Silencer® siRNA Starter Kit

Complete Kit for Inducing and Monitoring RNAi

Part Number AM1640

Silencer® siRNA Starter Kit

(Part Number AM1640)

Protocol

P/N 1640M Revision C **Revision Date: August 19, 2010**

For research use only. Not for use in diagnostic procedures. By use of this product, you accept the terms and conditions of all applicable Limited Label Licenses. For statement(s) and/or disclaimer(s) applicable to this product, see below.

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document.

Applied Biosystems disclaims all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Applied Biosystems be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *Silencer*® siRNA Starter Kit.

Warranty and Liability: Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

Patents and Licensing Notifications: The *Silencer*® siRNA Starter Kit is compatible with the 5' nuclease detection and dsDNA-binding dye processes covered by patents owned or licensable by Applied Biosystems. No license under these patents is conveyed expressly, by implication, or by estoppel to the purchaser by the purchase of this product. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA. This product is licensed under European Patents 1144623, 1214945 and foreign equivalents from Alnylam Pharmaceuticals, Inc., Cambridge, USA and is provided only for use in academic and commercial research including in vitro and in vivo identification and validation of drug targets (but excluding the evaluation or characterization of this product as the potential basis for a siRNA-based drug) and not for any other commercial purposes. Information about licenses for commercial use (including discovery and development of siRNA-based drugs) is available from Alnylam Pharmaceuticals, Inc., 300 Third Street, Cambridge, MA 02142, USA. This product is manufactured under license from the Massachusetts Institute of Technology to U.S. Patent Nos. 7,056,704 and 7,078,196 and pending counterparts.

Trademarks: Applied Biosystems, AB (Design), Ambion, RNAqueous, and *Silencer* are registered trademarks, and KDAlert, MagMAX, MessageSensor, siPORT and *NeoFX* are trademarks of Applied Biosystems Inc. or its subsidiaries in the US and/or certain other countries. All other trademarks are the sole property of their respective owners.

I. Introduction

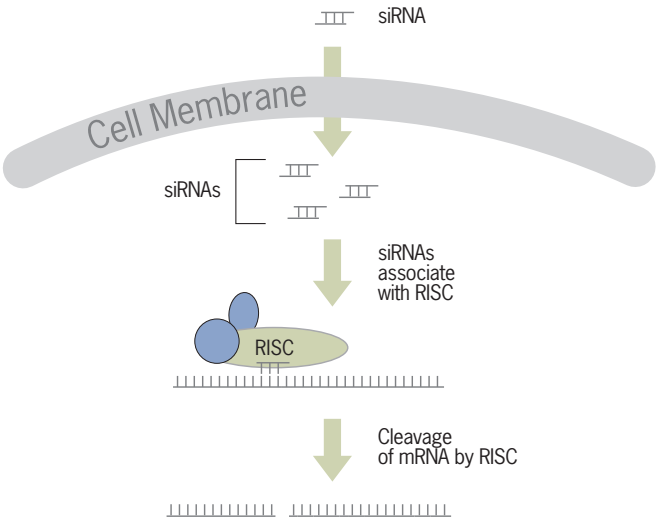
A. Background

RNA interference (RNAi), the biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation, is revolutionizing the way researchers study gene function. Scientists can quickly and easily reduce the expression of a particular gene in nearly all metazoan systems to analyze the effect that gene has on cellular function.

The RNAi mechanism

In non-mammalian systems, introducing or expressing long dsRNA triggers the RNAi pathway. The cytoplasmic nuclease Dicer first cleaves the long dsRNA into 21–23 bp small interfering RNAs (siRNAs), that then unwind and assemble into RNA-induced silencing complexes (RISCs). The antisense siRNA strand then guides the RISC to complementary RNA molecules, and the RISC cleaves the messenger RNA (mRNA), leading to specific gene silencing. Since most mammalian cells mount a potent antiviral response upon introduction of dsRNA longer than 30 bp, researchers transfect cells with 21–23 bp siRNAs to induce RNAi in these systems without eliciting the antiviral response (see Figure 1).

Figure 1. RNAi Triggered by siRNAs



Chemically synthesized siRNAs

Currently, the most widespread application of RNAi is to transiently transfect chemically synthesized siRNA into cultured mammalian cells, and assay the cells to monitor the RNAi effect. Ambion provides expert-designed, guaranteed-to-silence siRNAs to >100,000 human, mouse, and rat targets (>98% of all human, mouse, and rat genes in the RefSeq database).

The quality of siRNA can significantly influence RNAi experiments. siRNA should be free of reagents carried over from synthesis, such as salts and proteins. Also, dsRNA contaminants longer than 30 bp are known to cause cytotoxicity. We recommend using column, HPLC, or gel purified, chemically synthesized siRNAs to ensure quality and purity.

Ambion offers an extensive line of siRNAs available individually and in functional class-focused sets as *Silencer*® siRNA Libraries and *Silencer* CellReady™ Libraries. Individual siRNAs allow detailed analysis of an individual gene's role in one or more pathways, whereas sets of siRNAs (libraries) enable large scale screening experiments to tie genes to cellular function; for details, see our website at: www.ambion.com/siRNA.

B. Product Description

The *Silencer*® siRNA Starter Kit is ideal for researchers new to RNAi experiments. It contains the reagents and procedures to demonstrate gene silencing using RNAi, and it can also be used for siRNA delivery optimization experiments. Briefly, the kit includes control siRNAs, siPORT™ *NeoFX*™ Transfection Agent, and three different reagent sets to detect silencing of the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene, including the rapid KDalert™ GAPDH Assay Kit. The reagents in the kit are discussed in more detail below.

Delivery of siRNAs into cultured cells

For many immortalized cell lines, chemical transfection is the preferred means to deliver siRNA into your cells. The Ambion siPORT *NeoFX* Transfection Agent was designed expressly for this purpose. siPORT *NeoFX* is a proprietary mixture of lipids that functions by complexing with siRNAs to facilitate siRNA transfection in a broad range of cell types with high efficiency and reproducibility. It is easy to use and has minimal cytotoxic effects. However, the efficiency of siRNA transfection is strongly influenced by several parameters including the choice of transfection agent, the amount of transfection agent, and the number of cells plated.

GAPDH and Negative Control #1 siRNA

The ***GAPDH siRNA*** targets the abundant, ubiquitously-expressed housekeeping gene, GAPDH. It efficiently induces silencing in human, mouse, and rat cell lines, reducing both the mRNA and protein levels of GAPDH. The ***Negative Control #1 siRNA*** is a nontargeting sequence that has no significant homology to the sequences of human, mouse, or

rat transcripts. The Negative Control #1 siRNA should have no effect on the mRNA and protein levels of GAPDH; it serves as a baseline for measuring the effects of the GAPDH siRNA. The negative control can be used to identify nonspecific effects such as nonsequence-specific siRNA effects, cytotoxicity of the transfection agent and/or the siRNA, or suboptimal transfection conditions.

Reagents to detect gene silencing

In RNAi experiments, gene silencing can be measured either at the mRNA or protein level. The *Silencer* siRNA Starter Kit provides convenient methods for users to detect siRNA-mediated silencing of GAPDH gene expression at both the mRNA and protein levels. Quantitative measurements at the mRNA level can be obtained via qRT-PCR* using the ***GAPDH RT-PCR Primer Set***. For quantitative evaluation of GAPDH silencing at the protein level, we provide the ***KDalert GAPDH Assay***. For labs that routinely perform Western blot and/or immunofluorescence techniques, an ***Anti-GAPDH Antibody*** is included to demonstrate qualitative GAPDH knockdown. All the supplied detection reagents work equally well to detect silencing in human, mouse, and rat cells.

Reverse transfection

This Procedure provides guidelines for transfection optimization using reverse transfection. In reverse transfection, cells are transfected as they adhere to a plate after trypsinization (Figure 2 on page 7). This method bypasses several steps of the traditional "pre-plating" transfection method, making it faster, easier, and often more effective. We recommend reverse transfection for convenience and ease. Furthermore, it can be used with most chemical transfection agents and is effective in most cultured cell types. (A procedure for traditional transfection is provided in section V.A on page 31).

Kit applications

The *Silencer* siRNA Starter Kit is designed to provide an introduction to RNAi experiments in most types of adherent cultured cells. Note that for some cell lines, the siPORT *NeoFX* transfection agent provided with the kit may not be the most appropriate. Our website has a list of recommended siPORT Transfection Agents for different cell lines as well as information on transfection conditions compiled from recent scientific literature and reported to Ambion by customers who are using our products:

www.ambion.com/techlib/resources/delivery

* This product is compatible with the 5' nuclease detection and dsDNA-binding dye processes covered by patents owned or licensable by Applied Biosystems. No license under these patents is conveyed expressly, by implication, or by estoppel to the purchaser by the purchase of this product. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Reagents are provided for delivery of an extremely potent GAPDH siRNA and the nontargeting Negative Control #1 siRNA. Further, these reagents can be used for transfection optimization experiments. RNAi-induced gene silencing can be detected using any of three methods for detection of GAPDH.

C. *Silencer* siRNA Starter Kit Components and Storage Conditions

Properly stored kits are guaranteed for 6 months from the date received.

Amount	Component	Storage
1.75 mL	Nuclease-free Water	any temp*
400 µL	siPORT *NeoFX* Transfection Agent†	4°C
100 µg	Anti-GAPDH Monoclonal Antibody (MAb)	–20°C
250 µL	GAPDH PCR Primers (3 µM forward primer, 3 µM reverse primer)	–20°C
100 mL	KDalert Lysis Buffer	–20°C
12.5 mL	KDalert Solution A	–20°C
100 µL	KDalert Solution B	–20°C
75 µL	KDalert Solution C	–20°C
100 µL	GAPDH enzyme	below –70°C
50 µL	GAPDH siRNA, Human, Mouse, & Rat (50 µM)	below –70°C
50 µL	Negative Control #1 siRNA (50 µM)	below –70°C

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

† Keep the tube of siPORT *NeoFX* tightly closed to prevent evaporation.

D. Required and Optional Materials Not Provided with the Kit

General lab equipment and supplies

- Nuclease-free microcentrifuge tubes and barrier pipet tips (see www.ambion.com/prod/tubes for a complete listing)
- Constant temperature incubators

Cell culture material and equipment

- Opti-MEM® I Reduced-Serum Medium (Invitrogen Cat #31985)
- Routine tissue culture supplies and equipment

Material and equipment for KDalert GAPDH Assay (optional)

- 96 well plates: We recommend black U-bottom polypropylene 96 well plates (e.g., ABgene® Microplate 0796/k). Alternatively clear polystyrene plates can be used (recommended: BD Falcon Cat #353072 or Greiner Bio-One CellStar Cat #655180).
- Fluorescence plate reader (recommended) or UV-Vis plate reader
- General lab equipment and supplies such as 15 mL or microcentrifuge tubes, multichannel pipettors (recommended), vortex mixer, ice, etc.

Material and equipment for qRT-PCR (optional)

- Reagents for isolation of total RNA from transfected cells: Any method for isolating high quality total RNA suitable for qRT-PCR can be used. We recommend the following Ambion products:
  - MagMAX™-96 Total RNA Isolation Kit (P/N AM1830)
  - MagMAX-96 for Microarrays Total RNA Isolation Kit (P/N AM1839)
  - RNAqueous®-4PCR Kit (P/N AM1914)
- Reagents and supplies for two-step qRT-PCR using SYBR® Green I detection of amplified products: We recommend the following kits and reagents for qRT-PCR detection of siRNA-induced GAPDH knockdown:
  - MessageSensor™ RT Kit (P/N AM1745)
  - SuperTaq™† Thermostable DNA Polymerase (P/N AM2050, AM2052)
  - PCR primers for an endogenous control RNA such as 18S rRNA or cyclophilin
  - SYBR Green I or comparable nucleic acid stain
  - ROX normalization dye
  - Thermal cycler for real-time PCR
  - Optical PCR plates or tubes

E. Related Products Available from Applied Biosystems

siPORT™ *Amine* Transfection Agent P/N AM4502, AM4503	siPORT *Amine* is an easy-to-use proprietary blend of polyamines that delivers siRNA into mammalian cells with minimal cytotoxicity.
MessageSensor™ RT Kit P/N AM1745	The MessageSensor RT Kit for one-step qRT-PCR includes an optimized set of reagents for exceptionally sensitive reverse transcription. The kit is designed to be used for single-tube amplification of mRNA using either real-time or end-point amplification strategies.
Silencer® siRNA Transfection II Kit P/N AM1631	The *Silencer* siRNA Transfection II Kit contains both siPORT™ *NeoFX*™ and siPORT *Lipid* Transfection Agents in addition to a well-characterized siRNA targeting human, mouse, and rat GAPDH. This kit is ideal for developing an optimal transfection protocol for your cells. Also included are a highly validated non-targeting negative control siRNA and a detailed Protocol.

† Use of this product is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims), no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Silencer® CellReady™ siRNA Transfection Optimization Kit P/N AM86050	The *Silencer*® CellReady™ siRNA Transfection Optimization Kit facilitates identification of optimal siRNA delivery conditions in a high-throughput format. Developed as a companion kit to the *Silencer* CellReady siRNA Libraries, the kit includes three 96-well *Silencer* CellReady Optimization Plates, each with 48 wells containing *Silencer* GAPDH siRNA and 48 wells plated with *Silencer* Negative Control #1 siRNA. It also includes Ambion's lipid-based transfection agent, siPORT™ *NeoFX*™, as well as step-by-step instructions for rapid and efficient optimization of transfection conditions. The conditions identified using the kit are generally applicable for siRNA transfections in 96-well plates.
Silencer® siRNA Labeling Kits P/N AM1632, AM1634	The *Silencer* siRNA Labeling Kits are used for labeling siRNA synthesized with the *Silencer* siRNA Construction Kit or synthesized chemically. Labeled siRNA can be used to analyze the subcellular distribution of siRNA, in vivo stability, transfection efficiency, or the capability of the siRNA to attenuate target gene expression.
Silencer® siRNA Controls P/N AM4250–AM4639 see our web or print catalog www.ambion.com/siRNA	*Silencer* siRNA Controls are chemically synthesized siRNAs for genes commonly used as controls. Validated control siRNAs are available for genes such as GAPDH, β-actin, cyclophilin, KIF11 (Eg5), GFP, and luciferase. These siRNAs are ideal for developing and optimizing siRNA experiments and have been validated for use in human cells; many are also validated in mouse and rat cells.
Silencer® siRNAs see our web or print catalog www.ambion.com/siRNA	Ambion *Silencer* Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer* siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer* siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer* siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.

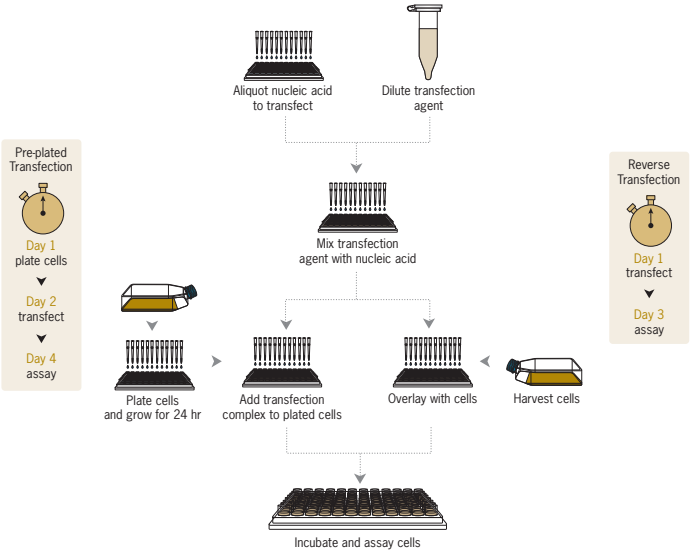
II. siRNA Transfection

A. Traditional and Reverse Transfection Methods

Delivery of synthetic siRNA molecules into adherent cells requires the use of chemical transfection agents. Lipid-based transfection agents such as siPORT *NeoFX* facilitate transfection by complexing into aggregates with the negatively charged siRNA molecules. These siRNA-transfection agent complexes are efficiently taken up by cells, presumably by endocytosis.

Historically, the first step in transfection of adherent mammalian cells is to pre-plate the cells 24 hr before transfection. During this time, the cells recover from trypsinization, grow, and adhere to the culture plate. Reverse transfection is a time-saving, effective alternative in which cells are transfected as they are passaged. Compared to the traditional pre-plating method, equivalent or improved transfection efficiency is seen for many of the cell types tested at Ambion. In addition, the reverse transfection process is an entire day shorter than traditional transfection (Figure 2). Because cells are in suspension, a larger amount of cell surface is exposed to transfection agent/siRNA complexes, and this is thought to contribute to the improved transfection efficiency. This procedure is written for reverse transfection, because it is the preferred method at Ambion; however, we also include a procedure for traditional pre-plated transfection in section V.A starting on page 31.

Figure 2.Traditional and Reverse Transfection Methods



B. Basic Transfection Procedure

Preparation and planning

Dilute the siRNAs to 2 µM

Follow the guidelines in the table below to prepare 2 µM solutions of the supplied siRNAs in nuclease-free microcentrifuge tubes:

Table 1. Preparation of 2 µM siRNA solutions

Amount	Component
16 µL	GAPDH or Negative Control #1 siRNA
384 µL	Nuclease-free water

Mix thoroughly and store the 2 µM siRNA solutions at –20°C for up to 3 months.

Planning for transfection

For each set of transfection conditions, plan to include 3 replicate transfections (i.e., a total of 9 transfections for each set of transfection conditions) with each of the following:

- GAPDH siRNA
- Negative Control #1 siRNA
- Nontransfected control: cells that are mock-transfected with Opti-MEM I medium, but no transfection agent and no siRNA

NOTE

In this section, suggested initial transfection conditions are listed first and reagent quantities for transfection optimization experiments follow. More detailed suggestions for how to optimize transfection conditions are provided in section III.F. Optimizing siRNA Transfection Conditions starting on page 22.

1. Prepare cells

a. Trypsinize adherent cells.

Trypsinize healthy, growing, adherent cells using your routine procedure. In general, healthy cells transfect better than poorly maintained cells. Routinely subculturing cells before they become overcrowded or unhealthy will minimize instability in continuous cell lines from experiment to experiment. Information on basic cell culture technique can be found in *Culture of Animal Cells: A Manual of Basic Technique* (2000) Freshney, NY:Wiley-Liss.

b. Resuspend cells in normal growth medium.

For ***initial experiments***, resuspend cells in normal growth medium to 1 x 10^5 cells/mL.

IMPORTANT

In order for cells to be transfected before they re-adhere; it is important to proceed immediately with the following steps.

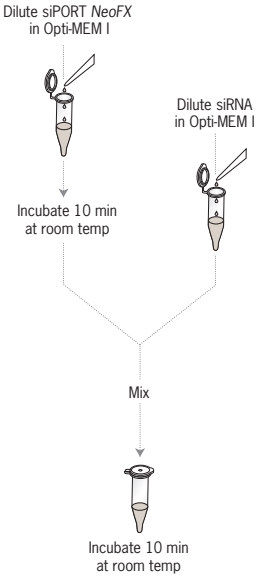
For ***subsequent transfection optimization experiments***, we recommend testing from 5 x 10^4 to 1.5 x 10^5 cells/mL. To do this, first suspend cells at 1.5 x 10^5 cells/mL and then dilute a portion of the suspension further to 1 x 10^5 cells/mL and 5 x 10^4 cells/mL.

Keep the cells at 37°C until they are needed in step 3.

The table below shows the volume of cells needed per well of different-sized culture plates. Count the number of wells in your experiment, and calculate the total volume of cells you will need. Plan to prepare ~5% overage to account for pipetting error.

	Culture Plate Type		
Amounts per transfection	96-well	24-well	6-well
Volume of cells	80 µL	400 µL	2.4 mL
Number of cells recommended for first expt (range for optimization)	8×10^3 ($4–12 \times 10^3$)	4×10^4 ($2–6 \times 10^4$)	2.4×10^5 ($1.2–3.6 \times 10^5$)

2. Prepare siRNA/siPORT *NeoFX* complexes and distribute into culture plate wells



Bring siPORT *NeoFX* or other transfection agent and the Opti-MEM I medium to room temp before use.

a. Dilute siPORT *NeoFX* in Opti-MEM I medium in a sterile conical tube.

Amounts per transfection	96-well	24-well	6-well
siPORT *NeoFX** recommended for first expt (range for optimization)	0.5 µL (0.2–0.8 µL)	1 µL (0.6–2 µL)	5 µL (2–6 µL)
Opti-MEM I to final volume:	10 µL	50 µL	300 µL

* Note that these volumes are appropriate for siPORT *NeoFX*, but may not be appropriate for other transfection agents. Use the manufacturer's volume recommendations for other transfection agents.

b. Incubate diluted siPORT *NeoFX* for 10 min at room temp.

c. Dilute the 2 µM GAPDH or Negative Control #1 siRNA in Opti-MEM I medium.

Be sure to dilute the siRNA supplied with the kit to 2 µM before use as described in *Preparation and planning* on page 8.

Amounts per transfection	96-well	24-well	6-well
2 µM siRNA* recommended for first expt (range for optimization)	1.5 µL (0.25–1.5 µL)	7.5 µL (1.5–7.5 µL)	45 µL (7.5–45 µL)
Opti-MEM I to final volume:	10 µL	50 µL	300 µL

* We recommend initially using 30 nM final siRNA concentration in the transfection mixture at the end of step 3 on page 10, and to test 5–30 nM final siRNA concentration in optimization experiments.

d. Mix diluted siRNA with diluted siPORT *NeoFX*. Incubate at room temp for 10 min.

Combine the diluted siPORT *NeoFX* (or other transfection agent) from step b with the diluted siRNA from step c. Mix gently by pipetting up and down or flicking the tube several times.

Incubate 10 min at room temp. siRNA/siPORT *NeoFX* complexes form during this incubation.

e. Dispense the siRNA/siPORT *NeoFX* complexes into the empty wells of a culture plate, and set up the nontransfected controls.

If you plan to evaluate GAPDH knockdown by Western blot or immunofluorescence, we recommend conducting transfections in 24-well plates, for immunofluorescence, plate cells onto 12mm round glass coverslips.

Aliquot siRNA/siPORT *NeoFX* transfection complexes from step d into the wells of the culture plate following the volume guidelines shown below. Include 3 ***nontransfected control*** wells, containing Opti-MEM I medium, but no siRNA and no transfection agent.

Amount per transfection	96-well	24-well	6-well
siRNA/siPORT *NeoFX* complex or Opti-MEM I for controls	20 µL	100 µL	600 µL

3. Add cells to the siRNA/siPORT *NeoFX* complexes (and control wells)

a. Transfer cells to the culture plate.

Gently mix the cells prepared in step 1 to resuspend, and pipet them into wells of the culture plate containing siRNA/siPORT *NeoFX* complexes or wells set up as nontransfected controls.

Amount per transfection	96-well	24-well	6-well
Volume of cells per well	80 µL	400 µL	2.4 mL

b. Gently mix the cells and siRNA/siPORT *NeoFX* complexes.

Rock the plate gently back and forth to evenly distribute the complexes; avoid swirling, as this can cause contents to aggregate in the center of the well.

4. Incubate at 37°C for 24 hr, then replace the culture medium

Incubate the transfection mixture at 37°C in normal cell culture conditions for 24 hr. Then, replace the culture medium with fresh normal growth medium.

5. Assay for transfection efficiency and cytotoxicity

As an initial screen for severe cytotoxic effects, check the visual appearance of transfected cells for evidence of cell necrosis and/or apoptosis before investing the time to evaluate GAPDH knockdown and cytotoxicity in more detail as described in the next section.

Assay for GAPDH knockdown ~48 hr after transfection.

III. Monitoring Cytotoxicity & GAPDH Knockdown

A. Quantitative and Qualitative Options for Evaluating RNAi

GAPDH knockdown or silencing can be measured at either the mRNA or protein level. For convenience, economy, and speed, we recommend monitoring GAPDH knockdown at the protein level using the KDalert GAPDH Assay. We also provide GAPDH PCR Primers designed for measuring GAPDH knockdown at the mRNA level by qRT-PCR. We recommend qRT-PCR if it is a routine procedure in your lab. Either the KDalert GAPDH Assay or qRT-PCR will yield data appropriate for quantitative analysis of your experimental results. If your goal is to simply evaluate RNAi qualitatively, we provide brief instructions for using the Anti-GAPDH Antibody to demonstrate GAPDH knockdown using Western blotting or immunofluorescence in section III.E starting on page 18.

KDalert GAPDH Assay

The KDalert GAPDH Assay Kit provides a rapid, convenient, fluorescence-based or colorimetric method for measuring the enzymatic activity of GAPDH in cultured cells. The ***GAPDH knockdown*** for a given transfection condition is determined from the ratio of GAPDH activity in cells transfected with GAPDH siRNA vs. cells transfected with Negative Control #1 siRNA.

Comparison of the GAPDH activity per cell in nontransfected cells and in cells transfected with Negative Control #1 siRNA is an indicator of ***cytotoxicity*** caused by transfection.

With the KDalert assay, we have found that evaluating cells for GAPDH knockdown 48 hr after transfection provides a good balance between reliable detection of protein knockdown and the elapsed time required to see such knockdown due to the half life of the protein. Slightly higher levels of GAPDH knockdown are typically seen 3 days after transfection.

qRT-PCR

qRT-PCR using the supplied GAPDH RT-PCR Primer Set can alternatively be used to analyze GAPDH knockdown and cytotoxicity. For these experiments, isolate total RNA from the transfected cells approximately 48 hr after transfection, then use the RNA in two-step qRT-PCR using SYBR Green-based amplicon detection.

qRT-PCR data are analyzed similarly to data from the using the KDalert GAPDH Assay. ***GAPDH knockdown*** is determined by comparing GAPDH mRNA levels in cells transfected with either the GAPDH siRNA or with Negative Control #1 siRNA, and ***cytotoxicity*** information is inferred from the GAPDH mRNA level in cells transfected with Negative Control #1 siRNA vs. nontransfected controls.

Comparison of KDalert and qRT-PCR methods

In general, successful knockdown will cause a slightly greater reduction in GAPDH mRNA levels than in GAPDH protein levels. This is probably because protein knockdown is influenced by the rates of protein synthesis and turnover, in addition to the rates of mRNA synthesis and turnover. For housekeeping genes such as GAPDH, the rate of cell division and concomitant protein synthesis during the transfection experiment also have an impact on knockdown levels.

	KDalert GAPDH Assay	qRT-PCR for GAPDH mRNA
Measures:	GAPDH activity (protein)	GAPDH mRNA
Assay timing	48 hr after transfection	48 hr after transfection
Expected result	≥50% knockdown	≥70% knockdown

Direct methods for analyzing transfection-induced cytotoxicity

KDalert and qRT-PCR provide ***indirect*** measurement of transfection-induced cytotoxicity, which is sufficient in most cases for demonstrations of RNAi and initial transfection optimization experiments. There are also many ways to ***directly*** assess cell viability, and any established method that is appropriate for the cells in the experiment can be used. If you choose to directly measure cell viability (or total cell number), some of the available methods are listed below:

- Trypan blue exclusion assay
- alamarBlue® assay
- Acid phosphatase or alkaline phosphatase assay
- Flow cytometry
- Fluorescence microscopy

B. KDalert GAPDH Assay Standard Fluorescence Procedure

The KDalert GAPDH Assay measures the conversion of NAD^+ to NADH by GAPDH in the presence of phosphate and glyceraldehyde-3-phosphate (G-3-P). The production of NADH under these conditions results in a fluorescence increase and a color change in the samples. If you have access to a fluorescence plate reader, we recommend that you use it to analyze results, but the reaction rate can alternatively be measured colorimetrically using a visible plate reader or spectrophotometer. Under the recommended assay conditions, the rate of NADH production is proportional to the amount of GAPDH enzyme present. Thus the assay can be used to accurately determine the amount of GAPDH protein in a sample.

1. Prepare KDalert Master Mix

a. On the day of the assay, 2 days after transfection, assemble a KDalert Master Mix as described in Table 2. Prepare Master Mix only for the samples to be assayed that day plus 5–10% overage (90 µL KDalert Master Mix per reaction).

Table 2. KDalert Master Mix

Component	Per sample	For one 96 well plate plus a standard curve*
KDalert Solution A	88.8 µL	12.34 mL
KDalert Solution B	0.68 µL	95 µL
KDalert Solution C	0.47 µL	65 µL

* Instructions for including a GAPDH Enzyme standard curve as a positive control is described in section IV.B starting on page 26.

b. Mix thoroughly by inversion or gentle vortexing.
The KDalert Master Mix can be stored at room temp for <60 min or on ice for several hours; allow it to warm up to room temp immediately before use.

2. Adjust the fluorescence plate reader settings

Turn on the fluorescence plate reader and set the data acquisition parameters as follows:

- Set the excitation wavelength at 560 nm and the emission wavelength at 590 nm.
  If the fluorometer uses preset filters, use the filters closest to these wavelengths. We have found that excitation and emission filters set to 545 and 575 nm, respectively, work well for the assay.
- Set the plate reader to kinetic mode, if available.
- Set the gain to autoscale, if available. Alternatively, set the gain to medium initially.
- Set the temperature to room temperature.

3. Remove culture medium from transfected cells

48 hr after siRNA transfection, aspirate the culture medium from transfected cells.

STOPPING POINT

The culture plate can be stored at –80°C after removing the culture medium. Thaw frozen cells on ice before proceeding.

4. Add KDalert Lysis Buffer

Add KDalert Lysis Buffer to each well containing cells following the volume guidelines in the table below.

(For the positive control reactions, add KDalert Lysis Buffer to empty wells of the assay plate.)

	Plate Type		
	96-well	24-well	6-well
Lysis Buffer Volume (per well)	200 µL	1 mL	4 mL

5. Incubate at 4°C for 20 min, then pipet the cell lysate up and down 4–5 times

a. Incubate at 4°C for 20 min to lyse the cells.
Alternatively, the lysis can be incubated on ice instead of at 4°C.

b. Pipet the cell lysate up and down 4–5 times (or shake the plate for 20 sec at room temp) to homogenize the lysate.

6. Transfer 10 µL of lysate to a 96 well plate

Transfer 10 µL of each lysate (for the positive control reactions described in section IV.B on page 26 transfer 10 µL of GAPDH Enzyme dilution–including the GAPDH Working Stock) to the wells of a clean 96 well plate. 96 well plate recommendations are provided in section *I.D. Required and Optional Materials Not Provided with the Kit* on page 4.

7. Add 90 µL of KDalert Master Mix to each sample

Working quickly, add 90 µL of KDalert Master Mix to each sample. It is important that all samples receive the KDalert Master Mix at approximately the same time. We recommend using a multichannel pipettor to dispense the KDalert Master Mix quickly.

8. Measure the increase in fluorescence at room temp

Measure the increase in fluorescence of the samples at room temp.

The fluorescence plate reader settings are provided in section B.3 on page 13.

If the plate reader is capable of ***real-time kinetic measurements***, immediately measure the increase in fluorescence over a 4 minute interval, collecting data every 1–2 min.

For ***endpoint fluorescence measurements,*** measure the fluorescence immediately after adding the KDalert Master Mix, then allow the reaction to proceed for 4 minutes at room temperature and measure the fluorescence again. Subtract the initial fluorescence reading from the second reading to determine the fluorescence increase for each sample.

Samples with a relatively high levels of GAPDH activity will acquire a magenta color approximately 15 min after the KDalert Master Mix is added to the samples. This color does not affect the fluorescence reading and can be considered a visible indicator of GAPDH activity.

C. qRT-PCR Instructions

We recommend the Ambion MessageSensor RT Kit (P/N AM1745) and SuperTaq® Polymerase (P/N AM2050) for qRT-PCR following the recommendations provided here and the instructions for two-step RT-PCR provided in the appendix of the MessageSensor RT Kit Protocol. Below we list the important protocol specifications for qRT-PCR of GAPDH mRNA using the GAPDH PCR Primers:

Isolate total RNA from transfected cells 48 hr after transfection

Any method for obtaining high quality RNA suitable for RT-PCR can be used to isolate total RNA from the transfected cells. Any of the following Ambion products are well-suited for this application as they provide high quality and purity RNA, and include procedures/reagents for removal of genomic DNA:

- MagMAX-96 Total RNA Isolation Kit (P/N AM1830)
- MagMAX-96 for Microarrays Total RNA Isolation Kit (P/N AM1839)
- RNAqueous-4PCR Kit (P/N AM1914)

Recommended controls and replicates

- Include reactions to amplify an endogenous control RNA such as 18S ribosomal RNA or cyclophilin for normalization of results.
- Include a no-template (no RNA) RT reaction that is carried through to become a no-template PCR negative control reaction.
- Include duplicate PCRs from each RT reaction.

Procedure recommendations

- Use a ***two-step*** qRT-PCR procedure.
- Use random primers for the reverse transcription.
- Detect amplification reaction products using SYBR Green I or a comparable nucleic acid stain.
- We recommend the following real-time PCR cycling conditions:

	Stage	Reps	Temp	Time
Initial denaturation	1	1	95°C	5 min
Amplification	2	40	95°C	15 sec
			60°C	30 sec
			72°C	30 sec

At the end of the PCR, perform dissociation analysis and use the thermal cycler software for C_t analysis

- Perform dissociation analysis (melt-curve) on the reactions to identify the characteristic peak associated with primer-dimers. Successful PCR amplification should exhibit a single prominent peak that is readily distinguishable from the primer-dimer observed in the no-template control reactions.

- Determine an appropriate cycle threshold (C_t) using the software supplied by the thermal cycler manufacturer. We recommend using the automatic baseline determination feature.

D. KDalert and qRT-PCR Data Interpretation

Data interpretation overview

Either the KDalert GAPDH Assay or qRT-PCR can be used to generate quantitative data about GAPDH knockdown. Successful transfection of GAPDH into cells causes a reduction in the levels of GAPDH mRNA compared to the GAPDH mRNA levels in cell transfected with Negative Control #1 siRNA; this can be quantitated by qRT-PCR. Of course a reduction in the amount of GAPDH mRNA will also result in knockdown of GAPDH protein in transfected cells. This reduction or knockdown of GAPDH at the protein level can be quantitatively evaluated using the KDalert assay. In this section we describe how to interpret data from both of these assays.

Analysis of KDalert GAPDH Assay data

For the KDalert GAPDH Assay, the GAPDH activity in each sample is defined as the fluorescence increase over 4 min, (Δfluorescence) in arbitrary units. This value is calculated automatically with a fluorescence plate reader set to kinetic mode, or it can be calculated from static fluorescence measurements by subtracting the fluorescence reading just after the reaction is started (t_0) from that at 4 minutes ($t_{4\ min}$).

Calculating % remaining gene expression and % knockdown

The percent remaining gene expression for a given transfection condition can be determined from the ratio of the fluorescence increase for samples transfected with GAPDH siRNA to the fluorescence increase for samples transfected with Negative Control #1 siRNA:

$$\%\ \text{remaining expression} = 100 \times \frac{\Delta\text{fluorescence}_{GAPDH}}{\Delta\text{fluorescence}_{Neg\ \#1}}$$

where $\Delta\text{fluorescence}_{GAPDH}$ and $\Delta\text{fluorescence}_{Neg\ \#1}$ are the mean fluorescence increases for a given transfection condition for samples transfected with GAPDH siRNA and Negative Control #1 siRNA, respectively.

The level of gene knockdown for a given transfection condition is calculated from the percent remaining gene expression:

$$\%\ \text{knockdown} = 100 - \%\ \text{remaining expression}$$

$$\%\ \text{knockdown} = 100 - \left(100 \times \frac{\Delta\text{fluorescence}_{GAPDH}}{\Delta\text{fluorescence}_{Neg\ \#1}}\right)$$

Transfection-associated toxicity

Transfection agents are somewhat toxic to cells. Since transfection with the Negative Control #1 siRNA will not induce RNAi, any decrease in the GAPDH levels in those cultures can be attributed to cytotoxicity. Specifically, compare the GAPDH activity in cells transfected with Negative Control #1 siRNA ($\Delta\text{fluorescence}_{\text{Neg \#1}}$) to that in nontransfected cells ($\Delta\text{fluorescence}_{\text{Nontransfected}}$) to examine the cytotoxic effects of the transfection conditions. Transfection conditions which do not cause any cytotoxicity would result in a ratio of 1, therefore, the closer this ratio is to 1, the less cytotoxic the transfection conditions.

$$\frac{\Delta\text{fluorescence}_{\text{Neg \#1}}}{\Delta\text{fluorescence}_{\text{Nontransfected}}} = \text{cytotoxicity ratio}$$

Calculating the best transfection condition tested

Typically, there is an inverse relationship between transfection efficiency (and associated gene knockdown) and cell viability. A useful way to describe the balance of transfection efficiency and cytotoxicity is the term Optimal Balance Factor, or OBF. The OBF is calculated ***for each transfection condition*** as follows:

$$\text{OBF} = \text{cytotoxicity ratio} \times \text{\% knockdown}$$

$$\text{OBF} = \frac{\Delta\text{fluorescence}_{\text{Neg \#1}}}{\Delta\text{fluorescence}_{\text{Nontransfected}}} \times \left[100 - \left(100 \times \frac{\Delta\text{fluorescence}_{\text{GAPDH}}}{\Delta\text{fluorescence}_{\text{Neg \#1}}}\right)\right]$$

Typically, optimal transfection condition(s) are those which exhibit the highest OBF value.

Analysis of qRT-PCR data

Calculating GAPDH knockdown from C_t values

For these calculations, use the mean C_T value from the duplicate PCRs. In qRT-PCR, the C_t of the experimental amplicon, GAPDH in these experiments, is proportional to the quantity of the target mRNA after normalization to the C_t value for an endogenous control RNA such as 18S ribosomal RNA or cyclophilin. This normalized C_t value is known as ΔC_T, and for the GAPDH amplicon, it is defined as follows:

$$\Delta C_T = C_T \text{for GAPDH PCR} - C_T \text{ for endogenous control PCR}$$

GAPDH knockdown is related to the difference in the ΔC_Tvalue for samples transfected with GAPDH siRNA (ΔC_TGAPDH) compared to the ΔC_T value for samples transfected with Negative Control #1 siRNA (ΔC_TNeg#1). This is known as the $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T\text{GAPDH} - \Delta C_T\text{Neg\#1}$$

The percent knockdown can then be determined using the relation:

$$\text{\% knockdown} = 100 - 100 \times 2^{-\Delta\Delta C_T}$$

Evaluating transfection-associated cytotoxicity from C_T values

Transfection agents are somewhat toxic to cells. Since transfection with the Negative Control #1 siRNA will not induce RNAi, any decrease in GAPDH expression levels in those cultures can be attributed to cytotoxicity. Specifically, compare the ΔC_T for GAPDH amplification in cells transfected with Negative Control #1 siRNA (ΔC_TNeg #1) to that in nontransfected cells (ΔC_Tnontransfected) to examine the cytotoxic effects of the transfection conditions. Transfection conditions which do not cause any cytotoxicity would result in a ratio of 1, therefore, the closer this ratio is to 1, the less cytotoxic the transfection conditions.

ΔC_TNeg #1 ÷ ΔC_Tnontransfected = cytotoxicity factor

Calculating the best transfection condition tested

Optimal conditions for siRNA transfection for a given cell type are those which simultaneously maximize the percent knockdown and minimize transfection-associated cytotoxicity. A useful way to describe the balance of transfection efficiency and cytotoxicity is the term Optimal Balance Factor, or OBF. The OBF is calculated ***for each transfection condition*** as follows:

OBF = cytotoxicity factor X % knockdown
OBF = (ΔC_T Neg #1 ÷ ΔC_T nontransfected) X (100 – 100 X $2^{-\Delta\Delta C_T}$)

Typically, optimal transfection condition(s) are those which exhibit the highest OBF value.

E. Demonstrating GAPDH Knockdown Using the Anti-GAPDH Antibody

The Anti-GAPDH Monoclonal Antibody (MAb) provided with the kit is a specific, sensitive reagent that can be used to show GAPDH knockdown at the protein level. We include it for users who are familiar with Western blotting and/or immunofluorescence and want to see qualitative evidence of GAPDH knockdown. As with the quantitative methods, compare the GAPDH signal in samples transfected with the GAPDH siRNA to that in samples transfected with Negative Control #1 siRNA and nontransfected control samples.

Anti-GAPDH Monoclonal Antibody description

The Anti-GAPDH MAb is a mouse monoclonal antibody specific for GAPDH expressed by human, mouse, and rat cells. We have successfully used it in Western blotting and immunofluorescence experiments; however, it also should be compatible with other routine procedures such as ELISA and immunoprecipitation.

- Use anti-mouse secondary antibodies for detection of Anti-GAPDH MAb.
- For Western blotting, use the Anti-GAPDH MAb at 1 µg/mL.
- For immunofluorescence, use the Anti-GAPDH MAb at 5 µg/mL.

Use the Anti-GAPDH MAb with your routine protocols

Many laboratories routinely use Western blot and immunofluorescence, and if your lab has standard protocols for these techniques, we encourage you to follow them. The procedures included here are used by some scientists at Ambion, and we provide them as a convenience, but they may not be detailed enough for someone who is has never used these techniques. *Current Protocols in Molecular Biology* Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) John Wiley & Sons, Inc. is an excellent source of detailed protocol information on these techniques.

E.I. Western Blot Procedure

Required reagents and materials

- Routine cell culture supplies and reagents such as 1X PBS, trypsin-EDTA, pipettes, plates, tubes, etc.
- Lysis buffer: 50 mM HEPES pH 8.3, 420 mM KCl, 0.1% NP-40, 1 mM EDTA
- Reagent to measure total protein concentration such as Lowry reagent
- Protein gel electrophoresis equipment and supplies: we recommend using 12% acrylamide/bisacrylamide (29:1) SDS gels with a stacking gel for good separation of the 36 kDa GAPDH protein
- Western blotting reagents and equipment such as transfer membrane and apparatus, rocker platform agitator, blocking buffer: e.g., 1% dry milk in 1X PBS, PBST: 0.1% Tween 20 in 1X PBS, anti-mouse secondary antibody conjugated to the enzyme or ligand of choice, and appropriate detection reagents/equipment

1. Collect cells and wash with 1X PBS

a. Collect the cells from a 24-well tissue culture plate using trypsin-EDTA and transfer to a 1.5 mL microcentrifuge tube.

b. Centrifuge the cells at 600 x g for 5 min, and discard the supernatant.

c. Wash with PBS by adding 500 µL 1X PBS and centrifuging as in the previous step. Discard the PBS wash.

2. Lyse the cells on ice for 15 min

a. Add 100 µL of lysis buffer to each sample and vortex for 15 sec.

b. Incubate for 15 min on ice.

3. Collect the lysate and determine the total protein concentration

a. Centrifuge at 16,000 x g at 4°C for 10 min to pellet cellular debris and transfer the supernatant to a fresh tube. Store the clarified lysate on ice for immediate use; alternatively store at –20°C.

b. Determine the total protein concentration.

4. Perform PAGE, and transfer protein to a blotting membrane

a. Mix clarified lysates with gel loading buffer. The protein amount and sample volume will depend on the size of the gel, for minigels, we recommend running samples containing ~5 µg total protein per well. Analyze equal protein amounts of each sample.

b. Heat samples to 95°C for 3 min to denature proteins, then store on ice while you set up the gel apparatus.

c. Load and run the gel.

d. Transfer the protein to a support membrane.

5. Detect GAPDH on the membrane using Anti-GAPDH MAb

a. Block nonspecific binding by immersing the membrane in blocking reagent for 1 hr.

b. Wash the membrane with PBST (0.1% Tween 20, 1X PBS) three times for 5 min each.

NOTE

Unless otherwise noted, all of these incubations are at room temp with rocking.

c. Add 1 µg/mL Anti-GAPDH MAb diluted in fresh blocking solution to the membrane and incubate for 1 hr.

d. Wash the membrane with PBST three times for 5 min each.

e. Add the secondary antibody diluted according to the supplier's recommendations in fresh blocking solution and incubate for 1 hr.

f. Wash the membrane with PBST three times for 5 min each.

g. Detect the Anti-GAPDH MAb using an appropriate detection method for the conjugated secondary antibody.

6. Expected result

First compare the GAPDH signal from cells transfected with the Negative Control #1 siRNA to that from nontransfected cells. The GAPDH signal should be equivalent indicating that the transfection process does not disrupt GAPDH expression. Then compare the GAPDH signal from cells transfected with the GAPDH siRNA to that from cells transfected with Negative Control #1 siRNA. Effective gene silencing will result in a lower signal (or no signal) from cells transfected with the GAPDH siRNA.

E.II. Immunofluorescence Procedure

IMPORTANT

This procedure is compatible with cells transfected and grown on 12 mm round glass cover slips in a 24 well tissue culture plate.

Required reagents and materials

- Routine cell culture supplies and reagents such as 1X PBS, pipettes, plates, tubes, etc.
- 4% paraformaldehyde/PBS: Freshly prepare this solution by mixing 0.4 g paraformaldehyde (powder) in 10 mL of 1X PBS, then add 25 µL of 5M NaOH. Heat to 65°C until dissolved, approximately 10 min, and cool to room temp before use
- 0.1% Triton X-100/PBS for cell permeabilization
- 3% BSA/PBS for blocking
- Anti-mouse secondary antibody conjugated with a fluorescent marker [e.g., donkey anti-mouse IgG labeled with fluorescein (FITC)]

NOTE

Use gentle agitation for all the incubation steps throughout the procedure

1. Wash and fix the cells

a. 48 hr after transfection, aspirate the media from the dish and wash the cells with 1 mL 1X PBS.

b. Remove the 1X PBS and add 400 µL of fresh 4% paraformaldehyde/PBS to each well.

c. Incubate for 7 min at room temp with gentle agitation.

2. Wash and permeabilize the cells

a. Discard the 4% paraformaldehyde/PBS and wash the cells with 1 mL of 1X PBS.

b. Remove the 1X PBS and add 500 µL of 0.1% Triton X-100/PBS.

c. Incubate for 7 min at room temp with gentle agitation to permeabilize cell membranes.

d. Remove 0.1% Triton X-100/PBS and wash the cells with 1 mL of 1X PBS.

3. Block and wash the cells

a. Remove the 1X PBS and block cells by adding 500 µL of 3% BSA/PBS (blocking solution).

b. Incubate for 1 hr at room temp with gentle agitation.

c. Remove the blocking solution and wash cells with 1 mL of 1X PBS.

4. Incubate the cells with 5 µg/mL Anti-GAPDH MAb in 1X PBS for 1 hr, and wash the cells

a. Remove the 1X PBS, and add 500 µL of Anti-GAPDH MAb diluted in 1X PBS at a final concentration of 5 µg/mL.

b. Incubate for 1 hr at room temp with gentle agitation.

c. Remove the diluted Anti-GAPDH MAb solution and wash the cells with 1 mL of 1X PBS.

5. Detect the Anti-GAPDH MAb

Use an anti-mouse secondary antibody with the detection technology of your choice to detect the Anti-GAPDH MAb. Follow the manufacturer's instructions for incubating with secondary antibody and detection. You may also want to stain nuclei (e.g., with DAPI) to help with interpretation of the immunofluorescence data.

6. Expected result

First compare the GAPDH signal from cells transfected with the Negative Control #1 siRNA to that from nontransfected cells. The signal should be equivalent indicating that the transfection process does not disrupt GAPDH expression. Then compare the GAPDH signal from cells transfected with the GAPDH siRNA to that from cells transfected with Negative Control #1 siRNA. Effective gene silencing will result in a lower signal (or no signal) from cells transfected with the GAPDH siRNA.

F. Optimizing siRNA Transfection Conditions

When is transfection optimization needed?

As a general guideline, we recommend optimizing transfection conditions so that ≥50% GAPDH knockdown as measured by KDalert or ≥70% GAPDH knockdown as measured by qRT-PCR with ≤25% cytotoxicity or cell death results. Figure 3 on page 23 shows an overview of the transfection optimization strategy described in this section.

Optimized transfection is a balance of target knockdown and cytotoxicity

The goal of transfection optimization is to identify the conditions that will provide good gene knockdown while minimizing transfection-induced cytotoxicity for the particular cell type. Typically conditions which improve gene knockdown (e.g., increase in amount of transfection agent) also result in increased cytotoxicity. Therefore, both gene knockdown and cytotoxicity must be considered when interpreting optimization experiments—with a balance between the two representing the ideal conditions for transfection. Once optimal conditions are established, they should be kept constant among experiments for a given cell type.

1. Choice of transfection agent

Ambion siPORT *NeoFX* reagent is a highly effective transfection agent with minimal cytotoxic effects. It is useful for a wide variety of adherent cultured mammalian cells. However, different cell types may vary in their response to a given transfection agent. Therefore, in some

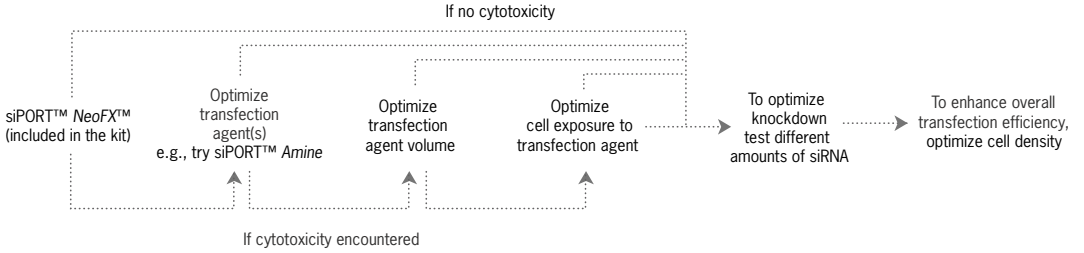


Figure 3. Transfection Optimization Strategy.

First, follow the procedure in section *B. Basic Transfection Procedure* on page 8 to evaluate the effectiveness of siPORT *NeoFX*. The goal is to achieve ≥50% GAPDH knockdown as measured by KDalert or ≥70% GAPDH knockdown as measured by qRT-PCR with ≤5% cytotoxicity. If these values are achieved, no further optimization is needed. Otherwise, optimize transfection using the strategy described here.

instances siPORT *NeoFX* may not efficiently transfect a particular cell type. In this case, test other transfection agents such as Ambion siPORT *Amine* (P/N AM4502, AM4503).

a. Follow the general procedure in section II.B (using 30 nM final concentration of siRNA) to test different transfection agents. Follow the transfection agent manufacturer's recommendations for the volume of transfection agent to use.

b. Assay for target knockdown and cytotoxicity.
   - If one of the transfection agents provides ≥50% GAPDH knockdown as measured by KDalert or ≥70% GAPDH knockdown as measured by qRT-PCR with ≤5% cytotoxicty, no further optimization is necessary.
   - If the observed results do not meet these criteria choose the transfection agent that gave better overall results, and proceed to step 2 below.

2. Amount of transfection agent

The volume of transfection agent used is a critical parameter to optimize; too little can limit transfection, but too much can be toxic. The overall transfection efficiency is influenced by the amount of transfection agent complexed to the siRNA.

a. Follow the procedure in section II.B (using 30 nM final concentration of siRNA) to test 4 different volumes of transfection agent in step II.B.2.

b. Assay for GAPDH knockdown and cytotoxicity.
   - If good GAPDH knockdown and minimal cytotoxicity (as defined above) are obtained, no further optimization is necessary.
   - If >25% cytotoxicity is observed, proceed to step 3.

- If acceptable levels of cytotoxicity are obtained, but GAPDH knockdown is insufficient, proceed to step 4.

3. Exposure time to transfection agent (if needed)

Although siPORT *NeoFX* was designed to minimize cytotoxicity, exposing cells to excessive amounts of transfection agent or for extended time periods can be detrimental to the overall health of the cell culture.

After determining the optimal volume of transfection agent for GAPDH knockdown, minimize cytotoxicity by adjusting the time that cells are exposed to transfection complexes.

a. Replace the medium at 6 hr and 12 hr after transfection by carefully aspirating the old medium from the well and adding fresh medium. It is usually not necessary to wash cells.

b. Re-evaluate GAPDH knockdown and cytotoxicity.
   - If good GAPDH knockdown and minimal cytotoxicity (as defined above) are obtained, no further optimization is necessary.
   - If GAPDH knockdown is insufficient, proceed to step 4.

4. Amount of siRNA

The optimal amount of siRNA and its capacity for gene silencing are influenced in part by properties of the target gene, including the following: mRNA localization, stability, and abundance, and target protein stability and abundance. If too much siRNA is used for transfection, it may lead to off-target effects. Conversely, if too little siRNA is transfected, reduction of target-gene expression may be undetectable. Because there are so many variables involved, it is important to optimize the siRNA amount for every cell line used, and in some cases, it may even be necessary to re-optimize for different targets.

To optimize the activity of transfected siRNAs, test 1, 3, 10, and 30 nM (final concentration) siRNA, using the transfection agent quantity and exposure time optimized in the experiments described above.

If GAPDH knockdown is still insufficient, even using 30 nM siRNA, try increasing the GAPDH siRNA concentration to 100 nM. If GAPDH knockdown is still insufficient, proceed to step 5.

5. Cell density

For most adherent cells, the optimal confluency for transfection is 30–80%. The cell amounts listed as the range for optimization in step II.B.1 on page 8 provide guidelines for seeding different sized culture plates to obtain 30–80% confluency after 24 hr of growth; these numbers are approximate because the exact number of cells required for seeding and transfection depends on cell type, size, and growth rate.

- Follow the transfection procedure in section II.B, using the conditions optimized in the steps above, while varying the cell plating density across the wells of the culture dish so that cells will reach between 30–80% confluency.

- Be sure to monitor cell viability during these experiments, as cell cultures can become unstable at low densities.
- Use the siRNA concentration optimized in step 4.

The optimal cell plating density results in the greatest reduction in GAPDH expression without creating instability in the cell line.

IV. Troubleshooting

A. Transfection Causes Extensive Cell Death

Too much transfection agent was used

Titrate transfection agent over a broad dilution range, and choose the most dilute concentration that still gives good gene knockdown.

Cells were exposed to transfection agent/siRNA complexes for too long

Sensitive cells may begin to die from exposure to the transfection agent after a few hours. If transfection causes excessive cell death with your cells, remove the transfection mixture, and replenish with fresh growth medium after 6–24 hr.

Cells are stressed

- Add fresh growth medium as early as 6 hr after transfection.
- Avoid using antibiotics when plating cells for transfection.
- Use healthy cells that have not been grown to the point of medium depletion between subculturing events.
- Avoid subjecting cells to frequent temperature and pH shifts.

B. Troubleshooting the KDalert GAPDH Assay

Positive control reaction for the KDalert GAPDH assay

As a positive control for the KDalert GAPDH Assay, we recommend preparing serial dilutions of the provided GAPDH enzyme, and subjecting them to the assay to produce a standard curve. Instructions for this experiment are provided below.

1. Prepare serial dilutions of GAPDH Enzyme
   To prepare a standard curve, make serial dilutions of the GAPDH Enzyme in KDalert Lysis Buffer over a 100-fold concentration range.

a. Thaw GAPDH Enzyme at room temperature, then immediately place on ice.

b. Mix 4 µL of GAPDH Enzyme with 396 µL KDalert Lysis Buffer to create a GAPDH Working Stock (see Table 3 below), then use it to prepare serial dilutions of GAPDH as shown in Table 3. Keep diluted GAPDH on ice until used.

Table 3. Dilutions for GAPDH standard curve

KDalert Lysis Buffer	GAPDH Enzyme or Dilution	Dilution	GAPDH concentration
396 µL	4 µL GAPDH Enzyme (provided with the kit)	GAPDH Working Stock	0.26 U/mL
50 µL	50 µL GAPDH Working Stock	1:2	0.133 U/mL
50 µL	50 µL 1:2 dilution	1:4	0.067 U/mL
50 µL	50 µL 1:4 dilution	1:8	0.033 U/mL
50 µL	50 µL 1:8 dilution	1:16	0.017 U/mL
50 µL	50 µL 1:16 dilution	1:32	0.0083 U/mL
50 µL	50 µL 1:32 dilution	1:64	0.0042 U/mL
50 µL	50 µL 1:64 dilution	1:128	0.0021 U/mL

2. Test GAPDH enzyme dilutions in the KDalert assay.
   Follow the procedure in section *III.B. KDalert GAPDH Assay Standard Fluorescence Procedure* starting on page 12 with the following exceptions:
   - Skip steps 3–5 on page 14.
   - In step 6, add 10 µL GAPDH enzyme dilution to the assay plate. We recommend creating a standard curve using duplicate samples of the GAPDH enzyme dilution plus two negative control wells that receive KDalert Lysis Buffer only (with no GAPDH enzyme).

Expected results from the positive control reactions

Figure 4 shows a typical standard curve obtained for a GAPDH enzyme dilution series. The observed GAPDH activity should show a linear relationship to the amount of enzyme present over a 100-fold range.

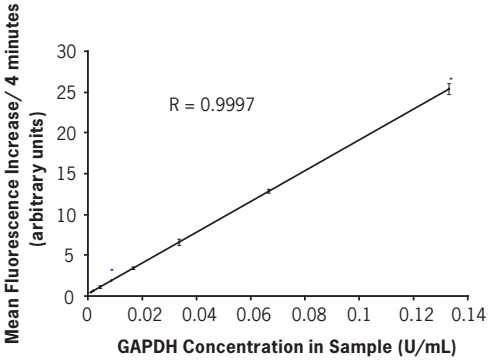


Figure 4. Typical Standard Curve for GAPDH enzyme

GAPDH enzyme was diluted as described in Table 3 on page 27, and assayed in duplicate using the KDalert GAPDH Assay Standard Fluorescence Procedure described in section III.B on page 12 using a fluorescence plate reader in kinetic mode.

No GAPDH activity seen in positive control reactions

If none of the diluted GAPDH enzyme reactions acquire a magenta color after 15 min, there was most likely an error in preparing the KDalert Master Mix. We suggest repeating the positive control experiment.

No fluorescence increase observed for either standard curve or samples

If several of the diluted GAPDH enzyme reactions acquire a magenta color after 15 min, but no fluorescence increase is detected by the fluorometer, check the settings for the appropriate excitation and emission wavelengths.

Low GAPDH activity

If the standard curve produces the expected result, but a low signal is seen from lysates that are expected to have relatively high GAPDH activity (i.e., lysates from nontransfected cells or cultures transfected with Negative Control #1 siRNA), there is probably not enough GAPDH in the cell extract used in the assay.

- For the fluorescence assay:
  - Increase the amount of cell lysate to 20 µL per reaction in step III.B.6 on page 14, and use only 80 µL of KDalert Master Mix in step III.B.7.
  - If fewer than 2,000 cells were plated per well, decrease the volume of KDalert Lysis Buffer used to lyse the cells step III.B.4 on page 14 proportionately.
- For the colorimetric assay: If fewer than 4,000 cells were plated per well, decrease the volume of KDalert Lysis Buffer used to lyse the cells proportionately.

No GAPDH gene knockdown

If the standard curve produces the expected result, and all cell lysates show GAPDH activity, even those from cultures transfected with the GAPDH siRNA, consider the following possible causes and suggestions:

- Problems with transfection: see next section *C. No Detectable Gene Silencing*.
- Too much cell lysate used in the KDalert reaction; this is indicated when reactions from Negative Control #1-transfected samples turn magenta in <5 min.
  - For the fluorescence assay: Reduce the lysate volume in the reaction to 2 µL.
  - For the colorimetric assay: Reduce the lysate volume to 10 µL.

C. No Detectable Gene Silencing

The transfection procedure requires optimization

We strongly recommend that you optimize the transfection procedure for each cell type using the GAPDH siRNA as described in section III.F starting on page 22.

Problems with siRNA/transfection agent complex formation

Follow the instructions for transfection complex formation closely; using the appropriate incubation times is important for good transfection efficiency.

Serum, polyanions, or other inhibitors were present during complexing.
Although siPORT *NeoFX* is compatible with serum during transfection, it is not compatible with serum during complex formation. Use Opti-MEM I reduced serum medium for siRNA/siPORT *NeoFX* transfection agent complex formation.

Do not overmix.
It is important to ***gently*** mix the siRNA with the diluted siPORT *NeoFX* in step II.B.2.d on page 10 of the reverse transfection procedure, or V.A.2.d on page 31 of the traditional transfection procedure.

Inactivated siPORT *NeoFX*

Store siPORT *NeoFX* at 4°C. Tightly cap tubes, because evaporation can significantly impact the activity of the transfection agents.

siRNA is degraded due to poor handling or storage

Check the integrity of the siRNA by running 4 µL (~2.5 µg) on a nondenaturing 15–20% acrylamide gel. Visualize the siRNA by staining with ethidium bromide, and verify that it is the expected size and intensity. The siRNA should migrate as a fairly tight band; smearing would indicate degradation.

Cells have been subcultured too many times or have undergone changes

Transfect cells within 10 passages of the optimization experiments. Since cells may gradually change in culture, we recommend transfecting cells within 10 passages of determining optimal transfection conditions. If transfection efficiency begins to drop, fresh cells should be thawed for subsequent experiments.

D. Gene Silencing Experiments Lack Reproducibility

Transfection complexes were not adequately mixed with cells

Distribute transfection agent/siRNA complex by gently rocking the plate back and forth. Do not swirl plates to mix, because this can concentrate cells and/or reagents in the center of the wells.

There were differences in the experimental procedure

The time of transfection after cell plating, incubation times, master mix volumes, and the order of component addition can all affect transfection efficiency. To obtain reproducible results in experiments involving transfection, conduct experiments exactly the same way every time.

Cell density is too low

Optimize cell density as described in section III.F.5 on page 24. When cell density is too low, cell cultures can become unstable. This instability can vary from well to well because conditions (pH, temperature, etc.) may not be uniform across a multi-well plate, and can differentially influence unstable cultures.

Cells were passaged too many times

Repeat experiment using cells that have been subcultured fewer times. Since cells may gradually change in culture, we recommend transfecting cells within 10 passages of determining optimal transfection conditions. If transfection efficiency begins to drop, fresh cells should be thawed for subsequent experiments.

V. Appendix

A. Traditional "Pre-plating" Transfection Procedure

The following procedure is a traditional "pre-plating" method. It requires more time than reverse transfection, but may be more effective with some cell types.

Table 4. Approximate Reagent Amounts per Well.

Procedure step	Reagent	96 well	24 well	6 well
1. Cell plating		0.2–1 x 10^4	2–10 x 10^4	1–5 x 10^5
2. Prepare siRNA/transfection agent complexes	Dilute Transfection agent in Opti-MEM I to:	0.15–1.2 µL to 10 µL	0.5–4 µL to 25 µL	3–9 µL to 100 µL
	Dilute small RNA (20 µM)* in Opti-MEM I to:	0.005–0.15 µL to 10 µL	0.025–0.75 µL to 25 µL	0.125–3.75 µL to 100 µL
3. Transfect cells	Adjust medium in wells to:	80 µL	450 µL	2300 µL
	Final transfection volume	100 µL	500 µL	2500 µL
	Add fresh normal growth medium after 8–48 hr	100 µL	0.5–1 mL	1–3 mL

* This gives a final concentration of 1–30 nM. If not preparing a master mix, we recommend diluting the stock siRNA to 1–2 µM using nuclease-free water or Opti-MEM I for easier handling.

IMPORTANT

The volumes and amounts in the following procedure are for transfection in a 24 well plate.

1. Cell plating

a. Approximately 24 hr before transfection, plate cells in normal growth medium so that they will be 30–80% confluent after 24 hr.

b. Incubate the cells overnight under normal cell culture conditions.

2. Prepare siRNA/transfection agent complexes

a. Just before using it, briefly vortex the siPORT *NeoFX*.

b. Dilute the siPORT *NeoFX* into Opti-MEM I medium.

   i. In a sterile, round-bottom (or V-bottom) 96-well tissue culture dish or in sterile polystyrene tube, dilute 1–3 µL of siPORT *NeoFX* dropwise into Opti-MEM I for a final volume of 25 µL.

   ii. Vortex well, and then incubate at room temp 10–15 min.

c. Dilute 0.25–7.5 µL of 2 µM siRNA (for a final concentration of 1–30 nM) into Opti-MEM I for a final volume of 25 µL.

d. Add diluted siRNA to diluted siPORT *NeoFX*; mix by gently flicking the tube or pipetting.

e. Incubate at room temp for 10–15 min.

3. Transfect cells

a. Adjust the volume of normal growth medium in wells containing cells to 450 µL.

b. Add the siPORT *NeoFX*/siRNA complex from step 2.e dropwise to the cells (the final transfection volume will be 500 µL).

c. Without swirling, gently rock the dish back and forth to evenly distribute the complexes.

d. Incubate cells under normal cell culture conditions for 48 hr.

e. 0.5–1 mL fresh normal growth medium may be added to each well after 8–48 hr to maximize cell growth and prevent potential cytotoxicity.

B. Alternate KDalert Colorimetric Assay Procedure

Notes about the colorimetric assay

- The colorimetric assay is slightly less sensitive than the fluorescence assay, so the reaction volume is increased from 100 µL to 200 µL to improve the signal.
- A water + KDalert Master Mix control is used as the reference for determining GAPDH activity.
- This procedure is written for assays conducted in 96-well plates.

Materials not provided with the assay

- Clear polystyrene 96 well plates. We recommend BD Falcon Cat #353072 or Greiner Bio-One CellStar Cat #655180.
- UV/vis plate reader.
- General lab equipment and supplies such as 15 mL or microcentrifuge tubes, multichannel pipettors, vortex mixer, ice, etc.

Colorimetric assay procedure

1. Set up the visible plate reader to read the absorbance of each sample at 615 nm.
2. Prepare enough KDalert Master Mix to use 180 µL per reaction.
3. 48 hr after transfection, remove the culture medium from the cells. The plate can be stored at –80°C. Thaw frozen cells on ice before proceeding.
4. Add 100 µL KDalert Lysis Buffer to each sample. (For 24-well plates add 500 µL KDalert Lysis Buffer; for 6-well plates, add 2 mL KDalert Lysis Buffer.)
5. Incubate on ice or at 4°C for 20 min.
6. Pipet the cell lysate up and down 4–5 times (or shake plate for 20 sec at room temp) to homogenize the lysate.
7. Transfer 20 µL aliquots of each cell extract, GAPDH Enzyme dilution including Working Stock (for positive control reaction–described in section IV.B starting on page 26), or water (water + Master Mix control) to wells in a clean 96-well microplate.

We recommend using duplicate aliquots of each GAPDH Enzyme dilution or water + Master Mix control.

8. Add 180 µL of KDalert Master Mix, and allow the reaction to proceed at room temp for 15 min.
9. Measure the absorbance of each reaction at 615 nm. Use a water reference blank in the measurement.
   GAPDH activity is indicated by a ***reduction*** in A_{615}.
   Some samples may acquire a magenta color over 15 min.

Determining optimal transfection conditions using colorimetric data

Calculating GAPDH activity using colorimetric data

During the assay, GAPDH activity causes a ***reduction*** in A_{615} of the samples, although they may eventually acquire a magenta color. For the colorimetric data, subtract the A_{615} for each sample ($A_{615\text{-sample}}$) from the A_{615} of the water + Master Mix control ($A_{615\text{-WMM}}$) to determine a GAPDH activity, $\Delta A_{615\text{-sample}}$, for that sample:

$$\Delta A_{615\text{-sample}} = A_{615\text{-WMM}} - A_{615\text{-sample}}$$

For example, the GAPDH activity $\Delta A_{615\text{-Neg}}$ is obtained by subtracting $A_{615\text{-WMM}}$ from A_{615} for negative control siRNA-transfected cells ($A_{615\text{-Neg}}$):

$$\Delta A_{615\text{-Neg \#1}} = A_{615\text{-WMM}} - A_{615\text{-Neg \#1}}$$

Construction of the GAPDH Enzyme standard curve

A graph of ΔA_{615}, calculated as described above, for each enzyme dilution should yield a line with a positive slope similar to that yielded by fluorescence data, e.g., Figure 4 on page 27.

Calculating % remaining gene expression and % knockdown of GAPDH expression

Knockdown is calculated in the same way as for the fluorescence-based assay, once the GAPDH activity, ΔA_{615}, is calculated. For each transfection condition, the % remaining expression and % knockdown is calculated from the ratio of ΔA_{615} for samples transfected with GAPDH siRNA to ΔA_{615} for samples transfected with negative control siRNA, as follows.

$$\%\ \text{remaining expression} = 100 \times \frac{\Delta A_{615\text{-GAPDH}}}{\Delta A_{615\text{-Neg \#1}}}$$

$$\%\ \text{knockdown} = 100 - \left(100 \times \frac{\Delta A_{615\text{-GAPDH}}}{\Delta A_{615\text{-Neg \#1}}}\right)$$

Measuring transfection associated toxicity

For each transfection condition, calculate the GAPDH activity, ΔA_{615}, as described above. Then compare the GAPDH activity in cells transfected with Negative Control #1 siRNA ($\Delta A_{615\text{-Neg \#1}}$) to that in non-transfected cells ($\Delta A_{615\text{-Nontransfected}}$) to examine the cytotoxic effects of the transfection conditions. Transfection conditions which do not cause any cytotoxicity would result in a ratio of 1, therefore, the closer this ratio is to 1, the less cytotoxic the transfection conditions.

$$\text{cytotoxicity ratio} = \frac{\Delta A_{615\text{-Neg \#1}}}{\Delta A_{615\text{-Nontransfected}}}$$

Calculating the best transfection condition tested

Typically, there is an inverse relationship between transfection efficiency (and associated gene knockdown) and cell viability. A useful way to describe the balance of transfection efficiency and cytotoxicity is the term Optimal Balance Factor, or OBF. The OBF for each transfection condition is calculated with colorimetric data in the same way as for fluorescence data:

OBF = % knockdown X cytotoxicity ratio

$$\text{OBF} = \left[100 - \left(100 \times \frac{\Delta A_{615\text{-GAPDH}}}{\Delta A_{615\text{-Neg \#1}}}\right)\right] \times \frac{\Delta A_{615\text{-Neg \#1}}}{\Delta A_{615\text{-Nontransfected}}}$$

Typically, optimal transfection condition(s) are those which exhibit the highest OBF value.

C. Quality Control

Functional testing

Cells were transfected with GAPDH siRNA or Negative Control siRNA using siPORT *NeoFX.* When measuring protein activity using KDalert, cells transfected with the GAPDH siRNA are shown to have a 50% reduction in GAPDH RNA signal compared to cells transfected with the Negative Control siRNA. A GAPDH Enzyme standard curve with an R-value of >0.94 was obtained.

RNase activity testing

The siPORT *NeoFX* and siPORT *Amine* are tested for RNase using Ambion RNaseAlert® assay.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

D. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.