Analysis

HeLa cells transfected with either control siRNA or Eg5 siRNA were harvested at 27 hours post-transfection (see Figure 1), and lysed with RIPA buffer. Approximately 40 µg of each lysate were loaded on an SDS-PAGE gel and proteins were fractionated and transferred to nitrocellulose membrane. The membrane was blocked with 5% BSA and immunoblotted using an antibody against Eg5 (Santa Cruz Biotechnology #SC-53691), and an antibody against GAPDH (Santa Cruz Biotechnology #SC-47724) as a loading control.

Bands were visualized using the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) kit. Results were scanned using an EpiChemII (UVP Bioimaging Systems); mean pixel intensities in equivalent areas bounding each band were background corrected and quantified using ImageJ Software (NIH). The extent of Eg5 protein knockdown was quantified by determining the ratio of the Eg5 to GAPDH band intensities (see Figure 3).

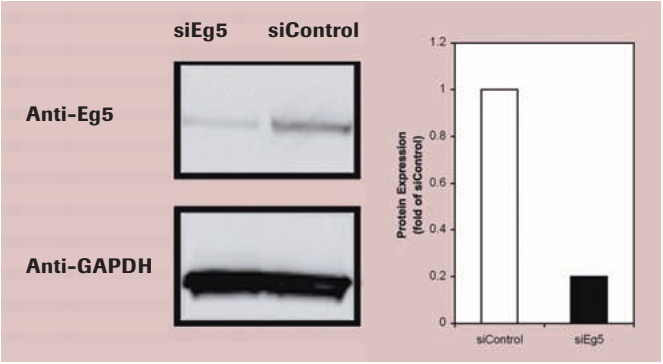


Figure 3. Protein expression analysis of Eg5 in siEg5 and siControl transfected cells. Protein lysates from the cells at 27 hours post-transfection were immunoblotted using antibodies against Eg5 and GAPDH. Bands were scanned densitometrically for quantitation, and the extent of Eg5 expression was expressed as the ratio of Eg5 to GAPDH levels.

The data shown above provide evidence that real-time cell monitoring using the xCELLigence System of Real-time Cell Analyzer (RTCA) Instruments is ideal for identifying the optimal time point for further molecular and biochemical analyses of cellular events. We have shown that transfection of siRNA targeted to Eg5 produces a transient cell phenotype, which is maximal in its expression at 27 hours post-transfection.

The real-time online aspect of the xCELLigence System allows the user to directly link phenotypic cellular events to the molecular and biochemical changes that have occurred after transfection with siRNA. Conventional analysis of the data, at time points prior or subsequent to the 27 hours after transfection, would have missed this optimal window of opportunity for carrying out the relevant gene and protein expression assays. Furthermore, the real-time data stream of the xCELLigence System provides a continuous, label-free measure of cell culture quality control for the transfection, simultaneously identifying potential off-target effects of the siRNA.

Ordering Information

Product	Cat. No.	Pack Size
X-tremeGENE siRNA Transfection Reagent	04 476 093 001 04 476 115 001	1 ml (400 transfections in a 24-well plate) 5 x 1 ml (2,000 transfections in a 24-well plate)
High Pure RNA Isolation Kit	11 828 665 001	1 kit (up to 50 reactions)
LightCycler® 480 Instrument II	05 015 278 001 05 015 243 001	1 instrument (96-well version) 1 instrument (384-well version)
LightCycler® 480 RNA Master Hydrolysis Probes	04 991 885 001	1 kit (for 5 x 100 reactions at 20 µl final volume)
RTCA SP Instrument	05 229 057 001 05 228 972 001 05 229 014 001	1 instrument (RTCA SP Station) 1 instrument (RTCA Analyzer) 1 instrument (RTCA Control Unit 1.1)
BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)	11 520 709 001	1 kit (2,000 cm^2 membrane)

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