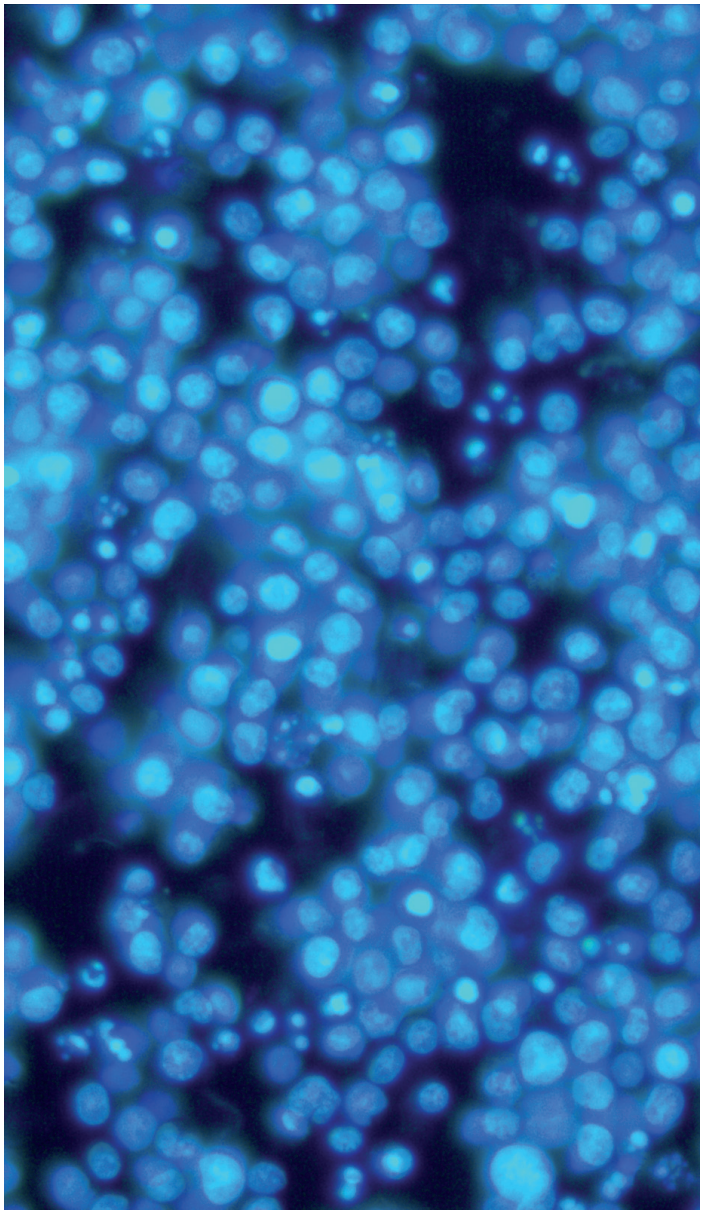
Lonza

White Paper

Efficient Delivery of Thermo Scientific Dharmacon SMARTpool siRNA Reagents in Difficult-to-Transfect Cell Lines Using Nucleofector® 96-well Shuttle® System



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Introduction

siRNA-mediated gene knockdown is a powerful tool that has been used to identify gene function and elucidate biological pathways. Successful siRNA experiments involving knockdown of individual genes or collections of gene targets require efficient delivery of highly functional and specific siRNA molecules into appropriate cells.

While lipid-mediated transfection is a common approach for siRNA delivery, many cell types, including suspension cell lines and primary cells, are not compatible with this technology. These limitations prevent analysis of more biologically-relevant cell types and confine studies to transformed, adherent cell lines that often exhibit phenotypic and genetic alterations after extended periods of culturing. In addition, several of the lipid delivery reagents can cause cytotoxicity and are capable of inducing a potent interferon response.[1] These unintended phenotypes can significantly affect experimental outcomes and drastically interfere with the understanding of a gene's function.

The combination of Dharmacon SMARTpool® siRNA Reagents with Lonza's Amaxa® Nucleofector® Technology overcomes the limitations associated with lipid reagent-mediated transfection. The technology is optimized for transfection of difficult-to-transfect cell types (in particular, primary and non-adherent cell lines) and can be linked to high throughput applications using the 96-well Shuttle® System.

Signature Thermo Fisher Scientific and Lonza

Thermo Scientific Dharmacon - Innovations in RNAi Technologies

- Predesigned, guaranteed siGENOME® siRNA Products to target every human, mouse, and rat gene in RefSeq database
- Next-generation ON-TARGET*plus*® siRNA Reagents reduce off-targets effects by up to 90% for highly accurate results
- All siGENOME® siRNA Products available as individual siRNA or SMARTpool ® Reagents for maximum experimental flexibility
- siGENOME® or ON-TARGET*plus*® siRNA Libraries grouped by pathway or gene ontology for high quality, efficient RNAi screens
- Validated siGENOME® Products to ensure experimental reproducibility and silencing specificity

Lonza's Amaxa® Nucleofector® Technology

- Non-viral Nucleofector® Technology for transfection of difficult-to-transfect cell types, such as suspension cell lines and primary cells
- Up to 99% transfection efficiency with siRNA duplexes, even in suspension cells
- Tested for high viability and maintenance of functionality
- Amaxa® Optimized Protocols for hundreds of cell lines and primary cells (see www.lonza.com/cell-database)
- Proven for siRNA applications with more than 100 publications
- Efficient co-transfection of different substrates, such as plasmid DNA and siRNA duplexes, e.g., for rescue experiments
- 96-well Shuttle® System for high throughput applications, such as siRNA library screening

In this Technical Note, Thermo Fisher Scientific and Lonza technologies were applied to Jurkat cells (clone E6-1, ATCC® TIB-152™) with the aim of performing future siRNA library screens. Jurkat cells are derived from a human acute T-cell leukemia line and are used extensively in the study of T-cell signaling and cancer drug development. As with any suspension cell line, Jurkat cells are difficult to transfect using reagent-mediated delivery (Figure 1).

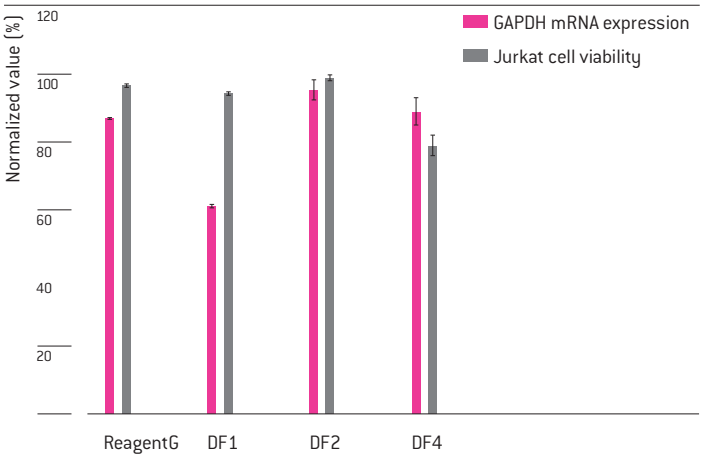


Figure 1. Lipid-mediated transfection of Jurkat cells is ineffective. Jurkat cells were transfected with 100nM GAPDH siRNA using various lipid-based reagents, DharmaFECT® 1, 2 and 4 (DF1, DF2, and DF4) are from Thermo Scientific Dharmacon, while reagent L is from another supplier. Cell viability was determined 24 hours post-transfection using the CellTiter-Blue® Assay (Promega) and normalized to untreated cells. GAPDH mRNA levels were analyzed 24 hours post-transfection by the QuantiGene® Branched-DNA Assay (Panomics) and normalized to siGENOME® Non-Targeting siRNA #1 (Dharmacon).

Critical Parameters for Successful siRNA Experiments

Prior to beginning siRNA experiments using Nucleofection®, multiple parameters associated with experimental design need to be optimized. In particular, the 1) Nucleofection® Conditions (i.e., Nucleofector® Solution and Program), 2) appropriate controls, 3) the most efficacious siRNA concentration and 4) the time point of analysis need to be determined. In addition, identifying the best readout assay for your particular application (i.e., mRNA, protein or phenotypic analysis) is critical. Each of these steps are outlined in Figure 2 and described in detail below.

Choose siRNA
- Select gene target(s)
- Select control siRNA
  - Negative control siRNA - e.g., siGENOME® Non-Targeting Pool
  - Positive control siRNA - e.g., siGENOME® GAPDH siRNA

Choose Cell Type and Transfection Protocol
- Select cell type(s) to maximize physiological relevance of results
- Find Optimized Nucleofection® Protocols at **www.lonza.com/cell-database**
- Select transfection controls
  - Untreated sample (no siRNA and transfection)
  - Mock-transfection (no siRNA, only transfection)

Confirm siRNA Delivery
- Confirm siRNA delivery efficiency using:
  - Fluorescently-labeled siRNA or
  - Fluorescent expression plasmid (e.g., pmaxGFP® Vector) or
  - pmaxGFP® Vector Vector and maxGFP® Reporter Protein siRNA or siRNA targeting housekeeping gene

Choose Detection Assay
- Select detection assay(s)
  - mRNA - branched-DNA, RT-PCR
  - Protein - ELISA, Western, FACS analysis
  - Phenotype - viability, apoptosis

Optimize Target Knockdown
- Determine optimal siRNA concentration
- Nucleofector® Device p0.2 – 200 pmol (2 nM – 2 µM)
- 96-well Shuttle® System p0.04 – 40 pmol (2 nM – 2 µM)

Adapt Assay Conditions
- Optimize detection assay(s) conditions for specific system
- Determine optimal cell densities for linear detection range
- Correlate results from multiple assays

Optimize Assay Conditions
- Perform detection time course or multiple assay
- mRNA p 12 – 72 hours
- Protein p 24 – 96 hours

Confirm specifity of silencing event
- Confirm gene knockdown results with different siRNA reagents
- If using Standard SMARTpool® siRNA Reagents, follow-up with ON-TARGET*plus*® SMARTpool® siRNA Reagents targeting same genes
- If using individual siGENOME® siRNA, use multiple siRNAs targeting same genes
- If possible, perform rescue experiments

Figure 2. Flowchart for successful siRNA experiments.

Optimized Programs for siRNA Delivery with the 96-well Shuttle® System

Lonza provides ready-to-use, cell type-specific kits and protocols for a large number of primary cells and cell lines. These detailed Amaxa® Optimized Protocols can be used in conjunction with either the standard Nucleofector® Device or the 96-well Shuttle® System and are applicable for delivery of any kind of nucleic acid substrate (DNA vectors, ssRNA, siRNA reagents) without any need for further optimization. The constantly growing list of delivery conditions (currently available for over 500 cell types) is referenced in Lonza's Amaxa® Cell Database: (www.lonza.com/cell-database).

The 96-well Nucleofection® Protocol for Jurkat clone E6-1 (ATCC® TIB-152™) recommends the 96-CL-120 Program and 96-well Nucleofector® Solution SE for efficient delivery of siRNAs. We have demonstrated that co-transfection of the ON-TARGET*plus*® SMARTpool® siRNA Reagent targeting maxGFP® Reporter Protein with pmaxGFP® Vector plasmid (Lonza) results in substantial knockdown of maxGFP® Reporter Protein (see Box 2). siRNA duplexes have been shown to be delivered at even greater efficiencies than plasmids.[2,3]

Similarly, co-transfection of a functional siRNA with a plasmid expressing a functional version of the target gene that is resistant to the siRNA can be performed with Nucleofection® to prove specificity of the RNAi phenotype (rescue experiments).

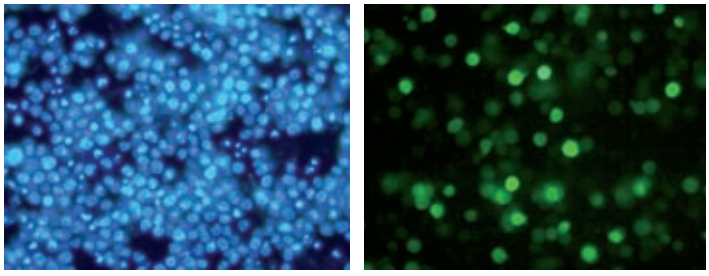
Identification of Appropriate Experimental Controls

To ensure that the conclusions drawn from siRNA experiments are accurate, it is necessary to include the appropriate experimental controls. Thermo Fisher Scientific and Lonza scientists recommend including at least four types of experimental controls in every RNAi experiment: positive and negative control samples, an untreated control sample, and a mock-treated control sample. These controls are described here and should be included in all parametric testing along with the siRNA targeting the gene(s) of interest. The Dharmacon portfolio is composed of both positive and negative control siRNAs in the siGENOME® Line of siRNA control reagents. Parallel testing of multiple controls under several conditions can be easily performed using the 96-well Shuttle® System.

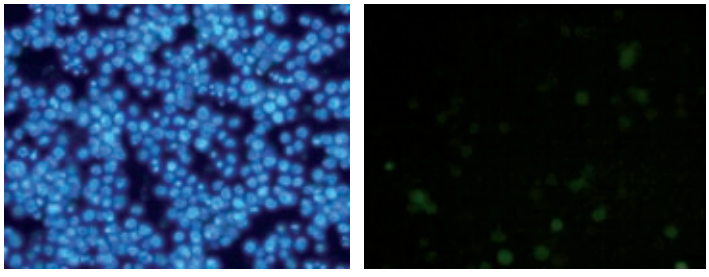
Visual Assessment of Nucleofection® Efficiency with pmaxGFP® Vector

Co-transfection of plasmid in conjunction with siRNA allows for a straightforward analysis of siRNA-mediated gene knockdown. Transfection of pmaxGFP® Vector alone provides a visual indication of transfection efficiency. Co-transfection of pmaxGFP® Vector together with Dharmacon ON-TARGET*plus*® SMARTpool® siRNA Reagent specifically targeting pmaxGFP® Vector provides a rapid and simple assessment of siRNA-mediated knockdown of maxGFP® Reporter Protein.

pmaxGFP® Vector alone



pmaxGFP® Vector and siRNA



Efficient co-transfection of plasmid and siRNA in Jurkat (clone E6-1, ATCC® TIB-152™) leads to significant down-regulation of maxGFP® Reporter Protein. Images were taken 24 hours post Nucleofection® of either pmaxGFP® Vector plasmid alone or plasmid together with the ON-TARGET*plus*® SMARTpool® siRNA Reagent targeting maxGFP® Reporter Protein. Cells were stained with the nuclear dye Hoechst.

1. **Positive control:** This should be a validated siRNA pool or individual siRNA targeting a well-characterized housekeeping gene, such as cyclophilin B (also known as PPIB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or Lamin. A good positive control reagent targeting a well-expressed but non-essential gene is useful for establishing experimental parameters without affecting cellular viability and can also be used as negative control that is unassociated with any particular pathway under study (i.e., it fails to generate a observable phenotype in the assay being employed).

2. **Negative control:** Negative siRNA control reagents are bioinformatically designed to have no known target in the cell line of choice. These reagents are important for distinguishing sequence-specific silencing from sequence-independent effects that are associated with the delivery of siRNA into the cell. Such sequence independent effects can include toxicity resulting from transfection in conjunction with nucleic acid delivery or hyper-sensitivity to introduction of double stranded RNA. Investigators are encouraged to test multiple candidates in their own experimental systems to empirically confirm that the negative controls do not result in any observable and unintended off-target effects.[4, 5] Taking this need into account, Thermo Scientific Dharmacon is a comprehensive portfolio of multiple negative controls, including the ON-TARGET*plus*® Non-Targeting Controls, which have been confirmed by microarray analysis to have little to no off-target signature in HeLa cells.

3. **Untreated transfection control:** The untreated control sample is comprised of cells that have neither been treated with siRNA nor subjected to the transfection process. This control serves as an indicator of baseline cellular activity to which all other conditions can be compared.

4. **Mock-treated control:** The mock-treated control sample is one in which the cells are subjected to the transfection procedure in the absence of siRNA. In the case of Nucleofection®, the cells would be exposed to the Nucleofector® Solution and subjected to the Nucleofection® Procedure in the absence of siRNA. The analysis of mock-treated cells will indicate whether the transfection process results in cytotoxicity or other non-specific effects.

Identifying Optimal Effective siRNA Reagent Concentrations

When performing siRNA-mediated knockdown experiments, it is advisable to conduct a dose-response (concentration) analysis to determine the minimum siRNA concentration necessary for sufficient target knockdown. For Nucleofection® in the 96-well Shuttle® System, the optimal siRNA concentration can range from 0.04 – 40 pmol (2 nM up to 2 µM), depending on multiple factors such as the cell type, and the half-life of the mRNA and/or protein of the gene target.

In the context of our optimization studies with Jurkat cells, Figure 3 shows that even the lowest siRNA concentration tested (0.5 pmol, 25 nM) reduces target gene (GAPDH) transcript levels by >75% with only small effects on cellular viability. Nucleofection® of greater amounts of siRNA confirms that maximal silencing is achieved at 5 pmol (250 nM) and that transfection at these or higher siRNA levels has no additional effects on cell viability.

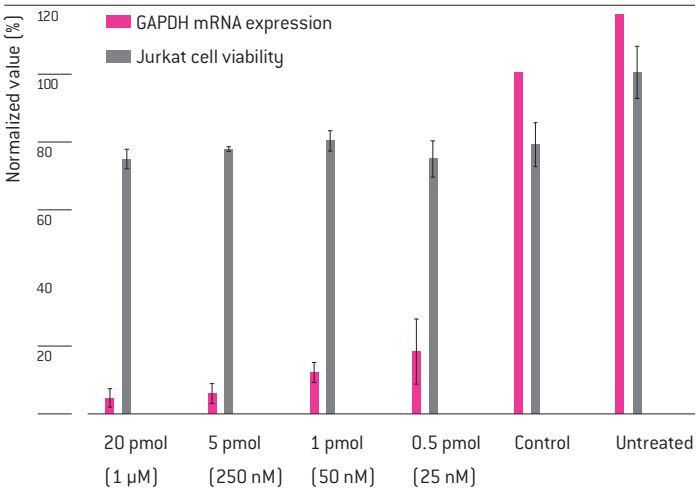


Figure 3. Effect of SMARTpool® siRNA Reagent concentration on GAPDH mRNA levels and cell viability in Jurkat cells clone E6-1 (ATCC® TIB-152™). Cells were transfected by Nucleofection® with various amounts of GAPDH SMARTpool® siRNA Reagent using the 96-well Shuttle® System with Program 96-CL-120. The negative control sample is the Dharmacon siGENOME® Non-Targeting siRNA Pool. Cell viability was determined 48 hours post-transfection using the CellTiter-Blue® Assay (Promega) and normalized to untreated cells. GAPDH mRNA levels were analyzed 24 hours post-transfection by the QuantiGene® Branched-DNA Assay (Panomics) and normalized to siGENOME® Non-Targeting siRNA #1 (Dharmacon).

Optimization of Read-out Assay(s)

Determining optimal analysis time points

As the stability and half-life of various mRNAs and their protein products varies, it is important to empirically determine the best time points for assessing target knockdown. For example, it has been documented that in mammalian cells, mRNA half-life can range from minutes to days, while the T1/2 of protein products can range from less than a few minutes to several days. Taking this into consideration, the experimental design should allow sufficient time for the siRNA to associate with RISC and deplete mRNA/protein concentrations to desired levels.

In general, the recommended time course ranges are 12 to 72 hours (to deplete target mRNA) and 24 to 96 hours to adequately knockdown target proteins and assess phenotypic outcomes. For GAPDH mRNA levels (half-life, 8 hours8) in Jurkats, 24 hours post Nucleofection® was the optimal time point for measuring knockdown (data not shown).

Selecting appropriate phenotypic read-outs

A variety of detection assays may be used to assess cell viability, mRNA levels, and associated phenotypes during the optimization and implementation of a siRNA experiment. Establishing robust assays for RNAi is important for meaningful results. Moreover, multiparametric measurements through the use of several complementary phenotypic assays are particularly helpful in interpreting biological results and performing hit stratification.[5]
We chose a well-defined and robust assay set for our experimentation using the 96-well Shuttle® System: the CellTiter-Blue® Assay (Promega) to indicate cell viability and the Apo-ONE® Assay (Promega) to monitor apoptosis (caspase 3/7 activity). The QuantiGene® Branched DNA Assay (Panomics) was utilized to quantitate transcript levels and correlate target knockdown with biological phenotype. Owing to the high throughput format, the 96-well Shuttle® System facilitates determination of optimal parameters for each of these assays by allowing systematic and parallel testing of several targets and controls under multiple conditions.

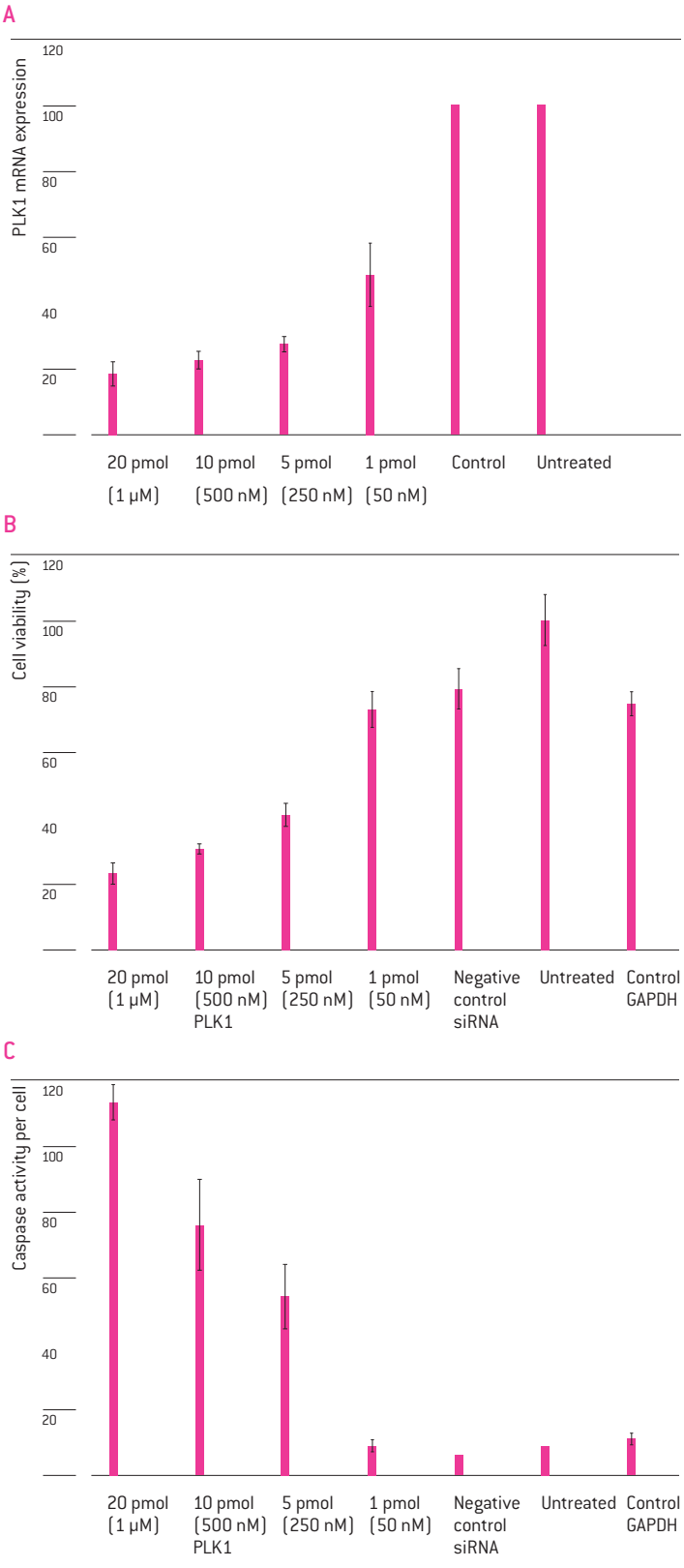


Figure 4. Effect of siRNA concentration on PLK-1 mRNA levels (A), induced cell loss (B) and caspase activity (C) in Jurkat clone E6-1 (ATCC® TIB-152™). Cells were transfected by Nucleofection® with various amounts of PLK-1 SMARTpool® siRNA Reagent using the Nucleofector® 96-well Shuttle® System with Program 96-CL-120. PLK-1 mRNA levels were determined 24 hours post Nucleofection® by Branched-DNA (Panomics) and normalized to siGENOME® Non-Targeting siRNA #1 (Dharmacon). 48 hours post-transfection, cell viability was determined by the CellTiter-Blue® Assay (Promega) and normalized to untreated cells, while caspase activity was determined by the Apo-ONE® (Promega) Assay and normalized to cell number and siGENOME® Non-Targeting siRNA #1 (Dharmacon). GAPDH SMARTpool® siRNA Reagent serves as another negative control.

To optimize assay conditions, Jurkat cells were transfected by Nucleofection® with a SMARTpool® siRNA Reagent targeting Polo-like kinase 1 (PLK-1). PLK-1 is a key regulator of mitotic progression in mammalian cells and the knockdown of PLK1 is known to induce apoptosis in cancer cells.[7, 9] As such, down-regulation of PLK-1 is expected to decrease cell viability and increase caspase 3/7 activity. Initial experiments identified 24 hours post Nucleofection® as optimal for measuring PLK-1 mRNA levels and 48 hours for viability and apoptotic measurements (data not shown).

Figures 4A, B and C demonstrate dose-dependent decreases in transcript levels and correlation with biological phenotype, cell viability and caspase 3/7 activity, respectively. The experiment shows that 5 pmol (250 nM) of siRNA is sufficient to silence ~75% of PLK-1 message and demonstrate a loss in viability and rise in apoptotic activity. Cells transfected by Nucleofection® with GAPDH siRNA (20 pmol, 1 µM) showed similar viability and cellular caspase activity as cells treated with negative control siRNA. While this knockdown of PLK-1 confirms effects described in the literature, it also serves to define experimental parameters for future siRNA screens using PLK-1 as a positive control for cell viability and caspase 3/7 activity in Jurkat cells.

Conclusions

The combination of the highly functional Dharmacon siGENOME® siRNA Reagents and the Lonza's Nucleofector® Technology provides a unique and powerful method for delivering SMARTpool® siRNA Reagents and siGENOME® or ON-TARGET*plus*® siRNA Libraries into cells that have been considered intractable to lipid reagent-based transfection methods. The 96-well Shuttle® System now expands the technology to large-scale screens by expediting assay optimization and enabling high throughput siRNA transfection in cell lines that previously have been inaccessible.

References

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Please note that the Amaxa® Nucleofector® Technology is not intended to be used for diagnostic purposes or for testing or treatment in humans.

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