PROTOCOL

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DharmaFECT™ Transfection Reagents—siRNA transfection protocol

The following is a general protocol for use of DharmaFECT™ transfection reagents to deliver siRNA into cultured mammalian cells. The examples given within the protocol are for 96-well plates, and (Table 1) provides the transfection reagent volumes for additional plate types. Table 2 presents recommended DharmaFECT formulations and conditions for the most efficient delivery of siRNA and subsequent silencing (assessed with GAPDH or PPIB siRNA) in cell lines for which we have performed transfection optimization (96-well format). These results are intended to serve as guidelines for carrying out your own experiments.

Successful transfection requires careful optimization of conditions. For optimization recommendations see the Transfection Optimization section on page 2. Below are steps for beginning a transfection experiment with known optimal conditions.

Each experiment should include the following samples in triplicate:

1. Untreated cells
2. Positive control siRNA (targeting an endogenous or reporter gene)
3. Negative control siRNA (non-targeting)
4. The desired test siRNA

Perform all steps of protocol in a laminar flow cell culture hood using sterile techniques

Cell plating

Optimal cell densities will vary with growth characteristics that are unique to each cell type and need to be determined empirically. See Table 2 for cell line specific cell density recommendations in 96-well format. For larger plate formats you can vary the number of cells plated in proportion to the difference in well surface area.

1. Trypsinize and count cells
2. Dilute cells in antibiotic-free complete medium to achieve the appropriate plating density in 100 µL of solution. (Complete medium is medium that the cells are maintained in, and may contain serum)
3. Plate 100 µL of cells into each well of a 96-well plate
4. Incubate cells at 37°C with 5 % CO_2 overnight

Transfection

The following steps will be used for positive control, negative control and test siRNAs. We recommend using 5–100 nM final siRNA concentrations. The volumes in this protocol are given for a single well in a 96-well plate format for 25 nM final siRNA concentration. To transfect triplicate wells and to account for loss during pipetting, multiply the volumes by 3.5.

1. Prepare 5 µM siRNA solution in 1× siRNA buffer or another appropriate RNase-free solution from your stock solution. (See Basic siRNA Resuspension Protocol for more details)
2. In separate tubes, dilute the siRNA (Tube 1) and the appropriate DharmaFECT transfection reagent (Tube 2) with serum-free medium.
   - Tube 1: Prepare 10 µL volume of the siRNA in serum-free medium by adding 0.5 µL of 5 µM siRNA to 9.5 µL of serum free medium.
   - Tube 2: Prepare 10 µL volume of diluted DharmaFECT transfection reagent in serum-free medium. Depending on the cell line and cell density the DharmaFECT reagent amount can vary between 0.05-0.5 µL (Table 1); for the cell lines that we have tested you can use the recommended volume of DharmaFECT reagent (Table 2).
   - Example: For HeLa cells at 5,000 cells/well, add 0.2 µL of DharmaFECT reagent to 9.8 µL of serum free medium. Final concentration of DharmaFECT reagent in Step 4 will be 0.2 µL/well. Note: For alternate plate formats, scale up the amount of DharmaFECT reagent according to the total transfection volume (Table 1).
3. Gently mix the contents of each tube by pipetting carefully up and down. Incubate for 5 minutes at room temperature.
4. Add the contents of Tube 1 to Tube 2, for a total volume of 20 µL. Mix by pipetting carefully up and down and incubate for 20 minutes at room temperature, and then add 80 µL of antibiotic-free complete medium for a total volume of 100 µL transfection medium.
5. Remove culture medium from the wells of the 96-well plate and add 100 µL of the appropriate transfection medium to each well.
6. Incubate cells at 37°C in 5% CO_2 for 24–48 hours (for mRNA analysis) or 48–96 hours (for protein analysis).

For best results, use samples that show >80% viability. If necessary, the transfection medium may be replaced with complete medium after 24 hours to reduce cytotoxicity. Continue incubation for an additional 24–72 hours and assay.

Transfection optimization

To obtain the highest transfection efficiency with minimal effects on cell viability we recommend the following guidelines in optimizing transfection conditions for each cell line:

The optimization experiment should include at least three cell densities and four DharmaFECT™ transfection reagent volumes within the range recommended in (Table 1).

- When selecting cell densities to assess, consider the assay and time-point requirements: lower cell densities for long term assays and higher cell numbers for short-term experiments.
- Use positive and negative control siRNAs at 25 nM final concentration as well as untreated cells to find conditions that show target mRNA knockdown of > 80% with the positive control siRNA and > 80% cell viability.
- Use these optimal conditions for your subsequent experiments with test siRNAs.
- Since the siRNA amount for optimal silencing can vary due to intrinsic properties of the target gene, we recommend performing a dose curve transfection with your test siRNA (using a range from 5 to 100 nM) to find the optimal siRNA concentration for your test siRNA.

For high throughput siRNA screening in easy-to-transfect cells, we recommend using a reverse transfection protocol (See this reverse transfection protocol). The DharmaFECT volumes and siRNA amounts for reverse transfection are usually lower than the amounts needed for traditional transfection. Therefore, a transfection optimization should be performed for the protocol that is going to be used for subsequent experiments.

Table 1. Recommended volumes per well for transfecting siRNA at 25 nM final concentrations

		Tube 1: diluted siRNA (µL/well)		Tube 2: diluted DharmaFECT (µL/well)			
Plating format (wells/plate)	Surface area (cm^2/well)	Volume of 5 µM siRNA (µL)	Serum-free medium (µL)	Volume of DharmaFECT reagent (µL)*	Serum-free medium (µL)	Complete medium (µL/well)	Total transfection volume (µL/well)
96	0.3	0.5	9.5	0.05–0.5	9.95–9.5	80	100
24	2	2.5	47.5	0.25–2.5	49.75–47.5	400	500
12	4	5	95	0.5–5.0	99.5–95.0	800	1000
6	10	10	190	1.0–10.0	199.0–190.0	1600	2000

*DharmaFECT volumes per well represent guidelines and need to be optimized. The optimal DharmaFECT reagent amount varies for different cell lines and is affected by the cell density. Easy-to-transfect cells and lower cell densities typically require lower amount of DharmaFECT reagent. During transfection optimization, vary the cell densities and DharmaFECT volumes in the recommended ranges above. **Note: 25 nM = 25 nmol/L = 25 pmol/mL = 25 fmol/µL**

Table 2. Transfection optimization in 96-well Plates using GAPDH or PPIB silencing as a measure of efficient transfection

Cell line	Cell type	Recommended DharmaFECT formulation	Relative GAPDH or PPIB silencing (%)	DharmaFECT® volume/ well (µl) in 96 well plate	Plating density in 96 well plate	Other successful formulations
Human						
786-0	Kidney adenocarcinoma	1	94	0.4	5×10^3	2
A549	Lung carcinoma	1	92	0.2	1×10^4	2, 3, 4
B×PC3	Pancreas adenocarcinoma	2	85	0.2	5×10^3	1, 3, 4
DLD-1	Colorectal adenocarcinoma	2	85	0.4	5×10^3	1, 3
DU 145	Prostate carcinoma	1	94	0.2	1×10^4	2, 3, 4
NCI-H1299	Lung carcinoma	2	93	0.2	1×10^4	4
HCT-116	Colorectal carcinoma	2	83	0.1	5×10^3	4
HEK293	Kidney transformed embryonic cells	1	92	0.2	1×10^4	2, 4
HeLa	Cervical epithelial adenocarcinoma	1	95	0.2	5×10^3	2, 3, 4
HeLa S3	Cervical epithelial adenocarcinoma	4	97	0.4	5×10^3	1, 2, 3
Hep G2	Hepatocellular carcinoma	4	91	0.4	1×10^4	1, 2
hMSC	Mesenchymal stem cells	1	94	0.4	5×10^3	2, 3, 4
HT-29	Colorectal carcinoma	1	99	0.2	5×10^3	2, 3, 4
HT1080	Fibrosarcoma	4	96	0.2	5×10^3	1, 2, 3
Huh-7	Hepatocarcinoma	4	76	0.05	5×10^3	1, 2
HUVEC	Umbilical vein endothelial cells	4	85	0.2	2×10^4	1, 2
LNCaP	Prostate carcinoma	3	80	0.2	1×10^4	1
MCF-10A	Breast adenocarcinoma	1	93	0.2	1×10^4	2
MCF-7	Breast adenocarcinoma	1	90	0.2	1×10^4	2, 4
MDA-MB-453	Breast adenocarcinoma	2	91	0.2	1×10^4	1, 3, 4
MDA-MB-231	Breast adenocarcinoma	4	87	0.1	5×10^3	1
OVCAR-3	Ovarian adenocarcinoma	1	90	0.1	5×10^3	2, 3, 4
PC-3	Prostate carcinoma	2	88	0.2	1×10^4	3
SK-BR3	Breast adenocarcinoma	2	90	0.2	1×10^4	1, 3, 4
SK-OV-3	Ovarian adenocarcinoma	3	90	0.4	1×10^4	1, 2, 4
u87MG	Brain glioblastoma	1	87	0.1	5×10^3	2, 3, 4
Rodent						
A7r5	Rat aortic smooth muscle	2	95	0.1	5×10^3	1
C2C12	Mouse myoblasts	1	87	0.2	5×10^3	2, 3, 4
CHO K1	Chinese hamster ovary cells	4	92	0.8	1×10^4	1, 2
ES-D3	Mouse embryonic stem cells	1	94	0.2	2×10^3	2
ES-E14TG2a	Mouse embryonic stem cells	1	93	0.2	2×10^3	2
H9c2	Rat myoblasts	1	96	0.2	1×10^4	2, 3, 4
J774A.1	Mouse macrophages	4	90	0.2	1×10^4	–
NIH/3T3	Mouse embryonic fibroblasts	1	91	0.2	1×10^4	3
NRK-49F	Rat kidney fibroblasts	2	92	0.2	1×10^4	1, 4
Rat2	Rat fibroblasts	1	75	0.2	2×10^4	2
3T3-L1	Mouse embryonic fibroblasts	1	80	0.2	5×10^3	3
Other						
COS-7	African green monkey kidney	2	94	0.4	5×10^3	1, 3, 4

Cells were transfected with 25–100 nM siRNA targeting either GAPDH or PPIB. Each cell line was tested at three plating densities (5×10^3, 1×10^4, 2×10^4 cells per well) with a range of DharmaFECT volumes (0.0–0.8 µL/well) for all four formulations of DharmaFECT transfection reagents.

The target mRNA knockdown and cell viability was assessed at 24 hours post-transfection. The GAPDH and PPIB Control pools have been validated for reducing mRNA level by 90% or more under optimal transfection conditions, therefore the knockdown observed represents a relative measure of transfection efficiency. The combination of conditions that gave optimal target mRNA knockdown (> 80%) with the least effect on cell viability [> 80% assessed by alamarBlue (BiosourceInternational) or resazurin (Acros Organics)] for each cell line is reported. For most cell lines there were multiple successful transfection conditions.

All experimental conditions resulted in cell viability and positive control gene silencing of 75% or better. All experiments were done in 96-well plates with Non-targeting control siRNA and PPIB (Cyclophilin B) or GAPD Control pools at 25 nM; alamarBlue (viability) and knockdown measured at 24 hr. Data normalized to untransfected for viability and both untransfected and Non-targeting control for knockdown. Transfection conditions should always be re-evaluated in the context of a new plate format or assay-specific requirements for cell density.

Frequently asked questions

Questions	Answers
Do you have a transfection protocol for 6-well plates?	This transfection protocol includes Table 1 to assist you with the appropriate volume range of DharmaFECT™ reagent for different plate formats. Cell density should be optimized based on well surface area and assay requirements. The formulation recommendations in Table 2 (DharmaFECT 1, 2, 3 or 4) may be applied to any plate format.
How soon may I replace the media/how long does the transfection take?	Transfection medium may be replaced 6 hours after transfection but this is not required. While the transfection is likely complete within this timeframe, gene silencing detection should not be carried out prior to 24 hours post-transfection.
Why is it necessary to use antibiotic-free media?	The avoidance of antibiotics is a general recommendation for transfection procedures. The rationale is that the cells are very sensitive in terms of permeability during transfection and the presence of antibiotics can greatly increase cell death due to uptake of antibiotic along with the transfection reagent/siRNA complex. If it is necessary to minimize the period of time that the cells are cultured in antibiotic-free medium, we recommend changing the antibiotic-free transfection medium to antibiotic-containing medium anytime beginning at 6 hours post-transfection.
May I use serum during the transfection? When do I need a serum-free medium in the protocol?	Serum-free medium is used for dilution of siRNA and DharmaFECT reagents in Steps 1 and 2. You may use complete medium after the complexing of siRNA and DharmaFECT reagent. After mixing contents of Tube 1 to Tube 2, we recommend adding antibiotic-free complete medium to the mix for obtaining the appropriate transfection volume (Table 1). (Complete medium is the medium that the cells are maintained in, and may contain serum).
What siRNA concentration should I use?	Due to our rational design and SMARTpool technology effective silencing can be achieved with our siRNA reagents at low nanomolar ranges. However, the optimal siRNA amount can vary for different siRNAs due to intrinsic properties of the target gene. Therefore, we recommend performing an siRNA dose curve (using a range from 5 to 100 nM) to determine the lowest functional siRNA concentration for each siRNA in the specific experimental setup.
If I double the amount of siRNA, do I double the amount of DharmaFECT transfection reagent?	The amount of DharmaFECT transfection reagent used is dependent on the cell density tested. If you have determined the transfection reagent concentration for a given cell density that delivers 50 nM siRNA efficiently without causing toxicity, we have found that this volume is adequate to deliver any amount of siRNA (0.1 nM-50 nM).
I don't see my cell line on Table 2. Which DharmaFECT formulation should I use?	DharmaFECT 1 is the most broadly-applicable reagent for effective siRNA delivery across cell types, and many researchers begin by assessing this formulation. However, in many cases another formulation may provide superior transfection efficienty or improved cell viability. For cell lines or conditions that we have not tested in-house, or if you plan to carry out a screen, the best recommendation is to use the DharmaFECT Set of 4 reagents and perform an assessment of all four formulations. Please also consider doing a literature search for publications that might have used DharmaFECT to transfect your cell line of interest.
Why do I get low transfection efficiency/ cytotoxicity after performing transfections?	Transfection conditions need to be optimized for each particular cell line in order to obtain the highest transfection efficiency with minimal effects on cell viability. Both cell density and the volume of the DharmaFECT reagent will affect the transfection efficiency. Our recommended conditions in Table 2 were obtained at 24 hrs post-transfection and should be used as starting guidelines for your transfection experiments. Different cell growth characteristics, assay time-point or different cell densities might affect the transfection efficiency and the cell viability. If careful transfection optimization was performed and no optimal conditions have been identified, then the cell line of interest might be difficult to transfect using conventional transfection reagents and alternative means of siRNA delivery might be necessary.

For additional Frequently Asked Questions (FAQs), please visit here.

For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

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