BioResearch

Lonza

Designing an RNAi Experiment Using Nucleofection™

Technical Reference Guide

The Nucleofector™ Technology is well suited for the transfection of siRNA duplexes or shRNA vectors into both primary cells and difficult-to-transfect cell lines.

Choose siRNA

- Select gene target(s)
- Select control siRNA
  - Negative control siRNA – e.g., Thermo Scientific siGENOME Non-Targeting Control
  - 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 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 → 0.2 – 200 pmol (2 nM – 2 µM)
  - 96-well Shuttle™ Device → 0.04 – 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 assays
  - mRNA → 12 – 72 hours
  - Protein → 24 – 96 hours

Confirm Specificity of Silencing Event

- Confirm gene knockdown results with different siRNA reagents
  - If using Thermo Scientific siGENOME SMARTpool siRNA Reagents, follow-up with Thermo Scientific 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

1. Establish/Verify Nucleofection™ Conditions with pmaxGFP™ Vector

Optimal Nucleofection™ Conditions for a particular cell type are identical whether you are transfecting DNA or RNA. We recommend performing a preliminary experiment with pmaxGFP™ Vector (our positive control plasmid, included in every kit) in order to establish/verify the optimal Nucleofector™ Solution and Program for your cells. Once these conditions have been determined, they remain the same whether you are transfecting DNA or RNA (or both together).

2. Identify Appropriate Experimental Controls

To make sure that the conclusions drawn from siRNA experiments are accurate, it is necessary to include the appropriate experimental controls. We recommend including at least four types of experimental controls in every RNAi experiment. Parallel testing of multiple controls under several conditions can be easily performed using the 96-well Shuttle™ System.

a) **Positive siRNA 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 aff ecting cellular viability. It can also be used as negative control that is not associated with any particular pathway under study (i.e., it fails to generate an observable phenotype in the assay being employed).

b) **Negative siRNA Control**
Negative siRNA control reagents are bioinformatically designed and validated 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 the process of transfection in conjunction with nucleic acid delivery or hypersensitivity 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. For that purpose, Thermo Fisher Scientific offers 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.

c) **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.

d) **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.

3. Fine Tune Specific siRNA Sequence/Concentrations

Using the same Nucleofection™ Conditions, simply substitute siRNA for the pmaxGFP™ Vector used in the preliminary experiment. We often include pmaxGFP™ Vector (either in a separate parallel sample or co-transfected by Nucleofection™ with the siRNA) as an easy means of comparing relative transfection efficiencies between experiments or selecting transfected cells. The transfection efficiency using DNA is usually substantially lower as compared to siRNA. If you are interested in using fluorescently labeled oligonucleotides, please first read our suggestions for working with these substrates or contact our Scientific Support
Team for specific suggestions.

a) **Selection of an Optimal siRNA Sequence**
If you are using siRNA sequences which have not been previously characterized, we recommend investing a considerable amount of time in their selection. The majority of siRNA providers offer an oligonucleotide optimization service, however, it is still often necessary to test several gene-specific siRNA oligonucleotides in order to find one which efficiently downregulates your target gene.

b) **Determination of the Optimal Effective siRNA Concentration**
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 on mRNA, protein or functional level.

For Nucleofection™, the optimal siRNA concentration can range from lower than 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. To determine the optimal concentration for your cell type and target, we suggest performing an initial titration of the siRNA concentration within the range of 2 nM – 2 µM (Nucleofector™ Device: 0.2 – 200 pmol in 100 µl; 96-well Shuttle™ Device: 0.04 – 40 pmol in 20 µl). Starting concentrations for a minimum titration would be 30 and 300 nM. If looking at concentrations, these values may seem higher than with lipid-based methods, but it is important to remember that Nucleofection™ occurs in a 5 x to 25 x lower volume (20 µl with 96-well Shuttle™ Device vs. 100 µl with 96-well lipofection; 100 µl standard Nucleofector™ Device vs. 1 – 2.5 ml with 6-well lipofection).

siRNA Concentration	Cell Type	Targets/Analysis Method	Knockdown	Reference
2 nM (0.2 pmol*)	COS-7 (monkey kidney fi broblast)	Bruton's tyrosine kinase / FACS	**96%**	Lindvall JM *et al.* (2005) Immunol Rev 203, 200-215.
7 nM (0.7 pmol*)	THP-1 (human monocytic leukaemia)	Interferon Regulatory Factor (IRF5) / RT-PCR	**»strongly reduced«**	Schoenemeyer A *et al.* (2005) J Biol Chem 280, 17005-17012.
1 nM (0.1 pmol*)	HUVEC (human umbilical vein endothelial cells)	Interferon Integrins 1 / 3 and Akt / Western Blot Migration	**> 90%**	Short SM *et al.* (2005) J Cell Biol 168, 643-653.

* Per cuvette (100 ml volume)

Table 1: Using low siRNA concentrations with Nucleofection™

The optimal effective siRNA concentration is dependent on the target and the cell type. Indeed, there are numerous publications in which Nucleofection™ of < 50 nM siRNA has been observed to elicit knockdown of the desired genes (see Table).
Some customers have also reported satisfying results with concentrations higher than 1 µM, but it is important to keep a balance between efficient knockdown and minimizing off-target effects. Although keeping siRNAs <30nt avoids activating the protein kinase (PKR) and 2',5'-oligoadenylate synthetase pathways, siRNAs have still been demonstrated to elicit non-specific effects, including both stimulation and repression of non-target genes[1].

4. Determine Optimal Analysis Time Point

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[2] 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 5 – 72 hours to deplete target mRNA and 24 – 96 hours to adequately knockdown target proteins and assess phenotypic outcomes.

5. Verification of siRNA Specificity

Keeping siRNA concentrations as low as possible helps to minimize non-specific effects, but it is also important to include appropriate controls in all experiments[3]. Monitoring gene knockdown at both the mRNA and protein levels verifies that the siRNA sequence is acting through the classical RNAi pathway, rather than as a microRNA (which, at least in part, inhibit translation of target mRNA, rather than targeting its destruction).
A good way to enhance confidence in RNAi data is to demonstrate a similar effect with two or more siRNAs targeted to different sites in the RNA transcript of interest. The rescue experiment as ultimate control: As suggested in the *Nature Cell Biology*[3] editorial, the control of choice for any RNAi experiment is rescue by expression of the target gene in a form refractory to the siRNA, usually achieved by utilizing one or more silent third codon point mutations or a deletion in the untranslated region within the targeted sequence. Translational effects can be avoided by using siRNAs targeted against 3'-untranslated regions. In practical terms, this means either co-transfecting the siRNA and a plasmid expressing the siRNA-resistant form of the target gene together, or using an shRNA-expression vector which co-expresses the siRNA-resistant target gene. Fortunately, the ability to transfect DNA and RNA using identical Nucleofection™ Conditions means that both of these types of experiments are quite straight forward and easy to perform using the Nucleofector™ Technology.

6. Additional Notes

Measuring transfection efficiency

a) Using Fluorescently-labeled siRNA

Experiments with fluorescently-labeled siRNAs have shown transfection efficiencies of up to 99% in some cell types. Unless a confocal microscope or FACS is available, the use of fluorescently-labeled siRNA for initial setup experiments is not advisable, as many fluorescent labels fade quickly following Nucleofection™. Likewise, the amount of fluorescently-labeled siRNA needed in order to adequately visualize fluorescent cells is often much higher than would be optimal for functional response, making this both an expensive and not highly informative experiment. Furthermore, microscopic analysis may lead to false positive results as a result of siRNA sticking to the membrane and not actually entering the cell.

We usually suggest including a sample transfecting pmaxGFP™ Vector in parallel to (or in the same sample as) the siRNA in order to provide a general estimate of relative transfection efficiency. Nevertheless, it has to be taken into account that the transfection efficiency of siRNA molecules is usually much higher.

If you wish to use labeled siRNA for your experiments, please contact our Scientific Support Team for suggestions to help your experiments run as smoothly as possible.

b) siRNA Test Kit

In order to quickly demonstrate general RNAi efficacy for a particular cell type, you can use our siRNA Test Kit for cell lines and adherent primary cells. This provides an siRNA against the GFP expressed by our pmaxGFP™ Vector control and is used in conjunction with the cell type-specific Nucleofector™ Solution and Parameters. For primary

human blood cells, we recommend examining downregulation of endogenous genes such as CD2, CD4 and vimentin. An Optimized Protocol (www.lonza.com/sirna-testkit) is available which lists the sequences we have used for examining these genes.

Enriching for Transfected Cells

One method of enriching for transfected cells is to co-transfect siRNA with a plasmid expressing a fluorescent reporter or surface marker, and then sorting for cells expressing the reporter. This approach has been used, for example, in Wu *et al.* (2005)[4]. Using shRNA-expressing vectors also allows you to use co-expressed fluorescent or antibiotic resistance markers to select for transfected cells (see below). However, transfection efficiencies for plasmid DNA are generally lower than those for siRNA duplexes.

Longterm RNAi Effects (siRNA Duplexes vs. shRNA-expressing Vectors)

Chemically synthesized siRNA duplexes offer a rapid means for determining the siRNA sequences that result in efficient knockdown of your target gene, but this downregulation is transient (generally persisting 2 – 5 days) and may not be sufficient when silencing targets with low turnover rates or in other applications where a longer duration of effect is required. Consequently, a number of different plasmid vectors that express siRNA, or shRNA (short hairpin RNA) are commercially available. In addition to enabling long-term expression of siRNA/shRNA, these vectors have the advantages that they can be grown, handled and stored as plasmid DNA, co-express fluorescent markers or antibiotic resistance genes (facilitating identification of transfected cells/selection of stably transfected cells) and can be engineered with inducible promoters to permit switching the knockdown phenotype on and off (such as pSuperior, OligoEngine). The ability of the Nucleofector™ Technology to transfect DNA into primary cells (and many cell lines which are difficult or impossible to transfect by other means) makes it possible to now use these vectors in virtually any cell type. Although do keep in mind that transfection efficiencies with siRNA oligonucleotides are generally higher than with plasmid DNA.

Stability of siRNA duplexes in the Nucleofector™ Solutions

The Nucleofector™ Solutions were tested for RNAse activity. Incubation of RNA in the solutions for 2 hours at 37°C did not affect RNA stability.

siRNA Amount-Concentration Comparison Chart

Amount	Weight Molecular weight of a 21 bp siRNA ds-oligonucleotide: 21 x 660 g/mol = 13860 g/mol = 13.86 ng/pmol ≈ 14 ng/pmol	Concentration Standard Nucleofector™ Device (100 µl)	Concentration 96-well Shuttle™ Device (20 µl)
1 pmol	14 ng	1 pmol/100 µl = 10 nmol/l = 10 nM	1 pmol/20 µl = 50 nmol/l = 50 nM
5 pmol	69 ng	5 pmol/100 µl = 50 nmol/l = 50 nM	5 pmol/20 µl = 250 nmol/l = 250 nM
10 pmol	140 ng	10 pmol/100 µl = 100 nmol/l = 100 nM	10 pmol/20 µl = 500 nmol/l = 500 nM
20 pmol	277 ng	20 pmol/100 µl = 200 nmol/l = 200 nM	20 pmol/20 µl = 1000 nmol/l = 1 µM
50 pmol	690 ng	50 pmol/100 µl = 500 nmol/l = 500 nM	50 pmol/20 µl = 2500 nmol/l = 2.5 µM
100 pmol	1.4 µg	100 pmol/100 µl = 1000 nmol/l = 1 µM	100 pmol/20 µl = 5000 nmol/l = 5 µM

For individual calculation, also refer to our website www.lonza.com/sirna-calculator

References

1. Persengiev SP, *et al.* RNA. 2004 Jan;10(1):12-8
2 Ross J, 1995, Microbiol Rev 59:423-50
3. Editorial (2003) Wither RNAi. Nat Cell Biol. 5 (6), 489-490.
4. Wu *et al.* (2005) MCB 25(22):9741-9752

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