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Instruction Manual

BLOCK-iT™ RNAi Basic Control Kit (Human)

To facilitate optimization of conditions for delivery of Stealth™ RNAi, siRNA, or diced siRNA into mammalian cells for RNAi analysis

Catalog nos. K4911-00

Version A
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Table of Contents

Kit Contents and Storage

Shipping and Storage

The BLOCK-iT™ RNAi Basic Control Kit (Human) is shipped as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Shipping	Storage
1	BLOCK-iT™ RNAi Basic Control Kit Reagents	Dry ice	**BLOCK-iT™ Fluorescent Oligo, Dead Cell Reagent, Nuclei Stain, and LUX™ Primers:** -20°C, protected from light **Stealth™ RNAi Controls, 1X RNA Annealing/Dilution Buffer:** -20°C
2	Lipofectamine™ 2000 Reagent	Blue ice	+4°C **(do not freeze)**

BLOCK-iT™ RNAi Basic Control Kit Reagents

The BLOCK-iT™ RNAi Basic Control Kit Reagents box (Box 1) includes the following items. The 1X RNA Annealing/Dilution Buffer is composed of 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, pH 8.0. **Store the BLOCK-iT™ Fluorescent Oligo, Dead Cell Reagent, Nuclei Stain, and LUX™ Primers at -20°C, protected from light. Store the other reagents at -20°C.**

Reagent	Composition	Amount
BLOCK-iT™ Fluorescent Oligo	20 µM fluorescein-labeled, double-stranded RNA oligomer in 1X RNA Annealing/Dilution Buffer	125 µl
Dead Cell Reagent	2 mM Ethidium homodimer-1 (EthD-1) in DMSO/H_2O 1:4 (v/v)	50 µl
Nuclei Stain	5 mM (3 mg/ml) Hoechst 33342 in water	50 µl
1X RNA Annealing/Dilution Buffer	10 mM Tris-HCl, pH 8.0 20 mM NaCl 1 mM EDTA, pH 8.0	1 ml
Human p53 Positive Control Stealth™ RNA	20 µM Stealth™ RNA in 1X RNA Annealing/Dilution Buffer	125 µl
Scrambled Negative Control Stealth™ RNA	20 µM Stealth™ RNA in 1X RNA Annealing/Dilution Buffer	125 µl
Human p53 Positive Control LUX™ Primer Set	10 µM FAM-labeled forward primer in TE Buffer, pH 8.0 10 µM unlabeled reverse primer in TE Buffer, pH 8.0	100 µl 100 µl
Human GAPDH Certified LUX™ Primer Set	10 µM FAM-labeled forward primer in TE Buffer, pH 8.0 10 µM unlabeled reverse primer in TE Buffer, pH 8.0	100 µl 100 µl

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Kit Contents and Storage, continued

Lipofectamine™ 2000 Reagent

The BLOCK-iT™ RNAi Basic Control Kit (Human) includes Lipofectamine™ 2000 Reagent (Box 2) for high-efficiency transfection of DNA and dsRNA oligomers (*i.e.* Stealth™ RNAi, siRNA, or diced siRNA) into a broad range of mammalian cell lines. Lipofectamine™ 2000 Reagent is supplied and should be stored as follows:

Amount supplied: 250 µl

Composition: Proprietary

Storage: +4°C (do not freeze)

Accessory Products

Accessory Products

Some of the reagents supplied in the BLOCK-iT™ RNAi Basic Control Kit (Human) as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 26).

Note: Some reagents are available in other sizes.

Item	Amount	Catalog no.
BLOCK-iT™ Fluorescent Oligo	2 x 125 µl (20 µM) 75 µl (1 mM)	2013 13750-062
Ethidium Homodimer-1 (Dead Cell Reagent)	1 mg	E-1169
Nuclei Stain (Hoechst 33342)	10 mg/ml	H-3570
Human GAPDH Certified LUX™ Primer Set	2 x 100 µl (FAM-labeled) 2 x 100 µl (JOE-labeled)	100H-01 100H-02
Lipofectamine™ 2000 Reagent	0.75 ml 1.5 ml	11668-027 11668-019
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985-062 31985-070
Concert™ 96 RNA Purification System	4 x 96 reactions	12173-011
mRNA Catcher™ Kit	1 x 96 reactions 10 x 96 reactions	7001 7003
TRIzol® Reagent	100 ml 500 ml	15596-026 15596-018
SuperScript™ III Platinum® One-Step qRT-PCR Kit	100 reactions 500 reactions	11732-020 11732-088
SuperScript™ III Platinum® Two-Step qRT-PCR Kit	100 reactions 500 reactions	11734-050 11734-068
ROX Reference Dye	500 µl	12223-023
DNase I, Amplification Grade (1 U/µl)	100 units	18068-015
DEPC-Treated Water	4 x 1.25 ml	10813-012

Introduction

Overview

Introduction

The BLOCK-iT™ RNAi Basic Control Kit (Human) is designed to facilitate optimization of conditions for delivery of Stealth™ RNAi, siRNA, or Dicer-generated siRNA (d-siRNA) to human cells for RNAi analysis. The kit provides reagents needed to assess transfection efficiency, cell viability, and control gene knockdown in human cell lines including:

- Lipofectamine™ 2000 Reagent for highly efficient delivery of DNA and double-stranded RNA (dsRNA) oligomers to a wide variety of mammalian cells
- BLOCK-iT™ Fluorescent Oligo, a fluorescein-labeled dsRNA oligomer for use as an indicator of transfection efficiency in RNAi experiments.
- A Stealth™ RNA molecule targeting the human p53 gene for use as a positive control for the RNAi response
- A Scrambled Stealth™ RNA molecule for use as a negative control for the RNAi response
- LUX™ fluorogenic primer set to the human p53 gene for assessing p53 knockdown (after transfection of the p53 Positive Stealth™ RNA control) using real-time quantitative RT-PCR (qRT-PCR). A LUX™ primer set for human GAPDH is included as an internal control to normalize your qRT-PCR experiment.
- Dead Cell Reagent and Nuclei Stain to visually or quantitatively assess mammalian cell viability after transfection of cells with RNAi molecules. Both reagents are fluorescent compounds that bind to DNA; however, Nuclei Stain binds to DNA in living cells, while Dead Cell Reagent only binds to DNA of dying cells.
- 1X RNA Annealing/Dilution Buffer for use in diluting RNAi molecules for transfection, if necessary.

For more information about each reagent, see pages 2-5.

Uses for the BLOCK-iT™ RNAi Basic Control Kit

Use the BLOCK-iT™ RNAi Basic Control Kit in your RNAi experiments for the following purposes:

- If you are transfecting RNAi molecules into a human cell line of interest for the first time, use the reagents provided to help you optimize your transfection conditions.

  Note: You may use some of the reagents supplied in the BLOCK-iT™ RNAi Basic Control Kit including Lipofectamine™ 2000, BLOCK-iT™ Fluorescent Oligo, Dead Cell Reagent, and Nuclei Stain to help you optimize transfection conditions in non-human mammalian cell lines.

- Once you have determined the optimal conditions to use for transfection, include the reagents provided in every RNAi experiment as an indicator of transfection efficiency and cell viability.

Components of the Kit

Introduction

This section provides more information about the reagents supplied in the BLOCK-iT™ RNAi Basic Control Kit (Human). For more information about the LUX™ Primer Sets supplied with the kit and how LUX™ Primers work, see the next section, page 6.

Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 Reagent is a proprietary, cationic lipid-based formulation suitable for delivery of nucleic acids including DNA, dsRNA oligomers, and the BLOCK-iT™ Fluorescent Oligo to mammalian cells for RNAi analysis (Gitlin *et al.*, 2002; Yu *et al.*, 2002). Using Lipofectamine™ 2000 to transfect eukaryotic cells offers the following advantages:

- Provides the highest transfection efficiency in a wide variety of mammalian cell types.
- Nucleic acid-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes or medium change or addition following transfection is not required, although complexes can be removed after 4-6 hours without loss of activity.

Lipofectamine™ 2000 is supplied with the BLOCK-iT™ RNAi Basic Control Kit, but is also available separately from Invitrogen (see page vii for ordering information).

BLOCK-iT™ Fluorescent Oligo

The BLOCK-iT™ Fluorescent Oligo allows strong, easy fluorescence-based assessment of dsRNA oligomer uptake into mammalian cells. The Oligo possesses the following characteristics:

- Is a fluorescein-labeled, double-stranded RNA duplex with the same length, charge, and configuration as standard siRNA.
- Contains chemical modifications that enhance the stability and allow assessment of fluorescence signal for a significantly longer time period than is obtained with other unmodified, fluorescently labeled RNA. **Example:** Fluorescence signal is readily detectable in HEK293 cells for at least 72 hours. Note that the strength of the fluorescence signal depends on the transfection efficiency, growth rate of the cells, and the amount of oligomer transfected.
- The sequence of the BLOCK-iT™ Fluorescent Oligo is not homologous to any known gene, ensuring against induction of non-specific cellular events caused by introduction of the Oligo into cells.
- Localizes primarily to the nucleus upon uptake (Fisher *et al.*, 1993).
- Fluorescence signal is detectable using any fluorescence microscope and a standard FITC filter set.

The BLOCK-iT™ Fluorescent Oligo is supplied with the BLOCK-iT™ RNAi Basic Control Kit, but is also available separately from Invitrogen (see page vii for ordering information).

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Components of the Kit, continued

Important

The BLOCK-iT™ Fluorescent Oligo is designed strictly for use as a tool for Stealth™ RNAi or siRNA uptake assessment, and is not meant to provide any information about the behavior of your Stealth™ RNAi or siRNA including its cellular localization, half-life, or stability.

Control Stealth™ RNA

The BLOCK-iT™ RNAi Basic Control Kit includes p53 and Scrambled Stealth™ RNA molecules for use as positive and negative controls, respectively, in an RNAi experiment targeting the human p53 gene. If p53 is expressed in your human cell line of interest, we recommend using the two Stealth™ RNA molecules to help you optimize your transfection conditions. For more information about Stealth™ RNAi, see below.

Note: Do not use the p53 and Scrambled Stealth™ RNA molecules as controls in non-human cell lines.

Stealth™ RNAi

Stealth™ RNAi is chemically modified dsRNA developed to overcome the limitations of traditional siRNA. Using Stealth™ RNAi for RNAi analysis offers the following advantages:

- Obtain effective target gene knockdown at levels that are equivalent to or greater than those achieved with traditional siRNA
- Reduces off-target effects caused by induction of cellular stress response pathways
- Exhibit enhanced stability for greater flexibility in RNAi analysis
- For more information about Stealth™ RNAi, see the RNAi resource page on our Web site (www.invitrogen.com/rnai) or contact Technical Service (see page 26). To design and order Stealth™ RNA molecules for your target genes, see the RNAi Designer on our Web site.

Control LUX™ Primer Sets

The BLOCK-iT™ RNAi Basic Control Kit includes the human p53 LUX™ Primer Set for measuring p53 knockdown (after transfecting human cells with the Positive p53 Stealth™ RNA Control) using real-time qRT-PCR. A LUX™ Primer Set for the human GAPDH gene is included for use as an internal control to normalize your qRT-PCR experiment. For more information about the primer and amplicon specifications for each primer set, see the next page. For general information about LUX™ Primers and qRT-PCR, see the next section.

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Components of the Kit, continued

Primer and Amplicon Specifications

The human p53 and GAPDH LUX™ Primer Sets amplify a region of each corresponding coding sequence, with amplicon specifications as follows. For more information about the p53 and GAPDH genes, see below.

Gene	CDS Location	Amplicon Melting Temp*	PCR Product Size Range
p53	Exons 6/7	T_m = 81.4°C	50-100 bp
GAPDH	Exons 4/5	T_m = 87°C	151-200 bp

*Note that this is the T_m of the amplicon, not the primers. T_m is approximate and dependent on experimental conditions.

p53

Tumor protein p53 (Harlow *et al.*, 1985; Matlashewski *et al.*, 1984; Zakut-Houri *et al.*, 1985) is a nuclear protein that plays an essential role in the regulation of cell cycle, specifically in the transition from G_0 to G_1. The protein is expressed in very low levels in normal cells; however, it is expressed at high levels in a variety of transformed cell lines, and is believed to contribute to transformation and malignancy. p53 is a DNA-binding protein containing DNA-binding, oligomerization and transcription activation domains. It is postulated to bind as a tetramer to a p53-binding site and activate expression of downstream genes that inhibit growth and/or invasion, and thus function as a tumor suppressor. Mutants of p53 that frequently occur in a number of different human cancers fail to bind the consensus DNA binding site, and hence cause the loss of tumor suppressor activity. Alterations of the p53 gene occur not only as somatic mutations in human malignancies, but also as germline mutations in some cancer-prone families with Li-Fraumeni syndrome (Varley, 2003). Database information for p53 is provided below.

Species	GenBank® Accession #	Entrez® Ref Seq #	OMIM™ #
Homo sapiens	BC003596	NM_000546	191170

GAPDH

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Arcari *et al.*, 1984; Piechaczyk *et al.*, 1984) catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). The enzyme is thought to be a tetramer of identical chains. GAPDH is highly expressed in all cells, and is used in this kit as a housekeeping control for normalizing qRT-PCR results. Database information for GAPDH is provided below.

Species	GenBank® Accession #	Entrez® Ref Seq #	OMIM™ #
Homo sapiens	M17851	NM_002046	138400

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Components of the Kit, continued

Dead Cell Reagent

Dead Cell Reagent is intended for use as an indicator of cell viability following transfection of mammalian cells with siRNA or Stealth™ RNAi, and is an ethidium dye (ethidium homodimer-1; EthD-1) with the following characteristics:

- Molecular formula: $C_{46}H_{50}Cl_4N_8$
- Molecular weight: 856.77

Dead Cell Reagent enters cells with damaged membranes (*i.e.* dying cells) and emits a red fluorescence signal upon binding to nucleic acids (λ_{ex} = 528 nm, λ_{em} = 617 nm). The dye can be excited with mercury- or xenon-arc lamps or with the argon-ion laser. The fluorescence signal is detectable using a fluorescence microscope and filters for propidium iodide or Texas Red®.

Note: Dead Cell Reagent is excluded by the intact plasma membrane of live cells.

Nuclei Stain

Nuclei Stain is intended for use as an indicator of cell viability following transfection of mammalian cells with siRNA or Stealth™ RNAi, and is a Hoechst dye (Hoechst 33342) with the following characteristics:

- Molecular formula: $C_{27}H_{37}Cl_3N_6O_4$
- Molecular weight: 615.99
- CAS Number/Name: 23491-52-3/2,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)

Nuclei Stain is a cell membrane-permeant, minor groove-binding DNA stain that emits a bright blue fluorescence signal upon binding to dsDNA in living cells (λ_{ex} = 350 nm, λ_{em} = 460 nm). The dye can be excited with the UV spectral lines of the argon-ion laser and by most conventional fluorescence excitation sources including a mercury lamp. The fluorescence signal is detectable using a fluorescence microscope and filters for DAPI (4′,6-diamidino-2-phenylindole).

1X RNA Annealing/Dilution Buffer

1X RNA Annealing/Dilution Buffer is supplied with the kit for use in diluting RNAi molecules, if necessary. For an RNAi molecule, the concentration of reagent required to induce effective target gene knockdown (if the RNAi molecule is active) can vary with each mammalian cell line and should be optimized. When optimizing your transfection conditions, you may use the 1X RNA Annealing/Dilution Buffer to dilute the RNAi reagent stock solution, if needed.

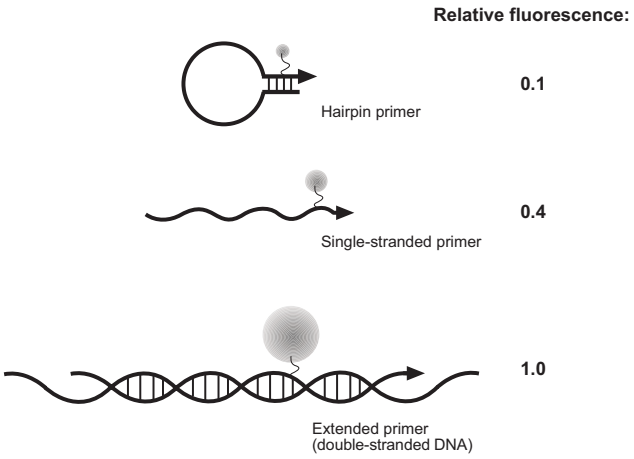
LUX™ Primer Sets

Introduction

This section provides more information about LUX™ Primers and how to use them for real-time quantitative PCR (qPCR) and RT-PCR (qRT-PCR). For more information about real-time qPCR or qRT-PCR, refer to published references (Edwards *et al.*, 2004; Higuchi *et al.*, 1993; Higuchi *et al.*, 1992; Schmittgen, 2001; Wittwer *et al.*, 2004).

LUX™ Primer Sets

LUX™ (Light Upon eXtension) Primers are a sensitive and efficient method for performing real-time quantitative PCR (qPCR) and RT-PCR (qRT-PCR). Each LUX™ primer pair includes a fluorogenic primer with a fluorophore attached to its 3′ end and a corresponding unlabeled primer (Nazarenko *et al.*, 2002; Nazarenko *et al.*, 2002). The fluorogenic primer has a short sequence tail of 4-6 nucleotides on the 5′ end that is complementary to the 3′ end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore (see figure below). When the primer is incorporated into double-stranded DNA during PCR, the fluorophore is dequenched and the signal increases by up to 10-fold. LUX™ primers combine high specificity with melting curve capability, have a broad dynamic range of 7-8 orders of magnitude, and are compatible with most real-time PCR instruments.



LUX™ Primer Applications

LUX™ Primers can be used in real-time qPCR and qRT-PCR to quantify 100 or fewer copies of a target gene in as little as 1 pg of template DNA or RNA. They have a broad dynamic range of 7–8 orders of magnitude.

To use LUX™ Primers for RNAi analysis, we generally perform real-time detection using qRT-PCR to quantitate mRNA levels (Javorschi *et al.*, 2004). See the guidelines and sample protocols for qRT-PCR on pages 15-18.

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LUX™ Primer Sets, continued

Instrument Compatibility

To perform real-time detection, you will need to have access to a real-time qPCR instrument. LUX™ Primers are compatible with a wide variety of real-time qPCR instruments with various detection capabilities, including but not limited to the ABI PRISM® 7700, 7000, and 7900 and GeneAmp® 5700; the Bio-Rad iCycler™; the Stratagene Mx4000™ and Mx3000™; the Cepheid Smart Cycler®; the Corbett Research Rotor-Gene; and the Roche LightCycler®. If you do not have one of the instruments listed above, note that at a minimum, the instrument used to perform real-time qPCR with LUX™ Primers must:

- Detect fluorescence at each PCR cycle
- Excite and detect FAM-labeled LUX™ Primers near their excitation/emission wavelengths of 490/520 nm

Instrument Settings

Follow the manufacturer's instructions to operate and configure your real-time qPCR instrument for use with LUX™ Primers. Note the following settings:

- LUX™ Primers are compatible with standard melting curve analysis, if your instrument software allows that option. Program your instrument accordingly.
- The quencher setting on the instrument should reflect the fact that LUX™ Primers do not contain a quencher.
- We recommend the use of ROX Reference Dye (see page 17) for normalization of well-to-well variability with instruments that are compatible with this option. Adjust your instrument settings accordingly.
- If you are performing one-step qRT-PCR, program the instrument to perform cDNA synthesis immediately followed by PCR amplification.

Additional guidelines and settings for specific instruments are available at www.invitrogen.com/lux; click on Instrument Protocols.

Other LUX™ Primer Sets

A large selection of Certified LUX™ Primer Sets for human housekeeping genes including GAPDH is available separately from Invitrogen. LUX™ Primer Sets are also available labeled with JOE (6-carboxy-4′, 5′-dichloro-2′, 7′-dimethyoxy-fluorescein) to allow multiplex applications. For more information about the Certified LUX™ Primer Sets available, see our Web site (www.invitrogen.com/lux) or contact Technical Service (see page 26).

Methods

Handling the Reagents

Introduction

Follow the guidelines below when handling the reagents supplied in the kit.

Handling the BLOCK-iT™ Fluorescent Oligo and Control Stealth™ RNA

The BLOCK-iT™ Fluorescent Oligo and the control Stealth™ RNA molecules are each supplied as a 20 µM stock solution in 1X RNA Annealing/Dilution Buffer. Follow the guidelines below when handling the BLOCK-iT™ Fluorescent Oligo and Stealth™ RNA stock solutions.

- **Storage:** Store the BLOCK-iT™ Fluorescent Oligo stock solution at -20°C, protected from light, and the control Stealth™ RNA stock solutions at -20°C. All stock solutions are stable for at least 6 months if stored properly.
- **Thawing:** When using, thaw the stock solution on ice or at room temperature. Once thawed, place the tube on ice until use. After use, return stock solution to -20°C storage.
- **Freeze/thaw cycles:** The stock solution may be frozen and thawed multiple times without loss of fluorescence signal (BLOCK-iT™ Fluorescent Oligo) or activity (Control Stealth™ RNAi) if handled properly.
- **RNase-free conditions:** Take precautions to ensure that the stock solution does not become contaminated with RNase.
  a. Use RNase-free sterile pipette tips and supplies for all manipulations.
  b. Wear gloves when handling reagents and solutions.

Handling the Dead Cell Reagent and the Nuclei Stain

The Dead Cell Reagent is supplied as a 2 mM stock solution in DMSO/H_2O 1:4 (v/v) while the Nuclei Stain is supplied as a 3 mg/ml stock solution in water. Follow the guidelines below when handling each stock solution.

- The Dead Cell Reagent and the Nuclei Stain are light sensitive. Store the stock solutions at -20°C, protected from light. The stock solutions are stable for at least 6 months if stored properly.
- When using, thaw each stock solution at room temperature. Tap the tube to mix the stock solution, and centrifuge briefly before opening. After use, return stock solution to -20°C storage.
- Each stock solution may be frozen and thawed multiple times without loss of fluorescence signal if handled properly.

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Handling the Reagents, continued

Handling the LUX™ Primers

The human p53 and GAPDH LUX™ Primer Sets contain a vial of 6-FAM-labeled primer and a vial of unlabeled primer, each at 10 µM concentration in TE Buffer. Volumes supplied are sufficient for 100 50-µl reactions or 250 20-µl reactions. Follow the guidelines below when handling the LUX™ Primers.

- The LUX™ Primers are light sensitive. Store the primers at -20°C, protected from light. The primers are stable for at least 6 months if stored properly.

  Note: Stability can be extended by storing at -80°C.

- When using, thaw each primer at room temperature. Tap the tube to mix the stock solution, and centrifuge briefly before opening. Be careful to minimize the exposure of labeled LUX™ Primers to direct light, as this can reduce their fluorescence intensity. Store on ice, protected from light for up to 4 hours. After use, return stock solution to -20°C storage.
- The stock solution may be frozen and thawed multiple times without loss of fluorescence signal if handled properly.

General Guidelines for Optimizing Transfection

Introduction

General guidelines are provided in this section to use Lipofectamine™ 2000 to transfect the BLOCK-iT™ Fluorescent Oligo and the control Stealth™ RNA molecules into mammalian cells. For procedures to transfect and stain cells, see pages 12-13.

We recommend using the reagents supplied in the BLOCK-iT™ RNAi Basic Control Kit for transfection optimization as follows:

- For each mammalian cell line used in your RNAi analysis, determine optimal transfection conditions using the BLOCK-iT™ Fluorescent Oligo. Once you have optimized transfection conditions, use these conditions as a starting point to transfect Stealth™ RNA molecules or siRNA, with additional optimization as necessary.
- For every transfection experiment, stain separate wells of untreated and Stealth™ RNAi or siRNA-treated cells with the Dead Cell Reagent and Nuclei Stain. Use fluorescence microscopy with the appropriate filter sets to assess cell viability by comparing the percentage of live cells and dead cells. If you are using optimal transfection conditions and if your Stealth™ RNAi or siRNA does not adversely affect your cells, you should see very few dead cells in RNAi molecule-treated wells.

General Guidelines for Transfection and Staining

Follow these general guidelines when using Lipofectamine™ 2000 to transfect the BLOCK-iT™ Fluorescent Oligo or the control Stealth™ RNA molecules into mammalian cells.

- Determine the appropriate amount of each reagent to use such that fluorescence signal (BLOCK-iT™ Fluorescent Oligo, Dead Cell Reagent, or Nuclei Stain) or gene knockdown effect (p53 Stealth™ RNA) is readily detectable. For recommended reagent amounts to use, see the next page.
- Use low-passage cells, and make sure that cells are healthy and greater than 90% viable before transfection.
- **Transfect cells at 30-50% confluence.** We recommend assessing BLOCK-iT™ Fluorescent Oligo uptake at 6 to 24 hours post-transfection; however, target gene knockdown levels (following Stealth™ RNAi or siRNA delivery) are generally assayed at a minimum of 24 to 72 hours following transfection. Transfecting cells at a lower density allows a longer time interval to elapse between transfection and assay time, and minimizes the loss of cell viability due to cell overgrowth. Depending on the nature of the target gene, transfecting cells at higher densities may be suitable with optimization of conditions.
- **Do not add antibiotics to the medium during transfection** as this reduces transfection efficiency and causes cell death.
- For optimal results, use Opti-MEM® I Reduced Serum Medium (Catalog no. 31985-062) to dilute Lipofectamine™ 2000 and the RNAi molecule prior to complex formation.

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General Guidelines for Optimizing Transfection, continued

Amount of BLOCK-iT™ Fluorescent Oligo to Use

The amount of BLOCK-iT™ Fluorescent Oligo to transfect depends on the growth rate and transfection efficiency of the mammalian cells. To optimize transfection conditions, evaluate several concentrations of lipid and vary the final concentration of the BLOCK-iT™ Fluorescent Oligo from 10 to 200 nM to determine the optimal amount of Oligo required to obtain a strong fluorescence signal. Use the 1X RNA Annealing/Dilution Buffer supplied with the kit to dilute the Oligo stock solution, if necessary.

Note: As a starting point, we recommend using 100 nM BLOCK-iT™ Fluorescent Oligo.

Amount of Control Stealth™ RNAi to Use

The amount of the p53 Positive Stealth™ RNA control to transfect to achieve optimal gene knockdown needs to be determined experimentally for each human cell line. To optimize transfection conditions, evaluate several concentrations of lipid and vary the final concentration of Stealth™ RNAi from 10 to 100 nM to determine the conditions required for optimal levels of gene knockdown. Use the 1X RNA Annealing/Dilution Buffer supplied with the kit to dilute the Oligo stock solution, if necessary. Use of higher concentrations of Stealth™ RNAi may be possible depending on the cell line.

Note: As a starting point, we recommend using 40 nM p53 Positive Stealth™ RNA control. Use the same concentration of the Scrambled Negative Stealth™ RNA control.

Transfecting and Staining Cells

Introduction

Use the procedure in this section to transfect the BLOCK-iT™ Fluorescent Oligo or control Stealth™ RNAi into mammalian cells using Lipofectamine™ 2000.

Materials Needed

Have the following reagents on hand before beginning:

- Mammalian cell line of interest cultured in the appropriate growth medium
- BLOCK-iT™ Fluorescent Oligo (supplied with the kit; 20 µM in 1X RNA Annealing/Dilution Buffer)
- p53 Positive Stealth™ RNA Control (supplied with the kit; 20 µM in 1X RNA Annealing/Dilution Buffer)
- Scrambled Negative Stealth™ RNA Control (supplied with the kit; 20 µM in 1X RNA Annealing/Dilution Buffer)
- Lipofectamine™ 2000 Reagent (supplied with the kit; store at +4°C until use)
- Opti-MEM® I Reduced Serum Medium (pre-warm to 37°C before use)
- Appropriate tissue culture plates and supplies

Transfection Procedure

Use this procedure to transfect the BLOCK-iT™ Fluorescent Oligo and the Control Stealth™ RNA molecules into mammalian cells using Lipofectamine™ 2000. Refer to the table in **Suggested Reagent Amounts and Volumes**, next page for the appropriate reagent amounts and volumes to add for different tissue culture formats. Use the suggested amounts as a starting point for your experiments, and optimize conditions for your cell line, as necessary.

1. One day before transfection, plate cells in the appropriate amount of growth medium **without antibiotics** such that they will be 30-50% confluent at the time of transfection.
2. For each transfection sample, prepare oligomer-Lipofectamine™ 2000 complexes as follows:
   a. Dilute the dsRNA oligomer in the appropriate amount of Opti-MEM® I Reduced Serum Medium without serum. Mix gently.
   b. Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in Opti-MEM® I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5-minute incubation, combine the diluted dsRNA oligomer with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur. The solution may appear cloudy, but this will not inhibit transfection.
3. Add the dsRNA oligomer-Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4. Incubate the cells at 37°C in a CO_2 incubator until you are ready to assess fluorescent uptake (see **Note** on the next page) or p53 gene knockdown. Removal of complexes or media change is not required; however, growth medium may be replaced after 4-6 hours without loss of transfection activity.

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Transfecting and Staining Cells, continued

Note

We recommend assessing fluorescence uptake at 6 to 24 hours post-transfection. The fluorescence signal may be detected at later time points depending on the transfection efficiency and growth rate of the cells.

Suggested Reagent Amounts and Volumes

The table below lists the recommended reagent amounts and volumes to use to transfect cells in various tissue culture formats. Use the recommended amounts of dsRNA oligomer (*e.g.* BLOCK-iT™ Fluorescent Oligo; see column 4) and Lipofectamine™ 2000 (see column 6) as a starting point for your experiments, and optimize conditions for your cell line.

Note: 20 µM BLOCK-iT™ Fluorescent Oligo or control Stealth™ RNAi = 20 pmol/µl.

Culture Vessel	Relative Surface Area (vs. 24-well)	Volume of Plating Medium	dsRNA Oligo (pmol) and Dilution Volume (µl)	dsRNA Oligo Amts (pmol) to Optimize	Lipid (µl) and Dilution Volume (µl)	Lipid Amts (µl) to Optimize
48-well	0.4	200 µl	25 pmol in 25 µl	1-50 pmol	0.5 µl in 25 µl	0.3-0.8 µl
24-well	1	500 µl	50 pmol in 50 µl	5-100 pmol	1 µl in 50 µl	0.5-1.5 µl
6-well	5	2 ml	250 pmol in 250 µl	25-500 pmol	5 µl in 250 µl	2.5-6 µl

Staining Cells with Dead Cell Reagent and Nuclei Stain

Follow this procedure to stain cells with Dead Cell Reagent and Nuclei Stain. Prepare a sufficient amount of the working solution based on the number of samples you wish to stain (see table below). Note that you may stain cells with the Dead Cell Reagent and Nuclei Stain simultaneously.

1. Thaw the 2 mM Dead Cell Reagent and 3 mg/ml Nuclei Stain stock solutions at room temperature. For each reagent, tap the tube to mix and centrifuge briefly before opening.
2. Prepare a Staining Solution by diluting the stock solutions of Dead Cell Reagent and Nuclei Stain into Opti-MEM® I Reduced Serum Medium to a final concentration of 2 µM and 3 µg/ml, respectively (1:1000 dilution of each stock solution).

   Example: To prepare 1 ml of a working solution, add 1 µl of Dead Cell Reagent and 1 µl of Nuclei Stain to 1 ml of Opti-MEM® I Reduced Serum Medium.
3. Aspirate the media from the cells and replace with the appropriate volume of Staining Solution (see table below).

Culture Vessel	Volume of Staining Solution per Well
48-well	0.25 ml
24-well	0.5 ml
6-well	2 ml

4. Incubate cells at 37°C in a CO_2 incubator for 10-15 minutes.
5. Remove the medium containing Dead Cell Reagent and Nuclei Stain. Replace with fresh Opti-MEM® I Reduced Serum Medium.
6. Evaluate fluorescence signal using a fluorescence microscope and the appropriate filter set (see the next page).

continued on next page

Detecting Fluorescence Signal

Once you have transfected your mammalian cells with the BLOCK-iT™ Fluorescent Oligo (and Stealth™ RNAi or siRNA) and have stained the cells with Dead Cell Reagent and Nuclei Stain, you may qualitatively assess Oligo uptake (*i.e.* transfection efficiency) and cell viability using any fluorescence microscope and the following filter sets:

- To assess transfection efficiency, use any standard FITC filter set (λ_{ex} = 494 nm, λ_{em} = 519) to detect the green fluorescence signal from the BLOCK-iT™ Fluorescent Oligo.
- To assess cell viability, use a filter set for propidium iodide or Texas Red® (λ_{ex} = 528 nm, λ_{em} = 617 nm) to detect the red fluorescence signal from the Dead Cell Reagent.
- To assess cell viability, use a filter set for DAPI (λ_{ex} = 350 nm, λ_{em} = 460 nm) to detect the blue fluorescence signal from the Nuclei Stain.

Detecting p53 mRNA or Protein

If you have included the p53 Positive Stealth™ RNA Control and the Scrambled Negative Stealth™ RNA Control in your transfection experiment, you may assay for human p53 knockdown using several methods:

- To assay for p53 mRNA levels, we recommend performing real-time quantitative RT-PCR (qRT-PCR) using the Human p53 LUX™ Primers supplied with the kit. Include the Human GAPDH LUX™ Primers supplied with the kit in your qRT-PCR experiment as an internal control for normalization.

  Note: Depending on your needs, you may perform either one-step or two-step real-time qRT-PCR. For RNAi applications, we generally perform two-step qRT-PCR. General guidelines and sample protocols to perform two-step qRT-PCR are provided in the next section, pages 15-18. For general guidelines and sample protocols to perform one-step qRT-PCR, see the **Appendix**, pages 23-25.
- If you have an antibody to human p53, you may assay for p53 protein levels using Western blot analysis. Remember to take into account the half-life of the protein when assessing RNAi effects at the protein level.

To successfully use the general guidelines and sample protocols provided in this manual to perform two-step qRT-PCR (or one-step qRT-PCR), you will need to have an understanding of the principles of real-time PCR, how to set up a real-time PCR experiment, use a qPCR instrument, and collect and analyze qPCR results. If you are performing this technique for the first time, or if you need more information about the topics above, refer to these published reference sources. Other reference sources are available.

- Edwards, K., Logan, J., and Saunders, N. (2004) Real-Time PCR: An Essential Guide. Horizon Scientific Press, Wymondham, Norfolk, UK.
- Schmittgen, T. (2001) Real-Time Quantitative PCR. In *Methods*, Vol. 25. (K. Adolph, P. Conn, M. Deutscher and J. Langone, eds.) Elsevier, San Diego, CA.
- Wittwer, C., Hahn, M., and Kaul, K. (2004) Rapid Cycle Real-Time PCR - Methods and Applications. Springer-Verlag, New York.

Performing Two-Step Real-Time qRT-PCR

Introduction

Real-time qRT-PCR is a technique used to quantitate the amount of specific mRNA transcripts in a given sample. Real-time qRT-PCR uses RNA as starting material in a reverse transcription reaction to generate first-strand cDNA. The cDNA is then quantified in a separate real-time qPCR reaction.

For optimal results, we recommend using Invitrogen's SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 or 11734-068) to facilitate qRT-PCR analysis. This section provides guidelines and an optimized protocol using LUX™ Primers to perform two-step qRT-PCR using the reagents supplied in the SuperScript™ III Platinum® Two-Step qRT-PCR Kit. If you are using another commercial kit, follow the manufacturer's instructions to perform qRT-PCR.

Note: For an example of qRT-PCR results obtained with the control reagents included in the kit, see page 19.

Template Specifications

To perform qRT-PCR, you may isolate either mRNA or total RNA from your untreated and RNAi reagent-treated cells. High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis and accurate quantification. The amount of starting material needed for cDNA synthesis can range from 10 pg to 1 µg mRNA or total RNA. After cDNA synthesis, use 10^2 to 10^7 copies or 1 pg to 10 µg of the cDNA from the first-strand reaction in the qPCR step.

Preparing mRNA or Total RNA

You may use any method of choice to isolate mRNA or total RNA. If you are isolating total RNA, we recommend using the Concert™ 96 RNA Purification System (Catalog no. 12173-011) or TRIzol® reagent (Catalog no. 15596-026). If you are isolating mRNA, we recommend using the mRNA Catcher™ Kit (Catalog no. 7001). Follow the instructions included with each kit to isolate high-quality RNA. To accurately quantitate RNA, particularly small amounts of RNA, we recommend using the RiboGreen® RNA Quantitation Kit (Catalog no. R-11490).

Important: The purity and integrity of the starting RNA directly impacts the efficiency of cDNA synthesis. RNase and genomic DNA contamination are the most common problems. We recommend including RNase inhibitors during purification. If you are isolating total RNA, we strongly recommend performing DNase I digestion of the RNA sample prior to amplification with LUX™ primers (see the next page for a protocol).

Enzyme Specifications

For two-step qRT-PCR, we recommend using a high-specificity, high-yield reverse transcriptase (RT) such as SuperScript™ III Reverse Transcriptase and a "hot-start" DNA polymerase such as Platinum® *Taq* DNA Polymerase. The SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068) includes SuperScript™ III RT, Platinum® *Taq* DNA Polymerase, and all the other necessary components to perform two-step qRT-PCR except the RNA. See the sample protocol on the next page.

For first-strand cDNA synthesis alone, we recommend using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Catalog no. 18080-051).

continued on next page

Performing Two-Step Real-Time qRT-PCR, continued

Removing Genomic DNA from RNA Samples

If you are starting with total RNA, we strongly recommend that you reduce the risk of genomic DNA contamination in your starting material by performing a digest with DNase I, Amplification Grade (Catalog no. 18068-015), as described below. Use this procedure to digest 10 pg to 1 µg of RNA. For larger amounts of RNA, increase volumes accordingly.

1. Combine the following in a tube on ice:

Component	Amount
RNA template (10 pg to 1 µg)	x µl
10X DNase reaction buffer	1 µl
DNase I, Amplification Grade (1 U/µl)	1 µl
DEPC-treated water	To a final volume of 10 µl

2. Incubate at room temperature for 15 minutes.
3. Add 1 µl of 25 mM EDTA solution to the reaction mixture and incubate at 65°C for 10 minutes to inactivate the DNase I.

Reverse Transcription Procedure

The following procedure for generating first-strand cDNA uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog nos. 11734-050 and 11734-068). The RT Enzyme Mix contains SuperScript™ III RT and RNaseOUT™. The 2X RT Reaction Mix contains 2.5 µM oligo(dT)$_{20}$, 2.5 ng/µl random hexamers, 10 mM $MgCl_2$, and dNTPs.

1. Combine the following kit components in a tube on ice. For multiple reactions, a master mix without RNA may be prepared:

2X RT Reaction Mix	10 µl
RT Enzyme Mix	2 µl
mRNA or RNA (10 pg to 1 µg)	x µl
DEPC-treated water	to 20 µl

2. Gently mix and incubate the sample at 25°C for 10 minutes.
3. Incubate tube at 42°C for 50 minutes.
4. Terminate the reaction by incubating at 85°C for 5 minutes, then chill the reaction on ice.
5. Add 1 µl (2 U) of *E. coli* RNase H and incubate at 37°C for 20 minutes.

   Note: The *E. coli* RNase H digestion step is included to remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis. This has been shown to increase PCR sensitivity.
6. Store the reaction at -20°C until use. Proceed to the real-time qPCR protocol on the next page if you are using a real-time qPCR instrument that uses PCR tubes or plates. If you are using the Roche LightCycler®, follow the protocol on page 18.

continued on next page

Performing Two-Step Real-Time qRT-PCR, continued

ROX Reference Dye

We recommend using ROX Reference Dye (Cat. no. 12223-023) to normalize the fluorescent reporter signal in real-time qPCR for instruments that are compatible with this option. ROX Reference Dye can be used to adjust for non-PCR-related fluctuations in fluorescence between reactions.

ROX is supplied at 50X concentration. To use, add 1 µl of ROX for every 50 µl of reaction volume (see below). For convenience and to reduce pipetting errors, you can premix a solution of ROX and the "hot start" DNA polymerase (*e.g.* Platinum® Quantitative PCR SuperMix-UDG). Add 1 µl of ROX for every 25 µl of SuperMix-UDG. Store mixture at either -20°C or +4°C in the dark.

Real-Time qPCR Protocol for Instruments Using PCR Tubes or Plates

The following real-time qPCR protocol uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit, and is optimized for use with real-time qPCR instruments that use tubes or plates (see page 7 for instrument setting guidelines). To use the Roche LightCycler®, see the protocol on the next page.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template.

Component	Vol/1 rxn	Vol/50 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	25 µl	1250 µl
ROX Reference Dye (optional)	1 µl	50 µl
Labeled LUX™ Primer (10 µM)	1 µl	50 µl
Unlabeled LUX™ primer (10 µM)	1 µl	50 µl
Sterile distilled water	to 45 µl	to 2250 µl

[1]Final concentration: 0.06 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 0.04 U/µl UDG

2. Program the real-time qPCR instrument as follows:

   Thermal Cycling
   50°C, 2 minute hold (UDG treatment)
   95°C, 2 minute hold
   45 cycles of:
   95°C, 15 seconds
   55°C, 30 seconds
   72°C, 30 seconds
   Melting Curve Analysis
   Refer to instrument documentation

3. Add 45 µl of the Master Mix to an optical PCR tube or each well of a 96-well PCR plate.
4. Add 5 µl (10^2 to 10^7 copies or 1 pg to 10 µg) of the cDNA from the first-strand synthesis reaction (Step 5, previous page) to each reaction sample. Cap or seal the tube/plate.
5. Gently mix and make sure that all components are at the bottom of the tube/plate wells. Centrifuge briefly if needed.
6. Place reaction in the real-time qPCR instrument and run the program. Collect and analyze results.

continued on next page

Protocol for the Roche LightCycler®

The following protocol uses components from the SuperScript™ III Platinum® Two-Step qRT-PCR Kit in a 20-µl reaction volume, and has been optimized for use with the Roche LightCycler®. Consult the LightCycler® documentation for detailed instructions to prepare the capillary tubes and operate the instrument. FAM-labeled LUX™ Primers are also compatible with Roche enzyme mixes.

Note: JOE-labeled LUX™ Primers (not supplied with the kit) are not compatible with the current version of the LightCycler®; use FAM-labeled primers only.

1. To reduce well-to-well variation, prepare a Master Mix of all the reaction ingredients except template. The following table provides volumes for one reaction and 34 reactions (scale as needed).

Component	Vol/1 rxn	Vol/34 rxns
Platinum® Quantitative PCR SuperMix-UDG[1]	10 µl	340 µl
FAM-labeled LUX™ Primer (10 µM)	1 µl	34 µl
Unlabeled LUX™ Primer (10 µM)	1 µl	34 µl
BSA, UltraPure (5 mg/ml)	1 µl	34 µl
Platinum® *Taq* DNA Polymerase[2]	0.12 µl	4 µl
Sterile distilled water	to 18 µl	to 612 µl

[1]Final concentration: 0.06 U/µl Platinum® *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 0.04 U/µl UDG

[2]Total units of Platinum® *Taq* DNA Polymerase in the reaction is 1.2 (including 0.6 U from Platinum® Quantitative PCR SuperMix-UDG)

2. Set the fluorescence on the Roche LightCycler® to the F1 channel.
3. Program the instrument as follows:

Thermal Cycling
Program choice: Amplification
Analysis mode: Quantification
Cycling:
50°C, 2 min hold (UDG treatment)
95°C, 2 min hold
45 cycles of:
  94°C, 5 sec
  55°C, 10 sec (single acquire)
  72°C, 10 sec

Melting Curve Analysis (optional)
Program choice: Melting curve
Analysis mode: Melting curves
Cycling:
95°C, 0 sec
55°C, 15 sec
95°C, 0 sec (increase 0.1°C/sec with continuous acquisition)
40°C, 0 sec

4. Add 18 µl of Master Mix to each capillary tube of the LightCycler®.
5. Add 2 µl (10^2 to 10^7 copies or 1 pg to 10 µg) of the cDNA from the first-strand synthesis reaction (Step 5, page 16) to each tube, and cap the tube.
6. Centrifuge the tubes at 700 × *g* for 5 seconds.
7. Place the reaction tubes in the rotor of the LightCycler® and run the program. Collect and analyze results.

continued on next page

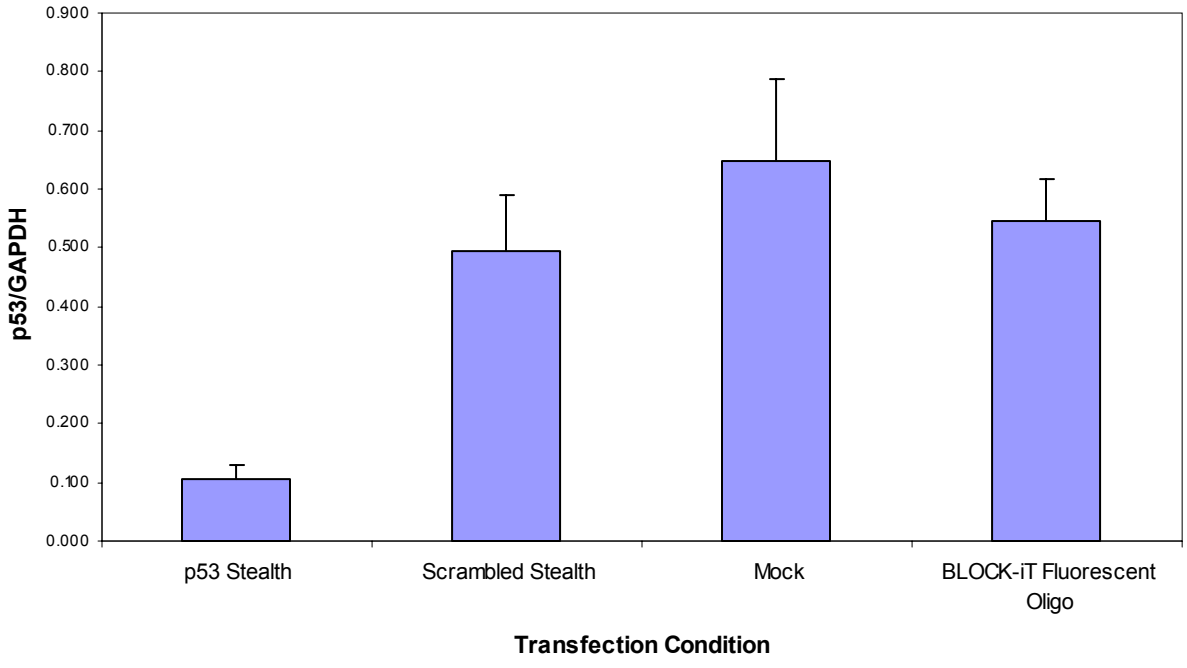
Example of Expected Results

In this experiment, A549 human lung carcinoma cells (ATCC, Catalog no. CCL-185) plated in 24-well plates were transfected with 50 pmoles of the p53 Positive Stealth™ RNA Control, Scrambled Negative Stealth™ RNA Control, or the BLOCK-iT™ Fluorescent Oligo using Lipofectamine™ 2000 and according to the protocol on page 12. A separate sample of mock-transfected cells was included. Cells were harvested 24 hours after transfection and mRNA was isolated using the mRNA Catcher™ Kit (Catalog no. 7001). Real-time qRT-PCR analysis was performed on the ABI Prism® 7700 instrument (Applied Biosystems, Foster City, CA) using the human p53 and GAPDH LUX™ Primers supplied with the kit. Twenty µl assays were performed using the reagents supplied in the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Catalog no. 11734-050) and following the protocols on pages 15-17. Melting curve analysis was performed according to conditions recommended by the instrument manufacturer. PCR efficiencies of the LUX™ primer sets were calculated from the slope of a 6-log dilution standard curve using the formula: $E = 10^{-1/slope} - 1$.

The graph below summarizes the data, with p53 expression presented as the ratio of p53 expression/GAPDH expression relative to mock-transfected cells.

Results:

- The p53 Positive Stealth™ RNA Control induces greater than 75% knockdown of p53 expression.
- The Scrambled Negative Stealth™ RNA Control and the BLOCK-iT™ Fluorescent Oligo induce minimal knockdown of p53 expression.



Troubleshooting

Introduction

Use the information in this section to troubleshoot your transfection and knockdown experiments.

Transfection and RNAi Analysis

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection and knockdown experiment.

Problem	Reason	Solution
Low levels of gene knockdown observed	Low transfection efficiency • Antibiotics added to the media during transfection • Cells were confluent at the time of transfection • Not enough siRNA or Stealth™ RNAi transfected • Not enough Lipofectamine™ 2000 used	• Do not add antibiotics to the media during transfection. • Plate cells such that they will be 30-50% confluent at the time of transfection. • Increase the amount of siRNA or Stealth™ RNAi transfected. • Optimize transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 used. • Use the BLOCK-iT™ Fluorescent Oligo to help you optimize transfection conditions for your cell line.
	Didn't wait long enough after transfection before assaying for gene knockdown	• Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown. • Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs.
	Target protein is stable (*i.e.* has a long half-life)	Perform qRT-PCR analysis using LUX™ Primers to assay for target gene knockdown at the mRNA level.
	Diced siRNA (d-siRNA) was degraded	• Make sure that the d-siRNA is stored in 1X RNA Annealing/Dilution Buffer. • Verify integrity of the d-siRNA using agarose gel electrophoresis before proceeding to transfection. • Aliquot purified d-siRNA and avoid repeated freeze/thaw cycles.
Cytotoxic effects observed after transfection (as indicated by staining with Dead Cell Reagent)	Too much Lipofectamine™ 2000 Reagent used	• Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used • Use the BLOCK-iT™ Fluorescent Oligo to help you optimize transfection conditions for your cell line.

continued on next page

Troubleshooting, continued

Transfection and RNAi Analysis, continued

Problem	Reason	Solution
Cytotoxic effects observed after transfection (as indicated by staining with Dead Cell Reagent), continued	Cells were not healthy or were seeded incorrectly at the time of transfection	Make sure that cells are > 90% viable before transfection. Plate cells such that they will be 30-50% confluent at the time of transfection.
	RNAi molecule targets an essential gene	Reduce the amount of RNAi molecule transfected. Note that doing so may negatively impact the level of knockdown observed.
	Transfection of siRNA or diced siRNA activates toxic pathways that result in cell death	• Convert the siRNA to a Stealth™ RNA molecule and repeat the RNAi analysis. • Test another target sequence to the same gene to confirm the toxicity response.
No gene knockdown observed	Stealth™ RNA molecule or siRNA was not active	Design a Stealth™ RNA molecule or siRNA to a different target region using the RNAi Designer on our Web site.
	Transfection conditions not optimized	• Use the BLOCK-iT™ Fluorescent Oligo to optimize transfection conditions for your cell line. • If you are using a human cell line, you may also use the Positive p53 Stealth™ RNA Control to help optimize transfection conditions.

qRT-PCR Analysis

The table below lists some potential problems and possible solutions that may help you troubleshoot your real-time qRT-PCR experiment using LUX™ Primers.

Problem	Reason	Solution
No PCR product is evident, either in the qPCR graph or on a gel	The protocol was not followed correctly	Verify that all steps have been followed and the correct reagents, dilutions, volumes, and cycling parameters have been used.
	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template.
PCR product is evident in the gel, but not on the qPCR graph	qPCR instrument settings are incorrect	Confirm that you are using the correct instrument settings (dye selection, reference dye, filters, acquisition points, etc.).
	Problems with your specific qPCR instrument	For instrument-specific tips and trouble-shooting using LUX™ Primers, see the instrument protocols at www.invitrogen.com/lux.

continued on next page

Troubleshooting, continued

qRT-PCR Analysis, continued

Problem	Reason	Solution
No amplification curve appears on the qPCR graph	There is no PCR product	Run the PCR product on a gel to determine whether PCR worked. Follow the other troubleshooting tips in this section, as appropriate.
Signals are present in no-template controls, and/or multiple peaks are present in the melting curve graph	Template or reagents are contaminated by nucleic acids (DNA, cDNA)	Use melting curve analysis and/or run the PCR products on a 4% agarose gel in an area separate from the reaction assembly area to identify contaminants. To reduce the risk of contamination, take standard precautions when preparing your PCR reactions. Assemble the amplification reaction in a DNA-free environment. Use aerosol-resistant barrier tips.
	Amplification of PCR carryover products	Analyze the PCR product on a 4% agarose gel in an area separate from the reaction assembly area to identify contaminants. We recommend using a UDG-based carryover prevention system such as Platinum® Quantitative PCR SuperMix-UDG or the SuperScript™ III Platinum® Two-Step qRT-PCR Kit. Since dUTP is substituted for dTTP in the reaction cocktail, any amplified DNA will contain uracil. UDG prevents reamplification of PCR carryover products by removing uracil residues from single or double stranded DNA. dU-containing DNA that has been digested with UDG is unable to serve as template in future PCRs. UDG is inactivated at high temperature during PCR thermal cycling, thereby allowing amplification of genuine target sequence(s).
PCR efficiency is above 110%	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template. Inhibitors in the template may result in changes in PCR efficiency between dilutions.
PCR efficiency is below 90%	The PCR conditions are suboptimal	• Verify that the amount of primers you are using is correct and that the labeled primer has not been exposed to direct light. • Verify that the reagents you are using have not been freeze-thawed multiple times and have not sat at room temperature for too long.

Appendix

Performing One-Step Real-Time qRT-PCR

Introduction

In one-step real-time qRT-PCR, cDNA synthesis and PCR are performed in a single reaction tube using total RNA or mRNA as starting material. The optimized enzyme mixture includes both a reverse transcriptase and a DNA polymerase. This section provides guidelines and an example protocol for performing one-step real-time qRT-PCR using LUX™ Primers. The example protocol uses the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System available from Invitrogen (Catalog nos. 11732-020 or 11732-088) for superior specificity and sensitivity with LUX™ Primers.

Primer Concentration

For optimal one-step qRT-PCR, primer titrations of 50–500 nM per LUX™ primer are recommended. The 50-µl sample reaction on the next page uses 200 nM of each primer (*i.e.* 1 µl of a 10 µM primer stock). Also see the note below.

In one-step qRT-PCR, the reverse LUX™ primer drives the reverse transcription reaction. We have found that doubling the concentration of the reverse LUX™ primer from 200 nM to 400 nM can in some cases decrease the cycle threshold for detecting a given target concentration, and thus increase sensitivity.

Template Specifications

The target template for one-step real-time qRT-PCR is mRNA or total RNA. See page 15 for recommended methods and kits to isolate mRNA or total RNA from your cells. If you are isolating total RNA, we recommend including a DNase I digestion to reduce the likelihood of genomic DNA contamination in your RNA sample (see page 16 for a protocol).

Enzyme Specifications

The one-step qRT-PCR enzyme mix should contain an optimized mixture of a high-specificity, high-yield reverse transcriptase and a hot-start DNA polymerase. If you are using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System, a SuperScript™ III reverse transcriptase/Platinum® *Taq* enzyme mix is included with the kit. See the sample reactions on pages 24-25.

Magnesium Concentration

The optimal $MgCl_2$ concentration for a given target/primer/polymerase combination can vary between 1 mM and 10 mM, but is usually in the range of 3 mM (see the sample reaction on page 24).

dNTP Concentration

The optimal concentration of dATP, dCTP, dGTP, and dTTP is 200 µM each. If dUTP is used in place of dTTP, its optimal concentration is 400 µM.

continued on next page

Protocol for Instruments Using PCR Tubes or Plates

The following protocol uses components from the SuperScript™ III Platinum® One-Step qRT-PCR Kit, and has been optimized for use with real-time qPCR instruments that use tubes or plates (see page 7 for instrument setting guidelines). Further optimization may be required. To use the Roche LightCycler®, see the protocol on the next page.

Note: Keep all components, reaction mixes and samples on ice. After assembly, transfer the reaction to a thermal cycler preheated to the cDNA synthesis temperature and immediately begin RT-PCR. We recommend performing the cDNA synthesis reaction at 50°C, but higher temperatures (up to 60°C) may be required for high GC content templates. RNase inhibitor proteins, such as RNaseOUT™ (Catalog no. 10777-019), may be added to the reaction to safeguard against degradation of RNA.

1. The following table provides Master Mix volumes for a standard 50-µl reaction size. Note that preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Component	Vol/1 rxn	Vol/100 rxns
SuperScript™ III RT/Platinum® *Taq* Mix	1 µl	100 µl
2X Reaction Mix[1]	25 µl	2500 µl
ROX Reference Dye (optional)	1 µl	100 µl
Labeled LUX™ Primer (10 µM)	1 µl	100 µl
Unlabeled LUX™ primer (10 µM)	1 µl	100 µl
RNaseOUT™ (optional)	1 µl	100 µl
Sterile distilled water	to 40 µl	to 4000 µl

[1]Supplied at 2X concentration: includes 0.4 mM of each dNTP and 6 mM $MgSO_4$

[2]See the **Important** note on primer concentration on page 23.

2. Program the instrument with the following thermal cycling protocol (for cDNA synthesis, use a 15-minute incubation at 50°C as a starting point):

   cDNA synthesis:
   50°C for 15 minute hold

   PCR:
   95°C for 2 minute hold
   40–50 cycles of:
   95°C, 15 seconds
   60°C, 30 seconds

   Melting Curve Analysis (optional)
   Program according to instrument instructions

3. For each reaction, add 40 µl of the Master Mix to a 0.2-ml microcentrifuge tube or each well of a 96-well PCR plate on ice.
4. Add 10 µl of sample RNA (1 pg to 1 µg total RNA) to each tube/plate well, and cap or seal.
5. Gently mix and make sure that all components are at the bottom of the tube/plate wells. Centrifuge briefly if needed.
6. Place reactions in a preheated thermal cycler programmed as described above. Collect data and analyze results.

continued on next page

Protocol for the Roche LightCycler®

The following protocol uses components from the SuperScript™ III Platinum® One-Step qRT-PCR Kit in a 20-µl reaction volume, and is optimized for use with the Roche LightCycler®. Further optimization may be required. FAM-labeled LUX™ Primers are also compatible with Roche enzyme mixes.

Note: JOE-labeled LUX™ Primers (not supplied with the kit) are not compatible with the current version of the LightCycler®; use FAM-labeled primers only.

Recommendation: After assembly, transfer the reaction to a thermal cycler preheated to the cDNA synthesis temperature and immediately begin RT-PCR. We recommend performing the cDNA synthesis reaction at 50°C, but higher temperatures (up to 60°C) may be required for high GC content templates. RNase inhibitor proteins, such as RNaseOUT™ (Catalog no. 10777-019), may be added to the reaction to safeguard against degradation of RNA.

1. The following table provides Master Mix volumes for a standard 20-µl reaction size. Note that preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Component	Vol/1 rxn	Vol/34 rxns
SuperScript™ III RT/Platinum® *Taq* Mix	0.8 µl	27.2 µl
2X Reaction Mix[1]	10 µl	340 µl
FAM-labeled LUX™ Primer (10 µM)[2]	1 µl	34 µl
Unlabeled LUX™ primer (10 µM)[3]	1 µl	34 µl
Bovine serum albumin (5 mg/ml)[4]	1 µl	34 µl
Sterile distilled water	to 18 µl	to 612 µl

[1]Includes 0.4 mM of each dNTP and 6 mM $MgSO_4$
[2]In the LightCycler® reaction, the LUX™ Fluorogenic Primer must be FAM labeled.
[3]See the **Important** note on primer concentration on page 23.
[4]Validated with non-acetylated Ultrapure BSA from Invitrogen (Cat. nos. P2489 and P2046)

2. Set the fluorescence on the Roche LightCycler® to the F1 channel.
3. Program the instrument as follows:

Thermal Cycling
Program choice: Amplification
Analysis mode: Quantification
Cycling:
45°C, 30 min hold (cDNA synthesis)
95°C, 2 min hold
50 cycles of:
95°C, 5 sec
55°C, 10 sec (single acquire)
72°C, 10 sec

Melting Curve Analysis (optional)
Program choice: Melting curve
Analysis mode: Melting curves
Cycling:
95°C, 0 sec
55°C, 15 sec
95°C, 0 sec (increase 0.1°C/s with continuous acquisition)
40°C, 0 sec

4. Add 18 µl of Master Mix to each capillary tube of the LightCycler® on ice.
5. Add 2 µl of sample RNA (1 pg to 1 µg total RNA) to each capillary tube and cap the tube.
6. Centrifuge the tubes at 700 × *g* for 5 seconds.
7. Place the reaction tubes in the rotor of the LightCycler® and run the program. Collect and analyze results.

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

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For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

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Technical Service, continued

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Product Qualification

Introduction

The components of the BLOCK-iT™ RNAi Basic Control Kit (Human) are qualified as described below.

Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 Reagent is tested for the absence of microbial contamination using blood agar plates, Sabaraud dextrose agar plates, and fluid thioglycolate medium, and functionally by transfection of CHO-K1 cells with a luciferase reporter-containing plasmid.

BLOCK-iT™ Fluorescent Oligo

The BLOCK-iT™ Fluorescent Oligo is qualified as follows:

- Concentration of the Oligo is verified by reading the optical density (OD)
- Oligo is functionally qualified by transient transfection into mammalian cells and assessment of fluorescence signal at 24 hours post-transfection

Stealth™ RNAi

Each Stealth™ RNA molecule is qualified as follows:

- Concentration of the Stealth™ RNA molecule is verified by reading the optical density (OD).
- The p53 Positive and Scrambled Negative Stealth™ RNA molecules are functionally qualified by transient transfection into A549 cells. At 24 hours post-transfection, mRNA is isolated from treated and untreated cells using the mRNA Catcher™ Kit, and qRT-PCR is performed using LUX™ primers for the human p53 gene. qRT-PCR analysis must demonstrate > 75% inhibition of human p53 expression levels in p53 Positive Stealth™ RNA control-treated cells and minimal inhibition in Scrambled Negative Stealth™ RNA control-treated cells.

LUX™ Primer Sets

The human p53 and GAPDH LUX™ Primer Sets are designed to discriminate between messages and known pseudogenes/different isoforms. Performance is functionally validated using a dilution series in a two-step real-time qRT-PCR with total HeLa RNA. The amplification efficiency based on the slope of the resulting standard curve is greater than 90%.

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