Application Note

Dharmacon™
RNAi, Gene Expression & Gene Editing

Reverse Transfection Format (RTF): a Rapid Method for RNAi-based High-throughput Studies of Biological Pathways

Abstract:

RNA interference (RNAi) screens are easier and more efficient with Dharmacon™ RTF™ small interfering RNA (siRNA) libraries, which combine optimized broad-spectrum transfection reagents and potent Dharmacon™ SMARTpool™ siRNAs into simple, assay-ready and automation-compatible formats.

Introduction:

The availability of siRNA collections targeting related genes offers the possibility of high-throughput studies; however, managing large-scale transfection of siRNA reagents is very time consuming and costly. To facilitate high-throughput analyses, a strategy was developed to streamline the transfection workflow, permitting rapid and economical screening. RTF siRNA libraries are SMARTpool siRNA reagents predispensed in a multiwell cell culture plate that is ready for resuspension and immediate use. The RTF siRNA libraries contain preselected groups of rationally designed SMARTpool siRNA reagents targeting genes confirmed to be relevant to a particular pathway or to be phylogenetically related to the indicated gene family as defined by the Gene Ontology Consortium (geneontology.org).

The distinguishing feature of RTF is that the SMARTpool reagents are provided in a pre-aliquoted format, such that cells are plated simultaneously into wells containing the individual SMARTpool silencing reagents rehydrated with a lipid-medium mixture. Here we illustrate this novel transfection strategy in the functional analysis of targets implicated in clathrin-mediated endocytosis (CME) demonstrating RTF siRNA Libraries as valuable tools for robust and reliable high-throughput screens.

Delivery: conventional transfection versus reverse transfection formats:

Conventional transfection, also known as forward transfection (FT), is the most common technique for delivering siRNA into cells for gene silencing. This standard approach involves preplating cells ~ 1 d before treatment. On day 2, target-specific siRNA is complexed with a transfection reagent for delivery into the cells. Silencing is then assessed 24 to 48 hours later (or more) depending on the target and the detection method. For multiple targets or high-throughput strategies, conventional FT requires significant handling of individual samples, such that screens with hundreds or thousands of genes quickly become laborious and cost prohibitive.

To overcome this challenge, a new reverse transfection format (RTF) was developed. RTF differs from FT by virtue of streamlined transfection preparation and setup (Figure 1). The SMARTpool reagents are pre-aliquoted as a RTF library in 96-well plates, supplied in qudruplicate and ready for a one-time transfection of 50 µM siRNA into the cells of interest. Each plate also contains three negative and three positive control siRNAs and is shipped complete with Dharmacon™ DharmaFECT™ Transfection Reagent and DharmaFECT Cell Culture Reagent (DCCR). Following a simple protocol, the SMARTpool reagents are rehydrated in each well with a mixture of DCCR and DharmaFECT Transfection Reagent. After a short incubation period (30-90 minutes), cells are added to each well and incubated under standard conditions. Silencing is then assessed using an assay appropriate for the pathway of interest.

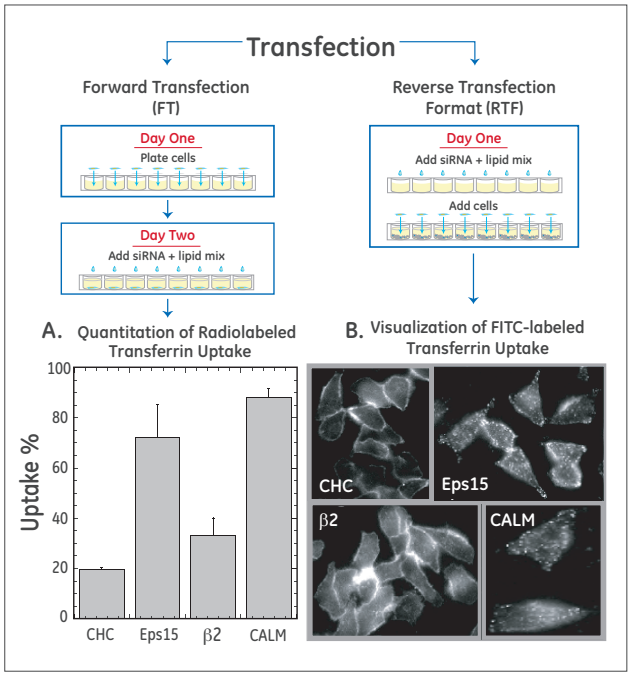

Figure 1. Workflow comparison of FT and RTF, illustrating the labor- and reagent saving features. **A.** FT siRNA-mediated silencing of specific CME genes affects internalization of radiolabeled Transferrin. **B.** RTF recapitulates phenotypes observed by FT of siRNAs targeting a colletions of genes involved in CME. Fluorescent images of FITC-transferrin internalization in HeLa cells treated with target-specific siRNAs.

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Under the conditions just described, RTF achieves efficient and specific target knockdown with minimal effect on cell viability, and at lower overall concentrations of transfection reagent and siRNA relative to FT. By eliminating complex and time-consuming manual processing, RTF reduces handling to merely rehydrating the pre-aliquoted SMARTpool siRNAs and adding the cells. Thus, RTF siRNA libraries minimize the potential for exposure to ribonucleases and provide means for reliable and robust targeted gene knockdown.

Application and experimental format:

To demonstrate the usefulness of the RTF approach, it was compared to FT in a functional analysis of genes involved in the CME pathway. CME is an important process in higher eukaryotes for the internalization of nutrients, macromolecules, viruses and plasma membrane proteins from the extracellular environment[1] and is of interest to those studying host-viral pathogen interactions.

In previous work, 13 genes (CHC, $ß_2$-adaptin, dynamin II, CALM, Eps15, Eps15*R*, epsin, EEA1, Rab5a, Rab5b, Rab5c, CLCa and CLCb) were targeted in HeLa cells by SMARTpool reagents using conventional FT. These targets were chosen because of their purported involvement in CME and the availability of antibodies for detection[2]. The consequence of RNAi-mediated gene knockdown was assessed by western blot analysis, and, where antibodies were lacking, by monitoring expression levels of yellow fluorescent protein-tagged constructs. The effect of targeted gene knockdown was also assessed quantitatively by monitoring the internalization of radiolabeled transferrin ([^{125}I]Tfn) (Figure 1A**)**. Tfn is a ligand that, upon binding to its receptor, is endocytosed constitutively through clathrin-coated pits. In all cases, the corresponding SMARTpool reagent and at least one of the individual siRNAs reduced protein levels by > 90% (all SMARTpool reagents and at least three of four individual siRNAs reduced protein levels by 75%)[2]. It was determined that the analysis of an expanded set of 44 genes involved in CME (Table 1) represented a suitable test case for the feasibility of RTF-based delivery and silencing. The corresponding Dharmacon RTF siRNA collection was rehydrated, and then HeLa cells were plated at a density of 15,000 cells/well. Uptake of fluorescein isothiocyanate-labeled Tfn (FITC-Tfn) was assessed in a short time-course assay at 37 °C to avoid the contribution of recycled ligand. The resulting phenotypes, determined by the presence or absence of labeled vesicles (endosomes) and labeled plasma membrane, were scored by visualization of FITC-Tfn internalization **(**Figure 1B**)**. For example, siRNA-mediated silencing of Eps15 had no effect on the endocytosis of FITC-Tfn, whereas knockdown of CHC and $ß_2$-adaptin resulted in strong visual phenotypes (Figure 1B). These results were consistent with those of FT, demonstrating the reproducibility of RTF siRNA libraries in rapid screens of large gene families.

Table 1. Collection of SMARTpool reagents targeting 44 genes implicated in CME.

AMPH	ARRB2	CLTC	HIP1	RAB11B	SYNJ1
AP1M1	CAV1	DNM2	HIP1R	RAB4A	SYNJ2
AP1M2	CAV2	DAB2	ITSN1	RAB4B	SYT1
AP2A1	CBL	EEA1	NEDD4	RAB5A	SYT2
AP2B1	CBLB	EPN1	NEDD4L	RAB5B	
AP2M1	CBLC	EPS15	NSF	RAB5C	
ARF6	CLTA	EPS15L1	PICALM	H3GLB1	
ARRB1	CLTB	GRB2	RAB11A	SH3GLB2	

Conclusions:

The application of RNAi-mediated silencing in high-throughput functional analyses can be a laborious endeavor by virtue of the number of samples to be processed. RTF siRNA Libraries represent an important advance in critical screening tools that combine optimized broad-spectrum transfection reagents and potent SMARTpool siRNAs into simple, assay-ready and automation-compatible formats. Depending on the screening assay, a screen of hundreds of targets could be completed within a matter of days.

As described here, RTF application distinguishes itself by permitting efficient transfection and rapid screens. The functional analysis of genes involved in CME was consistent with that achieved by FT, thus RTF providesa cost-effective strategy for quick, reliable high-throughput screening and target validation methods.

References:

1. C. Le Roy, J. Wrana, Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat. Rev. Mol. Cell Biol.* **6**, 112–126 (2005).
2. F. Huang, A. Khvorova, Analysis of clathrin-mediated endocytosis of EGF receptor by RNA interference. *J. Biol. Chem.* **279**, 16657-16661 (2004).

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