

HiPerFect Transfection Reagent Handbook

For transfection of eukaryotic cells with siRNA
and miRNA



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Kit Contents

HiPerFect Transfection Reagent	0.5 ml	1 ml	4 x 1 ml	100 ml
Catalog no.	301704	301705	301707	301709
Transfections per kit (in 24-well plates)	Up to 166	Up to 333	Up to 1332	Up to 1388 24-well plates
HiPerFect Transfection Reagent	0.5 ml	1 ml	4 x 1 ml	100 ml
Handbook	1	1	1	1

Shipping and Storage

HiPerFect Transfection Reagent is supplied as a ready-to-use solution and is shipped at ambient temperature without loss of stability. However, it should be stored at 2–8°C upon arrival. HiPerFect Transfection Reagent does not need to be stored on ice during the transfection procedure.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of HiPerFect Transfection Reagent is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

HiPerFect Transfection Reagent is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding HiPerFect Transfection Reagent or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The application of RNA interference (RNAi) to mammalian cells has revolutionized the field of functional genomics. The ability to simply, effectively, and specifically downregulate the expression of genes in mammalian cells holds enormous scientific, commercial, and therapeutic potential. Efficient transfection of siRNA is critical for effective gene silencing. HiPerFect Transfection Reagent has been developed for efficient transfection of cells even when low siRNA concentrations are used. Highly efficient transfection and silencing have been observed in some cases using as little as 100 pM siRNA. HiPerFect Transfection Reagent provides highly efficient siRNA transfection over a range of siRNA concentrations from low to high, allowing researchers to choose the siRNA concentration they wish to use. HiPerFect Reagent is also highly suited to miRNA mimic/miRNA inhibitor transfection for miRNA research.

Principle and procedure

Off-target effects in RNAi experiments

Studies have indicated that transfection of siRNA can result in off-target effects, in which siRNAs affect the expression of nonhomologous or partially homologous gene targets. Off-target effects can include mRNA degradation, inhibition of translation, or induction of an interferon response (1–4). The mechanisms of off-target effects are not fully understood. They may be caused by siRNA targeting mRNA with close homology to the target mRNA, by siRNAs functioning like miRNAs, or by a cellular response to siRNA toxicity. In addition, some researchers have observed an siRNA-mediated interferon response.

Research suggests that off-target effects, which may produce misleading results in RNAi experiments, can be largely avoided by using low siRNA concentrations (5, 6). By allowing highly efficient siRNA uptake and release within cells, HiPerFect Transfection Reagent enables gene silencing using low siRNA concentrations without compromising knockdown efficiency.

High knockdown and minimal risk of off-target effects in RNAi

HiPerFect Transfection Reagent enables highly efficient siRNA transfection, allowing gene silencing using low siRNA concentrations without compromising knockdown efficiency. In the data shown (Figure 1), efficient knockdown (>80%) is achieved with siRNA concentrations in the range of 1–50 nM. Transfection of 1 nM siRNA resulted in 86% knockdown and transfection of 5 nM siRNA increased the knockdown efficiency to 96%. Depending on the purpose of the RNAi experiment, the optimal concentration of siRNA to use may be 1 nM (minimal risk of off-target effects and efficient knockdown) or 5 nM (higher knockdown efficiency). To achieve the highest possible knockdown levels, it may be necessary to increase siRNA concentrations above the levels indicated in the optimization schemes in Tables 1 (page 15), 5 (page 55), 6 (page 56), and 11 (page 60). The optimal siRNA concentration will also depend on siRNA potency, cell type, and the target gene.

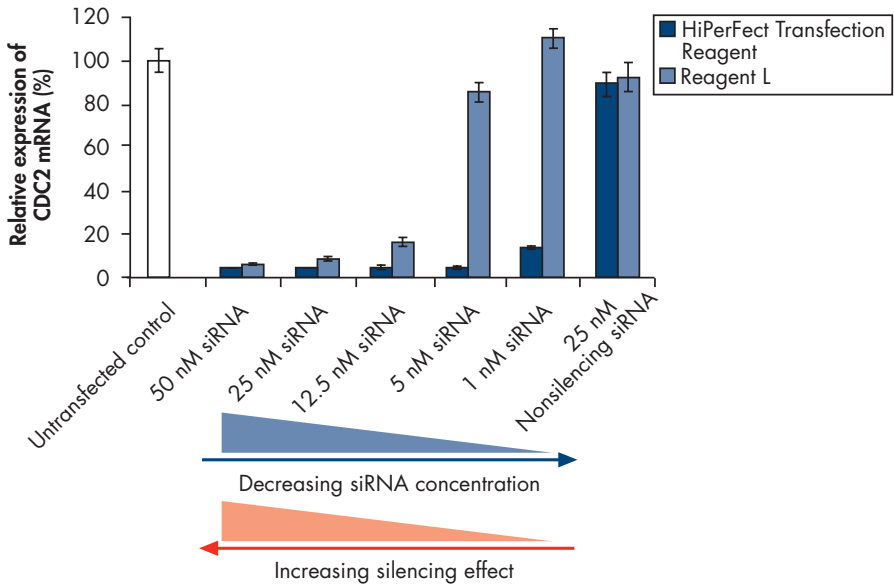


Figure 1. HiPerFect Reagent provides effective CDC2 knockdown even at low siRNA concentrations. HeLa S3 cells were transfected with a range of concentrations of siRNA targeted against CDC2 using HiPerFect Transfection Reagent from QIAGEN or Reagent L from another supplier. Nonsilencing siRNA was also transfected. After 48 hours, cells were harvested and total cellular RNA was purified using the RNeasy® system, and reverse transcribed using Omniscript® Reverse Transcriptase. The resultant cDNA was used for quantitative, real-time RT-PCR. Expression of CDC2 was normalized to expression of GAPDH. Values derived from real-time RT-PCR of control, untransfected cells were set at 100% and the relative expression levels of cells transfected with the experimental siRNA are shown.

miRNA research

microRNAs (miRNAs) are a class of endogenous small RNA molecules with similar characteristics to siRNAs. In recent years, it has been discovered that miRNAs play a role in many diverse biological processes such as development, differentiation, and apoptosis. Misregulation of miRNA expression is reported to be associated with several cancers and other diseases.

The miRNA system is an endogenous mechanism of regulation of gene expression. Mature miRNAs contribute to the regulation of endogenous genes, primarily by translational repression. In addition, miRNAs can mediate mRNA destruction by rapid deadenylation and/or decapping. Naturally occurring miRNA-binding sites are typically found in the 3' untranslated regions (UTRs) of target mRNAs. Their partial complementarity has made positive identification of true binding sites difficult and imprecise.

Transfection of synthetic miRNA mimics or miRNA inhibitors are techniques used to elucidate the targets and roles of particular miRNAs. miRNA mimics are chemically synthesized miRNAs which mimic naturally occurring miRNAs after transfection into the cell. miRNA inhibitors are single-stranded, modified RNAs which specifically inhibit miRNAs. Reduced gene expression after transfection of an miRNA mimic or increased expression after transfection of an miRNA inhibitor provides evidence that the miRNA is involved in regulation of that gene. Alternatively, the role of miRNAs in various pathways can be studied by examination of a specific phenotype after miRNA mimic or inhibitor transfection. Screening experiments can be performed to examine a phenotype after transfection of large numbers of synthetic miRNA mimics or inhibitors. HiPerFect Transfection Reagent is ideally suited to both low- and high-throughput transfection of miRNA mimics or miRNA inhibitors.

In addition to transfection, QIAGEN provides synthetic miRNA mimics and miRNA inhibitors, as well as innovative miRNeasy and miScript technologies for miRNA purification and detection. For more details, background information, and application data, visit www.qiagen.com/miRNA.

Protocols for various cell types and for special applications

This handbook contains protocols for adherent cells, suspension cells and macrophages, and primary cells.

Protocols for adherent cells

For adherent cells, two protocols are provided for siRNA/miRNA transfection in 24-well plates. In the Fast-Forward Protocol (page 20), cell seeding and transfection are carried out on the same day. In the Traditional Protocol (page 26), cell seeding is performed the day before transfection. The Fast-Forward Protocol is quicker and saves labor compared to the Traditional Protocol (Figures 2 and 3).

For high-throughput experiments with adherent cells, protocols are provided for reverse transfection in 96-well plates and 384-well plates (page 22 and page 24, respectively). In these protocols, cells are seeded and transfected on the same day. siRNA/miRNA is spotted into wells followed by the addition of HiPerFect Reagent. After complex formation, cells are added to the wells. Reverse transfection is rapid and convenient, and is frequently used for high-throughput experiments (Figures 2 and 3).

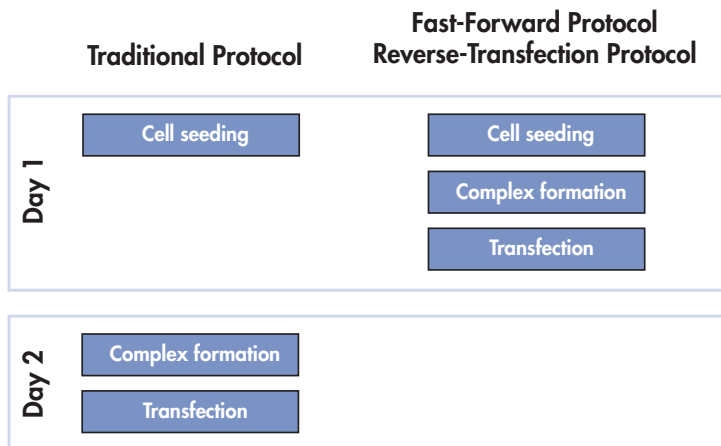


Figure 2. The Fast-Forward and Reverse-Transfection Protocols save time and labor. In the Reverse-Transfection and Fast-Forward Protocols, cell seeding, complex formation, and transfection are all performed on the same day. In the Traditional Protocol, cell seeding is performed the day before transfection.

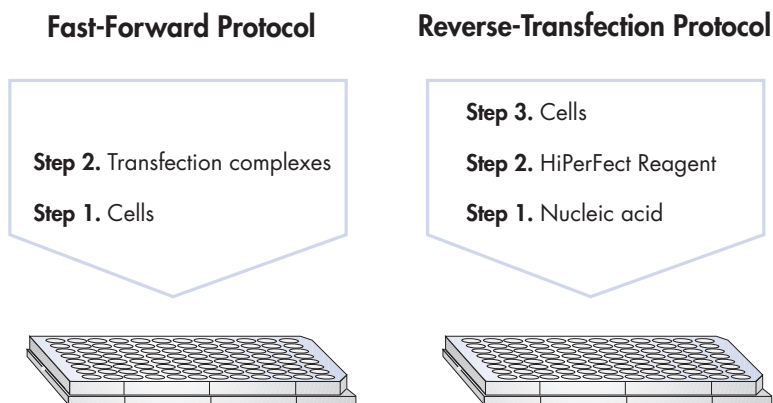


Figure 3. The order of steps differs between the Fast-Forward and Reverse Transfection Protocols. In the Fast-Forward Protocol, cells are added to plate wells first, followed by transfection complexes. In the Reverse-Transfection Protocol, siRNA/miRNA is added to plate wells, followed by HiPerFect Reagent. After complex formation in the wells, cells are added.

Protocols for suspension cells and macrophages

Protocols are also provided for siRNA/miRNA transfection of suspension cells (page 28), macrophages (page 30), and differentiated macrophages (page 32) in 24-well plates.

Protocols for primary cells

Protocols are provided for siRNA/miRNA transfection of HUVEC cells, fibroblasts, keratinocytes, epithelial cells, and smooth muscle cells in 24-well plates. In addition, guideline protocols are provided for transfection of primary neurons and primary hepatocytes in 96-well plates. These guidelines are standard parameters based on experiments with similar cell types and further optimization is recommended to achieve maximum transfection efficiency.

The primary-cell protocols provided have been mainly tested with human cells. However, they are applicable to all cell types.

Protocols for special applications with adherent cells

For large-scale transfection, a protocol is provided for transfection in 100 mm dishes on page 48. In this protocol, cell seeding and transfection are performed on the same day.

A protocol for long-term gene silencing is included on page 50. This protocol is for the study of phenotypic effects that require knockdown for periods longer than 3–4 days before they can be observed. Gene knockdown using siRNA is transient and expression usually increases again several days after transfection, especially when the cells are subcultured. However, the long-term protocol allows gene knockdown for over two weeks. The protocol uses HiPerFect Transfection Reagent to deliver siRNA/miRNA every time the cells are diluted and replated. This is possible because transfection with HiPerFect Reagent ensures extremely low levels of cytotoxicity, so cells remain viable and healthy after multiple cycles of dilution and transfection.

Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Culture medium
- siRNA of interest. We recommend functionally validated HP Validated siRNA or HP GenomeWide siRNA, which is available for every human, mouse, and rat gene. The GeneGlobe® Web portal allows convenient ordering (www.qiagen.com/GeneGlobe). QIAGEN also provides custom synthesis of highly pure HPP Grade siRNA and siRNA Sets for high-throughput RNAi. For more information, visit www.qiagen.com/siRNA .
- miRNA mimic or miRNA inhibitor of interest. For information about miRNA mimics and miRNA inhibitors from QIAGEN, visit www.qiagen.com/miRNA .
- For transfection of differentiated macrophages (page 32): a differentiation-inducing agent such as PMA
- For long-term transfection (page 50):
 - Sterile PBS (1x PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄; adjust to a final pH of 7.4). Store at room temperature (15–25°C)
 - Trypsin/EDTA (Hanks' Balanced Salt Solution containing 0.05% (w/v) porcine trypsin and 0.5 mM EDTA, or another appropriate trypsin or enzymatic solution)

Important Notes

Calculating concentrations of siRNA

Approximate values for a double-stranded, 21 nt siRNA molecule:

- 20 μ M siRNA is equivalent to approximately 0.25 μ g/ μ l
- The molecular weight of a 21 nt siRNA is approximately 13–15 μ g/nmol

To calculate the amount of siRNA to use for different formats, refer to Tables 3 and 4 for adherent cells (page 16), Table 8 for suspension cells (page 57), Table 9 for macrophages (page 57), Table 10 for differentiated macrophages (page 58), or Table 13 for primary cells (page 61).

Optimizing siRNA transfection

To achieve the best results in siRNA transfection of adherent cells, we recommend optimizing the following parameters. For details on optimizing siRNA transfection in suspension or macrophage cells, see Appendix A (page 55). For details on optimizing siRNA transfection in primary cells, see Appendix B (page 59). For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions.

Amount of siRNA

The amount of siRNA used is critical for efficient transfection and gene silencing. The recommended starting concentration for transfection of siRNA in 24-well plates is 5 nM. A pipetting scheme for optimizing siRNA transfection of adherent cells in 24-well plates is shown in Table 1 (page 15).

Ratio of HiPerFect Transfection Reagent to siRNA

The ratio of HiPerFect Transfection Reagent to siRNA should be optimized for every new cell type and siRNA combination used. As a starting point for optimization when using 24-well plates, we recommend 5 nM siRNA and 3 μ l HiPerFect Transfection Reagent for adherent cells. To optimize siRNA transfection in 24-well plates, prepare separate transfection mixtures according to Table 1 (page 15).

Please note that Table 1 is intended only as a guideline for starting amounts of siRNA and reagent. These amounts worked well as a starting point for transfection optimization in the range of cell lines that have been tested using HiPerFect Transfection Reagent. If necessary, it is also possible to further reduce the volume of HiPerFect Transfection Reagent without significantly compromising performance.

Table 1. Pipetting scheme for optimizing transfection of adherent cells in 24-well plates*

Amount (conc.) of siRNA	75 ng (10 nM)	75 ng (10 nM)	75 ng (10 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μ l	4.5 μ l
Amount (conc.) of siRNA	37.5 ng (5 nM)	37.5 ng (5 nM)	37.5 ng (5 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μl	4.5 μ l
Amount (conc.) of siRNA	7.5 ng (1 nM)	7.5 ng (1 nM)	7.5 ng (1 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μ l	4.5 μ l

* Amounts given are per well of a 24-well plate.

siRNA concentrations shown in brackets refer to the final siRNA concentration in each well.

For details on optimizing transfection in suspension or macrophage cells, see Appendix A (page 55) and in primary cells, see Appendix B (page 59).

Cell density at transfection

The optimal cell confluency for transfection should be determined for every new cell type to be transfected and kept constant in future experiments. This is achieved by counting cells before seeding and, in the case of the Traditional Protocol (page 26), by keeping the interval between seeding and transfection constant. This ensures that the cell density is not too high and that the cells are in optimal physiological condition at transfection. The recommended number of cells to seed for different formats is shown in Table 2.

Table 2. Recommended number of adherent cells to seed for different formats

Culture format	Fast-Forward Protocol	Traditional Protocol
	Reverse-Transfection Protocol (day of transfection)	(day before transfection)
384-well plate	4000–10,000	2000–5000
96-well plate	1–5 $\times 10^4$	0.5–3 $\times 10^4$
48-well plate	2–8 $\times 10^4$	1–4 $\times 10^4$
24-well plate	0.4–1.6 $\times 10^5$	2–8 $\times 10^4$
12-well plate	0.8–3 $\times 10^5$	0.4–1.6 $\times 10^5$
6-well plate	1.5–6 $\times 10^5$	0.8–3 $\times 10^5$
60 mm dish	0.3–1.2 $\times 10^6$	1.5–6 $\times 10^5$
100 mm dish	2–4 $\times 10^6$	1–2 $\times 10^6$

For recommended numbers of suspension or macrophage cells to seed, see Appendix A (page 55) and for primary cells, see Appendix B (page 59).

Calculating siRNA and reagent for different formats

Table 3 and Table 4 give starting points for optimization of siRNA transfection of adherent cells in different plate and dish formats for the Fast-Forward Protocol (page 20) and the Traditional Protocol (page 26). Starting points for 96-well, 384-well, and 100 mm formats are given in the respective protocols (pages 22–25, page 48).

Table 3. Starting points for optimizing transfection of adherent cells in different formats

Culture format	Volume of medium on cells (µl)	siRNA amount (ng)*	Final volume of diluted siRNA (µl)	Volume of HiPerFect Reagent (µl)	Final siRNA conc. (nM)
48-well plate	250	19	50	1.5	5
24-well plate	500	37.5	100	3	5
12-well plate	1100	75	100	6	5
6-well plate	2300	150	100	12	5
60 mm dish	4000	256	100	20	5

* Table 4 shows the corresponding volumes of siRNA stock to use for each siRNA amount.

For starting points for optimizing transfection of suspension or macrophage cells, see Appendix A (page 55) and for primary cells, see Appendix B (page 59).

Table 4. Volumes of siRNA stock for different siRNA amounts

Culture format	siRNA amount (ng)	Equivalent volume of 0.2 µM siRNA stock (µl)*	Equivalent volume of 2 µM siRNA stock (µl)*	Equivalent volume of 20 µM siRNA stock (µl)*
48-well plate	19	7.5	–	–
24-well plate	37.5	–	1.5	–
12-well plate	75	–	3	–
6-well plate	150	–	6.0	–
60 mm dish	256	–	–	1.0

* To make a 0.2 µM siRNA stock, dilute 10 µl of a 20 µM stock to a final volume of 1000 µl. To make a 2 µM siRNA stock, dilute 10 µl of a 20 µM stock to a final volume of 100 µl.

Transfection in multiwell plates — preparing a master mix

If you are performing transfection in multiwell plates, prepare a master mix of transfection complexes or of transfection reagent and culture medium (depending on the protocol) for distribution into plate wells.

- Calculate the required volumes of each component and the total volume before you prepare the master mix.
- Prepare 10% more master mix than is required to allow for pipetting errors (i.e., for a 48-well plate, prepare enough master mix for 53 wells).
- Add and mix the components of the master mix according to the instructions in the protocol.
- Use a repeat pipet to distribute the master mix.

Performing appropriate RNAi control experiments

It is important to perform suitable control experiments so that results can be correctly interpreted. A full range of control siRNAs is available at www.qiagen.com/AllStars. For information about appropriate miRNA control experiments, visit www.qiagen.com/miRNA. We recommend the following control experiments.

Positive control siRNA

This is an siRNA that is known to provide high knockdown of its target gene. For example, Mm/Hs_MAPK1 Control siRNA (cat. no. 1022564) efficiently knocks down human and mouse MAPK1 and Rn_Mapk1 Control siRNA (cat. no. 1027277) efficiently knocks down rat MAPK1. AllStars Hs Cell Death Control siRNA (cat. no. 1027298) provides high knockdown of ubiquitous human cell survival genes which results in a high degree of cell death which is visible by light microscopy. These siRNAs can be used as positive controls.

A positive control is used to establish that the experimental set up for transfection and knockdown analysis is working optimally. An siRNA that knocks down a gene resulting in the phenotypic effect under study may also be used as a positive control to ensure that the phenotypic assay is working optimally. A positive control siRNA should be transfected in every RNAi experiment.

Negative control siRNA

A negative control siRNA should be a nonsilencing siRNA with no homology to any known mammalian gene, such as AllStars Negative Control siRNA (5 nmol) from QIAGEN (cat. no. 1027280). AllStars Negative Control siRNA is the most thoroughly tested and validated negative control available. It has been shown to provide minimal nonspecific effects on gene expression and phenotype and is incorporated into RISC. For more details and to view data, visit www.qiagen.com/AllStars.

Transfection of negative control siRNA is used to determine whether changes in phenotype or gene expression are nonspecific. A negative control siRNA should be transfected in every RNAi experiment.

Transfection control siRNA

This control is used to measure the transfection efficiency. Transfection efficiency can be measured in several ways, for example by fluorescence microscopy after transfection of a fluorescently labeled siRNA or by observation of the level of cell death after transfection of siRNA that targets essential cell survival genes, such as AllStars Hs Cell Death Control siRNA. siRNA transfection efficiency should be as high as possible. This control should be performed for optimization, for example, when establishing RNAi in a new cell line.

Mock transfection control

Mock-transfected cells go through the transfection process without addition of siRNA (i.e., cells are treated with transfection reagent only). This control is used to determine any nonspecific effects that may be caused by the transfection reagent or process.

Untransfected cells control

Gene expression analysis should be carried out on cells that have not been treated to allow measurement of the normal, basal level of gene expression. Results from untreated cells can be used for comparison with results from all other samples. Untreated cells should be analyzed in every RNAi experiment.

Additional siRNAs for phenotype confirmation

A phenotypic effect caused by knockdown of a gene must be confirmed using at least one additional siRNA targeted against a different area of the mRNA.

Monitoring gene silencing at the mRNA or protein level

Gene silencing can be monitored at either the mRNA or the protein level. Protein analysis can be performed using western blotting, immunofluorescence, or FACS® analysis. More information about protein analysis and a protocol for western blotting can be found at www.qiagen.com/literature/BenchGuide. The AllPrep RNA/Protein Kit (cat. no. 80404) allows simultaneous purification of RNA and protein from the same sample for streamlined downstream analysis after knockdown. Protein analysis is the most comprehensive way of showing that a gene has been downregulated. However, it may not always be possible to perform protein analysis (e.g., if antibodies for the protein of interest are not available for western blotting).

Silencing is usually monitored at the mRNA level by real-time RT-PCR, microarray analysis, or northern blotting. Information about working with RNA and a northern blotting protocol are available at www.qiagen.com/literature/BenchGuide. Quantitative, real-time RT-PCR is an easy and routinely used method to monitor gene silencing at the mRNA level. QuantiTect® Primer Assays are bioinformatically validated primer sets that are available for every human, mouse, rat, dog, drosophila, chicken, and arabidopsis gene. QuantiTect Primer Assays are used in combination with QuantiTect or QuantiFast® SYBR® Green Kits for sensitive and specific one-step or two-step real-time RT-PCR using SYBR Green detection. Expression data should be compared with levels of a housekeeping gene, such as GAPDH, to normalize for variable amounts of RNA in different samples.

Protocol: Fast-Forward Transfection of Adherent Cells with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of adherent cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. This protocol can also be used as a starting point for optimizing transfection in other formats (e.g., 48-well plate, 12-well plate, 6-well plate, and 60 mm dish formats) using the siRNA and reagent amounts listed in Table 3 on page 16.

In this protocol, cell plating and transfection are performed on the same day. For a few sensitive cell types, it may be necessary to use the Traditional Protocol, where cells are plated the day before transfection (page 26).

Note: We recommend when using 24-well plates that transfection is performed in the order described in this Fast-Forward Protocol, with cells seeded in wells first followed by addition of siRNA/miRNA–reagent complexes. This ensures optimal mixing of cells and complexes. However, transfection can be performed using a Reverse-Transfection Protocol, with complexes added to wells and cells added on top of complexes, if desired. To perform a Reverse-Transfection Protocol, simply change the order in which cells and complexes are added to the plate (steps 1–5).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The optimal amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Shortly before transfection, seed $0.4\text{--}1.6 \times 10^5$ cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
Cells may alternatively be seeded after step 3 of this protocol.
2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and $5\% \text{CO}_2$).**
3. **Dilute 37.5 ng siRNA in 100 μl culture medium without serum (this will give a final siRNA concentration of 5 nM after adding complexes to cells in step 5). Add 3 μl of HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**
6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.**

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment. When using fluorescently labeled siRNA, microscopic analysis should be performed 4–24 h after transfection.

Protocol: Reverse Transfection of Adherent Cells with siRNA/miRNA in 96-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of adherent cells in a single well of a 96-well plate using HiPerFect Transfection Reagent. Starting points for optimizing transfection in other formats are listed in Table 3 on page 16. In this protocol, cell plating and transfection are performed on the same day.

Note: Transfection in 96-well plates can also be performed using a Fast-Forward Protocol, if desired. In this Reverse-Transfection Protocol, complex formation takes place in the plate wells first and then cells are added on top of the complexes. In the Fast-Forward Protocol, cells are added to plate wells followed by complexes (see Figure 3, page 11). To perform a Fast-Forward Protocol, simply change the order in which cells and complexes are added to the plate (steps 1–4). However, we recommend using this Reverse-Transfection Protocol when working with 96-well plates because it is quicker, has fewer pipetting steps, and uses fewer materials (the Fast-Forward Protocol requires two plates, one for complex formation and one for adding complexes on top of cells; the Reverse-Transfection Protocol requires only one plate).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The optimal amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Spot 12.5 ng siRNA in 1–3 μ l of siRNA Suspension Buffer/RNase-free water into a single well of a 96-well plate (this will give a final siRNA concentration of 5 nM after addition of cells to complexes in step 4).**

Note: After this step, siRNA may be stored at -20°C for long time periods. siRNA may be stored in solution or may be dried in the plate at room temperature (15 – 25°C) before storage.

Note: If preferred, siRNA can be spotted in $25 \mu\text{l}$ of siRNA Suspension Buffer/RNase-free water into each well. In this case, $150 \mu\text{l}$ culture medium (containing 1 – 5×10^4 cells) should be added in step 4.

2. **Add 0.75 μ l of HiPerFect Transfection Reagent to 24.25 μ l of culture medium without serum. Add the diluted HiPerFect Transfection Reagent to the prespotted siRNA.**

Note: To ensure accurate pipetting, diluted HiPerFect Reagent should be prepared in a larger volume for use in multiple wells (see “Transfection in multiwell plates — preparing a master mix” on page 17). Then add 25 μ l of the dilution to a single well.

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

3. **Incubate for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**
4. **Seed 1–5 $\times 10^4$ cells in 175 μ l of an appropriate culture medium (containing serum and antibiotics) into the well, on top of the siRNA–HiPerFect Reagent transfection complexes.**
5. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.**

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Reverse Transfection of Adherent Cells with siRNA/miRNA in 384-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of adherent cells in a single well of a 384-well plate using HiPerFect Transfection Reagent. Starting points for optimizing transfection in other formats are listed in Table 3 on page 16. In this protocol, cell plating and transfection are performed on the same day.

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Spot 3.125 ng siRNA in 1–3 μ l of siRNA Suspension Buffer/RNase-free water into a single well of a 384-well plate (this will give a final siRNA concentration of 5 nM after addition of cells to complexes in step 4).**

Note: After this step, siRNA may be stored at -20°C for long time periods. siRNA may be stored in solution or may be dried in the plate at room temperature (15 – 25°C) before storage.

2. **Add 0.5 μ l of HiPerFect Transfection Reagent to 9.5 μ l of culture medium without serum. Add the diluted HiPerFect Transfection Reagent to the prespotted siRNA.**

Note: To ensure accurate pipetting, diluted HiPerFect Reagent should be prepared in a larger volume for use in multiple wells (see “Transfection in multiwell plates — preparing a master mix” on page 17).

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

3. **Incubate for 5–10 min at room temperature (15 – 25°C) to allow formation of transfection complexes.**

4. Seed 4000–10,000 cells in 40 μ l of an appropriate culture medium (containing serum and antibiotics) into the well, on top of the siRNA–HiPerFect Reagent transfection complexes.
5. Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Transfection of Adherent Cells with siRNA/miRNA (Traditional Protocol)

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of adherent cells in a single well of a 24-well plate using HiPerFect Transfection Reagent if seeding cells the day before transfection is preferred. Starting points for optimizing transfection in other formats are listed in Table 3 on page 16. For some cell types, such as HepG2, the Fast-Forward Protocol (page 20) may give higher gene silencing effects at very low siRNA concentrations compared to this protocol.

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The optimal confluency for transfection of adherent cells using this protocol is 50–80%. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **The day before transfection, seed 2–8 x 10⁴ cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
2. **Incubate the cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **On the day of transfection, dilute 37.5 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 5 nM after adding complexes to cells in step 5). Add 3 µl of HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**

6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.**

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment. When using fluorescently labeled siRNA, microscopic analysis should be performed 4–24 h after transfection.

Protocol: Transfection of Suspension Cell Lines, Including Jurkat and K562, with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of suspension cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. Details about optimization of transfection of suspension cells and starting points for transfection in different formats are provided in Appendix A (page 55).

Cultivation of suspension cells

Optimal transfection efficiency is achieved when suspension cells are cultivated in a spinner flask for at least 24 hours prior to transfection. This ensures optimal gas exchange and that the cells are in logarithmic growth phase. The day before transfection, an aliquot of cells is mixed with trypan blue (which stains dead cells) and living (unstained) cells are counted using a counting chamber. An appropriate volume of cells is centrifuged and the cell pellet is resuspended in fresh culture medium and transferred to a spinner flask. The cell density in the spinner flask should be 3×10^5 cells per ml (protocol step 1).

Spinner flasks are available in various sizes (e.g., 50 ml, 250 ml, and 1000 ml). Pendulums inside the flask stir the cell culture. The volume of medium in the flask should be approximately one third of the flask size (e.g., a 250 ml spinner flask should contain ~85 ml cell suspension). The cap of the flask should be loose to allow air to enter. The cell suspension should be constantly stirred at ~60 stirs per minute.

The next day, cell number is determined. For most cell types, the cell number should have doubled. The appropriate volume of cells is then harvested for transfection (protocol step 3). If cells grow significantly slower than expected, it may indicate that cells are not healthy. In this case, repeat the overnight incubation with fresh cells and medium.

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. For recommended cell numbers to seed, see Table 7, page 56. The optimal amount of cells to seed depends on the cell line and the time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. The day before transfection, dilute cells at a density of 3×10^5 per ml in an appropriate culture medium containing serum and antibiotics in a spinner flask.
2. Incubate the cells under normal growth conditions (typically 37°C and $5\% \text{CO}_2$).
3. On the day of transfection, plate 2×10^5 cells per well of a 24-well plate in 100 μl culture medium containing serum and antibiotics.
4. Dilute 750 ng siRNA in 100 μl culture medium without serum (this will give a final siRNA concentration of 100 nM after adding medium in step 8). Add 6 μl HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target.

5. Incubate the samples for 5–10 min at room temperature ($15\text{--}25^\circ\text{C}$) to allow the formation of transfection complexes.
6. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

IMPORTANT: When performing transfections in 96-well plates, transfection efficiency can be significantly enhanced by thoroughly mixing the cells and transfection complexes by pipetting up and down 4–6 times.

7. Incubate the cells with the transfection complexes under their normal growth conditions for 6 h.
8. Add 400 μl culture medium containing serum and antibiotics to the cells and incubate until analysis of gene silencing (e.g., 6–72 h after transfection, depending on the experimental setup). Add fresh medium as required.

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Transfection of Macrophage Cell Lines, Including J774.A1 and RAW 264.7, with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of macrophage cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. Details about optimization of transfection of macrophage cells and starting points for transfection in different formats are provided in Appendix A (page 55).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. For recommended cell numbers to seed, see Table 7, page 56. The optimal amount of cells to seed depends on the cell line and the time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Shortly before transfection, seed $0.4\text{--}2 \times 10^5$ cells per well of a 24-well plate in 100 μl of an appropriate culture medium containing serum and antibiotics.**

Cells may alternatively be seeded after step 3 of this protocol.

2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 375 ng siRNA in 100 μl culture medium without serum (this will give a final siRNA concentration of 50 nM after adding medium in step 7). Add 6 μl HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target.

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**
6. **Incubate the cells with the transfection complexes under their normal growth conditions for 6 h.**

7. **Add 400 μ l culture medium containing serum and antibiotics to the cells and incubate until analysis of gene silencing (e.g., 6–72 h after transfection, depending on the experimental setup). Change the medium as required.**

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Transfection of Differentiated Macrophage Cell Lines, Including THP-1, with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of differentiated macrophages in a single well of a 24-well plate using HiPerFect Transfection Reagent. Details about optimization of transfection of differentiated macrophages and starting points for transfection in different formats are provided in Appendix A (page 55).

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. For recommended cell numbers to seed, see Table 7, page 56. The optimal amount of cells to seed depends on the cell line and the time point of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **24 h before transfection, seed 2×10^4 cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
2. **Add the appropriate amount of differentiation-inducing agent to the cells and incubate overnight under normal growth conditions (typically 37°C and 5% CO₂).**
Note: For differentiation of THP-1 cells, 100 ng/ml PMA was used.
3. **Shortly before transfection, remove the culture medium from the cells and add 100 µl fresh culture medium containing serum and antibiotics.**
4. **For the short time until transfection, incubate the cells under normal growth conditions.**
5. **Dilute 375 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 50 nM after adding medium in step 9). Add 6 µl HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target.

6. Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.
7. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
8. Incubate the cells with the transfection complexes under their normal growth conditions for 6 h.
9. Add 400 μ l culture medium containing serum and antibiotics to the cells and incubate until analysis of gene silencing (e.g., 6–72 h after transfection, depending on the experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Transfection of HUVEC Cells with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of HUVEC and other endothelial cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. Details about optimization of transfection of primary cells and starting points for transfection in different formats are provided in Appendix B (page 59).

Cells should be at a low passage number (do not exceed a passage number of 4).

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **On the day of transfection, seed 6×10^4 cells per well of a 24-well plate in 0.1 ml of an appropriate culture medium containing serum and antibiotics.**
Cells may also be seeded after step 3 of this protocol.
2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 75 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 10 nM). Mix by vortexing. Add 3 µl of HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent required for optimal performance may vary, depending on the exact cell type used. For specific cell types and targets, optimal conditions may be different from those described here.

The amount of siRNA required for optimal results may also vary. For many cell types it may be possible to reduce the amount of siRNA to 7.5 ng (1 nM). For some cell types it may be necessary to increase the amount of siRNA up to 225 ng (25 nM).

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
6. Incubate cells with the transfection complexes under their normal growth conditions.
7. After 3 h, add 400 μ l culture medium.
8. Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6-72 h after transfection, depending on experimental setup).

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time course experiment.

Protocol: Transfection of Fibroblasts with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of fibroblasts in a single well of a 24-well plate using HiPerFect Transfection Reagent. In this protocol, cell plating and transfection are performed on the same day. Details about optimization of transfection of primary cells and starting points for transfection in different formats are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **On the day of transfection, seed 6×10^4 cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
Cells may also be seeded after step 3 of this protocol.
2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 75 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 10 nM). Mix by vortexing. Add 3 µl of HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent required for optimal performance may vary, depending on the exact cell type used. For specific cell types and targets, optimal conditions may be different from those described here.

The amount of siRNA required for optimal results may also vary. For many cell types it may be possible to reduce the amount of siRNA to 7.5 ng (1 nM). For some cell types it may be necessary to increase the amount of siRNA up to 225 ng (25 nM).

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
6. Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time course experiment.

Protocol: Transfection of Keratinocytes with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of keratinocytes in a single well of a 24-well plate using HiPerFect Transfection Reagent. In this protocol, cell plating and transfection are performed on the same day. Details about optimization of transfection of primary cells and starting points for transfection in different formats are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **On the day of transfection, seed 6×10^4 cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**

Cells may also be seeded after step 3 of this protocol.

2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 37.5 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 5 nM). Mix by vortexing. Add 3 µl of HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent required for optimal performance may vary, depending on the exact cell type used. For specific cell types and targets, optimal conditions may be different from those described here.

The amount of siRNA required for optimal results may also vary. For many cell types it may be possible to reduce the amount of siRNA to 7.5 ng (1 nM). For some cell types it may be necessary to increase the amount of siRNA up to 225 ng (25 nM).

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
6. Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time course experiment.

Protocol: Transfection of Epithelial Cells with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of epithelial cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. In this protocol, cell plating and transfection are performed on the same day. Details about optimization of transfection of primary cells and starting points for transfection in different formats are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **On the day of transfection, seed 6×10^4 cells per well of a 24-well plate in 0.1 ml of an appropriate culture medium containing serum and antibiotics.**

Cells may also be seeded after step 3 of this protocol.

2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 75 ng siRNA in 100 μ l culture medium without serum (this will give a final siRNA concentration of 10 nM). Mix by vortexing. Add 3 μ l of HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent required for optimal performance may vary, depending on the exact cell type used. For specific cell types and targets, optimal conditions may be different from those described here.

The amount of siRNA required for optimal results may also vary. For many cell types it may be possible to reduce the amount of siRNA to 7.5 ng (1 nM). For some cell types it may be necessary to increase the amount of siRNA up to 225 ng (25 nM).

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
6. Incubate cells with the transfection complexes under their normal growth conditions.
7. After 3 h, add 400 μ l culture medium.
8. Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup).

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time course experiment.

Protocol: Transfection of Smooth Muscle Cells with siRNA/miRNA in 24-Well Plates

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of smooth muscle cells in a single well of a 24-well plate using HiPerFect Transfection Reagent. In this protocol, cell plating and transfection are performed on the same day. Details about optimization of transfection of primary cells and starting points for transfection in different formats are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition at the time of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **On the day of transfection, seed 6×10^4 cells per well of a 24-well plate in 0.1 ml of an appropriate culture medium containing serum and antibiotics.**

Cells may also be seeded after step 3 of this protocol.

2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **Dilute 75 ng siRNA in 100 μ l culture medium without serum (this will give a final siRNA concentration of 10 nM). Mix by vortexing. Add 3 μ l of HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent required for optimal performance may vary, depending on the exact cell type used. For specific cell types and targets, optimal conditions may be different from those described here.

The amount of siRNA required for optimal results may also vary. For many cell types it may be possible to reduce the amount of siRNA to 7.5 ng (1 nM). For some cell types it may be necessary to increase the amount of siRNA up to 225 ng (25 nM).

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
6. Incubate cells with the transfection complexes under their normal growth conditions.
7. After 3 h, add 400 μ l culture medium.
8. Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6-72 h after transfection, depending on experimental setup).

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time course experiment.

Guidelines for Transfection of Primary Neurons with siRNA/miRNA in 96-Well Plates

This protocol is a guideline for siRNA/miRNA transfection of primary neurons in a single well of a 96-well plate using HiPerFect Transfection Reagent. This guideline is based on experiments with similar cell types and may not be effective under the laboratory conditions used. The protocol will require further optimization to achieve maximum transfection efficiency. Details about optimization of transfection of primary cells are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Five days before transfection, seed 1.6×10^5 cells per well of a 96-well plate in 0.2 ml of an appropriate culture medium.**
2. **Incubate the cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **On the day of transfection, dilute 125 ng siRNA in 25 µl culture medium without serum (this will give a final siRNA concentration of 50 nM). Mix by vortexing.**
4. **Add 0.5 µl of HiPerFect Transfection Reagent to 24.5 µl of culture medium without serum. Add the diluted HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary. Depending on the exact cell type and gene target, it may be necessary to increase the amount of HiPerFect Reagent to 1 µl or 1.5 µl per well.

5. **Incubate for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
6. **Remove medium from cells and add 0.15 ml fresh medium per well.**
7. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**

8. Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Guidelines for Transfection of Primary Hepatocytes with siRNA/miRNA in 96-Well Plates

This protocol is a guideline for siRNA/miRNA transfection of primary hepatocytes in a single well of a 96-well plate using HiPerFect Transfection Reagent. This guideline is based on experiments with similar cell types and may not be effective under the laboratory conditions used. The protocol will require further optimization to achieve maximum transfection efficiency. Details about optimization of transfection of primary cells are provided in Appendix B (page 59).

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **The day before transfection, seed 8×10^4 cells per well of a 96-well plate in 0.15 ml of an appropriate culture medium.**

Note: The optimal cell density and time point of plating depends on the experimental requirements and the source of the cells. The number of cells to be plated may need to be adjusted.

2. **Incubate cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **On the day of transfection, dilute 125 ng siRNA in 25 µl culture medium without serum (this will give a final siRNA concentration of 50 nM). Mix by vortexing.**
4. **Add 0.75 µl of HiPerFect Transfection Reagent to 24.25 µl of culture medium without serum. Add the diluted HiPerFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times or by vortexing for 10 s.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary. Depending on the exact cell type and gene target, it may be necessary to increase the amount of HiPerFect Reagent to 1 µl or 1.5 µl per well.

5. **Incubate for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes.**

6. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.
7. Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.

Note: The optimal incubation time for gene silencing analysis depends on cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Large-Scale Transfection of Adherent Cells with siRNA/miRNA in 100 mm Dishes

This protocol is provided as a starting point for optimization of siRNA/miRNA transfection of adherent cells in a 100 mm dish using HiPerFect Transfection Reagent. Starting points for optimizing transfection in other formats are listed in Table 3 on page 16. In this protocol, cell plating and transfection are performed on the same day. For a few sensitive cell types, it may be necessary to plate cells the day before transfection (as in the Traditional Protocol on page 26).

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The optimal amount of cells seeded depends on the cell type and time of analysis.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **Shortly before transfection, seed $2\text{--}4 \times 10^6$ cells per 100 mm dish in 7 ml of an appropriate culture medium containing serum and antibiotics.**
Cells may alternatively be seeded after step 3 of this protocol.
2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and $5\% \text{CO}_2$).**
3. **Dilute 600 ng siRNA in 1 ml culture medium without serum (this will give a final siRNA concentration of 5 nM after adding complexes to cells in step 5). Add 40 μl of HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

4. **Incubate the samples for 5–10 min at room temperature ($15\text{--}25^\circ\text{C}$) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the dish to ensure uniform distribution of the transfection complexes.**

6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). Change the medium as required.**

Note: The optimal incubation time for gene silencing analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Long-Term Transfection of Adherent Cells with siRNA/miRNA

This protocol is for siRNA/miRNA transfection of adherent cells in a single well of a 24-well plate. It is provided as a starting point for optimization of siRNA/miRNA transfection in mammalian cells using HiPerFect Transfection Reagent.

Important points before starting

- Cells should be in optimal physiological condition on the day of transfection. The optimal confluency for transfection of adherent cells using this protocol is 50–80%. The amount of cells seeded depends on the cell type and time of analysis.
- This protocol requires sterile PBS and Trypsin/EDTA (not supplied). For details on preparation of PBS and Trypsin/EDTA, see “Reagents to Be Supplied by User”, page 13.
- If siRNA has been ordered from QIAGEN, it is delivered lyophilized and must be resuspended prior to transfection. To resuspend, follow the instructions provided with the siRNA.
- Nucleic acid amounts in the protocol refer to siRNA. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA in the protocol.

Procedure

1. **The day before the first transfection, seed $2-8 \times 10^4$ cells per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
2. **Incubate the cells under normal growth conditions (typically 37°C and 5% CO₂).**
3. **On the day of transfection, dilute 37.5 ng siRNA in 100 µl culture medium without serum (this will give a final siRNA concentration of 5 nM after adding complexes to cells in step 5). Add 3 µl of HiPerFect Transfection Reagent to the diluted siRNA and mix by vortexing.**

IMPORTANT: The amount of HiPerFect Transfection Reagent and siRNA required for optimal performance may vary, depending on the cell line and gene target. For details on optimization of the siRNA to HiPerFect Transfection Reagent ratio, see page 14.

4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**

6. **Incubate the cells with the transfection complexes under their normal growth conditions. After 6–24 h, change the medium.**

Note: Depending on the cell type, a medium change may not be necessary. Instead it may be possible to leave the complexes on the cells until they reach confluency.

7. **When the cells become confluent, they are split and retransfected as follows (steps 7–13). Remove the medium and wash the cells with 500 μ l PBS.**

8. **Add 100 μ l Trypsin/EDTA and incubate until the cells detach. Observe the cells closely to avoid extended incubation with trypsin.**

9. **Add 900 μ l medium containing serum and antibiotics to stop the trypsination.**

10. **Prepare transfection complexes as described in steps 3 and 4.**

11. **Dilute an aliquot of the trypsinated cells in a final volume of 500 μ l medium containing serum and antibiotics and transfer them to a fresh well of a 24-well plate.**

Note: The dilution factor is cell-type-specific and usually reflects the doubling time of the cell type.

12. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**

13. **Incubate the cells with the transfection complexes under their normal growth conditions. After 6–24 h, change the medium.**

Note: Depending on the cell type, a medium change may not be necessary. Instead it may be possible to leave the complexes on the cells until they reach confluency or until analysis.

14. **When the cells become confluent and need to be split, repeat the procedure from step 7 onward.**

15. **Monitor gene silencing after an appropriate time.**

Note: The optimal number of splitting cycles and transfections before gene silencing analysis depends on the gene targeted and the phenotype under study. This can be determined by performing a time-course experiment. Using this procedure, efficient knockdown without cytotoxicity has been observed up to 2 weeks after the initial transfection.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

Comments and suggestions

Low transfection efficiency

- | | |
|--|--|
| a) Suboptimal HiPerFect Transfection Reagent:siRNA ratio | Although fixed volumes of HiPerFect Transfection Reagent usually work very well with a range of siRNA concentrations, it could occur that the overall charge of the complexes is negative, neutral, or strongly positive, which can lead to inefficient adsorption to the cell surface. For optimal adsorption, complexes should be weakly positive. Optimize the HiPerFect Transfection Reagent to siRNA ratio using Tables 1 (page 15), 5 (page 55), 6 (page 56), or 11 (page 60), or perform systematic titrations of HiPerFect Transfection Reagent. |
| b) Suboptimal cell density | If cell density at the time of adding HiPerFect Transfection Reagent–siRNA complexes is not at an optimal level, cells may not be in the optimal growth phase for transfection. This can lead to insufficient uptake of complexes into the cells or inefficient processing of the siRNA. For adherent cells, the optimal confluency for transfection of siRNA is 50–80% if using the Traditional Protocol (page 26). |
| c) Poor siRNA quality | siRNA should be of high quality, as impurities can reduce transfection efficiency. We recommend HP GenomeWide siRNA and HP Validated siRNA for efficient gene silencing (www.qiagen.com/GeneGlobe). |

Excessive cell death

- | | |
|--|---|
| a) Concentration of HiPerFect Transfection Reagent–siRNA complexes is too high | Decrease the amount of HiPerFect Transfection Reagent–siRNA complexes added to the cells. |
| b) Cells are stressed | Avoid stressing cells with temperature shifts and long periods without medium during washing steps. It is particularly important for transfection of siRNA that the cells are in good condition. Therefore, ensure that cell density is not too low at transfection. For adherent cells, the optimal confluency for transfection is 50–80% if using the Traditional Protocol (page 26). |
| c) Poor siRNA quality | siRNA should be of high quality, as impurities can reduce transfection efficiency. We recommend HP GenomeWide siRNA and HP Validated siRNA for efficient gene silencing (www.qiagen.com/GeneGlobe). |
| d) Key gene is silenced | If the gene targeted is important for the survival of the cell, silencing this gene may lead to cell death. |

Variable transfection efficiencies in replicate experiments

- | | |
|--|---|
| a) Inconsistent cell confluencies in replicate experiments | Count cells before seeding to ensure that the same number of cells is seeded for each experiment. Keep the incubation time between seeding and complex addition consistent between experiments. |
| b) Possible mycoplasma contamination | Mycoplasma contamination influences transfection efficiency. Variations in the growth behavior of mycoplasma-infected cells will lead to different transfection efficiencies between replicate experiments. |

Comments and suggestions

- c) Cells have been passaged too many times Cells that have been passaged a large number of times tend to change their growth behavior and morphology, and are less susceptible to transfection. When cells with high passage numbers are used for replicate experiments, decreased transfection efficiencies may be observed in later experiments. We recommend using cells with a low passage number (<50 splitting cycles).
- d) Concentration of siRNA is too low Increase siRNA concentration used in transfection.

No or very small gene silencing effect

- a) Design of siRNA suboptimal The design of an siRNA can have a large effect on its gene silencing efficiency. We recommend HP GenomeWide siRNA and HP Validated siRNA for efficient gene silencing (www.qiagen.com/GeneGlobe).
- b) Incubation time after transfection too short The gene silencing effect observed at the protein level is dependent on the expression level of the protein and its rate of turnover within the cell. Perform a time-course experiment to determine the optimal time point for analysis.
- c) Problems with experimental design RNAi effects may not be seen for some genes targeted with certain siRNAs in some cell types. If possible, repeat experiments using a different cell type and/or siRNA. Where possible, include both positive and negative controls in your experiments. QIAGEN offers a range of control siRNAs at www.qiagen.com/AllStars .
- d) Concentration of siRNA is too low Increase siRNA concentration used in transfection.

Appendix A: Optimizing siRNA Transfection of Suspension and Macrophage Cells

To achieve the best results in siRNA transfection, we recommend optimizing the following parameters. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions.

Amount of siRNA

The amount of siRNA used is critical for efficient transfection and gene silencing. The recommended starting concentration for transfection of siRNA in 24-well plates is 100 nM for suspension cells or 50 nM for macrophages and differentiated macrophages. Pipetting schemes for optimizing siRNA transfection in 24-well plates are shown in Tables 5 and 6 (below).

Ratio of HiPerFect Transfection Reagent to siRNA

The ratio of HiPerFect Transfection Reagent to siRNA should be optimized for every new cell type and siRNA combination used. As a starting point for optimization when using 24-well plates, we recommend 100 nM siRNA and 6 μ l HiPerFect Transfection Reagent for suspension cells or 50 nM siRNA and 6 μ l HiPerFect Transfection Reagent for macrophages and differentiated macrophages. To optimize siRNA transfection in 24-well plates, prepare separate transfection mixtures according to Tables 5 and 6 (below). Please note that Tables 5 and 6 are intended only as a guideline for starting amounts of siRNA and reagent. These amounts worked well as a starting point for transfection optimization in the range of cell lines that have been tested using HiPerFect Transfection Reagent. If necessary, it is also possible to further reduce the volume of HiPerFect Transfection Reagent without significantly compromising performance.

Table 5. Pipetting scheme for optimizing transfection of suspension cells in 24-well plates*

Amount (final conc.) of siRNA	1.5 μ g (200 nM)	1.5 μ g (200 nM)	1.5 μ g (200 nM)
Volume of HiPerFect Reagent	3 μ l	6 μ l	9 μ l
Amount (final conc.) of siRNA	750 ng (100 nM)	750 ng (100 nM)	750 ng (100 nM)
Volume of HiPerFect Reagent	3 μ l	6 μl	9 μ l
Amount (final conc.) of siRNA	375 ng (50 nM)	375 ng (50 nM)	375 ng (50 nM)
Volume of HiPerFect Reagent	3 μ l	6 μ l	9 μ l

* Amounts given are per well of a 24-well plate.

Table 6. Pipetting scheme for optimizing transfection of macrophages and differentiated macrophages in 24-well plates*

Amount (final conc.) of siRNA	750 ng (100 nM)	750 ng (100 nM)	750 ng (100 nM)
Volume of HiPerFect Reagent	3 μ l	6 μ l	9 μ l
Amount (final conc.) of siRNA	375 ng (50 nM)	375 ng (50 nM)	375 ng (50 nM)
Volume of HiPerFect Reagent	3 μ l	6 μl	9 μ l
Amount (final conc.) of siRNA	187.5 ng (25 nM)	187.5 ng (25 nM)	187.5 ng (25 nM)
Volume of HiPerFect Reagent	3 μ l	6 μ l	9 μ l

* Amounts given are per well of a 24-well plate.

Cell density at transfection

The optimal cell confluency for transfection should be determined for every new cell type to be transfected and kept constant in future experiments. This is achieved by counting cells before seeding. This ensures that the cell density is not too high and that the cells are in optimal physiological condition at transfection. The recommended number of cells to seed for different formats is shown in Table 7.

Table 7. Recommended number of suspension or macrophage cells to seed for different formats

Culture format	Suggested number of suspension cells to seed
96-well plate	3–6 $\times 10^4$
24-well plate	1–2 $\times 10^5$
Suggested number of macrophage cells to seed	
96-well plate	1–6 $\times 10^4$
24-well plate	0.4–2 $\times 10^5$
Suggested number of differentiated macrophage cells to seed	
96-well plate	3–6 $\times 10^3$
24-well plate	1–2 $\times 10^4$

Calculating siRNA and reagent for different formats

Tables 8–10 give starting points for optimization of siRNA transfection in different plate and dish formats using suspension cells (Table 8), macrophages (Table 9), or differentiated macrophages (Table 10).

Table 8. Starting points for optimizing transfection of suspension cells in different formats

Culture format	Volume of cells plated (μl)	siRNA amount (ng)	Final volume of diluted siRNA (μl)	Volume of HiPerFect Reagent (μl)	Final siRNA conc. (nM)	Volume of medium added after 6 h (μl)
Protocol step	3	4	4	4	6	8
96-well plate	30	250	30	1	100	140
24-well plate	100	750	100	6	100	400

Table 9. Starting points for optimizing transfection of macrophage cells in different formats

Culture format	Volume of cells plated (μl)	siRNA amount (ng)	Final volume of diluted siRNA (μl)	Volume of HiPerFect Reagent (μl)	Final siRNA conc. (nM)	Volume of medium added after 6 h (μl)
Protocol step	1	3	3	3	5	7
96-well plate	30	125	30	1	50	140
24-well plate	100	375	100	6	50	400

Table 10. Starting points for optimizing transfection of differentiated macrophage cells in different formats

Culture format	Volume of medium on cells (µl)	siRNA amount (ng)	Final volume of diluted siRNA (µl)	Volume of HiPerFect Reagent (µl)	Final siRNA conc. (nM)	Volume of medium added after 6 h (µl)
Protocol step	3	5	5	5	7	9
96-well plate	30	125	30	1	50	140
24-well plate	100	375	100	6	50	400

Appendix B: Recommendations for Primary Cells

Recommendations for primary cell culture

Primary cells should always be handled with care and any unnecessary manipulation of the cells should be avoided. Some recommendations for handling primary cells are detailed below.

■ Culture medium

Always use prewarmed medium and avoid long incubation times at room temperature. Some primary-cell-specific media contain unstable components. For this reason, avoid using medium which was prepared over four weeks ago.

■ Passage number

Primary cells should be passaged for only a limited time before transfection. For fibroblasts, epithelial cells, keratinocytes, and smooth muscle cells, the passage number should not exceed 10 to 12. For endothelial cells, especially HUVEC, the passage number should not exceed 4 or at most 5.

■ Splitting cycles

When splitting primary cells, avoid long incubation times with trypsin (epithelial cells are an exception and usually require prolonged incubation times with trypsin at 37°C). For HUVEC and other endothelial cells, we recommend replacing trypsin with a gentler enzyme such as accutase. Stop trypsin incubation shortly after detachment of the cells by adding a neutralizing agent (e.g., medium containing significant amounts of serum). Cells should be harvested and centrifuged at low speed for 5 min and then resuspended in the appropriate medium.

As trypsinization causes stress to primary cells, cells should not be trypsinized on 2 consecutive days (e.g., do not split cells the day before seeding the cells).

■ Pipettes

Narrow pipettes should be avoided, especially for large cells like fibroblasts or smooth muscle cells, as they can lead to cell damage.

■ Cell confluency

The ideal cell confluency for gentle transfection varies depending on the cell type and cultivation method. The seeding numbers given in the protocols may have to be optimized if cytotoxicity is observed.

Optimizing siRNA transfection

To achieve the best results in siRNA transfection of primary cells, we recommend optimizing the following parameters. For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions.

Amount of siRNA

The amount of siRNA used is critical for efficient transfection and gene silencing. The recommended starting concentration for transfection of siRNA in 24-well plates is given on each protocol. An example of a pipetting scheme for optimizing siRNA transfection of keratinocytes in 24-well plates is shown in Table 11.

Ratio of HiPerFect Transfection Reagent to siRNA

The ratio of HiPerFect Transfection Reagent to siRNA should be optimized for every new cell type and siRNA combination used. Starting points for optimization for each cell type are given in the protocols. To optimize siRNA transfection, prepare separate reagent–siRNA mixtures, varying the amounts of siRNA and reagent. For example, to optimize siRNA transfection of keratinocytes in 24-well plates, prepare separate transfection mixtures according to Table 11.

Table 11. Pipetting scheme for optimizing keratinocyte transfection in 24-well plates*

Amount (conc.) of siRNA	75 ng (10 nM)	75 ng (10 nM)	75 ng (10 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μ l	4.5 μ l
Amount (conc.) of siRNA	37.5 ng (5 nM)	37.5 ng (5 nM)	37.5 ng (5 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μl	4.5 μ l
Amount (conc.) of siRNA	7.5 ng (1 nM)	7.5 ng (1 nM)	7.5 ng (1 nM)
Volume of HiPerFect Reagent	1.5 μ l	3 μ l	4.5 μ l

* Amounts given are per well of a 24-well plate.

Cell density at transfection

The optimal cell confluency for transfection should be determined for every new cell type to be transfected and kept constant in future experiments. This is achieved by counting cells before seeding and, where cells are seeded the day before transfection, by keeping the interval between seeding and transfection constant. This ensures that the cell density is not too high and that the cells are in optimal physiological condition at transfection. The recommended number of cells to seed for different formats is shown in Table 12.

For neurons and hepatocytes, we recommend using the cell number that is required for successful cultivation. This depends on the experimental conditions and may vary from experiment to experiment. For other primary cells, we recommend starting with the cell number given for the cell type that is most similar in morphology and growth behavior.

Table 12. Recommended number of cells to seed for different formats

Culture format	Cell number
96-well plate	20,000
48-well plate	40,000
24-well plate	60,000
12-well plate	120,000
6-well plate	200,000

These numbers refer to HUVEC, fibroblasts, keratinocytes, epithelial cells, or smooth muscle cells.

Calculating siRNA and reagent for different formats

Table 13 gives starting points (reagent volume and final siRNA concentration) for optimization of siRNA transfection of primary cells in different plate formats. The protocol on page 34 is recommended for HUVEC and other endothelial cells. Protocols are also provided for fibroblasts (page 36), keratinocytes (page 38), epithelial cells (page 40), smooth muscle cells (page 42), neurons (page 44), and hepatocytes (page 46). For all other cells, we recommend using the protocol for the cell type that is most similar in morphology and growth behavior.

Table 13. Starting points for optimizing transfection of primary cells in different formats*

Culture format	HUVEC	Fibroblasts	Keratinocytes	Epithelial cells	Smooth muscle cells	Neurons	Hepatocytes
96-well plate	0.75 μ l 10 nM	0.75 μ l 10 nM	1.5 μ l 5 nM	0.75 μ l 10 nM	0.75 μ l 10 nM	0.5 μ l 50 nM	0.75 μ l 50 nM
48-well plate	1.5 μ l 10 nM	1.5 μ l 10 nM	2 μ l 5 nM	1.5 μ l 10 nM	1.5 μ l 10 nM	–	–
24-well plate	3 μ l 10 nM	3 μ l 10 nM	3 μ l 5 nM	3 μ l 10 nM	3 μ l 10 nM	–	–
12-well plate	6 μ l 10 nM	6 μ l 10 nM	6 μ l 5 nM	6 μ l 10 nM	6 μ l 10 nM	–	–
6-well plate	12 μ l 10 nM	12 μ l 10 nM	12 μ l 5 nM	12 μ l 10 nM	12 μ l 10 nM	–	–

* Values given are volumes of HiPerFect Transfection Reagent (μ l) and final siRNA concentrations (nM).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

For an up-to-date list of cell lines successfully transfected using HiPerFect Transfection Reagent, visit the Transfection Cell Database at www.qiagen.com/TransfectionTools.

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Ordering Information

Product	Contents	Cat. no.
HiPerFect Transfection Reagent (0.5 ml)	HiPerFect Transfection Reagent for up to 166 transfections in 24-well plates	301704
HiPerFect Transfection Reagent (1 ml)	HiPerFect Transfection Reagent for up to 333 transfections in 24-well plates	301705
HiPerFect Transfection Reagent (4 x 1 ml)	HiPerFect Transfection Reagent for up to 1332 transfections in 24-well plates	301707
HiPerFect Transfection Reagent (100 ml)	HiPerFect Transfection Reagent for transfections in up to 1388 96-well plates	301709
Related products		
FlexiPlate siRNA	Custom siRNA set for customer-specified genes and siRNA controls; minimum order 36 siRNAs; 0.1 nmol, 0.25 nmol, or 1 nmol scale; plate layout chosen by the customer at GeneGlobe	Varies*
FlexiTube siRNA	1 nmol siRNA delivered in tubes (HP Validated siRNA or HP GenomeWide siRNA); minimum order of 4 siRNAs	Varies*
FlexiTube GeneSolution	A package of 4 siRNAs recommended for your gene; siRNAs delivered in tubes in 1-nmol amounts.	Varies*
HP Genomewide siRNA	Predesigned siRNAs for each gene of the human, mouse, and rat genomes	Varies*
HP Validated siRNA	siRNA that has been functionally tested for knockdown efficiency by quantitative RT-PCR	Varies*
RNAi Human/Mouse Starter Kit	0.5 ml HiPerFect Transfection Reagent, 9 ml siRNA Suspension Buffer, AllStars Negative Control siRNA (Alexa Fluor® 488 Labeled, 5 nmol), Hs/Mm_MAPK1 Control siRNA (5 nmol)	301799

* Visit www.qiagen.com/GeneGlobe to search for and order these products.

Ordering Information

Product	Contents	Cat. no.
AllStars Negative Control siRNA (5 nmol)	Validated siRNA with no homology to any known mammalian gene, for use as a nonsilencing control	1027280
Mm/Hs_MAPK1 control siRNA (5 nmol)	siRNA which knocks down both human and mouse MAPK1, for use as a positive control	1022564
Rn_Mapk1 Control siRNA (5 nmol)	siRNA which knocks down rat MAPK1, for use as a positive control	1027277
AllStars Hs Cell Death Control siRNA (5 nmol)	Positive cell death phenotype control	1027298
RNeasy Mini Kit (50) [†]	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74104
AllPrep RNA/Protein Kit (50)	50 AllPrep Mini Spin Columns, 50 RNeasy Mini Spin Columns, 50 Protein Cleanup Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	80404
QuantiTect Primer Assay (200)	For 200 x 50 µl reactions: lyophilized mix of forward and reverse primers	Varies*
QuantiTect SYBR Green RT-PCR Kit (200) [‡]	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green RT-PCR Master Mix, 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204243
QuantiTect SYBR Green PCR Kit (200) [‡]	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green PCR Master Mix, 2 x 2 ml RNase-Free Water	204143
QuantiFast SYBR Green RT-PCR Kit (400) [‡]	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204154

* Visit www.qiagen.com/GeneGlobe to search for and order these products.

[†] Also available in a larger size and in micro, midi, maxi, and 96-well formats; please inquire.

[‡] Also available in larger kit sizes; please inquire.

Ordering Information

Product	Contents	Cat. no.
QuantiFast SYBR Green PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water	204054
QuantiTect Reverse Transcription Kit (50)*	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, RNase-Free Water	205311
miRNeasy Mini Kit (50)	For 50 total RNA preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-Free Reagents and Buffers	217004
miRNeasy 96 Kit (4)	For 4 x 96 total RNA preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217061
miRNeasy FFPE Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Spin Columns, Collection Tubes, Proteinase K, and RNase-Free Reagents and Buffers.	217404
miScript Reverse Transcription Kit (10)	For 10 reactions: miScript Reverse Transcriptase Mix, miScript RT Buffer, RNase-Free Water	218060
miScript Reverse Transcription Kit (50)	For 50 reactions: miScript Reverse Transcriptase Mix, miScript RT Buffer, RNase-Free Water	218061
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218073

* Also available in larger kit sizes; please inquire.

Ordering Information

Product	Contents	Cat. no.
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe	Varies*
Human miScript Assay 96 Set V10.1 (100)	714 miScript Primer Assays targeting human miRNAs provided in 96-well plates	218411
Mouse miScript Assay 96 Set V10.1 (100)	561 miScript Primer Assays targeting mouse miRNAs provided in 96-well plates	218412
Rat miScript Assay 96 Set V10.1 (100)	346 miScript Primer Assays targeting rat miRNAs provided in 96-well plates	218413

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* Visit www.qiagen.com/GeneGlobe to search for and order these products.

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Bench Protocol: Fast-Forward Transfection of Adherent Cells with siRNA/miRNA in 24-Well Plates

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in the *HiPerFect Transfection Reagent Handbook*.

Important points before starting

- Cells should be in optimal physiological condition at transfection.
- For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA.

Procedure

1. Shortly before transfection, seed $0.4\text{--}1.6 \times 10^5$ cells per well in 0.5 ml culture medium containing serum and antibiotics.
2. Incubate cells under normal growth conditions.
3. Dilute 37.5 ng siRNA (1.5 μl of a 2 μM siRNA stock) in 100 μl culture medium without serum. Add 3 μl HiPerFect Reagent and mix by vortexing.
4. Incubate samples for 5–10 min at room temperature (15–25°C) for complex formation.
5. Add complexes drop-wise onto cells; gently swirl the plate.
6. Incubate under normal growth conditions and monitor gene silencing after an appropriate time. Change medium as required.



Bench Protocol: Reverse Transfection of Adherent Cells with siRNA/miRNA in 96-Well Plates

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in the *HiPerFect Transfection Reagent Handbook*.

Important points before starting

- Cells should be in optimal physiological condition at transfection.
- For appropriate amounts of miRNA mimic/miRNA inhibitor to transfect, refer to the manufacturer's instructions. Where possible, dilute miRNA mimic/miRNA inhibitor in the same volume as recommended for siRNA.

Procedure

1. Spot 12.5 ng siRNA (0.5 μ l of a 2 μ M siRNA stock) in 1–3 μ l of siRNA Suspension Buffer/RNase-free water into a single well of a 96-well plate.
At this point, siRNA can be stored at -20°C .
2. Add 0.75 μ l HiPerFect Reagent to 24.25 μ l culture medium without serum. Add the diluted HiPerFect Reagent to the prespotted siRNA.
3. Incubate for 5–10 min at room temperature (15–25 $^{\circ}\text{C}$) for complex formation.
4. Seed $1-5 \times 10^4$ cells in 175 μ l culture medium (containing serum and antibiotics) into the well, on top of the transfection complexes.
5. Incubate under normal growth conditions and monitor gene silencing after an appropriate time. Change medium as required.

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