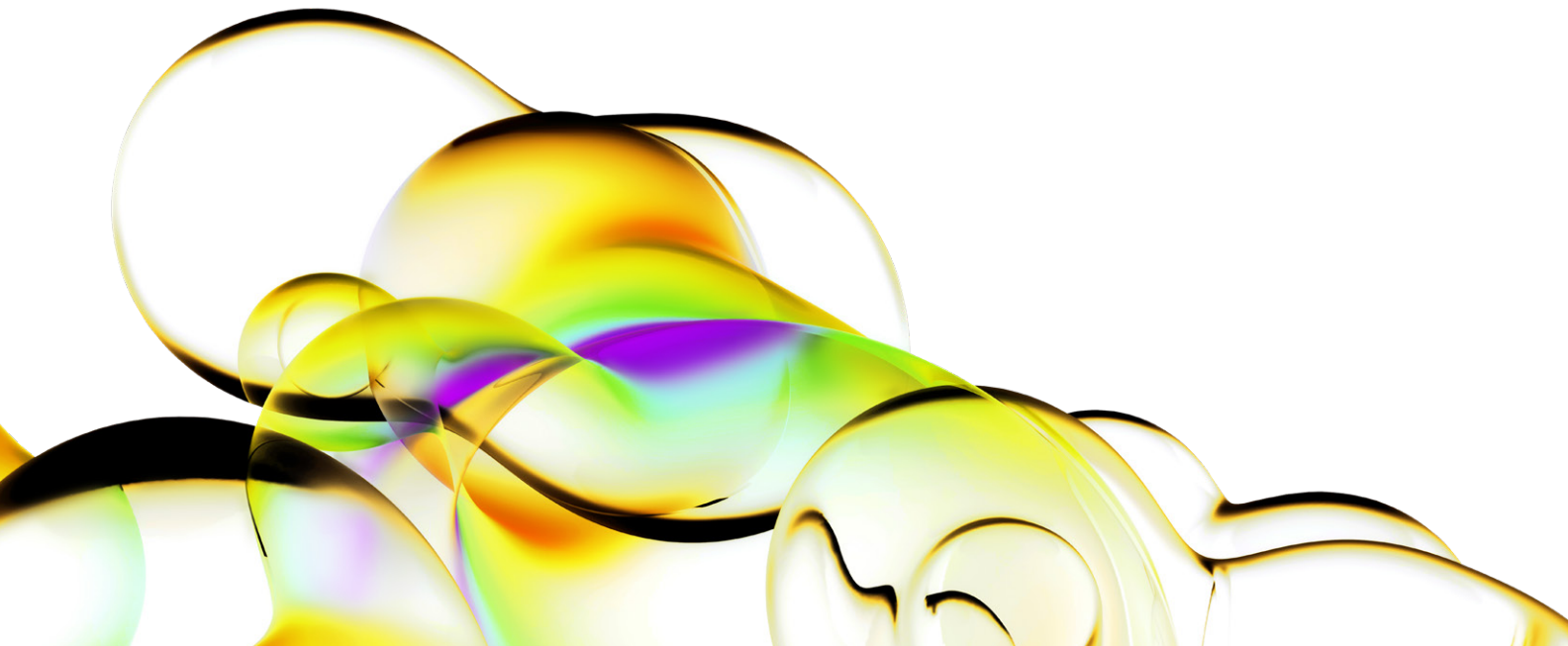
APPLICATION NOTE

High content analysis using targeted RNAi-mediated gene knockdown.

Introduction

Small interfering RNAs (siRNAs) are able to effectively down-regulate gene expression in mammalian cells through RNA interference (RNAi). Collections of siRNAs targeting gene families, biological processes, or even entire genomes provide a systems-based approach for identifying the role of particular genes in a cell. RNA-based gene function analysis is particularly powerful when combined with high content screening (HCS) where multi-parametric assays are used to better define the function of a specific gene target. In this study, parallel knockdown of 80 genes that are known or predicted to be involved in the cell cycle regulation was performed using Dharmacon™ SMARTpool™ reagents. Monitoring of four relevant phenotypes (cell cycle phase, nuclear area, cell count, and localization of phospho-ERK) using a high-content imager to identify gene targets involved in cell cycle regulation and cell cycle phase-specific arrest. These results clearly demonstrate that cell cycle status and phenotypic changes in cell populations can be easily assayed using a highly specific and functional SMARTpool siRNA library, followed by data acquisition using high content screening instrumentation and software. In addition, these results also show the utility of combining rationally designed SMARTpool reagents and high-content imaging technology for functional mapping of genes in specific disease states or model assays.



Materials and methods

siRNA library and transfection

The siRNAs used in this study were designed to target 80 individual cell cycle-related genes and are a subset of the human siGENOME™ siRNA library for cell cycle regulation. The complete library consists of SMARTpool siRNA reagents designed to target 111 genes involved in cell cycle regulation. Each SMARTpool reagent consists of four rationally designed siRNAs, targeting a distinct region of the target mRNA, thus assuring gene silencing and reducing the incidence of off-target effects. A549 cells were plated in 96-well microwell plates at a density of 5,000 cells per well 24 hours prior to transfection. Cells were transfected with the SMARTpool siRNA reagents (100 nM final concentration) using the DharmaFECT™ 1 at a final concentration of 0.15 µL/well. Transfection was monitored by microscopy and acquiring images on the ArrayScan VTI HCS Reader (version 5.5), using the siGLO RISC-Free™ siRNA (Cat# D-001600-01), a fluorescently labeled non-targeting control chemically modified to prevent uptake by the RISC complex. A validated SMARTpool reagent targeting the human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and an individual siRNA targeting the PPIB (cyclophilin B) gene (siGENOME Cyclophilin B Control siRNA, Cat #D-001136-01-05) were used as positive controls for transfection and knockdown efficiency. Untreated cells, cells treated with DharmaFECT 1 alone, and cells transfected with the siGENOME Non-targeting siRNA Pool (Cat# D-001206-14) or siGENOME RISC-Free Control siRNA were used as negative controls. The TOX Transfection Control (Cat #D-001500- 01-05) an RNA oligonucelotide designed to induce cell death, was used as a positive control for toxicity. Cells were then incubated for ~ 40 hours after transfection and fixed with 4% paraformaldehyde. Separate microwell plates were prepared for each transfection plate to determine gene expression and toxicity. Gene expression levels were determined using the branched DNA assay (Panomics, Fremont, CA). Toxicity was determined using Thermo Scientific™ alamarBlue™ staining (Cat #00-010).

High content screening and analysis

The transfected cells were fixed and analyzed for the following cell features: cell cycle phase characterization, nuclear area measurement, cell count per field, and ERK activation. The ERK Activation Thermo Scientific Cellomics™ HitKit™ HCS Reagent Kit (Cat #K0100071) was used for immunofluorescence staining of phosphorylated ERK (pERK) and a DNA specific stain, whose fluorescence is proportional to DNA content. The plates were imaged and quantitatively analyzed on the ArrayScan VTI HCS Reader using the Cell Cycle and Cytoplasm to Nucleus Bio-Application image analysis software. Numeric data generated by the ArrayScan VTI HCS Reader were evaluated with the vHCS™ Discovery Toolbox and exported to Spotfire™ Decision Site™ (Spotfire, Inc. Somerville, MA) for further analysis.

Results

Transfection of siRNAs and gene knockdown

The siGLO RISC-Free Control siRNA was utilized as a visual transfection control. The images were acquired on the ArrayScan VTI HCS Reader using 20x magnification. Nuclei were stained with 5 µg/mL Hoechst 33342 (Molecular Probes, Cat #H1399). The fluorescently tagged RISC-Free transfection control, observed as punctate spots and diffuse cytoplasmic fluorescence, suggests that there is efficient transfection of the siRNA into cells (Figure 1). To further demonstrate the efficiency of siRNA transfection, gene silencing was determined by analyzing the functional knockdown of a positive control SMARTpool reagent or siRNA targeting either human GAPDH or PPIB. Figure 2 shows the transfection efficiency and plate variability. Each plate showed efficient knockdown of PPIB (> 85%) in each well containing the PPIB siRNA and the GAPDH SMARTpool reagent showed similar transfection efficiency with > 90% knockdown of GAPDH.

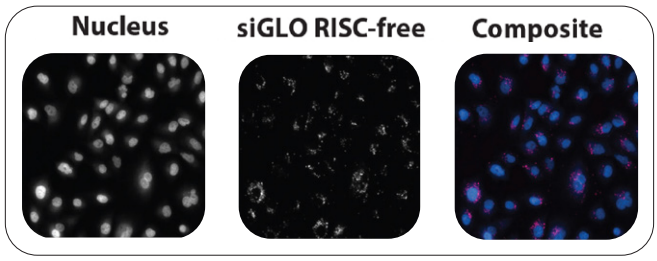


Figure 1: Individual fluorescent and composite images of A549 cells transfected with the fluorescently labeled siGLO RISC-Free control.

Images were generated by the ArrayScan VTI HCS Reader.

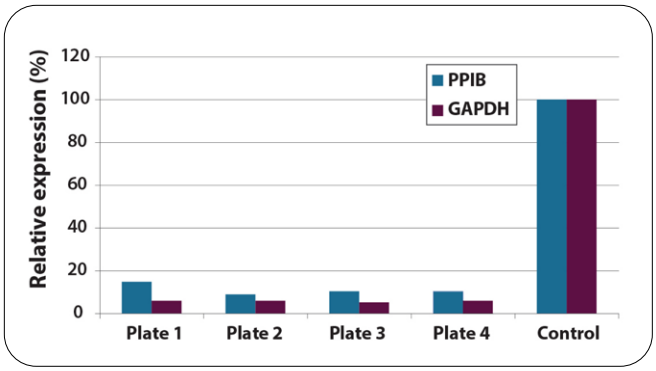


Figure 2: Efficiency of transfection was determined by functional knockdown of PPIB or GAPDH expression in the control wells containing either 100 nM PPIB siRNA or 100 nM GAPDH SMARTpool reagent. Expression levels were determined using a branched DNA assay and expression was normalized to the PPIB or GAPDH expression level in the lipid-alone negative control well.

Cell cycle analysis

Cells transfected with each of the 80 SMARTpool siRNA reagents were analyzed to determine the number of cells in each of five cell cycle-related stages (Figure 3) using the Cell Cycle BioApplication. This BioApplication is designed to classify cells and define cell cycle stage by measuring the nuclear DNA content using the total intensity of a DNA binding dye (Hoechst 33342) for each individual cell. Correspondingly, the cells are classified into one of five classes: less than 2N (apoptotic/damaged), 2N (G1), 2N-4N (S), 4N (G2/M), and > 4N (polyploid or necrotic).

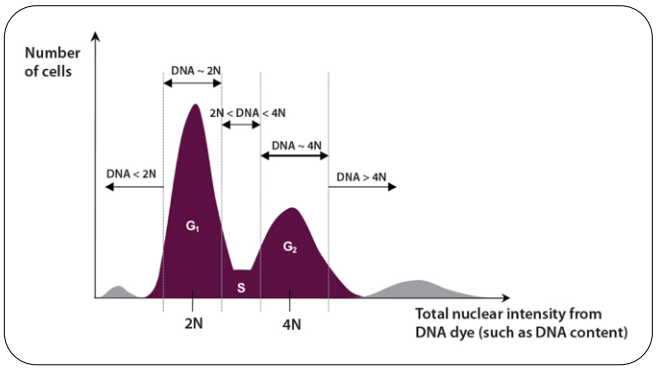


Figure 3: The Cellomics Cell Cycle BioApplication classifies cells into their cell cycle phase based on the total nuclear intensity of a DNA binding dye (Hoechst 33342).

The knockdown of Polo-like Kinase 1 (PLK1) resulted in an altered cell cycle distribution. Data in Figure 4 compares the distribution pattern of cells treated with the PLK1 siRNA SMARTpool and the siGENOME Non-targeting Pool. As expected, the knockdown of PLK1, a key regulator of mitotic progression in mammalian cells, results in an abnormal phenotype: an increase in the 4N population, consistent with an arrest in mitosis. Knockdown of PLK1 also induces poptosis as seen in the cell images and the increase in the < 2N population, as well as an increase in the > 4N population indicating possible necrosis.

Nuclear area analysis

The effect of gene silencing on nuclear area was determined using the Cytoplasm to Nucleus BioApplication. An increase in nuclear area is an indication of cell cycle arrest and results from the need to accommodate the increase in nuclear material. In Figure 5, the black line represents the average nuclear area among cells in control wells and the red and blue lines correspond to two standard deviations above and below the average. Data points above the red line indicate target silencing that results in larger than average nuclear area. Consistent with previous studies demonstrating that mutations in CDC2 result in an arrest in G2[1], silencing of CDC2 resulted in an increase in nuclear area. In addition, silencing of Cyclin A2 (CCNA2) also resulted in an increase in nuclear area. Cyclin A2 (CCNA2) promotes both G1/S and G2/M transitions[2]. The inset in Figure 5 shows images (10x magnification) of cells with larger nuclear area.

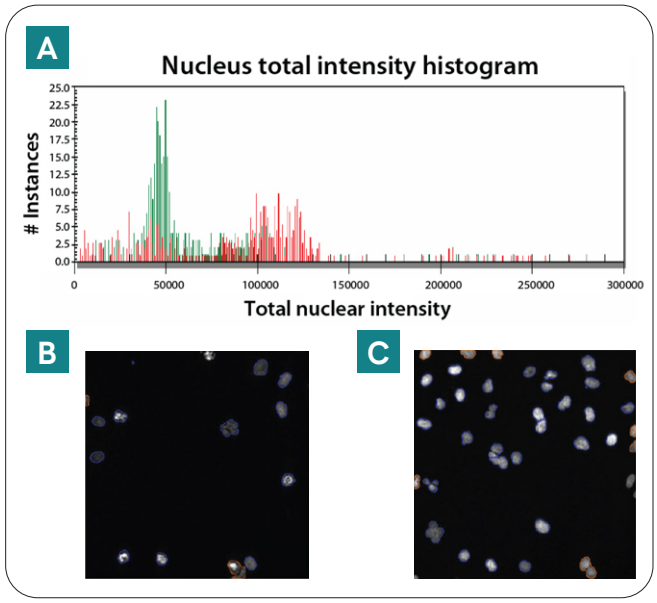


Figure 4: Cell cycle analysis of Non-targeting pool and PLK1 siRNAs. **A.** Cell cycle distribution histogram from the vHCS Discovery Toolbox of Non-targeting Pool (green) and PLK1 SMARTpool reagent (red) where the Y-axis represents the number of instances and the X-axis represents the total nuclear intensity. **B.** PLK1 and **C.** Non-targeting Pool: Nuclear images acquired on the ArrayScan VTI HCS Reader (10x magnification) from the Cell Cycle BioApplication. Nuclei are shown with either blue or red overlays where the blue overlays represent cells selected for analysis and red overlays represent cells rejected from analysis based on user set criteria.

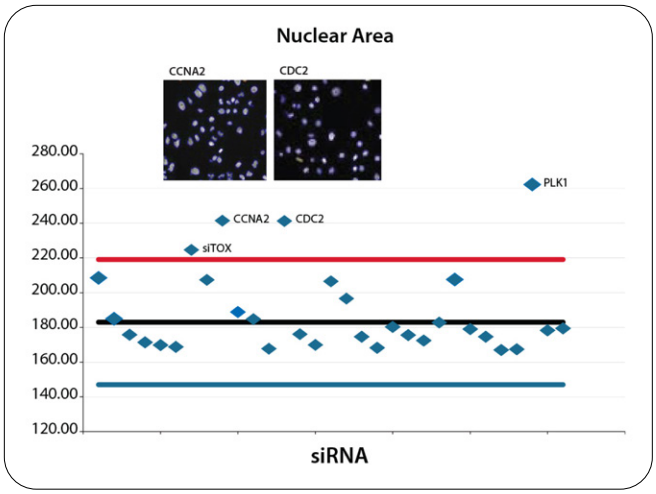


Figure 5: Nuclear area acquired on the ArrayScan VTI HCS reader. The X-axis represents wells transfected with SMARTpool reagents targeting individual genes and the Y-axis represents the mean nuclear area. The black line indicates the average nuclear area of the transfection controls and the red and blue lines correspond to two standard deviations above and below the average. The insets show images of nuclei from cells in which CDC2 or CCNA2 were silenced.

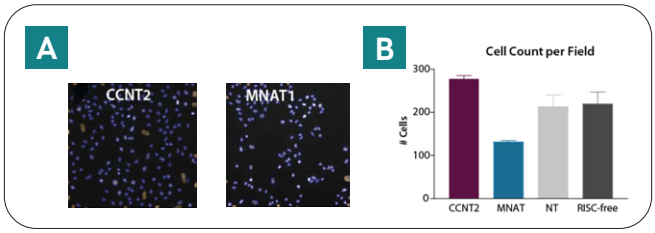


Figure 6: Cell Count analysis with representative images acquired on the ArrayScan VTI HCS Reader (10x magnification) from the Cell Cycle BioApplication. Nuclei are shown with either blue or red overlays where the blue overlays represent cells selected for analysis and red overlays represent cells rejected from analysis based on user set criteria. **B.** Bar graph representing the mean cell number per field with the error bars representing the standard deviation of the mean of replicate wells.

Cell count analysis

The effect of gene silencing on cell proliferation was determined by analyzing the cell count per field output using the Cell Cycle and Cytoplasm to Nucleus BioApplications. Figure 6 compares the representative phenotypes obtained by silencing of CCNT2, a component of positive transcription elongation factor b (P-TEFb) and MNAT1, a component of CDK-activating kinase (CAK). CAK is a multisubunit protein that activates cyclin-dependent kinases and functions in cell cycle progression, basal transcription and DNA repair[3]. Silencing of CCNT2 results in increased cell count while silencing of MNAT1 results in decreased cell count.

Phospho-ERK (pERK)

The Cytoplasm to Nucleus BioApplication can measure the activation and/or subcellular translocation of individual proteins from the cytoplasm to the nucleus in single cells. The BioApplication software is also programmed to analyze images and calculate the difference between fluorescence intensities in the nuclear and cytoplasmic regions on a single cell level and over the population of cells in a well (Figure 7).

Analysis of a downstream phenotypic marker for activation of a signal transduction pathway such as activated pERK was performed using the Cytoplasm to Nucleus BioApplication. ERK is phosphorylated in the nucleus upon activation of the mitogen-activated protein kinase (MAPK) pathways. The images in Figure 8 compare cells in which MCM4 or CDK10 was silenced. MCM4 is required for DNA replication; therefore, a loss of MCM4 does not result in nuclear phosphorylation of ERK (non-activated). CDK10 is a CDC2-related kinase that is proposed to play a role in regulating the G2/M phase of the cell cycle and silencing of CDK10 results in an increased level of phospho-ERK in the nucleus.

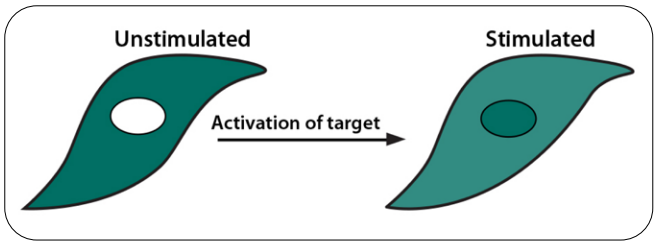


Figure 7: Example of the biology that is measured using the Cytoplasm to Nucleus BioApplication.

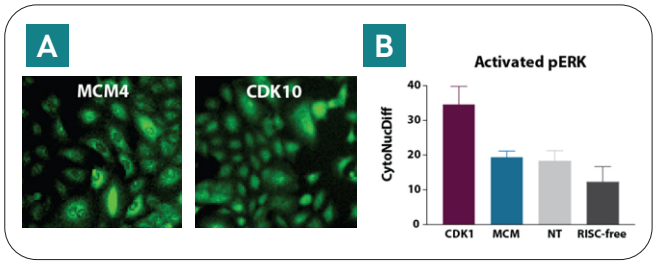


Figure 8: **A.** Composite images (10x magnification) acquired on the ArrayScan VTI HCS Reader from the Cytoplasm to Nucleus BioApplication demonstrate a lack of pERK in the nucleus of cells in which MCM4 is silenced and an increased activated nuclear pERK in cells in which CDK10 is silenced. **B.** Bar graph representing the mean difference between the cytoplasm and nuclear (CytoNucDiff) pERK with the error bars representing the standard deviation of the mean of replicate wells.

Conclusion

The results of this study demonstrate compatibility of SMARTpool siRNA reagents with high-content imaging and anaylsis, and reveal the power of using high content screening to assess multiple phenotypic changes due to specific RNAi-mediated gene silencing. Using highly specific and functional SMARTpool siRNA reagents such as the human siRNA library for cell cycle regulation, we show that cell cycle status and phenotypic changes in cell populations can be easily assayed using HCS instrumentation and software. Silencing of specific cell cycle genes (for example, PLK1) resulted in expected phenotypic changes and demonstrates that the integration of rationally designed siRNA libraries and the HCS platform offers the means for rapid and reliable determinations of gene function and for unprecedented high-throughput strategies.

References

1. G.R. Stark, W.R. Taylor, Analyzing the G2/M checkpoint. *Methods Mol. Biol.* 280, 51-82(2004).

2. M. Pagano, R. Pepperkok, Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11, 961-971 (1992).

3. E.A. Nigg, Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? *Curr. Opin. Cell Biol.* 8, 312-317 (1996).

For more information:

If you have any questions directly related to this document, contact our experts:

UK +44 (0) 1223 976 000
USA +1 800 235 9880, +1 303 604 9499
Website horizondiscovery.com/contact-us

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com