Dharmacon™ Reverse Transfection of siRNA

This protocol is for the Reverse Transfection of siRNA.

Three Methods for siRNA Transfection:
Successful gene silencing in siRNA-mediated RNA interference (RNAi) experiments requires efficient uptake of siRNA into the cells of interest. For in vitro experiments, transfection is an easy and rapid method of siRNA delivery. Three variations of transfection are currently being used: standard (forward), Reverse Transfection Format (RTF) plates from Dharmacon, and reverse. They differ in the order and timing of the addition of the three necessary components of transfection: siRNA, lipid-based transfection reagent, and cells. In standard transfection, siRNA and lipid are complexed and added to pre-plated cells (Figure 1A). In RTF transfection, cells are added to pre-plated, dried siRNA that is rehydrated and complexed with lipid (Figure 1B). In reverse transfection, all three components are added to the wells at essentially the same time (Figure 1C). Both RTF and reverse transfections reduce hands-on time for transfection from two days to only one. RTF plates offer the advantage of pre-dispensed siRNAs, while reverse transfection offers the flexibility of testing siRNA reagents at various concentrations or utilizing existing siRNA library resources in a standard transfection format.

<table>
<thead>
<tr>
<th>A. Standard Transfection</th>
<th>B. RTF Transfection</th>
<th>C. Reverse Transfection</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td>Plate Cells</td>
<td></td>
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<tr>
<td><strong>Day 1, Step 1</strong></td>
<td>Complex siRNA with Dharmacon DharmaFECT Transfection Reagent</td>
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<tr>
<td></td>
<td>sRNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DharmaFECT Transfection Reagent</td>
<td></td>
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<tr>
<td><strong>Day 1, Step 2</strong></td>
<td>Add siRNA: DharmaFECT complex to cells</td>
<td></td>
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<tr>
<td></td>
<td>wells containing sRNA</td>
<td></td>
</tr>
<tr>
<td><strong>Day 2 or 3</strong></td>
<td>Assay for knockdown</td>
<td></td>
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</table>

**Figure 1.** Three transfection methods.
Figure 2 shows optimization data for reverse transfection of $1 \times 10^4$ HeLa cells with 50 nM siRNA in a 96-well plate format. A threshold of 80% cell viability and 75% gene silencing is assigned for successful, non-toxic transfection of siRNA. Other cells and plate formats may require additional optimization to result in good cell viability and gene silencing. Typical parameters that may be varied are cell number, choice of transfection reagent, and the volume of transfection reagent. Table 1 lists recommended conditions for HeLa, HepG2 and MCF7 cell lines.

**Table 1.** Final conditions for reverse transfection of siRNA in 96-well plate.

<table>
<thead>
<tr>
<th>Condition</th>
<th>HeLa</th>
<th>HepG2</th>
<th>MCF7</th>
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</thead>
<tbody>
<tr>
<td>Cell number/well</td>
<td>$1 \times 10^4$</td>
<td>$1 \times 10^4$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>Dharmacon™ DharmaFECT™ formulation</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DharmaFECT volume/well</td>
<td>0.12 µL</td>
<td>0.12 µL</td>
<td>0.06 µL</td>
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</table>

Reverse Transfection Protocol:

It is recommended to include the control samples listed in Table 2 in every transfection experiment. All steps of the protocol should be performed in a laminar flow cell culture hood using sterile technique. Performing transfection experiments in triplicate is recommended to allow statistical analysis of the results. To account for normal loss during pipetting, all reagents should be prepared in excess.

**Table 2.** Recommended samples for siRNA transfection experiments.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Determine baseline phenotype, target gene level, and cell viability</td>
</tr>
<tr>
<td>Negative Control siRNA</td>
<td>Distinguish sequence-specific silencing from non-specific affects</td>
</tr>
<tr>
<td>Positive Control siRNA</td>
<td>Measure efficiency of siRNA uptake into cells</td>
</tr>
<tr>
<td>Test siRNA</td>
<td>Knockdown of target gene</td>
</tr>
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</table>

1. Prepare the following solutions:
   1.1. Stock siRNA solutions (100 µM) in an RNase-free, pH 7.4-buffered solution.
   1.2. Dilute DharmaFECT 1 by adding 1.8 µL DharmaFECT 1 in 373.2 µL DCCR. Total volume is 375 µL.
2. In three separate tubes, mix 100 µL diluted DharmaFECT 1 and 0.25 µL of the appropriate siRNA solutions (negative control, positive control and test siRNAs).
3. Aliquot 25 µL of the siRNA/DharmaFECT complex into triplicate wells in a 96-well plate.
4. Incubate plates for 30 minutes at room temperature.
5. Trypsinize HeLa cells and prepare a $1 \times 10^4$ cells/mL solution in antibiotic-free complete medium.
6. Add 0.1 mL cell solution to each well containing siRNA/DharmaFECT complex. The final volume is 125 µL/well.
7. Incubate cells at 37 °C in 5% CO₂ for 24-48 hours (for mRNA analysis) or 48-96 hours (for protein analysis).
8. If cell toxicity is observed after 24 hours, replace the transfection medium with complete medium and continue incubation. Cell viability may be determined with alamarBlue™, MTT, or other assays for metabolic activity. For best results, use samples that are at least 80% viable.
**Materials:**

Transfection experiments require standard cell culture reagents and instruments appropriate for maintenance of the cells being used. Reagents for assaying cell viability and gene silencing are also needed. Table 3 lists specific reagents required for the reverse transfection protocol described here. Assays for mRNA level, protein level, or other phenotypic change may be performed to assess silencing. Because RNAi is an mRNA-specific event, we highly recommend assaying for reduction at the mRNA level using reverse transcription quantitative real-time PCR (RT-qPCR), northern blot analysis, branched DNA, or other similar methods. Typical time points for detecting target knockdown with lipid-mediated siRNA delivery are 24-48 hours for mRNA and 48-96 hours for protein. Accell siRNA delivery is typically assessed at 72 hours. Time course studies are recommended to identify optimal time points for assessing knockdown of a specific gene of interest.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Description and Use</th>
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<tbody>
<tr>
<td>Cells</td>
<td>For best transfection efficiency, use cells in log-phase growth at passage number 20 or lower</td>
</tr>
<tr>
<td>Antibiotic-free complete medium</td>
<td>Medium in which the cells are normally maintained and may contain up to 20% serum, but does not contain antibiotics which may cause cell toxicity during transfection</td>
</tr>
<tr>
<td>DCCR (Cat #B-004500-100)</td>
<td>For dilution and optimal complexing of siRNA and DharmaFECT</td>
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<tr>
<td>Transfection Reagent</td>
<td>DharmaFECT 1 is recommended for most cell types, but additional formulations are offered for optimization studies. See the recommended formulation for your cell type at gelifesciences.com/dharmafect</td>
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<tr>
<td>Test siRNA</td>
<td>An siRNA that targets the gene of interest. Search for your gene at gelifesciences.com/geneius-product-search</td>
</tr>
<tr>
<td>Negative Control siRNA</td>
<td>An siRNA that does not target any gene in the cells being used, to distinguish sequence-specific silencing from non-specific effects</td>
</tr>
<tr>
<td>Positive Control siRNA</td>
<td>A validated siRNA that targets an abundantly expressed housekeeping or reference gene, to measure efficiency of siRNA uptake into cells.</td>
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