April 2004

# RNAi Human/Mouse Control Kit Handbook

For gene silencing in human and mouse cells

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## Kit Contents

| **RNAi Human/Mouse Control Kit** | |
|---|---|
| **Catalog no.** | **301698** |
| **Reactions per kit** | **>160 transfections** |
| Hs/Mm_MAPK1 control siRNA | 5 nmol |
| Non-silencing control siRNA AF 488* | 5 nmol |
| RNAiFect™ Transfection Reagent | 0.5 ml |
| Buffer EC-R | 15 ml |
| siRNA Suspension Buffer | 9 ml |
| Handbook | 1 |

* Light sensitive, see "Shipping and Storage", below.

## Shipping and Storage

RNAiFect Transfection Reagent and Buffer EC-R are supplied as ready-to-use solutions and are shipped at room temperature (15–25°C) without loss of stability. However, they should be stored at 2–8°C upon arrival. In contrast to many liposome-based reagents, RNAiFect Reagent is not sensitive to oxygen, so it does not require storage under an inert gas. Additionally, RNAiFect Reagent does not need to be stored on ice during the transfection procedure.

Non-silencing Alexa Fluor® 488-labeled control siRNA is light-sensitive and should be protected from light by wrapping tubes in aluminum foil.

siRNA is shipped at room temperature and should be stored at –20°C upon arrival. The siRNA should therefore be stored separately from the rest of the kit.

## Quality Control

As part of the stringent QIAGEN® quality assurance program, the performance of the RNAi Human/Mouse Control Kit is monitored routinely on a lot-to-lot basis. All kit components are tested separately to ensure highest performance and reliability.

## Product Use Limitations

The RNAi Human/Mouse Control Kit is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

## 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.

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 page).

## 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 molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the RNAi Human/Mouse Control Kit 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 call one of the QIAGEN Technical Service Departments or local distributors (see back page).

# 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/ts/msds.asp 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 RNAi Human/Mouse Control Kit

The RNAi Human/Mouse Control Kit allows easy establishment and optimization of gene silencing in a human or mouse cell line of interest. The kit contains positive and non-silencing control siRNAs, RNAiFect Transfection Reagent, and protocols intended as starting points for RNAi (RNA interference) experiments.

## RNAiFect Transfection Reagent

RNAiFect Transfection Reagent is based on a lipid formulation and has been designed for efficient transfection of cells with siRNA. RNAiFect Reagent is provided as a ready-to-use solution — just add the reagent to your diluted siRNA, mix, incubate, and pipet the complexes onto the cells. RNAiFect Reagent has been tested in a range of human and mouse cell lines. For an up-to-date list of cell lines successfully transfected using RNAiFect Reagent, please visit our cell database at www.qiagen.com/transfectiontools .

## Positive control siRNA targeted against MAPK1

The RNAi Human/Mouse Control Kit provides positive control siRNA targeted against the protein kinase MAPK1 (also called MAPK2 and Erk2). MAPK1 is ubiquitously expressed in both human and mouse cell lines, making it a suitable target gene for control experiments. Human and mouse MAPK1 can be easily detected by western blotting using the MAPK1-specific Tag·100 antibody (cat. no. 34680). The Tag·100 antibody was originally developed for protein detection when using QIAGEN's pQE-100 DoubleTag Vector for protein expression. However it is also effective for monitoring gene silencing of human and mouse MAPK1 at the protein level. The sequence of the Hs/Mm_MAPK1 control siRNA provided in this kit is homologous to both the human and mouse MAPK1 mRNA sequence (GenBank® accession number NM_002745 [human] and NM_011949 [mouse]). Western analysis of mouse and human cell lines after transfection with the Hs/Mm_MAPK1 control siRNA showed highly efficient gene silencing. As the Hs/Mm_MAPK1 control siRNA provides high levels of gene knockdown, it can be used to optimize experimental conditions, and it can be routinely transfected in parallel with the siRNA under study.

## Non-silencing control siRNA

Non-silencing controls, consisting of siRNAs that have no known homology with mammalian genes, are used to control for nonspecific silencing effects. The non-silencing control included in this kit is labeled with Alexa Fluor 488. The Alexa Fluor 488 label allows easy monitoring of transfection efficiency and enables optimization of transfection conditions. The absorption and emission spectra of Alexa Fluor 488 allow the use of equipment suitable for fluorescein excitement

and signal detection. The Alexa Fluor 488 fluorophore is brighter and more photostable than fluorescein. It is insensitive to changes over a wide range of pH values, making the fluorescence in living cells more stable than that of fluorescein.

## RNA interference

RNA interference (RNAi) is a biological process in which the introduction of double-stranded RNA (dsRNA) into a cell results in targeted post-transcriptional gene silencing. Historically, RNAi has been used as a tool for functional genomics research in *Caenorhabditis elegans* and Drosophila. Initial attempts to activate the RNAi pathway in mammalian cells were unsuccessful, since the introduction of dsRNA >30 nucleotides (nt) in length leads to nonspecific suppression of gene expression. However, as RNAi became better understood, scientists discovered that double-stranded short interfering RNA (siRNA) oligos of 23 nt could be used to mediate gene silencing in mammalian cells. The application of RNAi to mammalian cells is revolutionizing 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.

## Discovery of RNAi

The origins of RNAi involved a number of scientists working in different research fields, who observed a phenomenon that they did not immediately understand. Plant biologists attempting to boost the activity of the gene for chalcone synthase in petunias by introducing a powerful promoter sequence into a transgene observed that instead of the deep purple color they expected, flowers were variegated, or completely white. The researchers concluded that the introduced chalcone synthase transgene had somehow suppressed both itself and the endogenous petunia gene, and so named this phenomenon co-suppression (1).

Another research group working on genes expressed from the potato virus X in tobacco plants hoped that viral proteins produced by the plants would stimulate a defense mechanism, allowing the plants to resist subsequent attack by the virus. To their surprise, the plants with the strongest resistance to the virus were those in which the introduced gene was silent. The researchers concluded that the introduced gene was suppressing expression both of itself and of the same gene in the virus (2).

In fungi, gene silencing was observed during attempts to boost the production of an orange pigment by the mold *Neurospora crassa*. Extra copies of a gene involved in making a carotenoid pigment were introduced into mold cells. However, rather than turning a deeper orange, a third of the engineered mold appeared yellow or white. Something had suppressed the pigment genes. They called the observed phenomenon quelling (3, 4).

Other scientists working with *C. elegans* obtained strange results in their antisense RNA experiments. The antisense approach to gene silencing involves injecting an organism with RNA sequence complementary to mRNA transcribed from a target gene. The antisense RNA and sense mRNA hybridize and block production of the encoded protein. However in one case, a sense strand, injected as a control, led to gene silencing (5). This effect was later explained by the presence in the RNA preparation of very small amounts of the corresponding antisense strand. The presence of dsRNA duplex led to what we now recognize as an RNAi effect (6). Antisense experimental theory predicts that these small contaminants would have no effect on gene expression.

Using *C. elegans*, it was demonstrated that injection of double-stranded RNA was more effective in gene silencing than injection of sense or antisense strands alone (7). Only a few molecules of injected double-stranded RNA were required to shut down expression of protein in a cell. The dsRNA gene-silencing mechanism was found to be highly gene-specific and to be part of a complex biological regulation system. The phenomenon of gene silencing using dsRNA was termed "RNA interference" (7).

### RNAi in mammalian cells

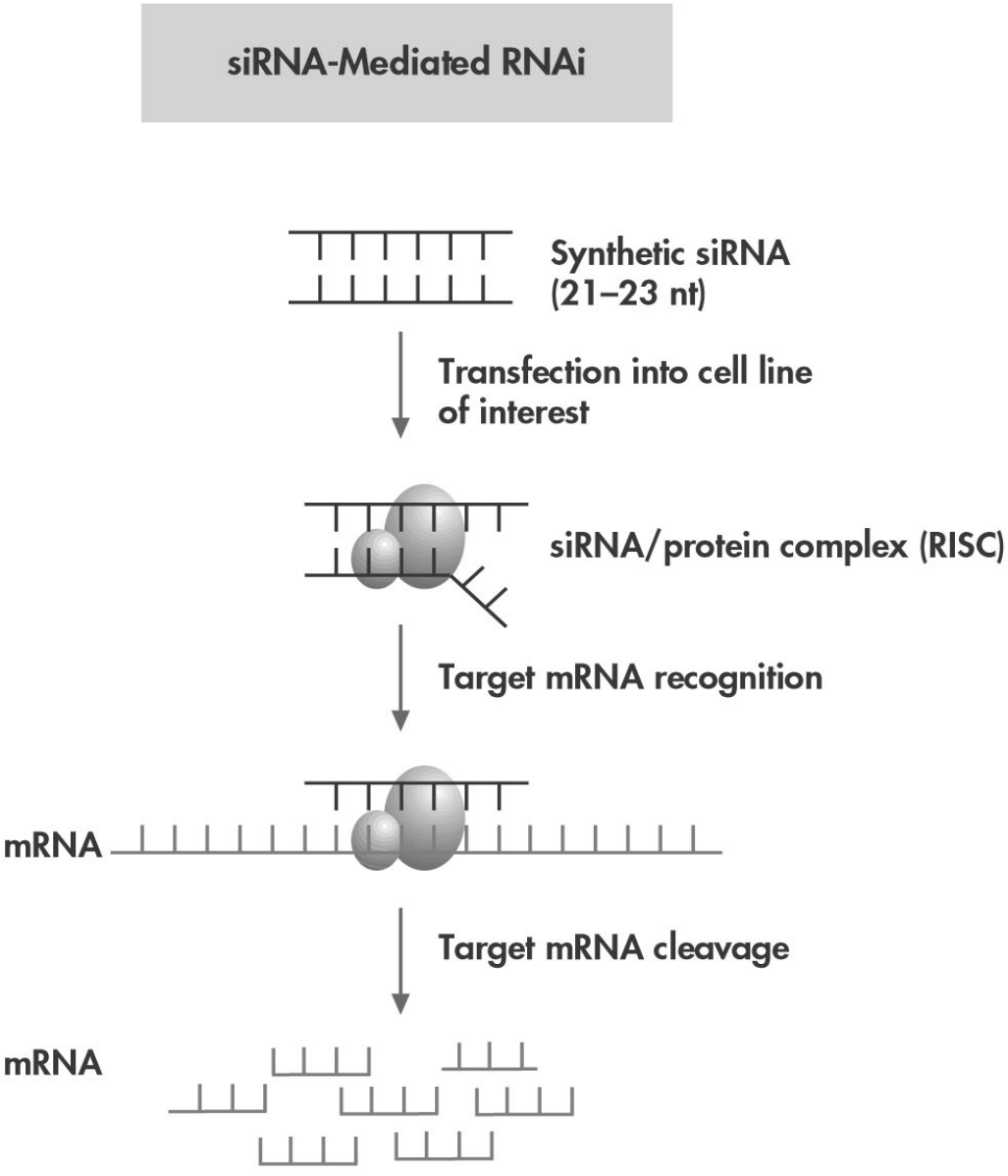
Initial attempts to activate the RNAi pathway in mammalian cells were unsuccessful, since the introduction of dsRNA leads to activation of the dsRNA-dependent protein kinase PKR and 2',5'-oligoadenylate synthetase (2',5'-AS). The activation of these two enzymes triggers a nonspecific shutdown of protein synthesis and nonspecific degradation of mRNA. Consequently, some researchers were led to believe that gene-specific RNAi was not possible in mammalian systems.

Elbashir et al. showed that 21–23 nt dsRNA fragments successfully trigger RNAi in an in vitro system using Drosophila lysate. In addition, they demonstrated that chemically synthesized 21 nt siRNA duplexes specifically suppress the expression of endogenous and heterologous genes in different mammalian cell lines, including human 293 and HeLa cells (8). A key discovery from these studies was that no nonspecific gene silencing effects were seen in mammalian cells by transfection of short dsRNA sequences (<30 nt). These results showed that 21 nt siRNA duplexes can be used as a new tool for studying gene function in mammalian cells, and may eventually find a use as gene-specific therapeutics.

Work by Caplen et al. (9) confirmed and extended the reports of siRNA-mediated RNAi in mammalian cell extracts. They demonstrated that identically sized synthetic siRNAs can induce gene-specific inhibition of expression in *C. elegans*, human, and mouse cells. Consistent with this hypothesis, numerous studies have since shown that dsRNA-induced gene silencing occurs in a number of different eukaryotic species (7, 11–21). The finding that the size of functional dsRNA fragments is conserved in plants and animals suggests a highly conserved mechanism in nature (10).

## How does RNAi lead to gene silencing?

The basic mechanism of RNAi is thought to be a multi-step process (see flowchart, below). In cultured mammalian cells, RNAi is mediated by 21nt RNA duplexes with symmetric 2-nt 3' overhangs. These siRNAs are introduced into a cell by transfection and lead to degradation of mRNA having the same sequence, thereby silencing gene expression. The specific pathways and mechanism of RNAi in mammalian cells are currently under intense investigation.



## 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.

### For all protocols

- Culture medium
- Sterile PBS (1x PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$; adjust to a final pH of 7.4). Store at room temperature (15–25°C).
- siRNA of interest. The QIAGEN siRNA Synthesis Service allows easy design and ordering of siRNA. Alternatively 2-for-Silencing siRNA Duplexes (cat. no. 1022562) are two siRNA duplexes, custom-designed by QIAGEN for guaranteed silencing of the target gene: visit www.qiagen.com/siRNA for more information.
- Tools for monitoring gene silencing at the mRNA or protein level. QIAGEN provides MAPK1-specific Tag·100 Antibody (cat. no. 34680) for western blot analysis of the MAPK1 protein. For quantitative real-time RT-PCR analysis the QuantiTect® Hs_MAPK1 Assay or the QuantiTect Mm_Mapk1 Assay are designed for human and mouse, respectively. For more information, visit www.qiagen.com/goto/assays .

# Important Notes

## Choosing a target sequence and designing an siRNA

Guidelines for designing an siRNA are provided at www.qiagen.com/goto/siRNAdesign .

## Quality of siRNA

Optimal results are achieved when high-purity siRNA is used for transfection. QIAGEN HPP Grade siRNA (>90% pure) is well suited for the transfection of many cell lines. For further information on QIAGEN custom siRNA, visit www.qiagen.com/siRNA .

## Calculating concentrations of siRNA

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

- 20 µM siRNA is equivalent to approximately 0.25 µg/µl
- Molecular weight of a 21 nt siRNA is approximately 13–15 µg/nmol (sequence-dependent)

## Optimizing siRNA transfection

For RNAi experiments, transfection conditions including amount of siRNA transfected, ratio of siRNA to transfection reagent, and cell density at transfection, should be optimized. The non-silencing Alexa Fluor 488-labeled siRNA included in this kit can be used to monitor transfection efficiency of individual cell lines. In transfections with the siRNA of interest, a positive control siRNA should be transfected in parallel. siRNA directed against the human and mouse MAPK1 genes, included in this kit, is a positive control for both human and mouse cell lines. To achieve good results in siRNA transfection, we recommend optimizing the following parameters.

### Amount of siRNA

The amount of siRNA used is critical for efficient transfection and gene silencing. The recommended starting amount for transfection of siRNA in 24-well plates is 0.5 µg per well when using a final cell culture volume of 400 µl. A pipetting scheme for optimizing the transfection of siRNA in adherent cells in a 24-well format is shown in Table 1 (see next page).

### Ratio of RNAiFect Transfection Reagent to siRNA

The ratio of RNAiFect Transfection Reagent to siRNA should be optimized for every new cell type and siRNA combination used. As a starting point for optimization, we recommend using an siRNA to RNAiFect Reagent ratio of 0.5 µg:3 µl when using 24-well plates. To optimize siRNA transfection in a 24-well format, prepare separate transfection mixtures according to Table 1.

**Table 1. Pipetting Scheme for Optimizing siRNA Transfection of Adherent Cells in a 24-Well Plate***

| | **Ratio of siRNA to RNAiFect Reagent (µg:µl)** | | |
|---|---|---|---|
| | 1:3 | 1:6 | 1:9 |
| Amount of siRNA | 0.25 µg | 0.25 µg | 0.25 µg |
| Volume of RNAiFect Reagent | 0.8 µl | 1.5 µl | 2.3 µl |
| Amount of siRNA | 0.5 µg | **0.5 µg** | 0.5 µg |
| Volume of RNAiFect Reagent | 1.5 µl | **3 µl** | 4.5 µl |
| Amount of siRNA | 1 µg | 1 µg | 1 µg |
| Volume of RNAiFect Reagent | 3 µl | 6 µl | 9 µl |

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

### Cell density at transfection

The optimal confluency for transfection of adherent cells with siRNA is 50–80%. The optimal confluency 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 by keeping the interval between seeding and transfection constant (minimum 24 hours). This ensures that the cell density is not too high and that the cells are in optimal physiological condition on the day of transfection. The recommended number of cells to seed for different formats is shown in Table 2 (see next page). For an up-to-date list of cell lines successfully transfected using RNAiFect Reagent, visit our cell database at www.qiagen.com/transfectiontools .

**Table 2. Recommended Number of Cells to Seed for Different Culture Formats**

| Culture format | Suggested number of adherent cells to seed (day before transfection) |
|---|---|
| 96-well plate | 1.0–3.0 x $10^4$ |
| 48-well plate | 2.0–4.0 x $10^4$ |
| 24-well plate | 4.0–8.0 x $10^4$ |
| 12-well plate | 0.8–2.0 x $10^5$ |
| 6-well plate | 1.5–4.0 x $10^5$ |

## Monitoring transfection efficiency using fluorescently labeled siRNA

When monitoring transfection efficiency by fluorescence microscopy, it is important to avoid stressing the cells. If problems appear when washing cells with PBS, cells should be washed in PBS containing 10% fetal calf serum, or in complete culture medium. If culture medium is used, the pH indicator phenol red should be omitted, as this dye interferes with fluorescence analysis.

During washing, a precipitate of siRNA–RNAiFect complexes may stick to the culture dish and to cell membranes, producing spots of fluorescence. Washing the cells will help to remove these complexes, but it is normal for some complexes to remain. These will not interfere with fluorescence analysis.

## Monitoring gene silencing at the protein level

Gene silencing can be monitored at either the protein or the mRNA level. Protein expression analysis is by western blotting, immunofluorescence, or FACS®. A mouse anti-Tag·100 antibody that recognizes endogenous mammalian MAPK1 is available from QIAGEN (cat. no. 34680). This antibody can be used to monitor MAPK1 gene silencing by western blot analysis. A general protocol for western blotting is given on page 19.

## Monitoring gene silencing at the mRNA level

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 . The RNeasy® system is recommended for purification of high-quality RNA. The RNAi Human/Mouse Control Kit can be used in combination with QuantiTect Gene Expression Assays and QuantiTect

Probe Kits for downstream analysis. The QuantiTect Hs_MAPK1 Assay or the QuantiTect Mm_Mapk1 Assay, for human and mouse respectively, can be used together with the QuantiTect Probe RT-PCR (cat. no. 204443) or PCR Kits (cat. no. 204343) for highly sensitive quantitative real-time RT-PCR (see appendix, page 27). Further information on products for gene expression analysis is available at www.qiagen.com/geneXpression/realtime_analysis . All expression data should be compared with levels of a "housekeeping" gene to normalize for variable amounts of RNA in different samples.

### Primer design

When designing custom primers for RT-PCR analysis, the following points should be considered:

- One or both primers should bind the cDNA at a position spanning an exon-exon boundary. This prevents amplification from contaminating genomic DNA.
- We have obtained good results with real-time analysis using the QuantiTect system. The QuantiTect SYBR® Green RT-PCR Kit (cat. no. 204243) has been used with custom primers designed to amplify a fragment between 90 and 250 base pairs in length. The GenBank accession numbers for MAPK1 are NM_002745 (human) and NM_011949 (mouse). Alternatively, the QuantiTect Hs_MAPK1 or Mm_Mapk1 Assays are functionally validated RT-PCR assays designed for detection of human or mouse MAPK1 mRNA, respectively.
- To minimize the amplification of incomplete cDNA sequences, oligo dT primers should be used for two-step RT-PCR.

Oligonucleotides are available from QIAGEN (visit www.qiagen.com/goto/oligos for further information).

# Protocol: Transfection of Adherent Cells with siRNA

This protocol is for transfection of adherent cells in a single well of a 24-well plate. It is provided as a starting point for optimization of siRNA transfection in mammalian cells using the RNAi Human/Mouse Control Kit. Starting points for optimizing the transfection of adherent cells in different formats are shown in Table 3 (page 18).

### Important points before starting

- Be sure to work in an RNase-free environment.
- Add 250 µl of siRNA Suspension Buffer (provided) to each tube containing 5 nmol lyophilized siRNA to obtain a 20 µM solution. (For approximate conversion to µg/µl, see "Calculating concentrations of siRNA", page 12.)

  Heat the tubes to 90°C for 1 min.

  Incubate at 37°C for 60 min.

  The incubation steps only need to be carried out the first time you use the siRNA. The procedure will disrupt aggregates, which may have formed during lyophilization, ensuring silencing efficiency. The solution can be stored frozen at –20°C and freeze-thawed numerous times.
- Cells should be in optimal physiological condition on the day of transfection. Plate the cells the day before transfection. The optimal confluency for transfection for most cell lines is 50–80%.

**Procedure**

1. **The day before transfection, seed 4–8 x $10^4$ cells (depending on the cell type and time of analysis) per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics.**
   Ensure that the cells are in good condition and are seeded 24 h before transfection. Cells should be 50–80% confluent on the day of transfection. For very fast growing cells, plating as few as 2 x $10^4$ cells per well may be sufficient.
2. **Incubate cells under normal growth conditions (typically 37°C and 5% $CO_2$).**
3. **On the day of transfection, dilute 0.5 µg siRNA in the appropriate volume of Buffer EC-R or culture medium containing serum and antibiotics to give a final volume of 100 µl, and mix by vortexing.**
   **Note:** In most cases, complex formation should be performed in Buffer EC-R for optimal results. However, for some cell lines complex formation in culture medium can improve results.
4. **For complex formation, add 3 µl of RNAiFect Transfection Reagent to the diluted siRNA. Mix by pipetting up and down 5 times, or by vortexing for 10 s.**
   **IMPORTANT**: The amount of RNAiFect Reagent required for optimal performance may vary, depending on the cell line and the amounts of siRNA used. For specific cell types and targets, optimal conditions may be different from those described here.
5. **Incubate the samples for 10–15 min at room temperature (15–25°C) to allow formation of transfection complexes.**
6. **While complex formation is taking place, gently aspirate the culture medium from the plate. Add 300 µl of culture medium containing serum and antibiotics to the cells.**
   **IMPORTANT**: Do not allow the cells to become dry. Keep the length of time they are without medium to a minimum.
7. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**

8. **Incubate cells with the transfection complexes under their normal growth conditions and monitor gene silencing at the mRNA or protein level after an appropriate time (see note below).**

   **Note**: In general, 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. We recommend monitoring gene silencing at the mRNA level by RT-PCR 24–72 h after transfection with MAPK1 siRNA, and at the protein level 3–5 days after transfection by western blotting (see protocol, page 19). When Alexa Fluor 488-labeled siRNA provided in this kit is used, the fluorescent signal may become weaker over time, although the siRNA remains stable in cells. We therefore recommend measuring the uptake of fluorescent siRNA after 4–8 h. If cytotoxic effects are visible, change the medium 6–24 h after transfection. We have not observed cytotoxic effects in cells transfected with MAPK1 siRNA for up to 5 days after transfection.

**Table 3. Starting Points for Optimizing the Transfection of Adherent Cells in Different Formats**

| Culture format | siRNA (µg) | Volume of 20 µM siRNA stock (µl) | Final volume of diluted siRNA (µl) | RNAiFect Reagent (µl) | Volume of medium on cells (µl) | Final siRNA conc. (nM) |
|---|---|---|---|---|---|---|
| Protocol step | 3 | 3 | 3 | 4 | 6 | |
| 96-well plate | 0.13 | 0.5 | 25 | 0.75 | 75 | 100 |
| 48-well plate | 0.25 | 1 | 50 | 1.5 | 150 | 100 |
| 24-well plate | 0.5 | 2 | 100 | 3 | 300 | 100 |
| 12-well plate | 1 | 4 | 100 | 6 | 600 | 100 |
| 6-well plate | 2.5 | 10 | 100 | 7.5 | 1900 | 100 |

## Transfection of suspension cells with siRNA

For transfection of suspension cell types with siRNA, we recommend amaxa Nucleofector™ Technology, described at www.amaxa.com .

# Protocol: Western Blot Analysis for Detection of MAPK1

This protocol is for detection of MAPK1 protein by western blotting using the Tag·100 Antibody (cat. no. 34680). It is optimized for a Mini Protean Chamber (BioRad) and is based on standard western blotting techniques (22). A detailed protocol for western blot analysis can be found in the *QIA*express® *Detection and Assay Handbook* supplied with the Tag·100 Antibody and is available at www.qiagen.com/literature/handbooks/INT/ProteinDetection.aspx . Further information about protein analysis can be found at www.qiagen.com/literature/BenchGuide .

**Procedure**

1. **Pour a 12% denaturing polyacrylamide* gel.**
2. **Prepare lysates from the cells under study using standard procedures for preparation of lysates under denaturing conditions (22). Heat the cell lysates in an appropriate SDS-sample buffer for 5 min at 95°C, then place on ice.**
   For detailed information about separation of proteins by SDS-PAGE, refer to the *QIA*express *Detection and Assay Handbook*.
3. **Spin down any condensed liquid and load approximately 3 µg total cell protein in each well.**
4. **Run the gel for 15 min at 80 V, then increase to 100 V for approximately 60–75 min.**
5. **Blot the gel on an appropriate membrane (e.g., Protran® Nitrocellulose, Schleicher and Schuell).**
   For detailed information about western transfer, refer to the *QIA*express *Detection and Assay Handbook*.
6. **Block the membrane by gently shaking in 5% (w/v) fat-free milk made in TBS (Tris-buffered saline)-Tween® for 60 min at room temperature (15–25°C).**
   TBS-Tween is 0.02% (v/v) Tween 20 in 10 mM Tris·Cl, 150 mM NaCl, pH 7.5.
7. **Wash the blot as follows:**
   **1 x 5 min in TBS**
   **2 x 5 min in TBS-Tween**
   **1 x 5 min in TBS**

* 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.

8. **Dilute the Tag·100 Antibody 1/1000 in 5% fat-free milk made in TBS-Tween and gently shake the membrane in the diluted antibody solution for 60 min at room temperature or overnight at 4°C.**
   **Note**: A 1/2000 dilution of monoclonal anti-α-tubulin antibody (Clone B-5-1-2, product no. T5168, Sigma) can be used for staining in parallel as a positive control.
9. **Wash the blot as described in step 7.**
10. **Perform detection using standard procedures (e.g., ECL Western Blot detection reagents, Amersham Biosciences).**
    For detailed information about detection go to www.qiagen.com/literature/BenchGuide .
    **Note**: The expected size of MAPK1 is approximately 41 kDa.

# 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 either the information and protocols in this handbook or molecular biology applications (see back page for contact information).

| | Comments and suggestions |
|---|---|
| **Low transfection efficiency** | |
| a) Sub-optimal RNAiFect:siRNA ratio | If the ratio of RNAiFect Transfection Reagent to siRNA is sub-optimal, the overall charge of the complexes may also be sub-optimal, which can lead to inefficient adsorption to the cell surface. Optimize the RNAiFect Transfection Reagent to siRNA ratio using Table 1, page 13. |
| b) Insufficient RNAiFect–siRNA complex | If the transfection efficiency is lower than expected and cytotoxicity is acceptably low, increase the overall amount of RNAiFect–siRNA complex added to the cells. We strongly recommend optimization of siRNA and RNAiFect amounts for every new siRNA and cell line used (Table 1, page 13). |
| c) Sub-optimal cell density | If cell density at the time of adding RNAiFect–siRNA complexes is not at an optimal level, 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%. Be sure to seed cells a minimum of 24 h before transfection. |

| | Comments and suggestions |
|---|---|
| d) Poor siRNA quality | siRNA should be of high quality, as impurities can lower transfection efficiency. HPP Grade siRNA from QIAGEN is >90% pure and provides efficient gene silencing. |
| e) Serum interferes with complex formation | For several cell lines, complex formation proceeds efficiently with RNAiFect Reagent in the presence of serum. However, in some cases serum may interfere with complex formation, and we then recommend diluting siRNA in Buffer EC-R. |
| **Excessive cell death** | |
| a) Concentration of RNAiFect–siRNA complexes is too high | Decrease the amount of RNAiFect–siRNA complexes added to 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%. Be sure to seed cells a minimum of 24 h before transfection. |
| c) Poor siRNA quality | siRNA should be of high quality, as impurities can lower transfection efficiency. HPP Grade siRNA from QIAGEN is >90% pure and provides efficient gene silencing. |
| d) RNA-related effects | Too much siRNA may be toxic to cells. Optimize the amount of siRNA used for each new siRNA and/or cell type (Table 1, page 13). |
| e) Essential gene is silenced | If the gene targeted is essential for the survival of the cell, silencing this gene will lead to cell death. |

| | Comments and suggestions |
|---|---|
| **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 incubation times between replicate seeding and complex addition consistent between experiments. Cells should be seeded at least 24 h before transfection. |
| 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. |
| c) Cells have been passaged too many times | Cells that have been passaged a large number of times tend to change their growth behavior, morphology, and potential for 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). |
| **Difficulties in monitoring transfection efficiency using fluorescent siRNA** | |
| a) Cells round up and detach from culture dish during washing and fluorescence microscopy | If this occurs, cells should be washed in PBS containing 10% fetal calf serum, or in complete culture medium. If culture medium is used, the pH indicator phenol red should be omitted, as this dye interferes with fluorescence analysis. |

| | Comments and suggestions |
|---|---|
| b) Spots of fluorescence seen, despite repeated washing | A precipitate of siRNA–RNAiFect complexes may stick to the culture dish and to cell membranes, producing spots of fluorescence. Washing the cells will help to remove these complexes, but it is normal for some complexes to remain. These will not interfere with fluorescence analysis. |
| c) Long incubation time after transfection | When fluorescently labeled siRNA is used, the fluorescent signal may become weaker over time, although the siRNA remains stable in cells. We therefore recommend measuring the uptake of siRNA after 4–8 h. |
| **No or very small gene silencing effect** | |
| a) Design of siRNA sub-optimal | The design of an siRNA can have a large effect on its gene silencing efficiency. Information on optimizing siRNA design is provided online at www.qiagen.com/goto/siRNAdesign . |
| b) Incubation time after transfection is too short | The gene silencing effect observed at the protein level is dependent on a protein's expression level 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. QIAGEN offers siRNA that has been functionally tested for specific gene silencing (see ordering information, page 29). |

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# Appendix: QuantiTect Gene Expression Assays

New QuantiTect Gene Expression Assays are an expanding range of functionally validated primer–probe sets that are ready-to-use in quantitative, real-time RT-PCR on any real-time cycler. Optimal results are guaranteed, and assays are available for human and mouse genes. QIAGEN provides QuantiTect Gene Expression Assays for a range of commonly analyzed genes and QuantiTect Custom Assays for any gene of choice. The QuantiTect Hs_MAPK1 Assay or the QuantiTect Mm_Mapk1 Assay can be used in combination with the RNAi Human/Mouse Control Kit to detect MAPK1 gene silencing at the RNA level for human and mouse MAPK1, respectively.

QuantiTect Gene Expression Assays, comprising two gene-specific primers and a dual-labeled QuantiProbe™, are provided in a convenient 10x Assay Mix. Assays have been optimized and validated to provide maximum sensitivity and a wide dynamic range. Each assay is validated together with QuantiTect Probe Kits, in both one-step and two-step RT-PCR, to ensure high PCR efficiency and accurate quantification of as few as 10 copies of template. In contrast to other commercial assays, primers are designed to cross exon/exon boundaries, enabling amplification and detection of RNA sequences only. This prevents co-amplification of genomic DNA, which can compromise assay sensitivity and efficiency by competition between the desired PCR product and the product derived from genomic DNA (Figure 1). Unique oligonucleotide technology makes this possible through the use of synthetic bases. QuantiProbes and primers frequently contain Superbases (SuperA™, SuperT™, and SuperG™) that stabilize primer and probe hybridization, even for sequences where hybridization is difficult, such as AT-rich regions. This enables the use of primers and probe at predefined sequences, such as splice sites, while still delivering optimal real-time performance — something that may not be possible with other combinations of primers and dual-labeled probes or FRET probes. QuantiProbes also carry a minor-groove binder moiety, enabling the use of short probes for highest specificity. For more information on QuantiTect Gene Expression Assays, visit www.qiagen.com/goto/assays or contact your local QIAGEN Technical Service Department.

## No Co-amplification of Genomic DNA

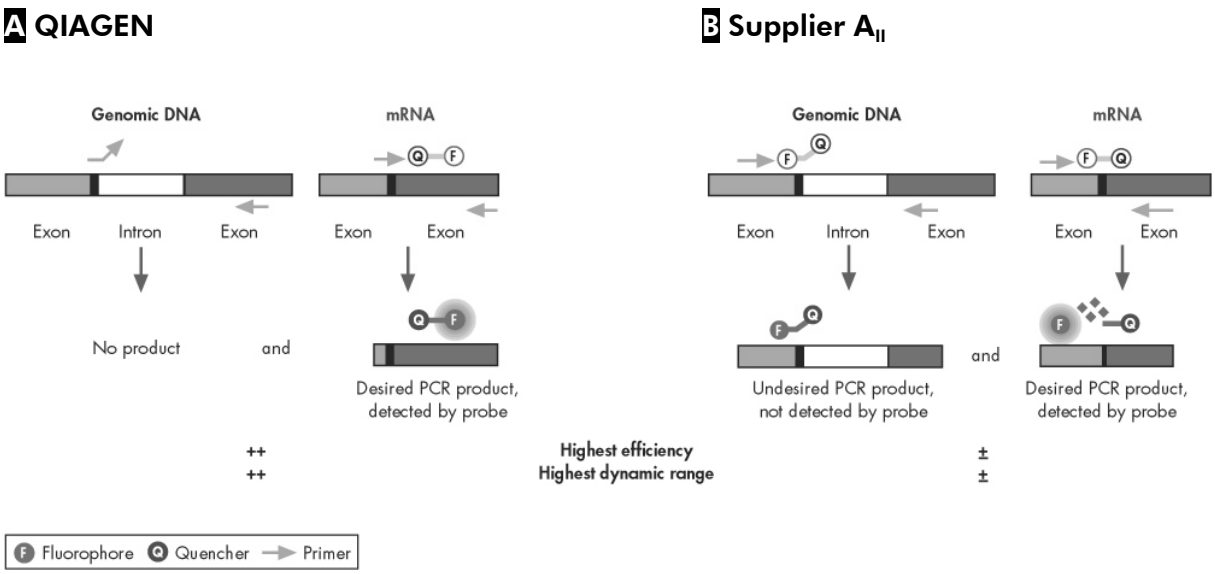


**Figure 1** Primer/probe design to eliminate signals from contaminating genomic DNA. Comparison of primer design for **A** QuantiTect Gene Expression Assays (**QIAGEN**). In contrast to Supplier $A_{II}$, primers in QuantiTect Gene Expression Assays are designed to cross exon/exon boundaries. **B** Pre-designed gene expression assays from Supplier $A_{II}$ (**Supplier $A_{II}$**).

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| RNAi Human/Mouse Control Kit | RNAiFect Reagent, siRNA Suspension Buffer, Buffer EC-R, MAPK1 siRNA, non-silencing labeled control siRNA | 301698 |
| **Accessories** | | |
| RNAiFect Transfection Reagent (1 ml) | RNAiFect Reagent and buffer, for up to 170 transfections in 24-well plates; up to 500 transfections in 96-well plates | 301605 |
| RNAiFect Transfection Reagent (4 x 1 ml) | RNAiFect Reagent and buffer, for up to 680 transfections in 24-well plates; up to 2000 transfections in 96-well plates | 301607 |
| RNAiFect Transfection Reagent (100 ml) | RNAiFect Reagent and buffer, for transfection of up to 520 96-well plates | 301608 |
| Tag·100 Antibody, BSA-free (100 µg) | 100 µg mouse anti-Tag·100 antibody, recognizes endogenous mammalian MAPK1 | 34680 |
| QuantiTect Hs_MAPK1 Assay | For 100 x 50 µl reactions (for use in a 96-well plate or single tubes) or 250 x 20 µl reactions (for use in a 384-well plate or single capillaries): 0.5 ml 10x QuantiTect Assay Mix (dyes available: FAM) | Inquire |
| QuantiTect Mm_Mapk1 Assay | For 100 x 50 µl reactions (for use in a 96-well plate or single tubes) or 250 x 20 µl reactions (for use in a 384-well plate or single capillaries): 0.5 ml 10x QuantiTect Assay Mix (dyes available: FAM) | Inquire |

For further details about QuantiTect Gene Expression Assays and QuantiTect Custom Assays, please visit www.qiagen.com/goto/assays .

| Product | Contents | Cat. no. |
|---|---|---|
| QuantiTect SYBR Green RT-PCR Kit (200)* | For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green RT-PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 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 QuantiTect SYBR Green PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 2 x 2 ml RNase-free water | 204143 |
| QuantiTect Probe RT-PCR Kit (200)* | For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-free water | 204443 |
| QuantiTect Probe PCR Kit (200)* | For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 2 x 2 ml RNase-free water | 204343 |
| RNeasy Mini Kit (50)† | 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers | 74104 |
| **Library siRNA duplexes** | | |
| Control (non-sil.) siRNA | 5 nmol duplex siRNA; for use as a non-silencing control in RNAi experiments | 1022076 |
| Control (non-sil.) siRNA, Fluorescein | 5 nmol duplex siRNA; for use as a non-silencing control in RNAi experiments | 1022079 |

* Larger kit sizes available, please inquire.

† RNeasy kits are also available in micro, midi, maxi, and 96-well formats; please inquire.

| Product | Contents | Cat. no. |
|---|---|---|
| Control (non-sil.) siRNA, Rhodamine | 5 nmol duplex siRNA; for use as a non-silencing control in RNAi experiments | 1022083 |

For a complete list of library siRNA available, please visit www.qiagen.com/siRNA .

**Custom siRNA**

| | | |
|---|---|---|
| HPP Grade siRNA | siRNA purified to >90%* | |
| 2-for-Silencing siRNA Duplexes | Two HPP Grade siRNAs (20 nmol), custom-designed by QIAGEN | 1022562 |

For an up-to-date list, and information on how to order custom siRNA, please visit www.qiagen.com/siRNA .

* Available in guaranteed yields of 20 and 40 nmol. Also available in 96-well plates; please inquire.

## Notes

## Notes

## Notes

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