ACEA Biosciences, Inc.

xCELLigence System

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Using the xCELLigence System for Functional Genomics: Assessing Gene Function in Real Time

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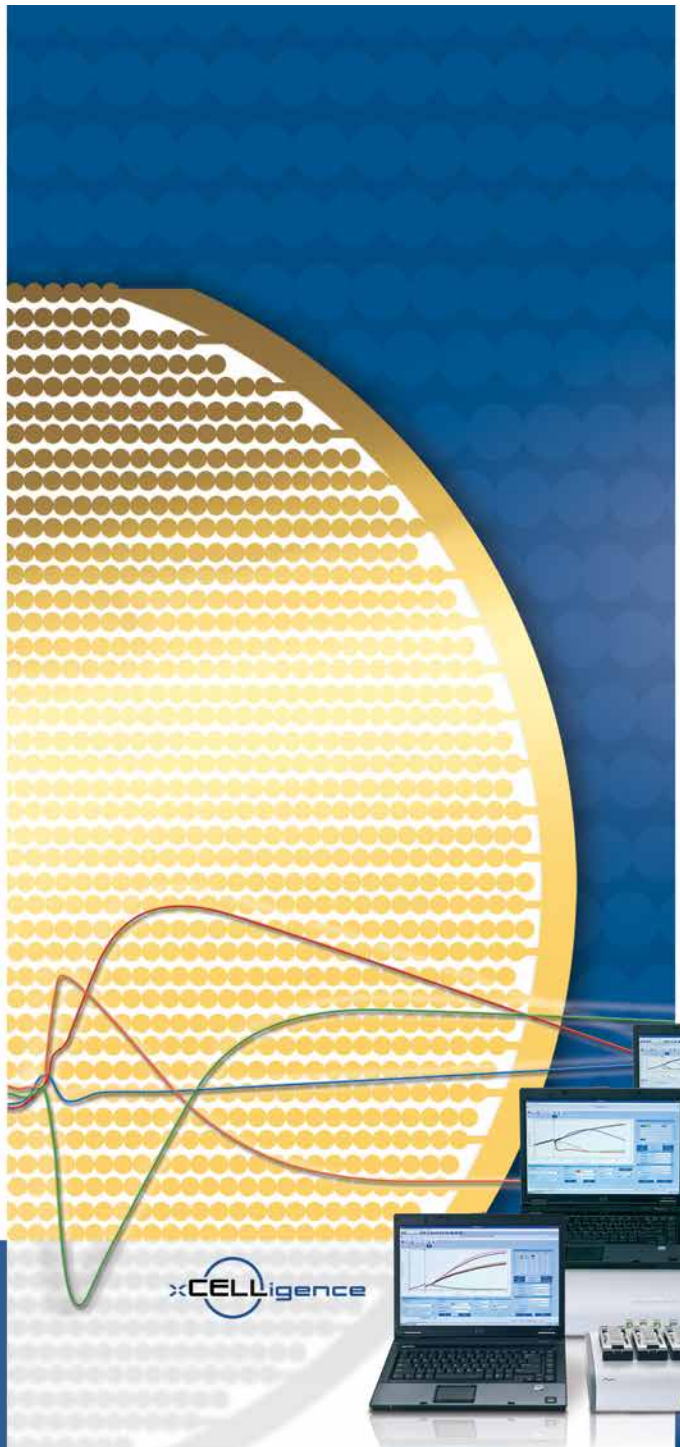

Using the xCELLigence System for Functional Genomics: Assessing Gene Function in Real Time

Introduction

To fully harness the wealth of genetic information acquired through the human genome project and realize its potential in delivering a new generation of therapeutic drugs and treatments, it is imperative that newly discovered genes be functionally characterized in a systematic manner. This can be done by introducing wild type or dominant negative forms of the gene into cells, or by downregulating the expression of specific genes using RNA interference (RNAi). RNAi has proven to be invaluable for understanding the functions of genes in biology and disease [1].

One of the challenges of RNAi is choosing an appropriate robust cell-based functional assay for obtaining incisive information about the phenotypic effect of targeted gene knockdown, discriminating off-target and toxic effects. Both off-target interactions and toxicity are an inherent part of the RNAi approach, dependent on the RNAi target sequence, concentration, and cell type used. A myriad of cell-based assays, including cell proliferation and survival, apoptosis, and reporter systems are used for functional genomics using RNAi.

All these assays are, however, endpoint assays measuring phenotypic changes at just a given time point. This means that significant phenotypic changes can be missed and not measured. Depending on the assay, it is also difficult to distinguish between a real effect of RNAi, and effect(s) induced by potential off-target and toxic interactions using siRNA transfection.

In the present study, the **xCELLigence System** was used for assessing RNAi-mediated knockdown of gene function. The xCELLigence family of Real-Time Cell Analyzer **(RTCA)** Instruments allows for label-free continuous monitoring of changes in cell phenotype using microelectrodes to record electrical impedance. Cells are seeded in standard 96-well microplates, manufactured with integrated microelectronic sensor arrays, called **E-Plates 96.**

The interaction of cells with the electronic biosensors generates cell-to-electrode impedance responses, measuring many aspects of cell status, including cell number, cell viability, cell morphology, and the quality of cell attachment. Real-time, continuous measurement ensures uninterrupted documentation of cell phenotype by producing time-dependent cell response profiles **(TCRPs).**

TCRPs provide predictive information about how cells and cellular pathways are responding *in vitro* [2]. Kinetic information revealed by TCRPs can discriminate siRNA-mediated off-target and toxic effects. The focus here is on several genes implicated in the mitotic pathway. The results demonstrate that the xCELLigence System provides incisive, reproducible, and quantitative data for the RNAi-mediated knockdown of gene targets and cellular pathways.

Materials and Methods

Cell culture

HeLa and A549 cells, obtained from ATCC, were maintained in DMEM media with 10% FBS and 1% penicillin and streptomycin, at +37°C with 5% CO_2.

Cell Index calculation

Cell attachment, spreading, and proliferation were continuously monitored every 30 minutes on E-Plates 96 using the xCELLigence System. The electronic readout of cell-sensor impedance is displayed continuously in real time as the **Cell Index** (CI). The CI value at each time point is defined as **Rn–Rb/Rb,** where Rn is the cell-electrode impedance of the well with the cells, and **Rb** is the background impedance of the well with media alone.

siRNA transfection, drug treatment, and cell proliferation assays

Silencer Select Validated siRNAs targeted against PLK1, KIF11, and control siRNA (Ambion), and Mad2 siRNA (Dharmacon) were used at 10 nM siRNA in most experiments, except in the dose-response experiments, in which threefold serially diluted siRNA was used. Reverse transfection with Lipofectamine RNAiMax (Invitrogen) was used for transfection following the manufacturer's procedure. Briefly, siRNA was mixed with lipid in 20 µl Opti-MEM, and incubated for 10 minutes in individual wells of a 96-well E-Plate 96; 2,500 to 5,000 cells in 100 µl media were then added to each well containing the siRNA-lipid mixture. For paclitaxel, monastrol, and staurosporine treatments, 3,000 cells were seeded; 24 hours later, serially diluted compounds were added; Cell Index values were recorded using the xCELLigence System.

Gene expression analysis

For RNA expression analysis of PLK1 and KIF11, HeLa cells were reverse transfected with 10 nM PLK1, KIF11, and the control siRNAs in either standard six-well plate (1.5 x 10^5 cells/well) or E-Plate 96 (5000 cells/well). Total cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche), 24 to 26 hours post transfection. Gene-specific primers and probes were designed using the Universal ProbeLibrary Assay Design Center, and purchased from IDTDNA and Roche, respectively. qRT-PCR was done using the LightCycler® 480 Real-Time PCR System with the RNA Master Hydrolysis Probe kit (Roche).

Mitotic index determination

For immunofluorescence studies, transfected cells grown on 16-well chamber slides (Labtec), were fixed in 100% ice-cold methanol, washed in PBS, and permeabilized in PBS with 0.25% Triton X-100. Cells were blocked with PBS containing 1% BSA and 0.1% TX-100. After washing with PBS, cells were stained with an anti-phospho-Histone H3 (p-H3) antibody (S-10), a FITC-conjugated anti-tubulin antibody, and DAPI (4, 6-diamidino-2-phenylindole). Visualization and imaging were done using a Nikon E400 epifluorescence microscope and Nikon ACT software. Mitotic indices were calculated by dividing the total number of p-H3-containing cells by the total number of DAPI-stained cells (70 to 150 cells counted per sample).

Results and Discussion

The xCELLigence System

The xCELLigence System of Real-Time Cell Analyzers is a family of three instruments differing primarily in throughput capacity (see Figure 1). The **RTCA SP Instrument** comprises four main components: (1) the electronic 96-well microplates, or **E-Plates 96;** (2) the **RTCA SP Station** accommodating one E-Plate 96 for placement inside a tissue culture incubator; (3) the **RTCA Analyzer** for sending and receiving the electronic cellular signals; and (4) the **RTCA Control Unit** which operates the software and continuously acquires and displays data in real time. In contrast, the **RTCA MP Instrument** has a higher throughput capability, accommodating up to six E-Plates 96 (for a total of 576 wells), independently controlled by up to six different users.

The **RTCA DP Instrument** is the lower throughput option, accommodating up to three 16-well E-Plates (for a total of 48 wells). Importantly, the key new feature of the RTCA DP Instrument is that it is also able to operate the new and quite different 16-well **Cell Invasion and Migration** culture plate, or **CIM-Plate 16.** The CIM-Plate 16 is essentially a modified Boyden chamber for monitoring cell migration and invasion in real time, in a continuous and automated fashion, using upper and lower chambers separated by a porous polyethylene terephthalate (PET) membrane in conjunction with microelectrodes.

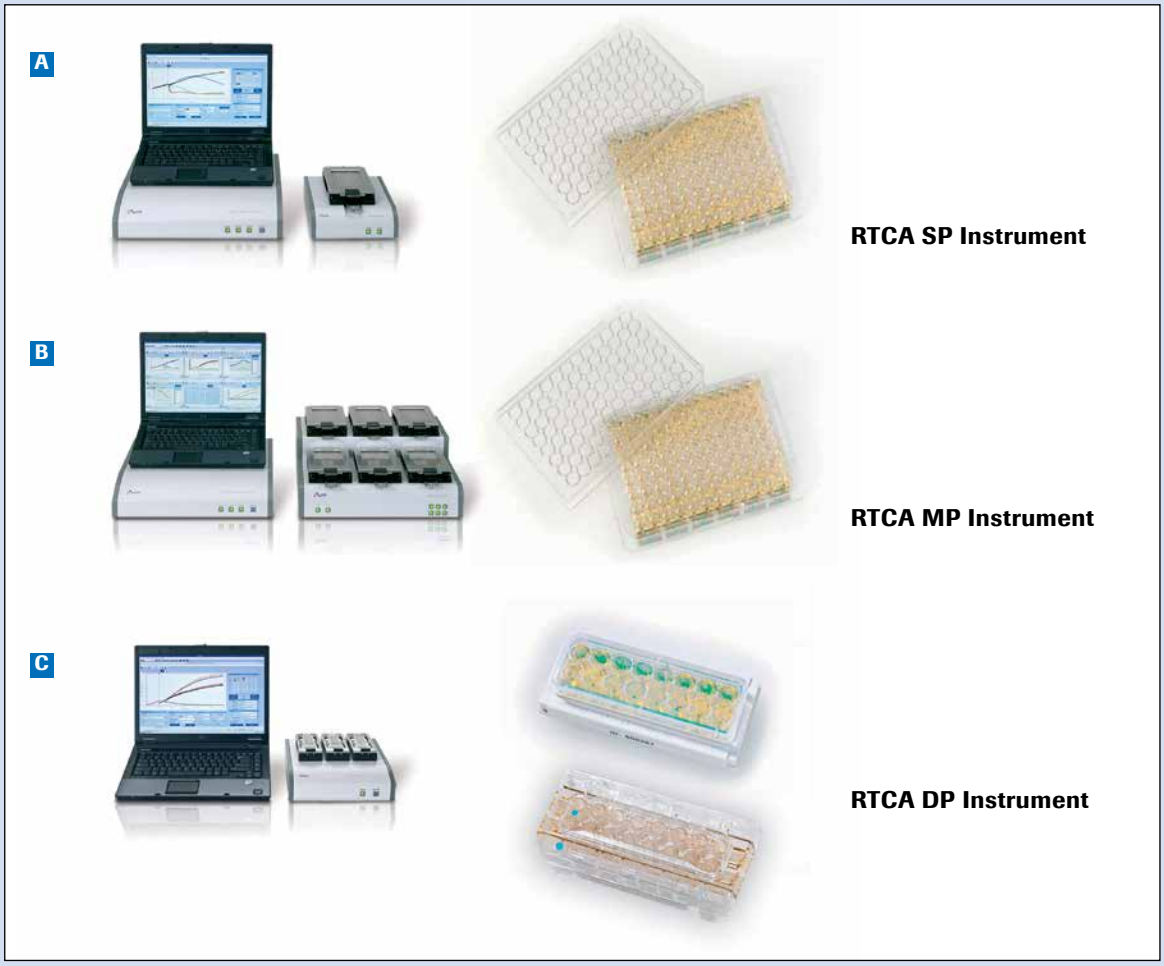

Figure 1: The xCELLigence System product line comprises three different Real-Time Cell Analyzer (RTCA) Instruments. (A) The **RTCA SP** (single plate) **Instrument:** control unit and analyzer (left), station (middle) and E-Plate 96 (right). **(B)** The **RTCA MP** (multiple plate) **Instrument:** control unit and analyzer (left), station (middle) and E-Plates 96 (right). **(C)** The **RTCA DP** (dual plate) **Instrument,** capable of assaying cell migration and invasion: control unit (left), analyzer (middle) and E-Plate 16 and CIM-Plate 16 (right).

Robust real-time phenotypic changes using siRNA targeting mitotic machinery

To demonstrate the utility of the xCELLigence System for functional genomics using RNAi, we targeted the cellular machinery regulating mitosis. Mitotic arrest is documented robustly by the xCELLigence System due to the morphological changes associated with mitosis, such as cell rounding and transient cell detachment [2, 3]. Furthermore, the mitotic regulatory pathway is well characterized using small molecules and siRNAs for functional studies.

We initially focused on KIF11, a member of the kinesin-like protein family encoding a motor protein required for the bipolar spindle during mitosis. KIF11 has been shown to play a pivotal role in mitosis and its inhibition; using either siRNA or small interfering molecules results in mitotic arrest and apoptosis [4, 5]. Proven siRNAs against KIF11 (Ambion), and control siRNAs with no homology match to known human genes, were reverse transfected into HeLa cells in E-Plates 96. Cells were monitored every 30 minutes for the entire duration of the experiment (see Figure 2A).

Transfection of KIF 11 siRNA produced a very distinct TCRP compared to controls. The impedance profile starts to differentiate from the control at 12 hours post transfection and the maximal effect is observed at 24 hours. Interestingly, the effect appears to be transient, ultimately leading to a recovery of the impedance signal by 48 hours. Analysis of cells by p-H3 staining at 24 hours post-transfection, which is coincident with the maximal impedance change compared to controls, revealed that the bulk of the cells are arrested at mitosis (see Figure 2B).

Treatment of HeLa cells with monastrol, a specific inhibitor of the KIF11 gene product, Eg5, produced a similar TCRP to that of the siRNA-mediated knockdown of KIF11 (see Figure 2C). Similarly, analysis of the cells using p-H3 staining at the point of maximal impedance change compared to control also revealed that the bulk of the cells are arrested at mitosis (see Figure 2D). The fact that the xCELLigence kinetic profiles are similar for both gene knockdown and small molecule inhibition of the same target is an excellent measure of the specificity of the proposed role of the target in regulating mitosis.

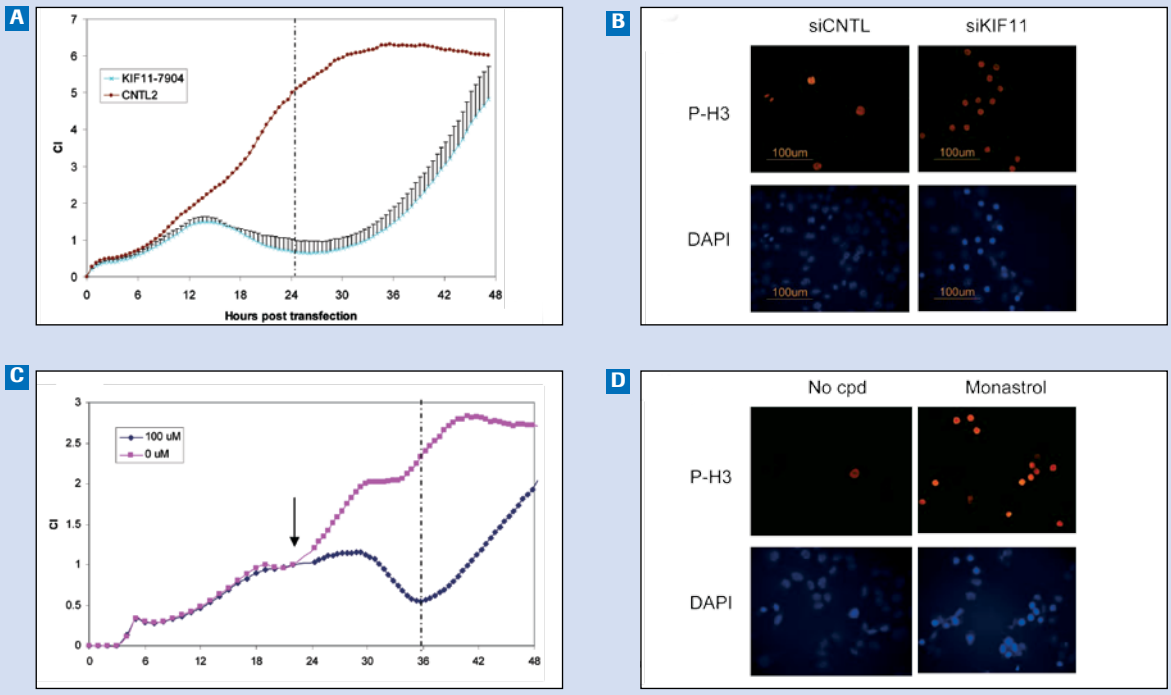

Figure 2: Similar TCRPs were obtained using both siRNA and the small molecule inhibitor, monastrol, targeted against KIF11. HeLa cells were reverse tranfected with KIF11 siRNA **(A)** or treated with monastrol **(C).** TCRPs were obtained in E-Plates 96 using the xCELLigence System. Parallel experiments were carried out in 16-well chamber slides **(B & D),** and stained with a p-H3 antibody (mitosis marker), and DAPI for cellular DNA. The dotted line in **(A & C)** indicates time of harvest for the immunofluorescence study; and the **arrow** in **(C)** indicates time of monastrol treatment. Error bars represent standard deviations of the mean.

Results and Discussion continued

These observations were extended in a more quantitative manner using KIF11 siRNA, as well as siRNAs targeting polo-like-kinase 1 (PLK1). PLK1 is a serine/threonine protein kinase involved in cell cycle progression, in particular the formation of the mitotic spindle, and activation of CDKs/cyclins during mitosis. A similar TCRP was obtained for all siRNAs targeting KIF11 and PLK1. However, PLK-1 knockdown using PLK1-448 siRNA at the concentration used, did not lead to similar recovery of CI as observed for the other PLK1 siRNA (PLK1-450), or both KIF11 siRNAs. This is most likely a concentration effect as transfection with lower concentrations of PLK1-448 siRNA showed similar TCRPs as KIF11 gene knockdown (see Figure 4E).

For KIF11, phenotypic changes start at 12 hours post transfection, and the maximal effect is observed at 24 hours, similar to previously tested siRNA. For PLK1, phenotypic changes start 16 hours post transfection, and the maximal effect is observed at 30 hours. Parallel experiments were done to quantitate target downregulation by qRT-PCR using the LightCycler® 480 Real-Time PCR System, and by p-H3 staining using immunofluorescence to quantitate mitotic arrest. Cells were harvested 26 hours post transfection for both experiments. Robust mRNA downregulation was observed for both targets, with a more than 90% downregulation for both KIF11 siRNA, and at least 70% downregulation for both PLK1 siRNAs (see Figures 3B and 3C).

Concurrently, mitotic arrest was indicated by an increased mitotic index after transfection of siRNAs targeting both KIF11 and PLK1 (see Figure 3D). A much higher mitotic index was observed when PLK1 (40.2% for PLK1-448 and 23.8% for PLK1-450) and KIF11 (48.6% for KIF11-7903 and 60.0% for KIF11-7904) mRNA levels were downregulated, compared to controls (4.7% for CNTL1 and 5.9% for CNTL2). Changes in mitotic index were highly correlated with impedance changes obtained using the xCELLigence System. For example, PLK1-450 showed both an intermediate impedance change and mitotic index increase, while the other three siRNAs gave more robust changes in both impedance and mitotic indices.

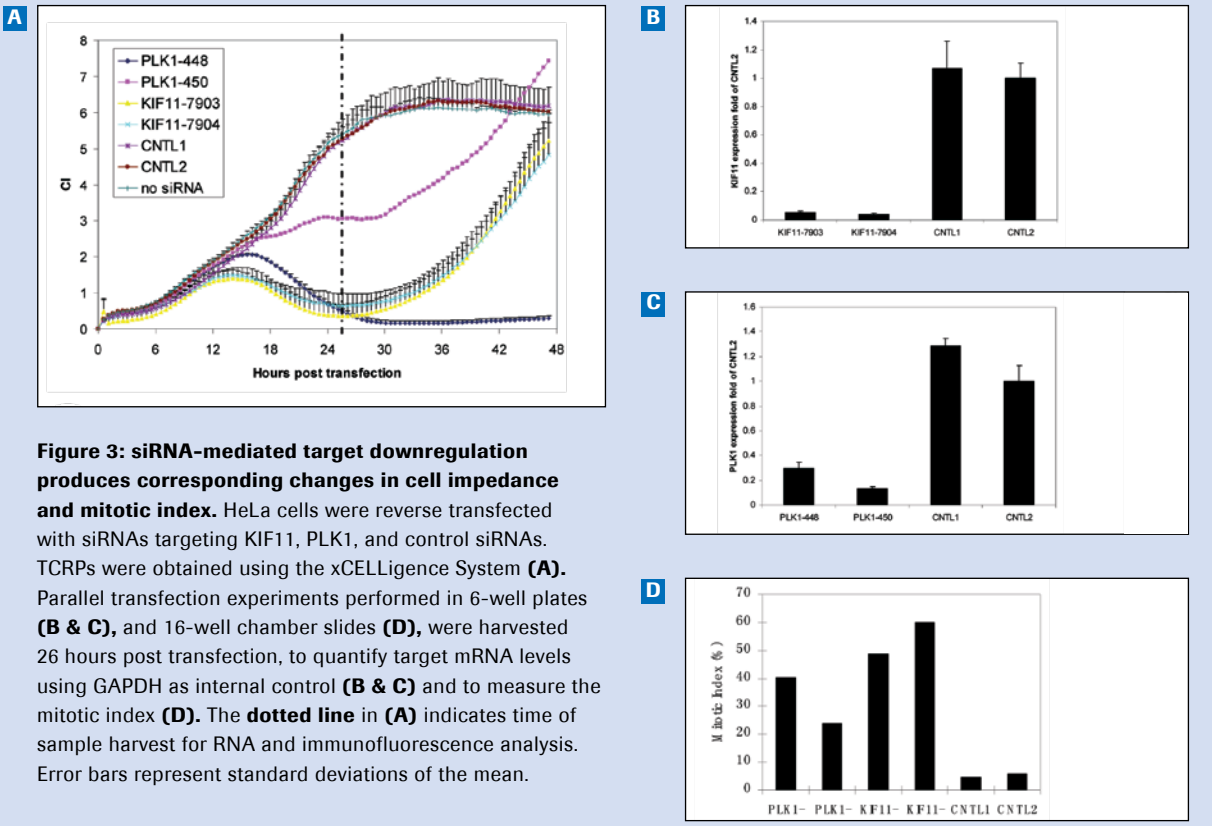

Figure 3: siRNA-mediated target downregulation produces corresponding changes in cell impedance and mitotic index. HeLa cells were reverse transfected with siRNAs targeting KIF11, PLK1, and control siRNAs. TCRPs were obtained using the xCELLigence System **(A).** Parallel transfection experiments performed in 6-well plates **(B & C),** and 16-well chamber slides **(D),** were harvested 26 hours post transfection, to quantify target mRNA levels using GAPDH as internal control **(B & C)** and to measure the mitotic index **(D).** The **dotted line** in **(A)** indicates time of sample harvest for RNA and immunofluorescence analysis. Error bars represent standard deviations of the mean.

Quantitative functional assessment of gene function after gene knockdown

To better document the correlation between target gene expression, CI changes, and mitotic arrest, threefold serial-diluted KIF11 siRNA (KIF11-7904) was transfected into HeLa cells in E-Plates 96 for Cell Index measurements, and 16-well chamber slides for quantifying mitotic index; 24 hours post transfection, cells were harvested from E-Plates 96 to quantify KIF11 mRNA levels, or fixed and stained using p-H3 antibody in chamber slides to determine mitotic indices. TCRPs showed time and concentration-dependent dose-response curves, with higher siRNA concentrations producing more CI changes (see Figures 4A and 4C).

The kinetics of CI changes were very similar for the different concentrations of siRNAs. CI values for transfected samples diverged from control samples, starting 9-12 hours post transfection, reaching the lowest level at 24 hours post transfection, before the CI started recovering. CI changes were highly correlated to target mRNA levels and mitotic indices, with higher siRNA concentrations producing more pronounced CI changes (see Figures 4A and 4C), resulting in higher target downregulation (see Figure 4B) and mitotic index (see Figure 4D).

Interestingly, siRNA transfected at a concentration of 0.01 nM still produced robust CI changes and target downregulation (see Figures 4A and 4B). Similar dose responses were also observed for PLK1-448 and PLK-450 siRNAs (see Figures 4E and 4F), while no dose response was seen for control siRNAs (data not shown). The dose-dependent TCRPs obtained underscore the significant advantage of using the xCELLigence System for functional genomics studies, allowing the determination of the efficacy of target knockdown on a particular phenotype.

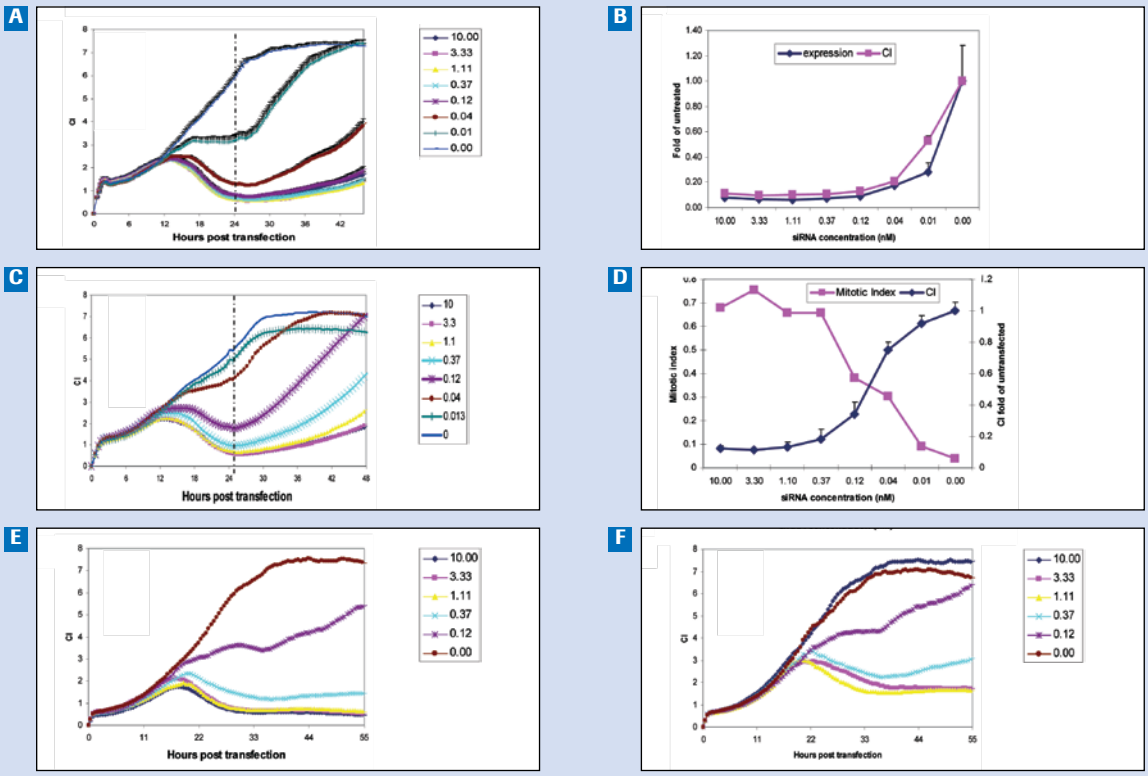

Figure 4: TCRPs and qRT-PCR show the siRNA dose-response effect of gene knockdown. siRNAs targeting KIF11 **(A & C)** and PLK1 (**E** for PLK1-448 & **F** for PLK1-450) were serially diluted and transfected into HeLa cells. TCRPs were obtained using the xCELLigence System. RNA samples were harvested from parallel experiments at **(A)** 24 hours post transfection, and subjected to qRT-PCR analysis using KIF11-specific primer/probes **(B).** Cells from parallel experiments **(C)** using 16-well chamber slides were harvested 24 hours post transfection, for immunofluorescence to determine the mitotic index **(D). Dotted lines** in **(A & C)** indicate time of sample harvest for analysis in **(B & D),** respectively.

Characterization of the mitotic arrest TCRPs

Mitotic arrest is mediated by spindle checkpoint proteins, including the Mad and Bub proteins. These proteins are the gatekeepers for mitosis. Disabling these proteins using small molecules or siRNA compromises their function, making cells insensitive to assault by antimitotic agents, such as paclitaxel. To determine whether TCRPs showing mitotic arrest are dependent on checkpoint mechanisms, we used a siRNA against Mad2 to disable the checkpoint machinery, to examine its effect on TCRP changes mediated by compounds inducing mitotic arrest.

Briefly, HeLa cells were transfected with either control or Mad2 siRNA; 24 hours later, cells were treated with compounds, paclitaxel, (see Figures 5A and 5B), monastrol, (see Figures 5C and 5D), and staurosporine (see Figures 5E and 5F). Paclitaxel is an antimitotic compound targeting tubulin polymerization and stabilizes microtubules; monastrol is also an antimitotic compound targeting the Eg5 kinesin motor protein; and staurosporine is a general kinase inhibitor. Real-time kinetic TCRPs were obtained showing CI values for 48 hours following treatment.

Cells transfected with control siRNA produced the expected mitotic arrest profile upon treatment with the antimitotic compounds (see Figures 5A and 5C), whereas cells transfected with Mad2 siRNA produced only minor effects after treatment with these same compounds (see Figures 5B and 5D). In stark contrast, the TCRPs for staurosporine were similar to cells transfected with control or Mad2 siRNAs (see Figures 5E and 5F), indicating the specificity of these TCRPs for mitotic arrest, underscoring their power to predict the underlying mechanisms of action.

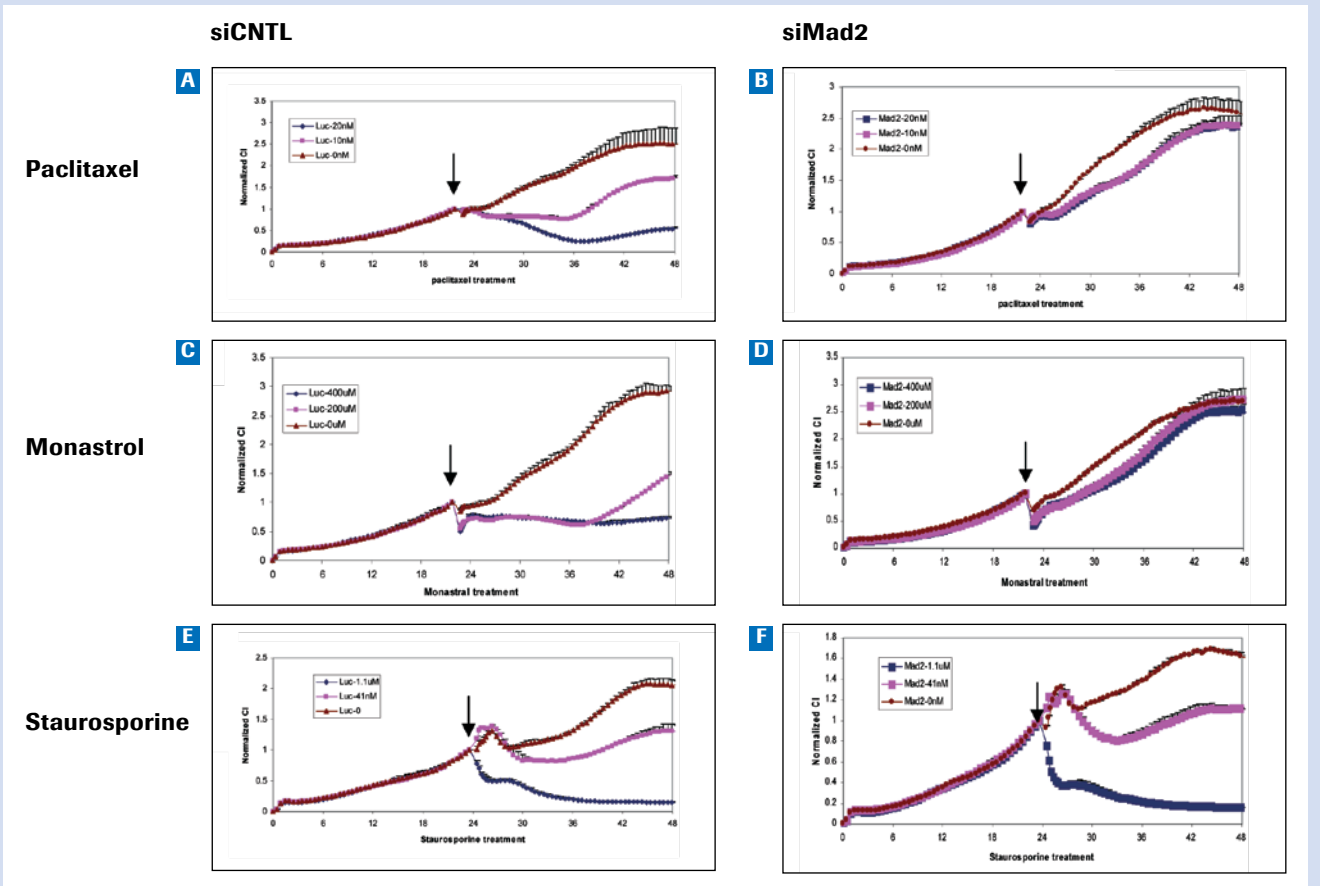

Figure 5: Mitotic TCRPs show dependence on the spindle checkpoint machinery. HeLa cells were reverse transfected with control or Mad2 siRNAs, and plated into E-plates 96; 22 hours later, cells were treated with paclitaxel **(A & B),** monastrol **(C & D)**, and staurosporine **(E & F).** Kinetic CI patterns (TCRPs) were obtained using the xCELLigence System, with **arrows** showing the time of compound addition.

Mitotic TCRP in other cell lines

The mitotic CI pattern was also determined for other cell lines using compounds or siRNA targeting mitotic machinery. Comparable kinetic CI pattern and dose-response curves were obtained for the NSCL cell line A549 with both KIF11 siRNA and monastrol (see Figures 6A and 6B), and the prostate cancer cell line PC3 and breast cancer cell line MCF7 (data not shown).

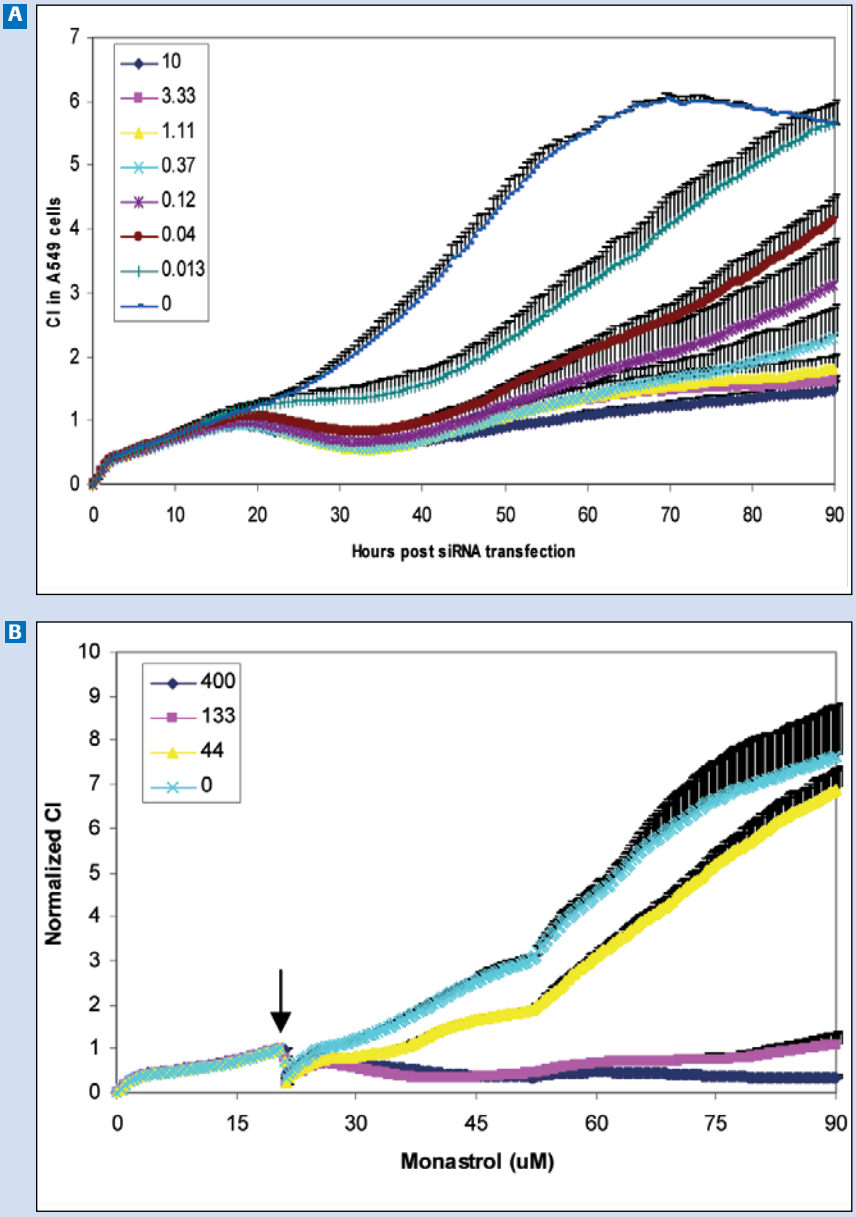

Figure 6: Mitotic TCRPs obtained using the xCELLigence System in A549 cells. A549 cells were transfected with different concentrations of KIF11 siRNA **(A)** or monastrol **(B).** TCRPs were recorded using the xCELLigence System, and show the time of compound addition **(arrow** in **B).**

Conclusions

Functional genomics studies using the xCELLigence System

The key to success is to optimize the cell culture conditions for assessing gene function using the xCELLigence System. This can vary depending on the cell lines used, the siRNA target, and the specific TCRP produced by the xCELLigence System. It can be helpful to refer to the following guidelines when working with siRNA:

1. **Mode of transfection:**
   Reverse transfection can produce more robust and reproducible phenotypic changes.
2. **Seeding density:**
   Phenotypic changes can vary with the seeding density, with a lower seeding density producing a more robust phenotypic change after transfection, probably due to more siRNA available per cell.
3. **Concentration of RNAi reagent used for transfection:**
   5 nM to 50 nM siRNA is an optimal range for transfection to elicit robust phenotypic responses. For some targets and siRNAs, robust CI changes are observed with much lower siRNA concentrations (see Results above).
4. **Observed phenotypic changes must be confirmed using other cell assays:**
   Meaningful interpretation of TCRPs is sup ported by conducting parallel experiments such as qRT-PCR and Western Blotting.

Combine real-time continuous cell monitoring with RNAi-mediated knockdown

The advantages of the xCELLigence System for functional genomics are:

1. Real-time, continuous measurement identifies both subtle and robust phenotypic changes.
2. Time-dependent phenotypic divergence from controls identifies the best time points for assaying changes in either mRNA and protein expression, or using other technologies such as high-throughput screening (HCS).
3. Time-dependent cell response profiles (TCRPs) identify culture wells with inappropriate cell numbers at the beginning of the assay, thus allowing the collection of more significant data.
4. Normalization of the cell index minimizes the role of cell seeding artifacts and culture plate edge effects
5. TCRPs can be predictive of the mechanisms of action during target modulation, providing an opportunity to identify specific pathway-target interactions.

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Ordering Information

Product	Cat. No.	Pack Size
xCELLigence RTCA DP Instrument	**00380601050**	**1 Bundled Package**
RTCA DP Analyzer	05469759001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA SP Instrument	**00380601030**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA SP Station	05229057001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA MP Instrument	**00380601040**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA MP Station	05331625001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
E-Plate 16	05469830001	6 Plates
	05469813001	6 x 6 Plates
E-Plate VIEW 16	06324738001	6 Plates
	06324746001	6 x 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)
CIM-Plate 16	05665817001	6 Plates
	05665825001	6 x 6 Plates
E-Plate 96	05232368001	6 Plates
	05232376001	6 x 6 Plates
E-Plate VIEW 96	06472451001	6 Plates
	06472460001	6 x 6 Plates
E-Plate Insert 96	06465412001	1 x 6 Devices (36 16-Well Inserts)
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