APPLICATION NOTE

Successful RNA interference experiments in difficult-to-transfect cell lines.

Author

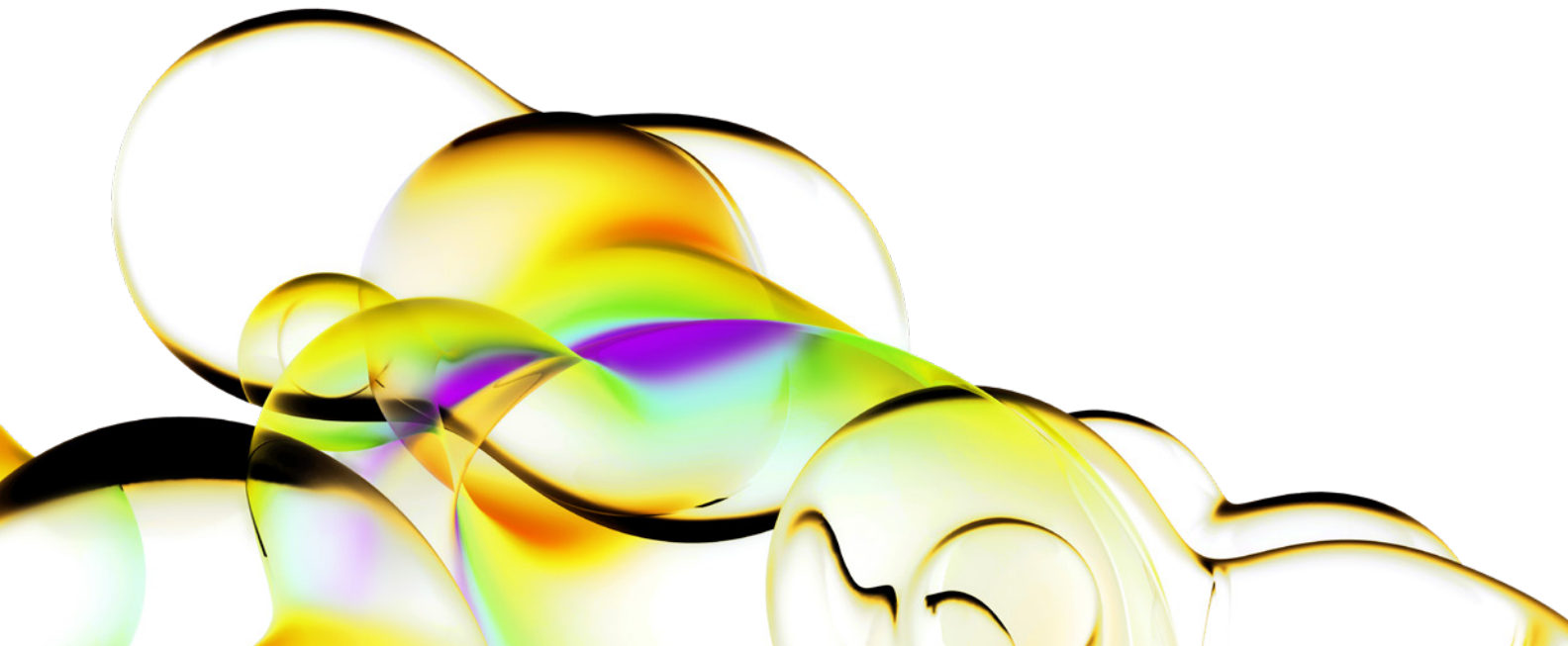
Annaleen Vermeulen

Revvity, Inc.

Successful gene silencing experiments that help clarify a biological pathway rely on good experimental planning and design. The main components of a well-planned experiment include optimal delivery of the functional silencing reagents into a cell type and accurate confirmation of gene knockdown. RNA interference (RNAi) silencing methods borrow an elegant endogenous biological pathway that can be applied to modulate gene expression thus permitting the detailed study of a particular gene target, a defined biological pathway or a high-throughput screen of an entire genome. By this one method, a researcher can characterize a specific phenotype or disease state. Regardless of the experimental approach (targeted knockdown or high-throughput screen), RNAi experiments can be described with a general workflow that includes[1] identification of functional siRNAs,[2] optimization of siRNA delivery, followed by[3] detection of gene down-regulation and finally,[4] investigation of the biological phenotype (Figure 1).

Delivery solutions

Early hurdles of finding potent siRNAs have essentially been eliminated with the availability of rational design methods for functional siRNAs as with the Dharmacon™ SMARTselection™-designed siRNAs. However, delivery of these functional siRNAs remains a significant challenge in certain cell types. Lipid-based transfection and electroporation are widely utilized, well-validated methods for many standard cell lines. Unfortunately, these methods are often inefficient for more biologically relevant model systems represented by cell types that are refractory to traditional transfection strategies.



For example, human suspension (hemapoetic cell lines) and neural cell types, of interest in immune and neurological studies, are typically intractable to standard lipid-mediated siRNA delivery. A variety of alternative vehicles or delivery modes have been developed specifically for these difficult to-transfect cell types. Revvity's Dharmaconl™ Accell™ siRNA technology represents one such innovation that successfully combines bioinformatics and chemical modification strategies to provide functional and specific siRNAs that are enhanced for delivery in the absence of lipid. Accell siRNA represents a new delivery technology to empower RNAi experiments in many difficult-to-transfect cell types, without transfection reagents, viral vectors or instrumentation. Accell siRNAs are shown to be effective in a wide range of human, mouse and rat cell types that have been traditionally identified as difficult-to-transfect by conventional lipid delivery methods.

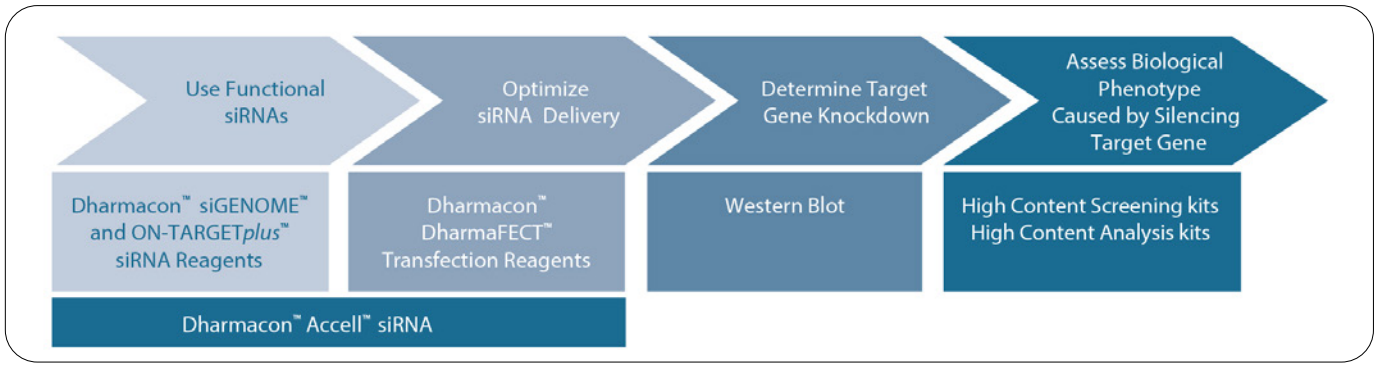


Figure 1: Workflow for successful RNAi gene silencing experiments

Detection solutions

Detection of the level of gene knockdown represents yet another challenge during the optimization of RNAi experiments. Reverse transcription quantitative real-time PCR (RT-qPCR) represents the most commonly recommended method for mRNA detection. While often perceived as an easy, quick and reliable process, RT-qPCR methods often suffer from variability and poor reproducibility. The Thermo Scientific™ Verso™ QRT-PCR SYBR Green kits incorporate a unique blend of high performance reaction components optimized to eliminate variability in detection—giving reliable and consistent mRNA level assessments.

Here we provide a step by step description of an experimental workflow that combines the use of functional siRNAs enhanced for delivery and potent target knockdown and a robust method for determining gene expression levels (Figure 2). Specifically, we highlight: (1) Delivery of Accell control siRNAs into two difficult-to-transfect cell lines: SH-SY5Y (adherent neuroblastoma cells) and Jurkat (suspension T-cells), and. (2) Detection of target gene knockdown using a Verso qRT-PCR SYBR Green kit.

Workflow and result

Accell application in neuroblastoma cells and T-cells

Delivery optimization is a balance between potent knockdown and the maintenance of cell viability. Application of Accell siRNA to your cell line of interest is relatively straightforward (Figure 2) and is described in more detail in the Accell siRNA Protocol (horizondiscovery.com/en/gene-modulation/knockdown/sirna/accell-sirna). Briefly, cells were either pre-plated in their specific growth medium the day before (SH-SY5Y) or just prior to Accell transfection in Accell Delivery Media (Jurkat) in 96-well formats at densities optimized for growth conditions, viability, and gene knockdown assays (see Materials and Methods for details). Target-specific, positive, and negative control Accell siRNAs were each reconstituted to 100 µM stocks in 1x siRNA buffer, (diluted from 5x siRNA buffer; Dharmacon, Cat #B-002000-UB-100). For delivery, the Accell siRNA stocks were mixed with Accell Delivery Media (Dharmacon, Cat #B-005000-100) and added to cells at a final concentration of 1 µM in 100 µL volume per 96-well. Delivery, viability and gene knockdown was determined after a 72 hours incubation period.

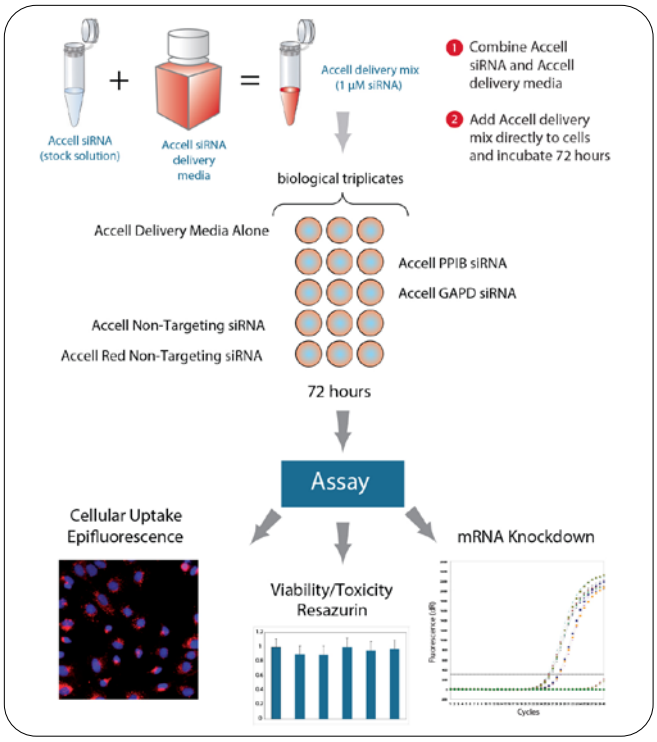


Figure 2: Experimental workflow for Accell siRNA-mediated gene silencing and detection of mRNA knockdown.

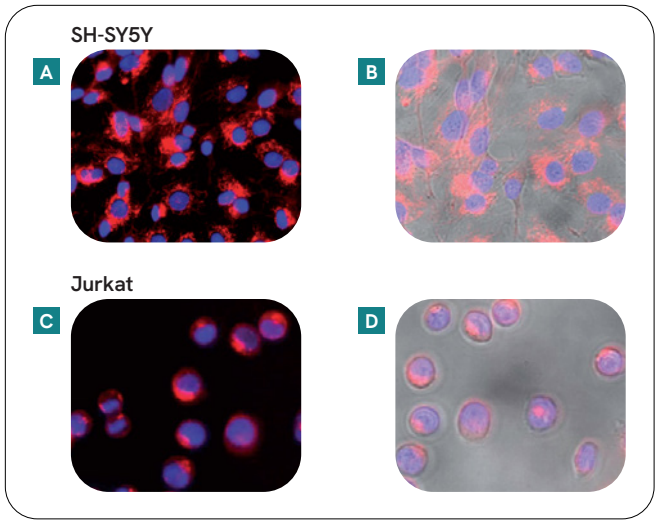


Figure 3: **Cellular Uptake of Accell Red Non-targeting siRNA in SH-SY5Y and Jurkat Cells.** Accell Red Non-targeting siRNA was delivered to SH-SY5Y (A, B) and Jurkat (C, D) cells. Cellular uptake (red) in live cells was visualized using epifluorescence microscopy. Nuclei were stained with Hoechst 33342 (blue). Accell Red Non-targeting siRNA localizes in the cytoplasm of both cell types. Images (A) and (C) are merged epifluorescence micrographs of Accell Red Non-targeting siRNA and Hoechst 33342. Images (B) and (D) are epifluorescence micrographs merged with phase contrast micrographs.

Qualitative visualization of Accell siRNA cellular uptake

A qualitative assessment of delivery can be made throughout the 72 hours incubation period. Uptake of dye-labeled Accell Red Non-targeting siRNA into cells was visualized by fluorescent microscopy. Accell siRNA accumulates mainly in the cytoplasm of the cells and is observed as early as 24 hours* but increases and persists by 72 hours (Figure 3).

*Note: While uptake of dye-labeled siRNA is observed 24 hours post delivery, the optimal time point for detecting mRNA knockdown is usually 72 hours post delivery.

Assessment of cell viability

It is important to determine whether the cell line was adversely affected by Accell application using a cell viability assay. Although Accell siRNAs do not induce the gene responses that are common with lipid-mediated delivery, it is always necessary to see if a delivery method or medium condition is compatible with your biological system. In this report, cell viability was assessed using a method that measures the reduction of resazurin (non-fluorescent blue to rusorufin (fluorescent pink) based on the metabolic capacity of the cells. Accell siRNA delivery had little to no effect on viability as compared to untreated cells for both SH-SY5Y and Jurkat cells (data not shown). All experiments described here showed cell viability of > 95% compared to untreated cells.

Workflow and results

Relative quantification of mRNA with Verso™ SYBR Green qRT-PCR Kits

While the ultimate goal for RNAi experiments is to assess affects on phenotype, the level of gene silencing must be determined to demonstrate working experimental design. Gene knockdown can be determined by both mRNA and protein detection, although the standard for detection of gene silencing is RT-qPCR.

Detection results of target mRNA knockdown by RT-qPCR are shown in Figures 4 and 5. Amplification curves for both target (PPIB or GAPD and housekeeping (ACTB) genes are presented. Successful delivery of Accell GAPD siRNA resulted in robust silencing of GAPD expression. GAPD expression was silenced 95% in SH-SY5Y and 93% in Jurkat cells. Furthermore, delivery of Accell PPIB siRNA into either cell line also resulted in knockdown of PPIB mRNA expression. PPIB expression was reduced by 75% in SH-SY5Y and 60% in Jurkats. In both SH-SY5Y and Jurkat cells there was no significant effect on the expression of the non-targeted housekeeping gene, ACTB, in either control or target wells.

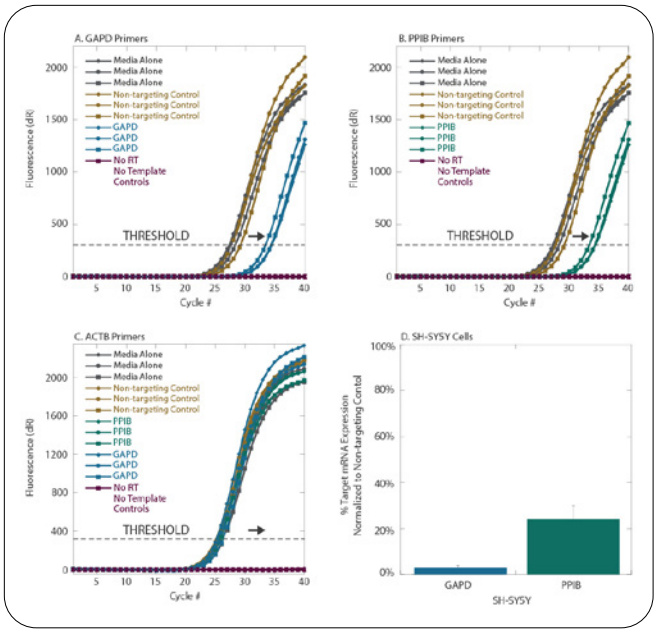


Figure 4: **RT-qPCR quantification of Accell siRNA Gene knockdown in SH-SY5Y Cells.** Experiments were performed in biological triplicate for all samples. (A) qPCR amplification curves detecting GAPD in all samples. (B) qPCR amplification curves detecting Cyclophilin B (PPIB) in all samples. (C) qPCR amplification curves detecting ACTB in all samples. (D) Calculated results of GAPD and PPIB knockdown in SH-SY5Y cells normalized to housekeeping gene (ACTB) and Non-targeting control #1.

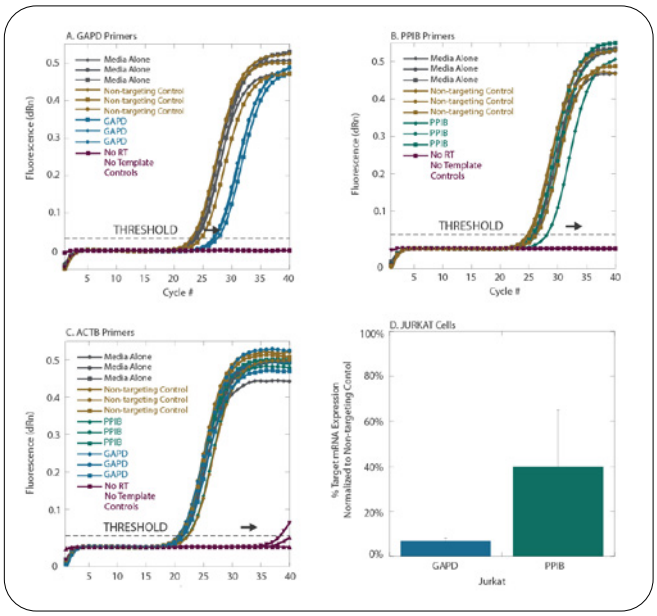


Figure 5: **RT-qPCR Quantification of Accell siRNA Gene Knockdown in Jurkat Cells.** Experiments were performed in biological triplicate for all samples. (A) qPCR amplification curves detecting GAPD in all samples. (B) qPCR amplification curves detecting Cyclophilin B (PPIB) in all samples. (C) qPCR amplification curves detecting ACTB in all samples. (D) Calculated results of GAPD and PPIB knockdown in Jurkat cells normalized to housekeeping gene (ACTB) and Non-targeting control #1.

Summary

The ultimate goal of RNAi experiments is to investigate the biological impact of target gene knockdown in a particular biological system. However, significant and relevant results depend on a number of factors that we have addressed in this basic workflow including delivery, detection and analysis. Of these, optimal delivery of functional siRNAs and accurate confirmation of gene knockdown remain paramount for correctly interpreting and assigning functional roles to specific genes. After optimizing siRNA delivery and the parameters to assess mRNA knockdown in a given cell type, researchers are able to confidently proceed to the study of the resulting biological phenotype through high content or endpoint assays.

Materials and methods

Accell siRNA delivery

SH-SY5Y cells were plated at 10,000 cells per well in a 96-well plate and allowed to adhere overnight. Growth medium for SH-SY5Y cells was removed by aspiration. Prior to transfection, SH-SY5Y cells were washed in Accell Delivery Media as serum has been identified as a growth medium component that interferes with efficient Accell siRNA uptake. Accell siRNA was added to Accell Delivery Media resulting in a final concentration of 1 µM. 100 µL of the Accell siRNA and Delivery Media mixture per well was then added to SH-SY5Y cells in a 96-well plate. Jurkat cells were plated at 30,000 cells in 50 µL of Accell Delivery Media per well in a 96-well plate on the day of delivery. Accell siRNA was added to Accell Delivery Media resulting in a final concentration of 2 µM. 50 µL of the Accell siRNA and Delivery Media mixture per well was then added to Jurkat cells in a 96-well plate. Cells were incubated for 72 hours at 37 °C and 5% CO_2. A sample 96-well plate map is shown in (Figure 2). It is highly recommended to have biological triplicates for each Accell siRNA and controls. Optimal delivery and siRNA knockdown is observed 72 hours after the original application of Accell siRNAs.

Table 1: Dharmacon Accell siRNA reagents

Accell control siRNA kits	Species	Cat #
Accell control siRNA kits (Red)	H	K-005000-R1-01
	M	K-005000-R1-02
	R	K-005000-R1-03
Accell positive control reagents	**Species**	**Cat #**
Accell cyclophilin B control siRNA	H	D-001920-01
	M	D-001920-02
	R	D-001920-03
Accell GAPD control siRNA	H	D-001930-01
	M	D-001930-02
	R	D-001930-03
Accell red cyclophilin B control siRNA	H	D-001975-01
	M	D-001975-02
	R	D-001975-03
Accell negative control reagents	**Species**	**Cat #**
Accell red non-targeting siRNA	—	D-001960-01
Accell control siRNA kits	**Species**	**Cat #**
Accell control siRNA kits (Red)	H	K-005000-R1-01
	M	K-005000-R1-02
	R	K-005000-R1-03

Viability assay

Resazurin was added to cells at a final concentration of 25 µg/mL 72 hours post-delivery. Cells were returned to the incubator for 1 to 3 hours. Plates were read on a Wallac™ VICTOR 2 (Revvity Life Sciences) plate reader (Excitation 530 nM, Emission 590 nM and 1 second exposure).

Microscopy

Although it is possible to observe Accell Red siRNA fluorescence directly in experimental wells there is a large amount of background fluorescence due to auto-fluorescence of the medium. It is recommended to replace medium with pre-warmed 1x PBS with or without a nuclear counterstain dye (Hoechst 33342) to clearly visualize uptake. Briefly, 72 hours post-delivery cells were washed once with 1x PBS and then incubated for 5-10 minutes with 1x PBS plus Hoechst 33342 (1 µg/mL) for staining of the nuclei. Epifluoresence was visualized with a Leica™ DM IL Microscope (Meyer Instruments™). Excitation/Emission maxima for Hoechst 33342 is 350/461 nM and for Accell Red is 557/574 nM. Photos were taken with SPOT Insight™ camera and software (Diagnostic Instruments Inc.).

mRNA detection by RT-qPCR

Primers were designed using Primer3 software (frodo.wi.mit.edu) and synthesized by Operon. The sequences are listed in Table 2. All primers were tested for technical reproducibility (standard deviation +/- 0.3 Cq), dynamic range (50 ng to 80 pg total RNA input) and amplification efficiency (> 90% and 110%; data not shown). It is recommended for researchers to optimize all primers before assessing percent gene silencing. Cells were harvested for RNA purification (Qiagen™ RNeasy™ Plus) 72 hours post-delivery. Average amount of RNA purified from each well was ~ 50 ng total.

Verso™ SYBR Green 1- and 2- Step qRT-PCR Master Mixes were used in a final reaction volume of 25 µL. Gene-specific primers with a final concentration of 87.5 nM and ~ 15 ng of template were added to each reaction. 1- Step RT-qPCR cycling was performed in a Stratagene™ Mx3005P thermocycler. The thermal cycling program consisted of: 50 °C for 30 minutes (1 cycle); 95 °C for 15 minutes (1 cycle); 95 °C for 15 seconds/60 °C for 30 seconds/72 °C for 30 seconds (40 cycles); plus a melt curve profile.

Table 2: Primer pairs for each target

Target (human)	Accn No.	Forward (5'→3')	Reverse (5'→3')
beta-Actin (ACTB)	NM_001101	tggacatccgcaaagacctg	ccgatccacacggagtactt
Cyclophilin B (PPIB)	NM_00942	gatggcacaggaggaaagag	agccaggctgtcttgactgt
Glyceraldhyde-3-phosphate dehydrogenase (GAPD)	NM_002046	tgcaccaccaactgcttag	ggatgcagggatgatgtt

For 2-Step RT-qPCR, the RT thermal cycling program consisted of 42°C for 30 minutes (1 cycle); 95 °C for 2 minutes (1 cycle); 4 °C hold, and the thermal cycling qPCR Program was 95 °C for 15 minutes (1 cycle); 95 °C for 15 seconds/60 °C for 30 seconds/72 °C for 30 seconds (40 cycles); plus a melt curve profile.

The ΔΔCt method[1] was used to calculate relative expression. The housekeeping gene, beta-Actin, was used to normalize gene expression among wells. Expression levels were further normalized to non-targeting control and are reported as a percentage of the non-targeting control expression level.

References

1. Bustin, Stephen A. A-Z of Quantitative PCR. LaJolla: *International University Line,* 2004.

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

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