Knockdown of ZFX by siRNA in C42B

LY 20160606

Day 0: Cell Plating

The cell density recommendation for LNCap is $1*10^4$ in 96-well format (see table 2 in DharmaFECT ™ Transfection Reagents protocol). Since the proportion of surface area between 10cm and 96-well plate is 55 cm^2: 0.32cm^2 =172, the cell density recommendation for LNCap should be $1.72*10^6$ in 10cm plate

C42B cells were cultivated in 15cm dish before cell plating. Average cell yield in 15cm dish is $1.52*10^7$ when they reach about 80% confluence.

1. Trypsinize C42B cells and resuspend cells with antibiotic-free complete media in each 15cm plate.

In this case, we resuspend cells with 10mL antibiotic-free complete media.

Calculation: ~100cells/square $*1*10^4$= ~10^6cells/mL =~10^7cells/10mL, so I diluted them in 10mL medium.

2. Count cells: assume the average cell number/ square is X**,** which is between 40-100.

Cells per mL= X $*1*10^4$

The amount of cells plated in 10cm plate= ($1.72*10^6$ / X $*1*10^4$) mL. The estimating amount is 2-3mL

In summary, in each 10cm plate: 10mL antibiotic-free complete media + C42B cells (2-3mL)

3. Incubate cells at 37°C with 5% CO_2 overnight

Day 1: Transfection

- siRNA ZFX vendor: Dharmacon

  Product: ON-TARGETplus Human ZFX (7543) siRNA - SMARTpool, 5 nmol

  Catalogue number: L-006572-00-0005

- siRNA control vendor: Dharmacon

  Product: ON-TARGETplus Non-targeting Pool

  Catalogue number: D-001810-10-05

- Transfection reagent:

  DharmaFECT 3 Transfection Reagent

Table 1: Recommended Volumes Per Plate For Transfecting siRNA at 50nM Final Concentrations:

(See DharmaFECT ™ Transfection Reagents Table 1)

		Tube 1: diluted siRNA (µL/plate)		Tube 2: diluted DharmaFECT (µL/plate)			
Plating Format	Surface Area (cm^2)	Volume of 5µM siRNA (µL)	Serum-free Medium (µL)	Volume of DharmaFECT reagent (µL)	Serum-free Medium (µL)	antibiotic-free Complete Medium (mL/plate)	Total transfection volume (mL/plate)
10cm plate	55	110	890	11	989	9	11

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)

In this case, nuclear free water was used.

2. In separate tubes, dilute the siRNA (Tube 1) and the appropriate DharmaFECT transfection reagent (Tube 2) with serum-free medium.

• Tube 1: Prepare 1mL volume of the siRNA in serum-free medium by adding 110µL of 5 µM siRNA to 890µL of serum free medium.

• Tube 2: Prepare 1mL volume of diluted DharmaFECT transfection reagent in serum-free medium. Add 11µL DharmaFECT to 989µL of serum free medium.

Depending on the cell line and cell density the DharmaFECT reagent amount can vary between 0.05-0.5 µL (See Basic_Dharmafect_Resuspension_Protocol Table 1); for the cell lines that we have tested you can use the recommended volume of DharmaFECT reagent (See Basic Dharmafect Resuspension Protocol 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 (See DharmaFECT ™ Transfection Reagents Table 1). In this case, for 10cm plate, the total transfection volume is 11mL.

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 2mL. Mix by pipetting carefully up and down and incubate for 20 minutes at room temperature.

5. During 20-minute wait, remove old culture medium from 10cm plates and add 9mL of antibiotic-free complete medium to each 10cm plate.

6. After 20 minutes, add the mix (2mL) in step 4 to 9mL of antibiotic-free complete medium for a final volume of 11mL transfection medium and a final siRNA concentration of 50nM.

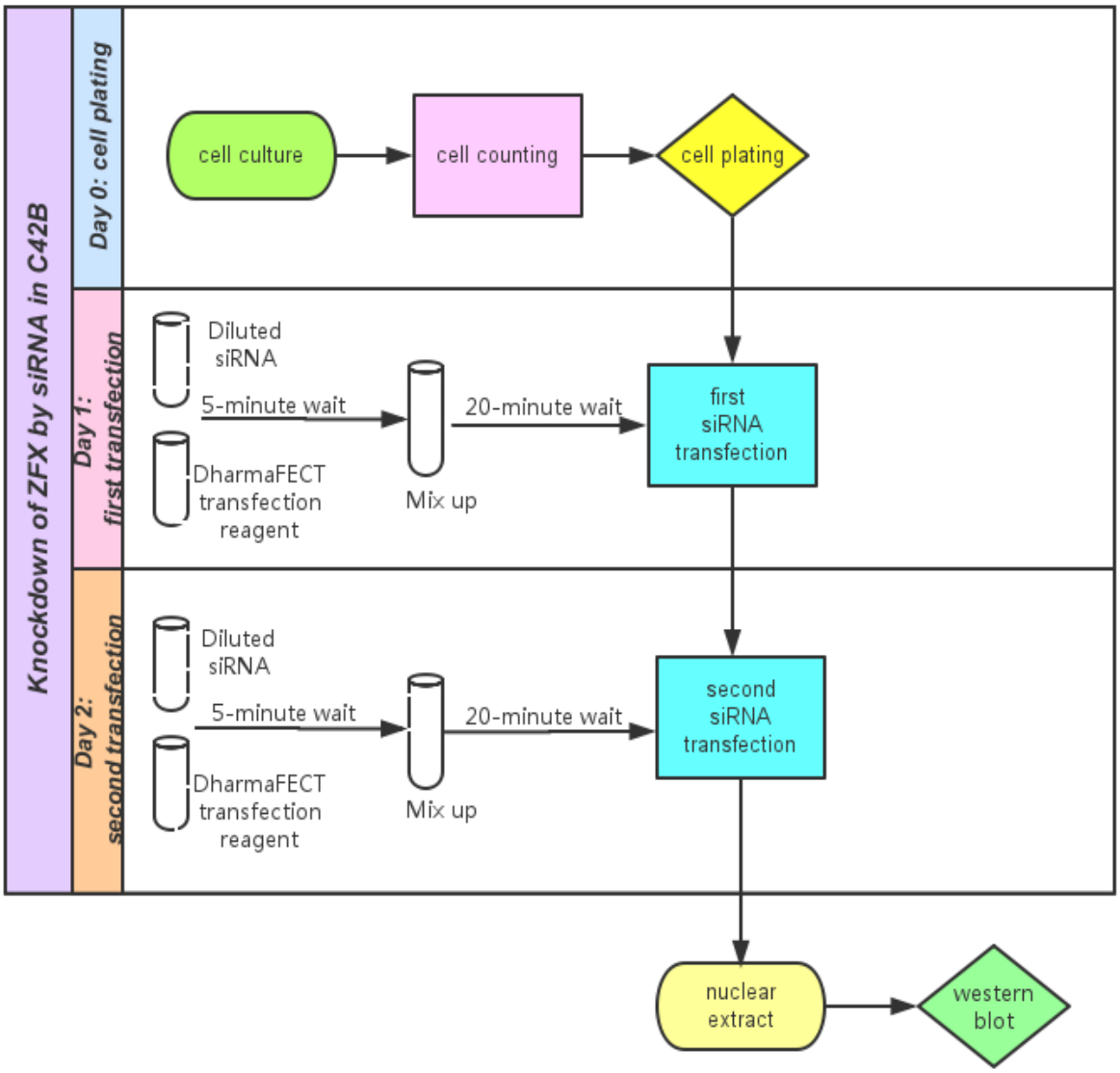
7. Incubate cells at 37°C in 5% CO_2 for 24 hours.

Day 2: Repeat Transfection

Repeat transfection for the second time. Extract nuclear after 24-48 hrs after the second transfection for western blot.

Ref:

1. Dharmacon Reverse Transfection of siRNA
2. DharmaFECT ™ Transfection Reagents
3. Dharmacon ™ Basic siRNA Resuspension Protocol


Knockdown of ZFX by siRNA in C42B
Day 0: cell plating
cell culture
cell counting
cell plating
Day 1: first transfection
Diluted siRNA
5-minute wait
DharmaFECT transfection reagent
Mix up
20-minute wait
first siRNA transfection
Day 2: second transfection
Diluted siRNA
5-minute wait
DharmaFECT transfection reagent
Mix up
20-minute wait
second siRNA transfection
nuclear extract
western blot

Protocol

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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 tranfection format.

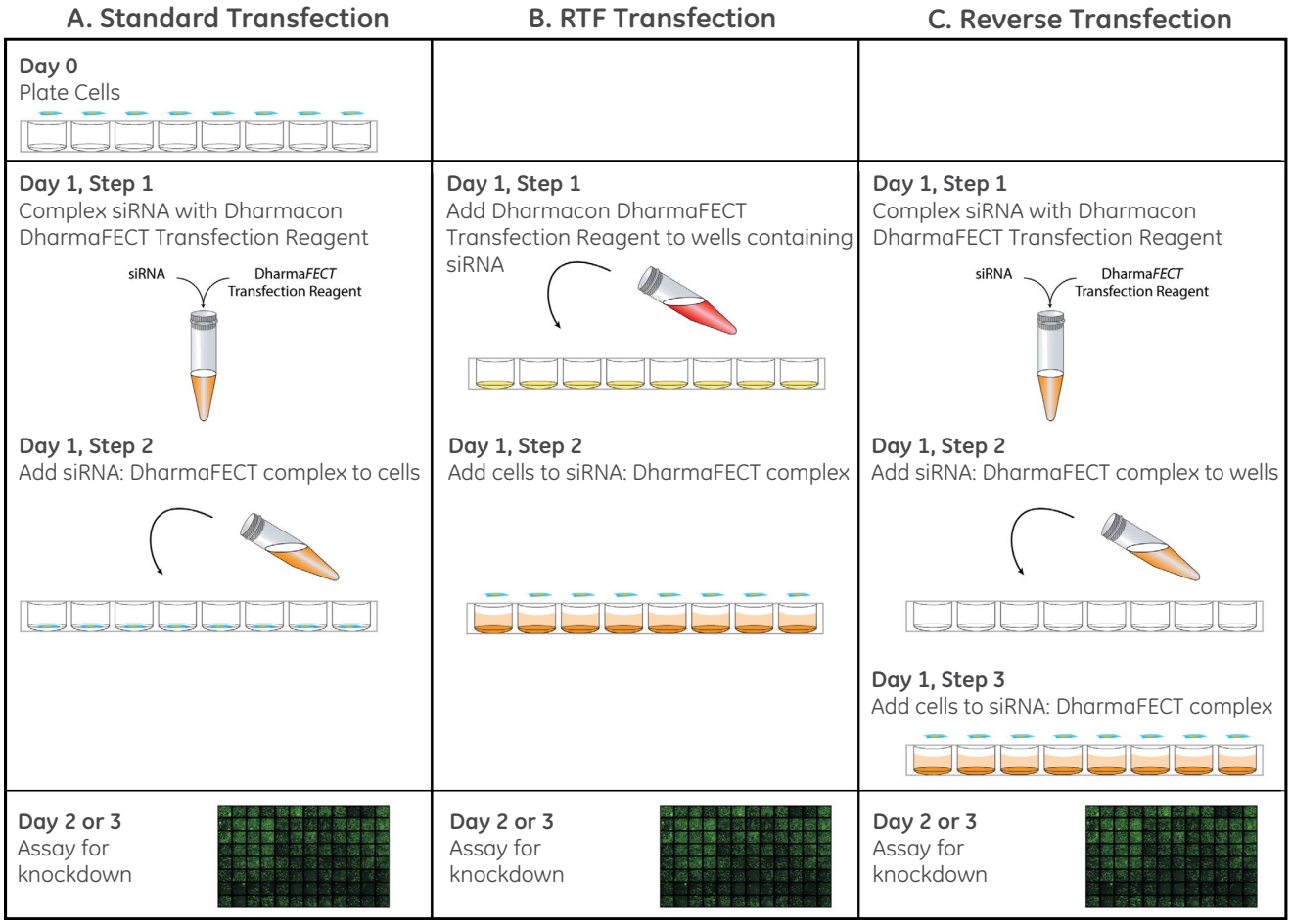

Figure 1. Three transfection methods.

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Figure 2 shows optimization data for reverse transfection of 1 × 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.

Condition	HeLa	HepG2	MCF7
Cell number/well	1 × 10^4	1 × 10^4	1 × 10^4
Dharmacon™ DharmaFECT™ formulation	1	4	4
DharmaFECT volume/well	0.12 µL	0.12 µL	0.06 µL

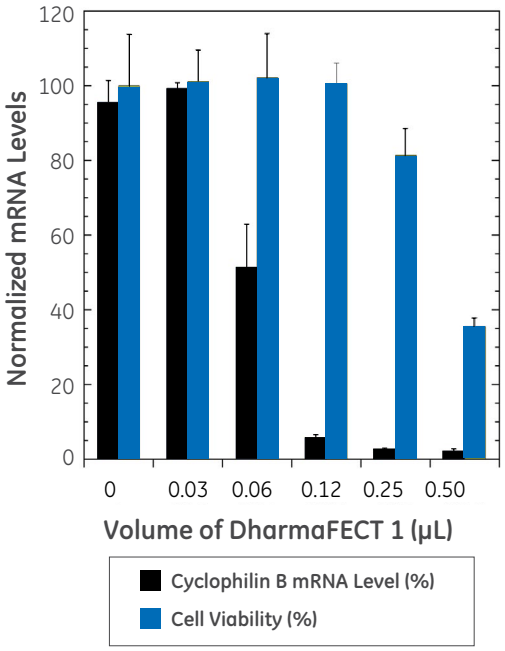

Figure 2. Reverse transfection optimization. HeLa cells (1 × 10^4 cells/well) were transfected with 50 nM Dharmacon™ siGENOME™ Cyclophilin B control siRNA (Cat #D-001136-01) and varying DharmaFECT 1 (Cat #T-2001) volumes (0.03-0.50 µL/well) in 96-well plates using the protocol described on the next page. Cyclophilin B mRNA level was measured using bDNA at 48 hours and expressed as percent of sample treated with siGENOME Non-targeting siRNA (Cat #D-001210-01). Cell viability was measured by Thermo Scientific™ alamarBlue™ assay at 48 hours and expressed as percent of untreated sample. All values are expressed as mean ± SD, n=3.

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.

Samples	Purpose
Untreated	Determine baseline phenotype, target gene level, and cell viability
Negative Control siRNA	Distinguish sequence-specific silencing from non-specific affects
Positive Control siRNA	Measure efficiency of siRNA uptake into cells
Test siRNA	Knockdown of target gene

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 × 10^5 cells/mL solution in antibiotic-free complete medium.
6. Add 0.1 mL cell solution to each well containing siRNA: Dharmacon DharmaFECT complex. The final volume is 125 µL/well.
7. Incubate cells at 37 °C in 5% CO_2 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 list 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 3. Reagents for siRNA reverse transfection protocol.

Reagents	Description and Use
Cells	For best transfection efficiency, use cells in log-phase growth at passage number 20 or lower
Antibiotic-free complete medium	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
DCCR (Cat #B-004500-100)	For dilution and optimal complexing of siRNA and DharmaFECT
Transfection Reagent	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
Test siRNA	An siRNA that targets the gene of interest. Search for your gene at gelifesciences.com/geneius-product-search
Negative Control siRNA	An siRNA that does not target any gene in the cells being used, to distinguish sequence-specific silencing from non-specific effects
Positive Control siRNA	A validated siRNA that targets an abundantly expressed housekeeping or reference gene, to measure efficiency of siRNA uptake into cells.

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V3-0615

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Protocol

DharmaFECT™ Transfection Reagents

siRNA Transfection Protocol

The following is a general protocol for use of Dharmacon 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 5-50 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.
5. Add 80 µL of antibiotic-free complete medium to the mix in step 4, for a final volume of 100 µL transfection medium and a final siRNA concentration of 25 nM.

6. Remove culture medium from the wells of the 96-well plate and add 100 µL of the appropriate transfection medium to each well.
7. Incubate cells at 37°C in 5% CO_2 for 24–48 hours (for mRNA analysis) or 48–96 hours (for protein analysis).

Note: 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 50 nM) to find the optimal siRNA concentration for your test siRNA.

Note: For high throughput siRNA screening in easy-to-transfect cells, we recommend using a wet reverse transfection protocol (See Wet 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. Final concentrations of 0.05–5 µL DharmaFECT/100 µL transfection medium are shown. 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 PPI B 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 (0.05 - 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 SMART pool 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 50 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.

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V2-0614

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Protocol

Dharmacon™ Basic siRNA Resuspension

This protocol is for the resuspension of synthetic siRNA

Note: This protocol is written for siRNA, but may also be applied to applied to microRNA mimic resuspension. For detailed recommendations for resuspension of siRNA/microRNA in plates, please see our siRNA Library Guidelines.

1. Briefly centrifuge tubes containing siRNA to ensure that the siRNA pellet is collected at the bottom of the tube.
2. Resuspend in RNase-free 1x siRNA Buffer (See note below) for the desired final concentration using volumes listed in Table 1.
   a. For example: for 10 nmol of siRNA and a 20 µM stock concentration, add 500 µL 1x siRNA Buffer.
3. Pipette the solution up and down 3-5 times, avoiding the introduction of bubbles and securely seal tubes (or multi-well plates).
4. Place the solution on an orbital mixer/shaker for 30 minutes at room temperature.
5. Briefly centrifuge tubes containing siRNA to ensure that the solution is collected at the bottom of the tube.
6. Verify the concentration of siRNA using UV spectrophotometry at 260 nm. For siRNA, 1 µM = 13.3 ng/µL. For microRNA mimic, 1 µM=14.1 ng/µl, see FAQs for additional information.
7. RNA may be used immediately, or aliquoted into smaller volumes to limit the number of freeze-thaw cycles. Resuspended siRNA should be stored at -20 °C in a manual defrost or non-cycling freezer. Storage at 4 °C is suitable for up to 6 weeks.

Table 1. Recommended siRNA resuspension volumes and concentrations.

siRNA Amount (nmol)	1x siRNA Buffer to be added (µL) for desired final concentration	
	100 µM Stock	20 µM Stock
1.0	10	50
2.0	20	100
5.0	50	250
10	100	500
20	200	1000
50	500	Exceeds tube volume
100	1000	

Notes:

- **siRNA (and microRNA mimic) should be resuspended in RNase-free solutions. We recommend 1x siRNA Buffer (diluted from Dharmacon 5x siRNA Buffer, Cat #B-002000-UB-100). For short-term storage, RNase-free water (Cat # B-003000-WB-100) is also appropriate for resuspension of concentrated stocks.**
- **Salts present in buffer are known to affect the absorbance reading of RNA. For the most precise readings, dissolve in 4 volumes of sterile RNase-free water for spectrophotometric analysis. Then adjust with addition of 5x siRNA Buffer appropriately to desired final concentration of siRNA adjusting to 1x siRNA Buffer. Please see the FAQ section on page 2.**
- **To dilute the 5x siRNA Buffer to 1x siRNA Buffer, mix four volumes of sterile RNase-free water with one volume of 5x siRNA Buffer. The composition of the 1x siRNA Buffer is 60 mM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM $MgCl_2$.**
- **5x siRNA Buffer is not intended for *in vivo* applications, as it has not been optimized for physiological conditions. Instead, an appropriately buffered RNase-free solution should be used.**

Technical Considerations:

- For efficient Dharmacon™ siGENOME™ siRNA, ON-TARGET*plus*™ siRNA, or miRIDIAN™ microRNA Mimic delivery, we strongly recommend following the instructions provided by the manufacturer for the delivery method of choice (such as transfection reagent, or electroporation) and taking measures to test and optimize the conditions best suited for the cell line or culture selected. For protocols using DharmaFECT™ transfection reagents or Dharmacon™ Accell™ siRNA delivery, click here.
- Assays for mRNA level, protein level, or phenotypic change may be performed to assess silencing effects. 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). Typical time points for detecting target knockdown with lipid-mediated siRNA or microRNA mimic delivery are 24-48 hours for mRNA and 48-96 hours for protein. Accell siRNA delivery is typically assessed at 72 hours or longer. Time course studies are recommended to identify optimal time points for assessing knockdown.

Frequently Asked Questions:

Question	Answer
How do I quantitate the resuspended siRNA?	RNA is most accurately quantified by measuring its absorbance at 260 nm (A_{260}) with a dual beam spectrophotometer.
How do I calculate the concentration of the siRNA sample?	Use Beer's Law, $A_{260} = (\varepsilon)(C)(L)$ where ε is the extinction coefficient (from the Product Transfer Form), C is the siRNA concentration, and L is the path length of the cuvette. Calculate the final concentration of the resuspended siRNA by solving for C and multiplying by the dilution factor.
Why does the calculated amount of RNA in solution differ from that on the Product Transfer Form?	Salts present in 1x siRNA Buffer (or other resuspension solution) are known to cause a decrease in the absorbance reading of RNA. Differences in instrumentation for quantifying RNA may lead to differences in apparent values. Dual beam UV-VIS spectrophotometers are recommended. Sample is too concentrated. Absorbance values are most accurate between 0.15 and 0.6 and within the linear range of a standard curve. Sample is too diluted. Measurements with dilutions of small volumes (1-1.5 µL) are more susceptible to variation. Sample may not be fully resuspended. Heat samples to 95 °C for 1-3 minutes and allow to cool for 30-45 minutes to reanneal complementary strands.
The siRNA has been at room temperature for a week. Will the siRNA still be okay?	Yes. Samples are shipped as dried pellets and are stable at room temperature for 2-4 weeks. Upon receipt, we recommend that all samples should be stored at -20 °C or -70 °C to -80 °C.
What is the average molecular weight of a siRNA or miRIDIAN™ Mimic?	The average molecular weight (MW) of a siRNA is 13,300 g/mol. The average MW of a miRIDIAN mimic is 14,100 g/mol.
How do I convert between nmol to µg of siRNA?	Multiply the number of moles by the MW on the Product Transfer Form, or the average MW for your oligo. For example, 5 nmol of siRNA would be: $(5\ nmol)(13{,}300\ g/mol)(mol/10^9\ nmol)(10^6\ \mu g/g) = 66.5\ \mu g$

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

Orders can be placed at:
gelifesciences.com/dharmacon

Customer Support: cs.dharmacon@ge.com
Technical Support: ts.dharmacon@ge.com or
1.800.235.9880; 303.604.9499 if you have any questions.

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