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Validation of Short Interfering RNA Knockdowns by Quantitative Real-Time PCR

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Summary

RNA interference (RNAi) is a natural mechanism, that is triggered by the introduction of double-stranded RNA into a cell. The long double-stranded RNA is then processed into short interfering RNA (siRNA) that mediates sequence-specific degradation of homologous transcripts. This phenomenon can be exploited to experimentally trigger RNAi and downregulate gene expression by transfecting mammalian cells with synthetic siRNA. Thus, siRNAs can be designed to specifically silence the expression of genes bearing a particular target sequence. In this chapter, we present methods and procedures for validating the effects of siRNA-based gene silencing on target gene expression. To illustrate our approach, we use examples from our analysis of a Cancer Gene Library of 278 siRNAs targeting 139 classic oncogenes and tumor suppressor genes (Qiagen Inc., Germantown, MD). Specifically, this library was used for high-throughput RNAi phenotype analysis followed by gene expression analysis to validate gene silencing for siRNA that produced a phenotype. Methods and protocols are presented that illustrate how sequence-specific gene silencing of effective siRNAs are analyzed and validated by quantitative real-time PCR assays to measure the extent of target gene silencing, as well as effects on various gene expression end points.

Key Words: Dicer; gene expression; gene knockdown; gene quantification; gene silencing; housekeeping genes; nonradioactive analysis; quantitative real-time PCR; reference genes; relative quantification; RISC; RNA; RNAi; siRNA; transcription.

1. Introduction

Posttranscriptional gene silencing mediates resistance to both endogenous and exogenous pathogenic nucleic acids, such as double-stranded RNA, and manipulates the expression of functional genes by way of a mechanism known as RNA interference (RNAi; **refs. *1*** and ***2***). RNAi has facilitated the identification

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of gene function in a number of species ***(3)***. This natural phenomenon can be experimentally exploited to manipulate gene expression in a number of ways. These include transfecting mammalian cells with synthetically made, double-stranded, short interfering RNA (siRNA) molecules of approx 20 to 25 nucleotides, in vitro transcribing RNAs, using RNase III-derived products, and making use of in vivo expressed single-stranded transcripts that form a hairpin corresponding to a siRNA, short hairpin RNA. RNAi has been rapidly adopted as a functional genomics tool in a broad range of species and has been adapted to allow for the transient or stable knockdown of gene expression generation in cell lines and animals. Applications for RNAi in mammalian cells include determination of the function of uncharacterized genes, study of the interaction of proteins within pathways, and the development of transgenic cell lines and animals ***(4–6)***. Additionally, the RNAi mechanism has enormous potential as a therapeutic agent ***(7–11)***. RNAi has rapidly been adapted for the high-throughput analysis of gene function ***(12–14)***. These large-scale RNAi screens have facilitated the functional assignment of a number of genes with previously undefined function, and have identified a new role for certain genes (both characterized and uncharacterized) in critical pathways.

The mechanism of RNAi is mediated by RNA-induced silencing complex (RISC), which binds to complementary messenger RNA (mRNA) sequence on the target RNA molecule by aligning with the antisense strand of the siRNA and destroying the mRNAs homologous to the double-stranded siRNAs ***(15,16)***. The sequence-specific mRNA degradation is mediated by a member of the RNase III family of nucleases, commonly known as Dicer, which enzymatically cleaves long double-stranded RNAs into short siRNAs (**Fig. 1**; **refs.** ***17*** and ***18***). Consequently, siRNAs can be designed to specifically knockdown the expression of genes bearing a particular target sequence, although not all target sequences are amenable to knock-down. Specific bioinformatics strategies to design siRNAs, which increase the potency of silencing and minimize the chance of nonspecific or off target effects, have been developed by a number of commercial and academic laboratories. We have used such a strategy to design a Cancer Gene Library of 278 synthetic siRNAs against 139 known oncogenes and tumor suppressor genes (Qiagen Inc., Germantown, MD). This library has been synthesized by using TOM -amidite chemistry to yield 21-nucleotide RNA oligonucleotides, which were purified by high-performance liquid chromatography and annealed to produce high-purity and high-quality siRNA with 2-base 3′ overhangs with dTdT on both strands (Qiagen Inc.).

High-throughput transfection of HeLa cells with the Cancer Gene Library of 278 synthetic siRNAs, as well as with negative- and positive-control siRNAs, on a 96-well plate, was used to screen for RNAi phenotype of decreased cell proliferation. Quantitative real-time PCR was used to validate the siRNA-mediated decrease in the level of the targeted transcript. In this chapter, we present specific

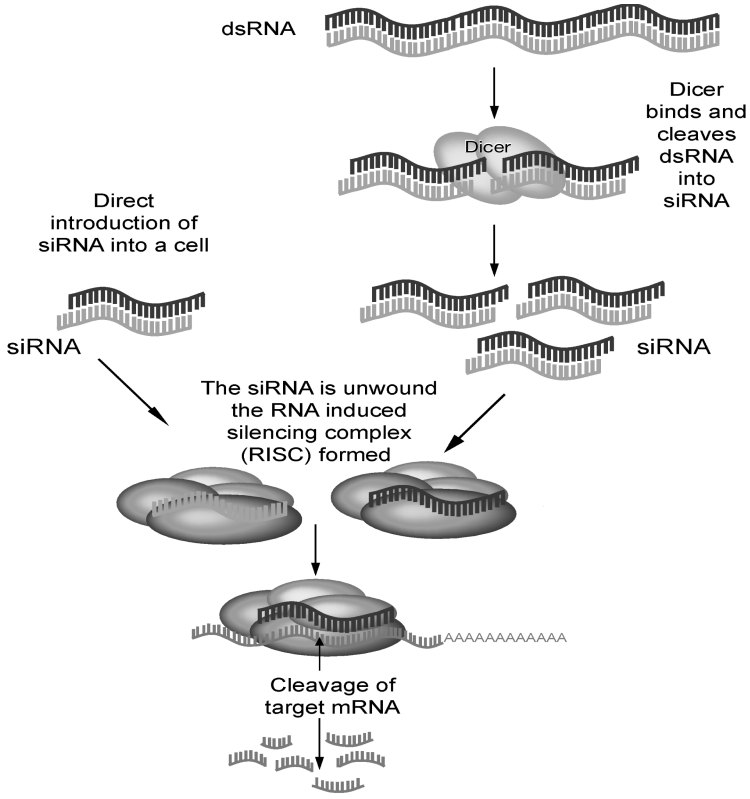

Fig. 1. RNAi mechanism. (Adapted from **ref. *18*.**)

methods and procedures for quantitative real-time PCR-based validation of the siRNA-mediated decrease in the target gene mRNA expression.

To date, many methodologies have been described as tools to investigate gene expression analysis. Common techniques for studying transcriptional analysis include: Northern blotting, *in situ* hybridization, semiquantitative quantitative real-time PCR, RNase protection assay, competitive quantitative real-time PCR, microarray analysis, and quantitative real-time PCR ***(19)***. There are significant advantages of quantitative real-time PCR compared with the rest of the gene expression quantification methods. These include enhanced sensitivity and accuracy, a large dynamic range, high-throughput capability, the ability to perform multiplex reactions, faster and more reliable amplification, and lack of post-PCR manipulations. This powerful technique has become the method of choice for rapid and quantitative monitoring of specific gene expression levels. Currently, the commercial availability of a wide range of gene expression assays for quantitative real-time PCR validation of siRNA hits and high-throughput application of these assays in 96- or 384-well formats also make this technology attractive for end point analysis.

2. Materials

2.1. Cell Culture

1. siRNA cancer gene library (Qiagen Inc.; *see* **Note 1**).
2. HeLa Cells (human cervical carcinoma cell line; ATCC, Manassas, VA).
3. MCF-7 Cells (human breast carcinoma cell line; ATCC).
4. Dulbecco's Modified Eagle's Medium: high glucose, with pyridoxine hydrochloride, without L-glutamine, without sodium pyruvate.
5. 5% fetal bovine serum.
6. 1% penicillin/streptomycin.
7. L-Glutamine.
8. Lipofectamine 2000 (Gibco, Invitrogen Corporation, Frederick, MD).
9. Opti-MEM I: Reduced serum medium, modification of MEM (Eagle's) 1X, with HEPES buffer, with 2400 mg/L sodium bicarbonate, with L-glutamine (Gibco).
10. 96-well plates.
11. CellTiter-Blue Cell Viability Assay (Promega, Madison, WI).
12. Fluorescent plate reader.

2.2. Quantitative Real-Time PCR

1. RNA extraction system (e.g., RNeasy Mini total RNA extraction kit, Qiagen Inc.).
2. Spectrophotometer.
3. Agarose gels and gel equipment.
4. Reverse transcriptase (e.g., ThermoScript RT-PCR System, Invitrogen).
5. TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA; *see* **Note 2**).
6. TaqMan® Universal PCR Master Mix (Applied Biosystems; *see* **Note 2**).
7. Hard-shell, thin-wall, 96-well microplates, and optical 96-well fast thermal cycling plates.
8. Real-time PCR machine (e.g., DNA Engine, Opticon 2, MJ Research Inc., ABI 7900HT Fast Real-Time PCR System).

3. Methods

Description of the methods outline the reverse transfection of siRNAs into HeLa cells, the measurement of cell proliferation (the cell viability assay) and phenotype assessment of siRNA hits, the extraction of total RNA, the synthesis of complementary DNA (cDNA), the quantitative real-time PCR assay, and the analysis of gene expression data using quantitative real-time PCR.

3.1. Reverse Transfection

1. Spot individual siRNAs (Qiagen Inc.) at a 13 n*M* concentration into black clear-bottom 96-well plates.
2. Add 15 µL of freshly prepared dilution of Lipofectamine 2000/serum-free DMEM at a 1:150 ratio (13.2 µL of Lipofectamine to 1966.8 µL of serum-free DMEM per 96-well plate) to allow for lipid/siRNA complex formation for 30 min (*see* **Note 3**).

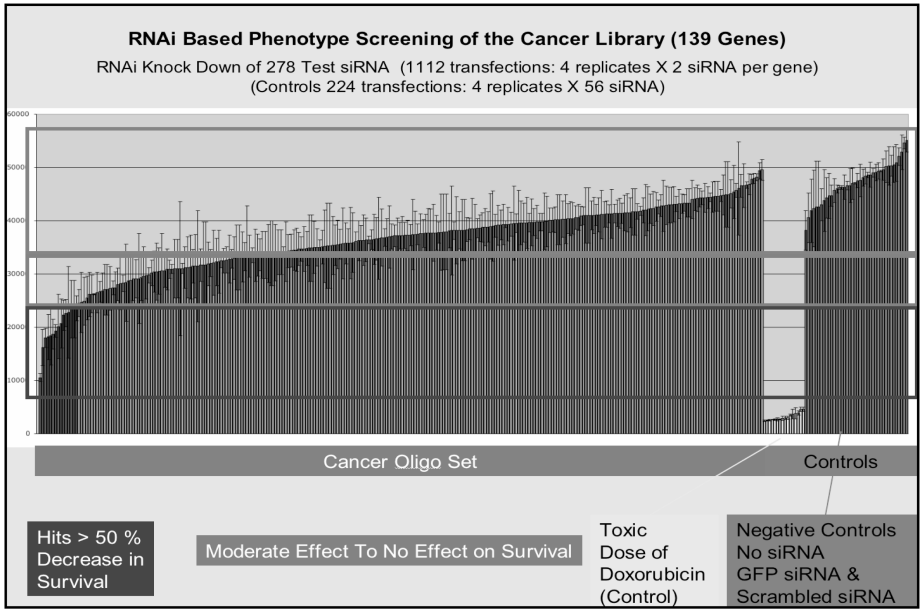

Fig. 2. RNAi-based phenotype screening.

3. Add HeLa cells at a concentration of 4000 cells/well on top of the prepared complex of 278 cancer-associated siRNAs (Qiagen Inc.).
4. Incubate the HeLa cells with lipid/siRNA complex for 72 h at 37°C under 5% CO_2. **Figure 2** describes the RNAi-based phenotype screening of the siRNA cancer library in HeLa cells (*see* **Note 3**).

3.2. Cell Viability Assay

Monitor the HeLa cell viability at 72 h via a proliferation assay, using the CellTiter-Blue Cell Viability Assay (Promega; *see* **Note 4**).

1. Add 20 µL of CellTiter-Blue reagent (at 37°C) directly to each well.
2. Incubate the plates at 37°C for 4 h (*see* **Note 5**).
3. Determine the fluorescence intensity in relative fluorescent units using a fluorescence plate reader (BMG), with filters set at 544 nm excitation and 590 nm emission wavelengths (*see* **Note 6**).

3.3. Total RNA Extraction

Completely aspirate the cell culture media after determining the fluorescent intensity of the samples from **Subheading 3.2**. Great care should be taken to avoid introducing RNases into the RNA sample during or after the isolation of RNA procedure (*see* **Note 7**). At this stage, either store the samples at –80°C

for later use or proceed to the total RNA extraction in Step 3 using an RNeasy Mini Kit (Qiagen Inc.; *see* **Note 8**).

1. Disrupt and lyse the cells in 96-well plates (growth area ~0.32–0.6 cm^2; ~4–5 × 10^4 cells) by adding 100 µL of guanidine isothiocyanate (GITC)-containing buffer (Buffer RLT, Qiagen Inc.) into each well (*see* **Note 8**).
2. Pipet up and down to disrupt and lyse the cells in each well and collect the total volume in individual eppendorf tubes per siRNA treatment.
3. Pipet the pooled samples from **step 2** with the same siRNA treatment directly onto individual QIAshredder spin columns (Qiagen Inc.) placed in 2-mL collection tubes, and centrifuge for 2 min at maximum speed to homogenize the lysate (*see* **Note 8**).
4. Add an equal volume of 70% ethanol to the homogenized lysate and mix well by pipetting to adjust binding conditions (*see* **Note 9**). Do not centrifuge. If some lysate is lost during homogenization, adjust the volume of ethanol accordingly. Visible precipitates may form after the addition of ethanol when preparing RNA, but the RNeasy procedure is not affected by this (Qiagen Inc.).
5. Apply up to 700 µL of the sample, including any precipitates that may have formed, to RNeasy Spin Columns placed in 2-mL collection tubes to adsorb RNA to the membrane of the columns.
6. Close the tube gently and centrifuge for 15 s at 8000*g*. Discard the flow-through. Reuse the collection tubes in Step 7. At this step, on-column RNase-free DNase digestion during RNA purification can be performed according to certain RNA applications (e.g., RNase–Free DNase Set, Qiagen Inc.; *see* **Notes 10** and **11**).
7. Re-assemble the column and collection tube and add 700 µL Buffer RW1 to the columns (*see* **Note 12**).
8. Close the tube gently, and centrifuge for 15 s at greater than 8000*g* to wash the columns. Discard the flow-through and collection tubes (*see* **Note 12**).
9. Transfer the columns into new 2-mL collection tubes.
10. Pipet 500 µL of Buffer RPE onto the columns (*see* **Note 13**).
11. Close the tubes gently and centrifuge for 15 s at greater than 8000*g* to wash the columns. Discard the flow-through.
12. Add another 500 µL of Buffer RPE to the column.
13. Close the tubes gently and centrifuge for 2 min at full speed to dry the membrane (*see* **Note 14**).
14. To elute the ready-to-use total RNA, transfer the columns to new 1.5-mL collection tubes.
15. Pipet 30 to 50 µL of RNase-free water directly onto the membrane (*see* **Note 15**).
16. Close the tube gently, and centrifuge for 1 min at 8000*g* to elute the total RNA (*see* **Note 16**).

3.4. cDNA Synthesis

Synthesis of cDNA from total RNA samples is the first step in the analysis of determining the degree of silencing exerted by siRNAs on the target genes in

Table 1
cDNA Synthesis

Step 1: Reagent volumes for cDNA master mix			
Components	One reaction (100 µL)	One reaction (20 µL)	No. of reactions times single-reaction volume
50 µ*M* oligo-dT_{20} (50 pmol/µL)	5 µL	1 µL	
10 m*M* dNTP mix	10 µL	2 µL	
10 pg to 5 µg RNA	— µL	— µL	
DEPC-treated water	— µL	— µL	
Final volume	60 µL	12 µL	
Step 2: Reagent volumes for cDNA master mix			
Components	One reaction (100 µL)	One reaction (20 µL)	No. of reactions times single-reaction volume
5X cDNA synthesis buffer	20 µL	4 µL	
0.1 *M* DTT	5 µL	1 µL	
40 U/µL RNaseOUT	5 µL	1 µL	
DEPC-treated water	5 µL	1 µL	
15 U/µL ThermoScript RT	5 µL	1 µL	
Final volume	40 µL	8 µL	

question. Using the ThermoScript RT-PCR System (Invitrogen), set up cDNA reactions as outlined (*see* also **Note 17**):

1. Denature the RNA and primer at 65°C for 5 min, then place on ice.
2. Prepare the master mix in **Table 1**, step 1 based on whether 20- or 100-µL reactions are to be performed. Multiply each given volume by the number of reactions to be performed to get the total volume of each reagent.
3. Vortex the 5X cDNA synthesis buffer for 5 s just before use and keep on ice.
4. Prepare another master reaction mix on ice (**Table 1**, step 2).
5. Pipet 8 µL (20-µL reaction) or 40 µL (100-µL reaction) of master mix into each reaction tube on ice.
6. Transfer the sample to a thermal cycler, preheated to the appropriate cDNA synthesis temperature, and incubate as follows, depending on the priming method: 50 pmol/µL oligo-dT_{20} primer is primed for 50 min at 50°C; 50 ng/µL random-hexamer is primed for 10 min at 25°C, followed by 50 min at 50°C; and 10 pmol/µL gene-specific primer is primed for 50 min at 50°C.
7. Terminate the reaction by incubating for 5 min at 85°C.
8. Add 1 µL (20 µL reaction) or 5 µL (100 µL reaction) of RNase H and incubate for 20 min at 37°C (*see* **Note 17**).

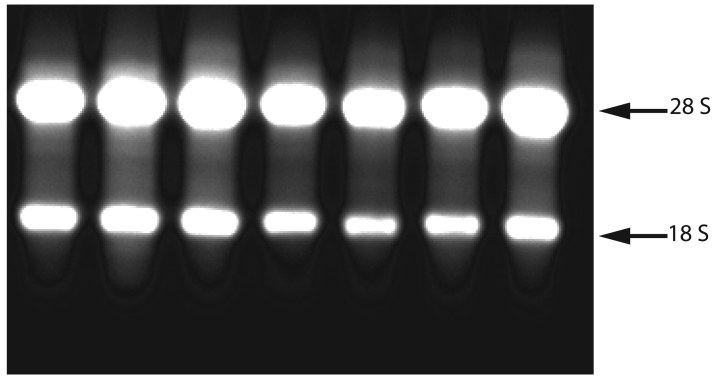

Fig. 3. Agarose gel of total RNA isolated (1–2 µg) from Hela cells.

9. The completed cDNA synthesis reaction can be stored at –20°C or used for quantitative real-time PCR reactions immediately.

3.5. Quantitative Real-Time PCR Assay

After the synthesis of cDNA, perform a standard quantitative real-time PCR for amplifying the target, using the cDNA as the template in a 20-µL reaction (we use TaqMan® Gene Expression Assays, from ABI) (*see* **Notes 2** and **18**, **Fig. 4**, and **Table 2**). Additionally, before starting the quantitative real-time PCR run, *see* **Note 19** for the amplification efficiency of quantitative real-time PCR reactions, a description of the mechanism of the TaqMan® probe cleavage resulting in a fluorescent signal, the quantitative real-time PCR instrument of choice, the quantitative real-time PCR reaction set-up, and quantitative real-time PCR tips.

3.6. Analysis of Gene Expression Data Using Quantitative Real-Time PCR

The two most routinely used methods of data analyses from quantitative real-time PCR are absolute quantification and relative quantification ***(20–22)***. Absolute quantification assesses the exact (insofar as the defined standards) copy number of the target gene, using a standard curve quantification ***(23–28)***, whereas relative quantification presents the relative change in gene expression compared with an untreated control/scrambled siRNA control sample (**ref. *21***, *see* **Note 20**). The $2^{-\Delta\Delta Ct}$ method ***(21)*** is an example of a relative quantification method. Use this formula to calculate the relative gene expression changes from quantitative real-time PCR experiments (*see* **Note 21**). The article by Livak and Schmitten ***(21)*** describes the derivation, assumptions, and examples of the $2^{-\Delta\Delta Ct}$ method of relative gene quantification. The schematic presentation of the quantitative real-time PCR and the relative quantification data of gene expression after siRNA treatment is illustrated in **Fig. 4** and **Table 4**, respectively.

Table 2
Quantitative Real-Time PCR Reaction Preparation

Components	Single reaction volume (μL)
20X Target assay mix or 20X endogenous control assay mix	1
2X TaqMan® universal master mix (with or without Amp Erase UNG)	10
Template DNA + RNase/DNase-free water	9
Final volume	20
PCR conditions for DNA Engine Opticon 2 (MJ Research)	Time
1. 50°C	2 min
2. 95°C	10 min
3. 95°C	15 s
4. 60°C	1 min
5. Plate read	
6. Go to line 3 for 44 more cycles	
7. Incubate at 72°C	7 min
8. Incubate at 10°C	Forever
9. End	

4. Notes

1. A Cancer Gene Library of 278 siRNAs against 139 known oncogenes and tumor suppressor genes was designed in collaboration with Qiagen Inc., applying extensive bioinformatics strategies to ensure that siRNAs target only the transcripts under study and minimize the chance of nonspecific, off-target effects (http://www1.qiagen.com/Products/GeneSilencing/LibrarySiRna/SiRnaSets/CancerSiRnaSet. aspx?ShowInfo=1).
2. The information for a comprehensive collection of predesigned primer and probe sets of TaqMan® Gene Expression Assays is available on the Applied Biosystems Web site:http://myscience.appliedbiosystems.com/cdsEntry/Form/ gene_expression _keyword.jsp. TaqMan® Gene Expression Assays are built on Applied Biosystems 5′ nuclease chemistry. Each assay consists of two unlabeled PCR primers for amplifying the gene of interest (used at a final concentration of 900 n*M* each) and a TaqMan® probe with a FAM dye-labeled reporter and a Minor Groove Binder attached to a nonfluorescent quencher probe (*see* **Fig. 4**). The Minor Groove Binder increases the probe melting temperature by binding in the minor groove of a DNA duplex. This increase enables the use of probes as short as 13 bases. These shorter probes offer superior primer/probe design for improved specificity. They also provide more flexibility when designing assays for closely related sequences, such as gene family members or species-specific assays. The incorporation of a

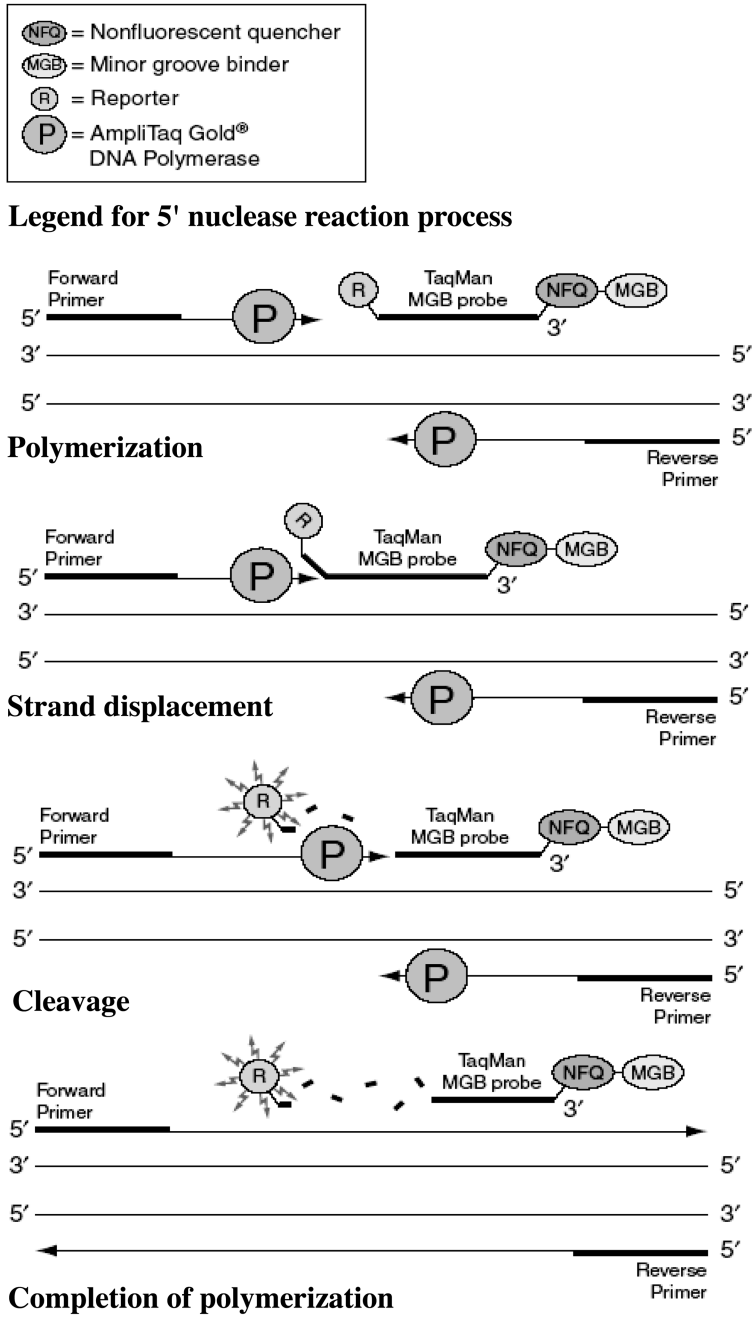

Fig. 4. 5′ nuclease assay illustrating the mechanism of probe cleavage resulting in a fluorescent signal.

nonfluorescent quencher virtually eliminates the background fluorescence associated with traditional quenchers (e.g., TAMRA), providing better sensitivity and quantification precision. All components are formulated as a single ready-to-use 20X mix. TaqMan® Gene Expression Assays are designed to:

a. Run under universal conditions for two-step quantitative real-time PCR.
b. Work with TaqMan® Universal Master Mix (with or without AmpErase Uracil N-glycosylase [UNG]). UNG uses an enzymatic reaction analogous to the restriction-modification and excision-repair systems of cells to specifically degrade contaminating PCR products from previous PCR amplifications in which dUTP has been incorporated, without degrading native nucleic acid templates ***(29–32)***.
c. Amplify target cDNA without amplifying genomic DNA, if possible. This is achieved by designing probes that cross exon–exon junctions. Further information on specific product uses is available on the ABI website (http://www.allgenes.com).

3. Spotting of the individual siRNAs and the lipid reagent into black clear-bottom 96-well plates can either be performed manually with a multichannel pipet or robotically. Perform duplicate transfections at two different positions to overcome position effects. The reverse transfection enhances the delivery of the siRNAs into HeLA cells compared with the conventional transfection (personal observation), in which the cells are first grown at the bottom of the 96-well plates and the lipid/siRNA complex is prepared in a separate tube and then added to the already plated cells. Additionally, this procedure accelerates the experimental process by eliminating the extra 24 h of incubation involved in the conventional transfection protocol. The experiments performed on HeLa cells have also been repeated for MCF-7 cells. The percent cell survival data of the cancer set siRNA treatments on HeLa and MCF-7 cells are graphically depicted in the multidimensional phenotype analysis (*see* **Fig. 5**). Three of the genes, A, B, and C, have been chosen as examples for subsequent validation of siRNA-mediated decrease in the targeted mRNA. **Figure 6** illustrates the relative quantification analysis for genes A, B, and C.
4. The CellTiter-Blue Cell Viability Assay (Promega) provides a fluorometric method for estimating the number of viable cells present in a multiwell format. The indicator dye, resazurin, present in the buffered solution of CellTiter-Blue, is used to measure the metabolic capacity of cells (cell viability). Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and, thus, do not generate a fluorescent signal. The spectral properties of CellTiter-Blue Reagent change after reduction of resazurin to resorufin. Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin. The absorbance maximum of resazurin is 605 nm, and that of resorufin is 573 nm. Either fluorescence or absorbance may be used to record results; however, fluorescence is the preferred method because it is more sensitive.
5. Incubation of the cells at 37°C allows the cells to convert resazurin to resorufin, after which, the fluorescent signal is measured. Different cell types may take different times from reduction of resazurin to resorufin. For example, the MCF-7

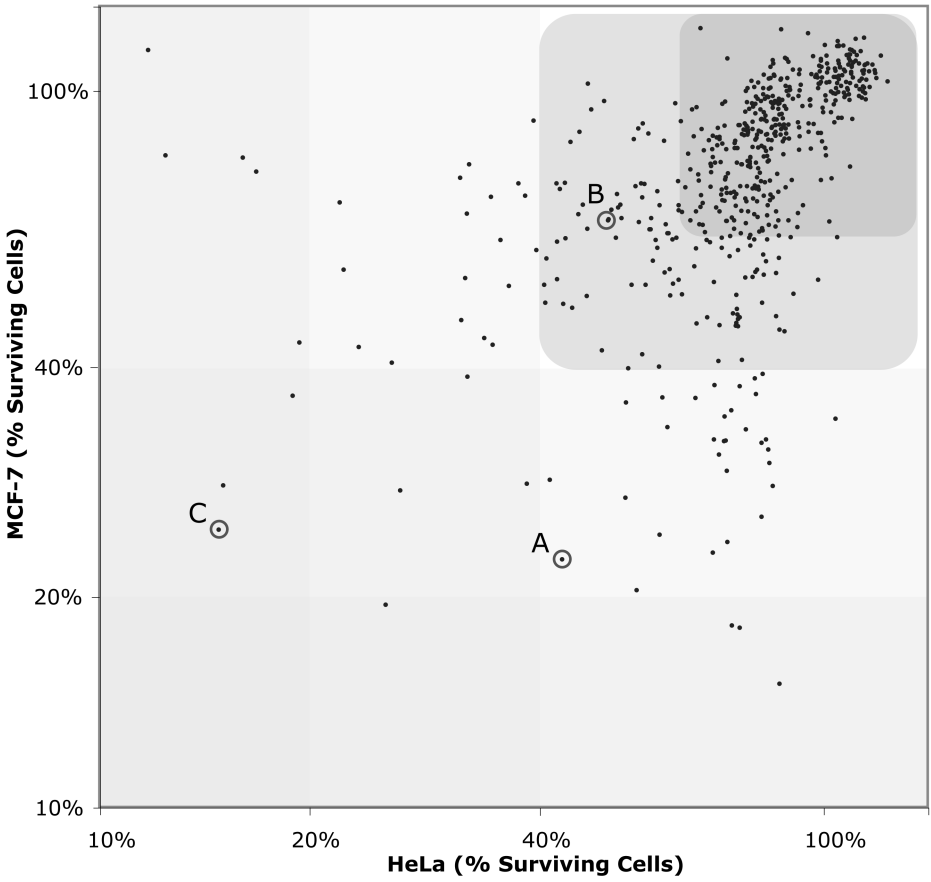

Fig. 5. Multidimentional phenotype analysis.

cells have a longer reduction time than HeLa cells; therefore, the user will have to empirically determine the amount of time for each cell line.

6. Relative fluorescent units are measured at 544 nm excitation and 590 nm emission wavelengths. All siRNA treatments, including controls (*see* **Subheading 3.1.**), were repeated in four different wells, and the average of the four replicates were later used to plot the effect for each siRNA; the standard deviation of the replicates were used to plot the error bars. Controls include "no siRNA," "no cell," and a universal nonspecific "scrambled siRNA sequence" control (Qiagen Inc.). Data are normalized to the scrambled siRNA sequence control and presented as a percentage decrease in cell viability. The efficacy of the siRNAs on phenotypic end points in cancer cells reveals that approx 5% of the siRNAs have a significant effect on HeLa cell survival (**Fig. 2**). siRNAs that had a significant effect on HeLa cell proliferation/survival were determined, and the specific sample wells were pooled for further total RNA extraction.

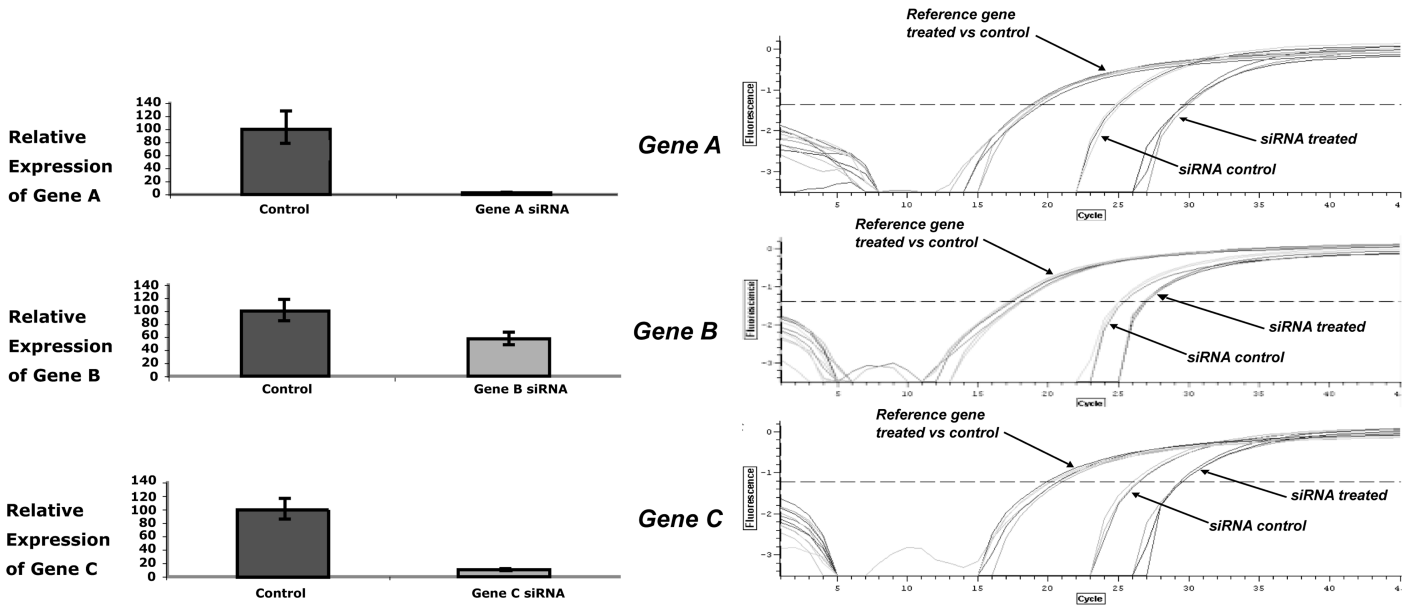

Fig. 6. Relative quantification of gene expression after siRNA treatment.

7. Handling of RNA: RNases are very stable and active enzymes that generally do not require cofactors to function. Because RNases are difficult to inactivate, and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA. Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice while aliquots are pipetted for downstream applications. The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases. Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 *M* NaOH and 1 m*M* EDTA followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for 4 h or longer (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC (DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using DEPC). Fill glassware with 0.1% DEPC (0.1% v/v in water), allow to stand overnight (12 h) at 37°C, and autoclave or heat to 100°C for 15 min to eliminate residual DEPC. Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% sodium dodecyl sulfate), thoroughly rinsed with RNase-free water, and then rinsed with ethanol (plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions) and allowed to dry. Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 mL DEPC to 100 mL of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency

in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 min. Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are, therefore, free of any DEPC contamination (Qiagen Inc.). Purified RNA may be stored at –20 or –80°C in water. Under these conditions, we have observed no degradation of RNA after 1 yr. The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA/mL. This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be performed in water. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvets are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing the cuvets with 0.1*M* NaOH and 1 m*M* EDTA followed by washing with RNase-free water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is:

Volume of RNA sample = 100 µL.
1/50 Dilution = 10 µL RNA sample + 490 µL distilled water.
Measure absorbance of diluted sample in an RNase-free, 1-mL cuvet;
$A_{260} = 0.23$.
Concentration of RNA sample = $40 \times A_{260} \times$ dilution factor = $40 \times 0.23 \times 50$ = 460 µg/mL.
Total yield = concentration × volume of sample in milliliters = 460 µg/mL × 0.1 mL = 46 µg.

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV range, such as proteins. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Because water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination ***(33)***. For accurate values, measuring absorbance in 10 m*M* Tris-HCl, pH 7.5, is recommended. Pure RNA has an A_{260}/A_{280} ratio of 1.9 to 2.1. Values up to 2.3 are routinely obtained for pure RNA (in 10 m*M* Tris-HCl, pH 7.5), with some spectrophotometers. Always be sure to calibrate the spectrophotometer with the same solution. For determination of RNA concentration, however, dilution of the sample in water is recommended (Qiagen Inc.) because the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/mL RNA) is based on an extinction coefficient calculated for RNA in water.

8. Incomplete removal of the cell culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy silica-gel membrane. Both effects may reduce RNA yield. The RNeasy Mini procedure combines the selective binding properties of a silica-gel-based membrane with the speed of

microspin technology. A specialized high-salt buffer system allows up to 600 to 750 ng (per well of a 96-well plate) of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. First, lyse the HeLa cells in each well of the 96-well plates by adding GITC-containing lysis buffer (Buffer RLT). Buffer RLT may form a precipitate after storage. If necessary, redissolve by warming, and then place at room temperature. Buffer RLT is added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane and to inactivate RNAses. Buffer RLT contains GITC and is, therefore, not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling. β-mercaptoethanol (β-ME; commercially available solutions are usually 14.3 *M*) must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µL of β-ME/1 mL of buffer RLT. Buffer RLT is stable for 1 mo after addition of β-ME. Pipet up and down several times and pool the quadruplicate samples of similar siRNA treatment together in a single 1.5-mL Eppendorf tube after adding Buffer RLT. Homogenize the pooled lysate using QIAshredders (Qiagen Inc.) in the presence of the highly denaturing buffer RLT, which immediately inactivates RNases, to ensure isolation of intact RNA. Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Disruption and homogenization are considered two distinct steps. Complete disruption of cells walls and plasma membranes of cells and organelles is absolutely required to release all of the RNA contained in the samples. Different samples require different methods to achieve complete disruption. Here, the HeLa cells are disrupted by the addition of the lysis buffer RLT and repeated pipetting. Incomplete disruption results in significantly reduced yield. On the other hand, homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. The QIA shredders (Qiagen Inc.) are used to homogenize the HeLa cells. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy membrane and, therefore, significantly reduced yield.

9. Add ethanol to provide appropriate binding conditions and then apply the sample to an RNeasy mini column, in which the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in at least 30 µL of water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA because most RNAs with fewer than 200 nucleotides (such as 5.8S ribosomal RNA, 5S ribosomal RNA, and transfer RNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.
10. DNA contamination: No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, designing primers

that anneal at intron splice junctions, so that genomic DNA will not be amplified, is recommended. Alternatively, DNA contamination can be detected on agarose gels after RT-PCR by performing control experiments in which no reverse transcriptase is added before the PCR step or by using intron-spanning primers. For sensitive applications, such as differential display, or if it is not practical to use splice-junction primers, DNase digestion of the RNA on the column with RNase-free DNase is recommended. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the RNeasy procedure, the eluate containing the RNA can be treated with DNase. The RNA can then be repurified with the RNeasy cleanup protocol (Qiagen Inc.), or, after heat inactivation of the DNase, the RNA can be used directly in downstream applications. The RNeasy Mini Protocol for Isolation of Cytoplasmic RNA from Animal Cells is particularly advantageous in applications in which the absence of DNA contamination is critical, because the intact nuclei are removed. Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and the RNeasy silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., quantitative real-time PCR analysis with a low-abundance target). Use of the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive quantitative real-time PCR analyses. The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel (formaldehyde agarose gel), electrophoresis, and ethidium bromide staining. The respective ribosomal bands (**Fig. 3**) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with intensity approximately twice that of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller-sized RNAs, it is likely that the RNA sample suffered major degradation during preparation. The smears just above 28S and in between 28S and 18S ribosomal bands represent the mRNA population.

11. Cell pellets can be stored at –80°C for later use or can be used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that cell pellets can be dislodged by flicking. Homogenized cell lysates (in Buffer RLT) can be stored at –80°C for several months. To process frozen lysates, thaw samples for 15 to 20 min at 37°C in a water bath to dissolve salts. If any insoluble material is visible, centrifuge for 5 min at 3000*g* to 5000*g*. Transfer the supernatant to a new RNase-free glass or polypropylene tube and continue with the next step.

    Generally, DNase digestion is not required (*see* **Note 10**). However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., quantitative real-time PCR analysis with a low-abundance target). In these cases, the small residual amounts of DNA remaining can be removed using RNase-free DNase I (e.g., the RNase-Free DNase, Qiagen Inc.) for the optional on-column DNase digestion, or by a DNase digestion after RNA isolation.

12. Buffer RLT and Buffer RW1 contain a guanidine salt (guanidinium thiocyanate, 25–50% and 2.5–10%, respectively); in addition, Buffer RW1 contains 2.5 to 10% ethanol. They are, therefore, not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling guanidine.
13. Buffer RPE is used at the washing step and is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
14. Residual ethanol may interfere with the downstream reactions, therefore, centrifugation at **Subheading 3.3**, **step 13** is very important.
15. To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate from **Subheading 3.3.**, **step 15**. The yield will be approx 15 to 30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.
16. All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
17. mRNA can also be used depending on the purpose of the experiment. Depending on the yield of the total RNA from the cell lines (HeLa and MCF-7) grown and the siRNA treated in 96-well plates, a concentration between 100 ng and 1 µg of total RNA is used in the 20 to 100 µL cDNA reactions. The cDNA synthesis experiments described throughout this chapter are performed using oligo-dT_{20} primers. This allows the detection of multiple transcripts from a single first-strand reaction. The denaturation step (**Subheading 3.4.**, **step 1**) is optional, but the users are encouraged to include this step as part of their cDNA synthesis reaction. Heating the RNA in the absence of reaction buffer and RT enzyme before cDNA synthesis at temperatures as high as 65°C can enhance the unwinding of the secondary structures in the RNA molecule, and full-length cDNA synthesis may be achieved as a result. We use Thermoscript RT at 50°C for oligo-dT_{20} to reduce secondary structure or to improve specificity. For convenience, the components of the cDNA synthesis reaction have been calculated not only for 20-µL but also for 100-µL reaction volumes. The cDNA synthesis reagents are recommended to be prepared as two separate master mixes before combination (**Steps 1** and **2**), and to be kept on ice at all times. Preparation of the master mixes allows homogenous distribution of the reaction contents to keep the conditions of the cDNA synthesis the same for all samples. Treatment of cDNA with RNase H to remove the complementary RNA before quantitative real-time PCR is optional (**Subheading 3.4.**, **step 8**), but recommended, because digestion with RNase H will improve the quantitative real-time PCR signal of many targets and, in some cases, is required for efficient and consistent amplification of long quantitative real-time PCR templates.
18. Fluorescent reporters are the common entities for all of the quantitative real-time PCR assays. The analysis of amplification during quantitative real-time PCR is achieved by detecting the direct or indirect accumulation of the fluorescence of the newly amplified cDNA. Among the most common dyes and fluorescent detection chemistries used in quantitative real-time PCR are SYBR Green I (Molecular

Probes), TaqMan® Probes (Roche Molecular Systems), and Molecular Beacons (Research Genetics). Choosing a detection system can be a major consideration in developing a quantitative real-time PCR assay and these are described more fully in Chapter 17. Each system may have its advantages and disadvantages. Specificity, assay optimization time, and cost may be some of the concerns involved before making a decision regarding an assay of choice. The TaqMan® chemistry, an indirect assay system, is the method of choice for our study (**ref. *34***; **Note 2** and **Fig. 4**). The TaqMan® Gene Expression Assays (Applied Biosystems) are ready-to-use, prevalidated, and are available for more than 300,000 quantitative real-time PCR assays for measuring the expression of genes in human, mouse, and rat. In our experience, these assays have been consistent between batches as well as in sample replicates. They eliminate the labor, expense, and bioinformatics expertise previously needed for generating high-quality quantitative gene expression data, especially important in the process of validation of large number of siRNA knockdowns.

19. One of the major concerns of the researchers involved with quantitative real-time PCR is the amplification efficiency of the PCR reaction. Efficiency estimates can vary significantly if rigorous measurement procedures are not met. Measurements must be acquired over a broad range of dilutions (5–6 logs) and should include replicates (e.g., triplicates) to reduce the effects of handling errors (e.g., pipetting accuracy). Efficiency of PCR reactions can be affected by substances such as heme, and detergents that are known to inhibit PCR. Researchers must make sure that cDNA templates are free of inhibitors. One other important issue regarding the efficiency validation of the quantitative real-time PCR is ensuring that the two amplicons have the same efficiency, if the $2^{-\Delta\Delta Ct}$ method is used ***(21)***. For example, the efficiency of an internal control (e.g., reference gene) should be the same as the target gene, in which case, the normalization factor comes into question, especially if multiplexing in the same reaction tube. If the efficiencies of the two particular genes in question are not equal, then new primers can be designed and/or optimized to achieve a similar efficiency for both the target and the reference control (*see* **Subheading 3.6.**; **ref. *21***). A sensitive method of measuring whether two amplification products have the same efficiency is to check how the cycle threshold (Ct) changes (ΔCt) with template dilution ***(21)***. For this, cDNA dilutions over a 100-fold range are prepared for both the target and the reference gene. For each dilution sample, amplifications are performed using TaqMan® gene expression assays or other available fluorogenic probes. The average Ct is calculated for both the template and the reference gene, and ΔCt is determined. A plot of the log cDNA dilution vs ΔCt is made. If the absolute value of the slope is close to zero, the efficiencies of the target and the reference genes are considered similar, and the ΔΔCt calculation for the relative quantification of target may be used to analyze the data ***(21)***.

    TaqMan® assays use a fluorescence resonance energy transfer probe as a reporter system with a 6-FAM dye-labeled reporter (*see* **Note 2**). During PCR, the TaqMan® probe anneals specifically to a complementary sequence between the sense and antisense primer sites (polymerization; *see* **Fig. 4**). If the probe is intact (at the start

of the polymerization step), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, mainly by fluorescent resonance energy transfer ***(35)***. The DNA polymerase cleaves only probes that bind to the target (strand displacement). Cleavage separates the reporter dye from the quencher dye, resulting in increased fluorescent signal by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to the probe and is amplified during the PCR process. Because of these stringent requirements, any nonspecific amplification is not detected. Polymerization of the strand continues, but the 3′-end of the probe is blocked to prevent extension of the probe during PCR (cleavage and completion of polymerization).

The two quantitative real-time PCR instruments used in our laboratory are MJ Research's Opticon 2 and ABI's 7900HT Fast Real-Time PCR System. Other qRT-PCR instruments can be used according to the researchers' needs or availability.

Table 2 describes a typical quantitative real-time PCR reaction setup in our laboratory. Each siRNA-treated sample, the suitable reference gene for the particular siRNA treatment and cell line, the nontemplate control, and the necessary group of internal and external controls are prepared in triplicate. Special care should be taken when handling the light-sensitive TaqMan® probes. It is recommended that after light-protected thawing, i.e., by holding in the hand with gloves until thawed, the probes are immediately placed on ice and covered with aluminum foil. Care should be taken to avoid light exposure to preserve the life of the fluorescent probes. The calculation for components of a 20-µL TaqMan® reaction is given in **Table 2**. The amount of total RNA in each TaqMan® reaction is typically 10 to 50 ng. The number of reactions to be prepared can be calculated accordingly. Preparation of a master mix per siRNA-treated sample to be validated (per particular gene assay) and the corresponding reference genes are recommended. Sample distribution into each well of the 96-well plate is aided considerably by the guidance of a Pipetter Guide (e.g., Diversified Biotech). Attention should be paid to cover over the wells of 96-well plates with aluminum foil, during the process of pipetting other components of the quantitative real-time PCR reaction. Use of an automatic pipet is highly recommended for sample distribution in the wells, for well-to-well accuracy and consistency. With our MJ Research's Opticon 2 instrument, hard-shell, thin-wall, 96-well microplates with white shell and white wells are used, together with eight-strip ultraclear caps for the amplification process. Dilution of the cDNA with DNase/RNase-free water to make up for 9 µL of volume per sample would ease the quantitative real-time PCR preparation step, especially when handling large amount of samples. Depending on the range of total RNA amount needed (10–50 ng) per quantitative real-time PCR reaction of 20 µL, use the total volume of cDNA reaction (i.e., 20 µL) to make up a working cDNA dilution per sample. For example, if 10 ng of total RNA/20 µL of quantitative real-time PCR reaction is needed from a 20-µL cDNA reaction with 1 µg of initial total RNA input, take 0.2 µL of cDNA and add 8.8 µL of DNase/RNase-free water to prepare 9 µL of volume per quantitative real-time PCR reaction. For ease of pipetting, to a total of 20 µL cDNA reaction, add 880 µL of DNase/RNase-free water for the final working

dilution (9 µL of diluted cDNA per quantitative real-time PCR reaction). This, in turn, would allow for more than 100 quantitative real-time PCR reactions-worth of cDNA dilutions per siRNA-treated sample.

After the preparation of all the components of the quantitative real-time PCR reaction, pipet the contents, as listed in **Table 2**, and cap the 96-well plates that are ready for the quantitative real-time PCR reaction at the set thermal cycler conditions for the MJ Research DNA Engine Opticon 2 (*see* **Table 2**). The thermal cycler temperature conditions for other quantitative real-time PCR instruments can be set according to the needs of researchers and the manufacturer's recommendations. Before placing the 96-well plate in the thermal cycler, it is strongly recommended that the 96-well plate be briefly centrifuged to mix the components of the quantitative real-time PCR reaction. Pipetting up and down is not recommended while distributing the reagents. This may cause pipetting errors and loss of the already pipetted small volume of reaction components. Centrifuges that accommodate rotors with 96-well plates can be used, but we find that a quicker and easier way of briefly centrifuging the 96-well plates is to use a DNA Speed Vac with a 96-well rotor (DNA 110, Savant), without turning the vacuum on. This allows a shorter time of handling the quantitative real-time PCR reactions outside just before placing the 96-well plates in the thermal cycler.

20. Unless it is necessary to report the absolute transcript copy number, determining the relative change in gene expression profiles of the transcripts should be sufficient. There are drawbacks in using standard curve quantification if a lot of sample screening is required, as in the case of siRNA validation via quantitative real-time PCR. If using absolute quantification, each target requires a standard curve quantification at least with triplicates of each dilution series over a range of 5 to 6 logs of dilutions. This would take much space on a 96-well plate, not leaving enough room for a large number of siRNA target sample analysis. This problem may be somewhat eased on a 384-well system, but, again, the more target genes to be analyzed, the larger will be the number of dilution series to be considered. The necessity for using cDNA, RNA, plasmid, oligonucleotide, or other sources for standard curve analysis introduces extra steps and further variation, causing difficulty in comparison of experiments run at different times ***(22)***. Variation can be reduced with additional controls, but this further decreases the number of target samples per plate. Moreover, in different laboratory settings, the data analysis may be difficult because of the variations in the samples used to generate standard curves, unless the data are normalized against a standard cell line. An additional source of error can also be introduced at the cDNA synthesis step, because different RNA samples may have varying efficiencies of reverse transcription. For studies with small number of samples, absolute quantification using the standard curve method may be considered. A large degree of assay optimization would not be required. Any concerns regarding the changes introduced on the internal control genes because of a specific treatment on the cells will be eliminated, but PCR efficiency should always be calculated and optimized as often as possible ***(22)***. The choice of gene expression data analysis method in our laboratory

is relative quantification. This allows us to screen and validate a large number of siRNA knockdowns in a 96-well format. By using quantitative real-time PCR in our RNAi validation experiments, we have faced some challenges, which we were able to address by testing an array of endogenous controls or reference genes that are not affected transcriptionally by the treatments with different siRNAs. These transcriptional changes and challenges are usually observed when the siRNA has an effect on the cell growth or survival. For the data obtained from the siRNA knockdowns to reflect the truth regarding the extent of silencing by the siRNAs and to be devoid of erroneous results, it is required that the expression levels of the chosen reference gene be kept unchanged between the siRNA-treated and siRNA control samples ***(36)***. **Table 3** lists the different flavors of 11 reference genes that have been used in our high-throughput RNAi validation experiments for quantitative real-time PCR normalization purposes. In the event that all of these in-house reference genes are afzzfected by our treatment of siRNAs, we will start looking for additional reference genes that are least or not at all affected. An alternative solution to the problem of variable gene expression levels between the reference genes and the target gene of interest caused by a particular treatment would be to use a software that would correct for the changes in the levels of reference genes after treatment of the cells ***(37)***. Additionally, websites, such as www.gene-quantification.info, are a valuable resource in obtaining information regarding quantitative real-time PCR in general.

21. Data analysis varies depending on the quantitative real-time PCR instruments used. Therefore, refer to the appropriate user's guide for instructions on how to analyze your data. As in the case of the conventional PCR ***(38)***, the more copies of the nucleic acid that are present at a start of a given quantitative real-time PCR reaction, the fewer cycles of the template amplification it will take to synthesize a specific number of amplicons. The Ct value, which is the number of cycles necessary for an amplification-related fluorescence to reach a specific threshold, is inversely proportional to the amount of template that was present at the start of a quantitative real-time PCR reaction. Because the amplification process in a given quantitative real-time PCR is monitored in real-time, during the exponential phase of a quantitative real-time PCR reaction—when the amplification efficiency is at its peak and least affected by reaction-limiting conditions—determination of the Ct values can be achieved ***(39)***. It is possible to roughly quantify a Ct value without having to plot a standard curve. During the exponential phase of a quantitative real-time PCR, the number of amplicons theoretically doubles during each cycle, assuming that the efficiency of the amplification is 100%. Hence, a sample with twice the number of starting copies compared with another sample will need one less cycle of amplification to build up an equal number of products. Therefore, using the difference between the Ct values of two samples, the relative difference of different samples can be determined ***(40)***. When using $2^{-\Delta\Delta Ct}$ method (relative quantification method), the amplification efficiencies of the target and reference genes must be approximately equal. To assess whether the amplification efficiencies of the target and control genes are the same, look at how ΔCt varies with the

Table 3
Reference Genes Used in Quantitative Real-Time PCR Assays for the Validation of High-Throughput RNAi[a]

Gene symbol	RefSeq	Gene name	Gene function	Assay ID (ABI)
B2M	NM_004048	β2-Microglobulin	β-Chain of major histocompatibility complex class I molecules	Hs00187842_m1
SDHA	NM_004168	Succinate dehydrogenase complex, subunit A	Electron transporter in the TCA cycle and respiratory chain	Hs00417200_m1
HMBS	NM_000190	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	Hs00609297_m1
YWHAZ	NM_003406	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, ζ-polypeptide	Signal transduction via binding to phosphorylated serine residues on various signaling molecules	Hs00237047_m1
UBC	NM_021009	Ubiquitin C	Protein degradation	Hs00824723_m1
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	Hs99999905_m1
ACTB	NM_001101	β-actin	Cytoskeletal structural protein	Hs99999903_m1
TBP	NM_003194	TATA box-binding protein	General RNA polymerase II transcription factor	Hs99999910_m1
HPRT1	NM_000194	Hypoxanthine phosphoribosyl-transferase I	Purine synthesis in salvage pathway	Hs99999909_m1
POLR 2A	NM_000937	Polymerase RNA II (DNA directed) polypeptide A, 220 kD	DNA-directed RNA polymerase II activity	Hs00172187_m1
PPIA	NM_021130	Peptidylprolyl isomerase A (cyclophilin A)	Accelerate the folding of proteins and may play a role in cyclosporin A-mediated immunosuppression	Hs99999904_m1

[a]Assay IDs are quotations from TaqMan® Gene Expression Assays (ABI).

template dilutions ***(21)***. Calculate the average Ct value for both the target gene and the reference gene. Then calculate ΔCt (ΔCt = Ct of target – Ct of reference). Finally, plot the log cDNA dilution vs ΔCt. If the absolute value of the slope is close to zero, then the efficiencies of the target and the reference genes are similar. Consequently, the ΔΔCt method for calculating relative quantifications may be used ***(21)***. In situations in which the efficiencies of the target and the reference genes are not equal, either design new primers or try to find another reference gene to meet the ΔΔCt criteria.

To perform an accurate validation of the siRNA knockdowns, it is absolutely essential that the selection of the internal controls/calibrators for the relative quantification method (ΔΔCt method) be fulfilled cautiously. The reference genes must be properly validated for each experiment to determine that experimental treatments (e.g., siRNA treatments) will not affect the gene expression of the reference gene for the normalization purpose. Untreated control samples or, in our case, scrambled siRNA-treated control samples can be considered to be the calibrator (a control sample against which the treated samples are normalized) for the relative quantification method using the $2^{-\Delta\Delta Ct}$ formula.

A typical quantitative real-time PCR run of siRNA-treated samples is prepared using triplicates of each siRNA treatment together with the appropriate controls (*see* **Fig. 6**). The Ct values obtained from quantitative real-time PCR instrumentation (e.g., Opticon 2, MJ Research) can be easily imported into a Microsoft Excel spreadsheet program. *See* **Table 4** for a demonstration of a data analysis of siRNA treatment using relative expression method ($2^{-\Delta\Delta Ct}$). Quadruplicate samples of cells were pooled and quantitative real-time PCR was performed in triplicate on cDNA synthesized from total RNA extracted from HeLa and MCF-7 cells treated with gene A-, gene B-, and gene C-specific siRNAs. Controls included scrambled siRNA controls and β-actin as the reference control gene for normalization purposes. The data are analyzed as the fold change in gene expression normalized to an endogenous reference gene (β-actin) and relative to the scrambled siRNA-treated control using the following equations:

ΔCt of treated = average target gene Ct – average β-actin Ct
ΔCt of control = average target gene Ct – average β-actin Ct
ΔΔCt = ΔCt of treated – ΔCt of control

The amount of target normalized to β-actin and relative to the calibrator is equal to $2^{-\Delta\Delta Ct}$ where "treated" refers to the target gene, and "control" refers to the scrambled siRNA control. The scrambled siRNA control is chosen as the calibrator because no change is expected to be seen as a result of scrambled siRNA treatment. In other words, this should behave as the untreated control. The choice of calibrator for the relative quantification of gene expression using $2^{-\Delta\Delta Ct}$ method depends on the type of experiment that is planned ***(21)***. Ct is exponentially correlated to copy number. Hence, as part of the final calculation, the standard error is estimated by calculating the $2^{-(\Delta\Delta Ct + SD)}$ and $2^{-(\Delta\Delta Ct - SD)}$ terms, where SD is the standard deviation. Thus, a range of values that are asymmetrically distributed relative

Table 4
Data Analysis Using $2^{-\Delta\Delta Ct}$ Method[a]

ΔCt of treated = average target gene Ct – average β-actin Ct = 29.75 – 18.84 = 10.91
ΔCt of control = average target gene Ct – average β-actin Ct = 24.96 – 19.29 = 5.67
$\Delta\Delta Ct$ of treated = ΔCt of treated – ΔCt of control = 10.91 – 5.67 = 5.24
$\Delta\Delta Ct$ of control = ΔCt of control – ΔCt of control = 10.91 – 10.91 = 0.00 (the calibrator)

Relative quantification of gene	Relative quantification of scrambled
$A = 2^{\Delta\Delta Ct} = 2^{-5.24} = 0.03$	control $= 2^{\Delta\Delta Ct} = 2^{-0.00} = 1.00$

SD[b] $(\Delta\Delta Ct)_{treated} = [(SD\Delta Ct_{treated})^2 + (SD\Delta Ct_{control})^2]^{0.5} = (0.19^2 + 0.30^2)^{0.5} = 0.36$
SD[c] $(\Delta\Delta Ct)_{control} = [(SD\Delta Ct_{treated})^2 + (SD\Delta Ct_{control})^2]^{0.5} = (0.19^2 + 0.30^2)^{0.5} = 0.36$

[a]Relative quantification of gene expression analysis in HeLa cells using gene A siRNAs relative to scrambled siRNA controls and normalized to β-actin (an endogenous reference gene; *see* **Note 20**; ref. ***21***).

[b]Standard deviation (*see* **Note 21**): SD of gene A siRNA-treated sample: $2^{-(\Delta\Delta Ct + SD)} = 0.02$; and $2^{-(\Delta\Delta Ct - SD)} = 0.03$. Gene A: (+) SD = $2^{-(\Delta\Delta Ct - SD)} - 2^{-\Delta\Delta Ct} = 0.01$; and (–) SD = $2^{-\Delta\Delta Ct} - 2^{-(\Delta\Delta Ct + SD)} = 0.01$.

[c]SD of scrambled siRNA control: $2^{-(\Delta\Delta Ct + SD)} = 0.78$; and $2^{-(\Delta\Delta Ct - SD)} = 1.28$. Scrambled siRNA control, (+) SD = $2^{-(\Delta\Delta Ct - SD)} - 2^{-\Delta\Delta Ct} = 0.28$; and (–) SD = $2^{-\Delta\Delta Ct} - 2^{-(\Delta\Delta Ct + SD)} = 0.22$.

to the average value is obtained. The asymmetric distribution arises from converting the results of an exponential outcome into a linear comparison of amounts ***(21)***. The above equations from an actual quantitative real-time PCR validation of siRNA experiment (*see* **Table 4**).

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