



BioModuleTM Transfection and Control Unit with BLOCK-iTTM Technology

**For delivery of StealthTM RNAi or siRNA into
mammalian cells for RNAi analysis**

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User Manual

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Experienced Users Procedure

Introduction

A brief experienced user's procedure for transfecting Stealth™ RNAi or siRNA molecules into mammalian cells using Lipofectamine™ 2000 Reagent is described below. Refer to this manual for a detailed protocol.

Step	Procedure								
Preparing Cells	Grow your mammalian cell line of choice in the recommended medium. Use low-passage cells, and make sure that cells are healthy and greater than 90% viable before transfection.								
Amount of RNAi to Transfect	<p>The recommended starting amount of each duplex used for transfection is listed below. To achieve optimal target gene knockdown, you may need to experimentally determine the amount of RNAi required for each mammalian cell line.</p> <table> <tr> <th>Duplex</th><th>Recommended Conc.</th></tr> <tr> <td>Stealth™ RNAi or siRNA</td><td>40 nM</td></tr> <tr> <td>Stealth™ RNAi Negative Control Duplex</td><td>40 nM</td></tr> <tr> <td>BLOCK-iT™ Fluorescent Oligo</td><td>100 nM</td></tr> </table>	Duplex	Recommended Conc.	Stealth™ RNAi or siRNA	40 nM	Stealth™ RNAi Negative Control Duplex	40 nM	BLOCK-iT™ Fluorescent Oligo	100 nM
Duplex	Recommended Conc.								
Stealth™ RNAi or siRNA	40 nM								
Stealth™ RNAi Negative Control Duplex	40 nM								
BLOCK-iT™ Fluorescent Oligo	100 nM								
Perform Transfection	<p>Use this procedure to transfect Stealth™ RNAi or siRNA into mammalian cells in a 24-well format. All amounts and volumes are given on a per well basis.</p> <p>For other formats, see Scaling Up or Down Transfections (page 18). Use this procedure as a starting point and optimize transfections as described below.</p> <ol style="list-style-type: none"> One day before transfection, plate cells in 500 µl of growth medium without antibiotics such that they will be 30-50% confluent at the time of transfection. For each transfection sample, prepare as follows: <ol style="list-style-type: none"> Dilute 20 pmol (1 µl of 20 µM solution) Stealth™ RNAi or siRNA oligomer in 50 µl Opti-MEM® I Reduced Serum Medium without serum (resulting concentration of RNA is 40 nM). Mix gently. Dilute 60 pmol (3 µl of 20 µM solution) BLOCK-iT™ Fluorescent Oligo in 50 µl Opti-MEM® I Reduced Serum Medium without serum (resulting concentration of the Fluorescent Oligo is 100 nM). Mix gently. Mix Lipofectamine™ 2000 gently before use, then dilute 1 µl in 50 µl Opti-MEM® I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature. After the 5-minute incubation, combine the diluted oligomer with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy). Add the oligomer-Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth. Incubate the cells at 37°C in a CO₂ incubator for 24-96 hours until you are ready to assay for gene knockdown. Medium may be changed after 4-6 hours. 								
Optimizing Transfection	<p>To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions as described below.</p> <ul style="list-style-type: none"> Use a range of 0.5 µl to 1.5 µl Lipofectamine™ 2000 Reagent for 24-well format. Use a range of 20-100 nM Stealth™ RNAi or siRNA for 24-well format. Depending on the nature of the target gene, you may transfect cells at higher densities. 								

Kit Contents and Storage

Shipping and Storage

The shipping conditions for each component are listed in the table below. Upon receipt, store the components as described below.

Components	Shipping	Storage
Lipofectamine™ 2000 Reagent	Blue ice	4°C (do not freeze)
BLOCK-iT™ Fluorescent Oligo	Blue ice	-20°C (protect from light)
Stealth™ RNAi Negative Control Duplex (Medium GC)	Dry ice	-20°C
Opti-MEM® I Reduced Serum Medium	Room temperature	2°C to 8°C (in the dark)

Kit Contents

The components included with the BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology are described below.

Box	Item	Composition	Amount
1	Lipofectamine™ 2000 Reagent	Proprietary formulation	0.75 ml
2-3	BLOCK-iT™ Fluorescent Oligo	20 µM stock of fluorescein-labeled double-stranded RNA (dsRNA) oligomer in: 100 mM potassium acetate 30 mM HEPES-KOH, pH 7.4 2 mM Magnesium acetate	2 x 125 µl
4	Stealth™ RNAi Negative Control Duplex (Medium GC)	20 µM in 1X RNA Annealing/Dilution Buffer	250 µl
	1X RNA Annealing/Dilution Buffer	10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, pH 8.0	1 ml
5	Opti-MEM® I Reduced Serum Medium	See below for formulation	100 ml

Formulation of Opti-MEM® I Reduced Serum Medium

Opti-MEM® I Reduced Serum Medium is a modification of Eagle's Minimal Essential Medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine or GLUTAMAX™, trace elements, and growth factors. The protein level is minimal (15 µg/ml); insulin and transferrin are the only protein supplements. Phenol red is included at a reduced concentration as a pH indicator.



Note

- Some reagents in the unit may be provided in excess of the amount needed.
- Individual documentation detailing general use are included with some of the products supplied in the BioModule™ Transfection and Control Unit. **To use the products specifically with the BioModule™ Transfection and Control Unit, follow the recommended protocols in this manual.**

Introduction

Overview

Introduction

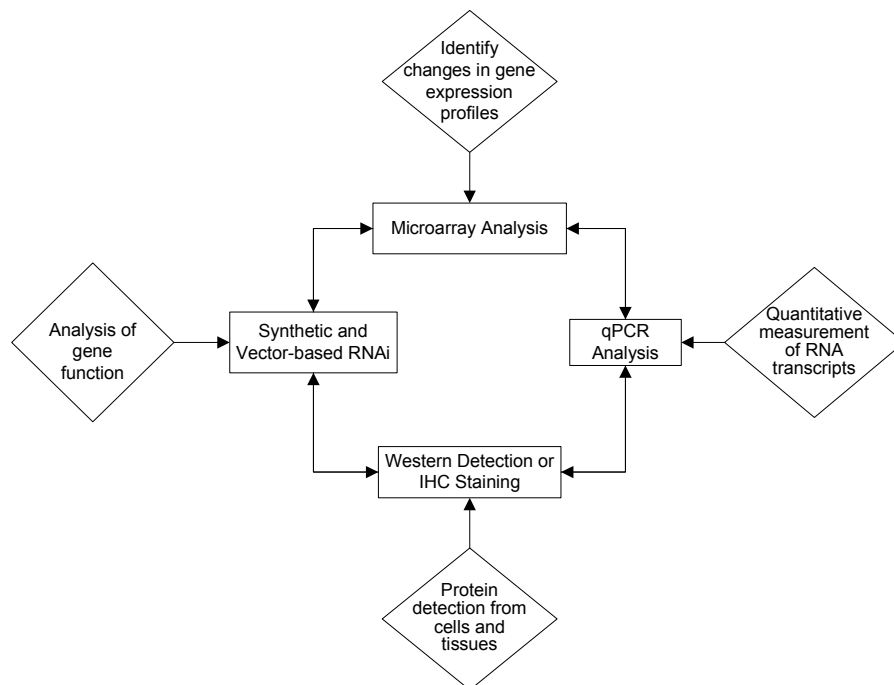
The BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology provides qualified reagents and validated protocols to deliver Stealth™ RNAi (see page 3 for details) or siRNA (short interfering RNA) molecules into mammalian cells for RNA interference (RNAi) studies. The BioModule™ Transfection and Control Unit provides reagents and controls required to perform transfection and assess transfection efficiency for RNAi analysis in mammalian cell lines.

The BLOCK-iT™ Technology is a next generation RNAi technology employing synthetic Stealth™ RNAi or expression vectors containing an RNAi cassette to perform RNAi studies in mammalian cells. A variety of RNAi products with BLOCK-iT™ Technology are available from Invitrogen to facilitate RNAi analysis. For more information, visit the RNAi Central portal at www.invitrogen.com/rnai or contact Technical Service (page 24).

BioModule™ Units for Gene Expression Profiling

The BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology is one of the several BioModule™ Units available from Invitrogen (page 26) for gene expression profiling. Each of the BioModule™ Units for gene expression profiling includes high-quality reagents and validated protocols with relevant controls for each step of the workflow (see below). Each unit is designed to provide an integrated workflow that allows you to perform various steps seamlessly during expression analysis.

Gene expression profiling comprises multiple steps employing various technologies such as microarray analysis or quantitative PCR (qPCR) for analysis at the nucleic acid level; western immunodetection and immunohistochemistry for analysis at the protein level; and RNAi for functional analysis.



Continued on next page

Overview, Continued

System Components

The BioModule™ Transfection and Control Unit includes:

- Lipofectamine™ 2000 Reagent for highly efficient delivery of dsRNA oligomers into a wide variety of mammalian cells
- BLOCK-iT™ Fluorescent Oligo, a fluorescein-labeled double-stranded RNA (dsRNA) oligomer for use as an indicator of transfection efficiency in RNAi experiments with Stealth™ RNA or siRNA
- Stealth™ RNAi Negative Control Duplex (Medium GC) for use as a negative control for the RNAi response
- Opti-MEM® I Reduced Serum Medium is a multi-purpose, proven medium used for diluting the Lipofectamine™ 2000 Reagent and nucleic acid during transfection. The medium is also useful in reducing serum requirements for a wide variety of cell lines and applications, and has been effective in the growth and maintenance of adherent and non-adherent cell lines
- 1X RNA Annealing/Dilution Buffer for use in diluting RNAi molecules for transfection, if necessary

For more information about each component, see page 5.

System Overview

To use the BioModule™ Transfection and Control Unit, you will:

- Obtain synthetic Stealth™ RNAi or siRNA duplex to the target gene of interest
 - Transfect mammalian cell line of choice with the Stealth™ RNAi or siRNA using Lipofectamine™ 2000 Reagent and Opti-MEM® I Reduced Serum Medium
 - Assay for gene knockdown using an assay of choice
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Overview, Continued

Uses for the BioModule™ Transfection and Control Unit

Use the BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology in your RNAi experiments for the following purposes:

- Use Lipofectamine™ 2000 Reagent for highly efficient delivery of dsRNA oligomers including the BLOCK-iT™ Fluorescent Oligo and Stealth™ RNAi or standard siRNA into your mammalian cell line of interest for RNAi analysis.
- Include the BLOCK-iT™ Fluorescent Oligo in your RNAi experiments to help you optimize your transfection conditions and to assess transfection efficiency in your mammalian cell line. Once you have optimized your transfection conditions, include the BLOCK-iT™ Fluorescent Oligo in every RNAi experiment as an indicator of transfection efficiency.
- If you are transfecting RNAi molecules into a mammalian cell line of interest for the first time, use the reagents provided to help you optimize your transfection conditions.

Stealth™ RNAi

Stealth™ RNAi is chemically modified, blunt end dsRNA developed to overcome the limitations of traditional siRNA. The two strands of Stealth™ RNAi are modified in a manner that prevents sense strand activity, eliminating sense strand mediated-off-target activity. The Stealth™ RNAi interacts with the RNAi machinery to elicit gene silencing, similar to traditional siRNA. See next page for a description of the RNAi pathway.

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 non-specific effects caused by induction of cellular stress response pathways
- Exhibits enhanced stability for greater flexibility in RNAi analysis

A large variety of ready-to-order, pre-designed Stealth™ RNAi products are available from Invitrogen to facilitate RNAi analysis (page 27) or you may design and order custom Stealth™ RNAi for any target gene of interest using the BLOCK-iT™ RNAi Designer from Invitrogen (www.invitrogen.com/rnaidesigner).

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Overview, Continued

RNAi Pathway

RNAi describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et al.*, 1994; Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990) and quelling in fungi (Cogoni & Macino, 1997; Cogoni & Macino, 1999; Romano & Macino, 1992). In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li & Ding, 2001; Voinnet *et al.*, 1999).

In eukaryotic organisms, dsRNA produced *in vivo* or introduced by pathogens is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001). The siRNA is then incorporated into an RNA-induced silencing complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation (Hammond *et al.*, 2000; Nykanen *et al.*, 2001). In addition to dsRNA, other endogenous RNA molecules including short temporal RNA (stRNA) and micro RNA (miRNA) (Ambros, 2001; Carrington & Ambros, 2003) have been identified and shown to be capable of triggering gene silencing.

For more information about the RNAi pathway, refer to recent reviews (Bosher & Labouesse, 2000; Dykxhoorn *et al.*, 2003; Hannon, 2002; Plasterk & Ketting, 2000; Zamore, 2001).

Purpose of this Manual

This manual provides the following information:

- An overview of Stealth™ RNAi and the RNAi pathway
- General guidelines for transfection and performing expression/screening assays
- Transfection protocol for transfecting the Stealth™ RNAi or siRNA duplex into mammalian cells for RNAi analysis
- Example of expected results
- Troubleshooting

Protocols for screening or expression assays are not included in this manual.

Description of Components

Introduction

Brief description of the components included with the BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology is described in this section.

Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 Reagent is a proprietary formulation for the delivery of the BLOCK-iT™ Fluorescent Oligo and Stealth™ RNAi or standard siRNA oligomers 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:

- Highest transfection efficiency in many cell types and formats (e.g. 96-well). Refer to the Cell Lines database at www.invitrogen.com for a list of cell types successfully transfected.
 - Nucleic acid-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium, in the presence or absence of serum.
 - It is not necessary to remove complexes or change/add medium after transfection, but complexes may be removed after 4-6 hours.
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Stealth™ RNAi Negative Control Duplex

Stealth™ RNAi Negative Control Duplex (Medium GC) is ideal for use in RNA interference (RNAi) experiments as a control for sequence independent effects following Stealth™ RNAi delivery in any vertebrate cell line.

The Stealth™ RNAi Negative Control Duplex is designed to minimize sequence homology to any known vertebrate transcript, does not induce the interferon-mediated stress response pathways as measured by real-time quantitative RT-PCR, and demonstrates minimal knockdown of vertebrate target genes. The Stealth™ RNAi Negative Control Duplex (Medium GC) contains 48% GC content and is suitable for use with Stealth™ RNAi duplexes containing 45-55% GC.

The Stealth™ RNAi Negative Control Duplex is supplied in a ready-to-use format.

BLOCK-iT™ Fluorescent Oligo

The BLOCK-iT™ Fluorescent Oligo is a fluorescein-labeled dsRNA oligomer designed for use in RNAi analysis to facilitate assessment and optimization of cationic lipid-mediated delivery or electroporation of dsRNA oligonucleotides into mammalian cells.

Using the BLOCK-iT™ Fluorescent Oligo in RNAi studies offers the following advantages:

- Provides a good indication of the transfection efficiency with Invitrogen's Stealth™ RNAi, standard unmodified siRNA, or purified Dicer-generated siRNA.
 - Allows strong, easy fluorescence-based indication of transfection efficiency in every RNAi experiment.
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Description of Components, Continued

Characteristics of the BLOCK-iT™ Fluorescent Oligo

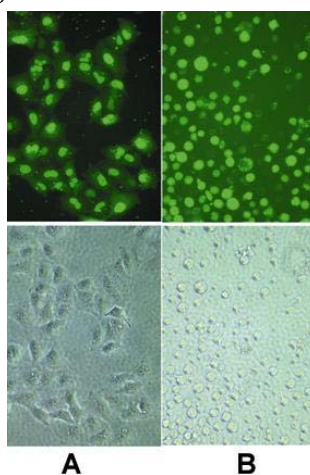
The BLOCK-iT™ Fluorescent Oligo possesses the following characteristics:

- 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 and growth rate of the cells.

- 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)).

For examples of fluorescent uptake in adherent (panel A) and suspension (panel B) cells, see the figure below.



Important: The BLOCK-iT™ Fluorescent Oligo is designed strictly for use as a tool for 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.

Continued on next page

Description of Components, Continued

Opti-MEM® I Reduced Serum Medium

Opti-MEM® I Reduced Serum Medium is a versatile, chemically-defined medium used for diluting the lipid and nucleic acid during transfection.

The Opti-MEM® I Reduced Serum Medium is a multi-purpose medium proven to be useful in reducing serum requirements for a wide variety of cell lines and applications and has been effective in the growth and maintenance of adherent and non-adherent cell lines. When supplemented with 2-4% fetal bovine serum or alternative sera, Opti-MEM® I Reduced Serum Medium supports proliferative rates and maximal cell densities comparable to, and in some cases superior to, conventional media supplemented with 10% fetal bovine serum. Relatively non-fastidious cell lines may be maintained in long-term culture with even more substantial serum reduction. If using adherent cell lines and less than 2% serum supplementation or in an agitated system such as in roller bottles, the medium should be further supplemented with 100 mg/l CaCl₂.

The versatility of Opti-MEM® I Reduced Serum Medium in the propagation of various cell types makes this medium the optimal choice for many cell culture requirements. For details on using the medium to reduce serum requirements for various cell lines, download the Opti-MEM® I Reduced Serum Medium manual from www.invitrogen.com.

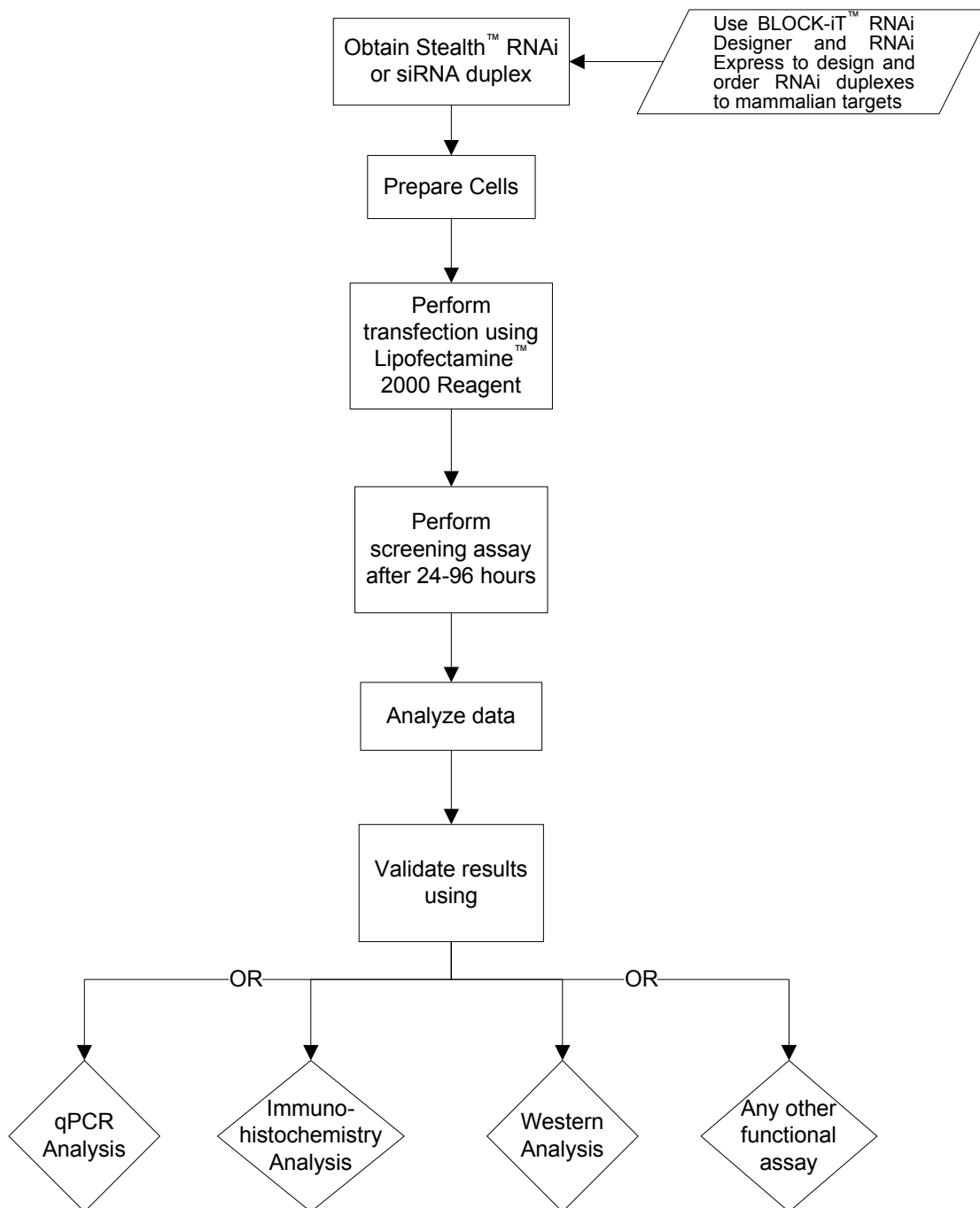
1X RNA Annealing/Dilution Buffer

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 requires optimization. When optimizing your transfection conditions, use the 1X RNA Annealing/Dilution Buffer to dilute the RNAi reagent stock solution, if needed.

Experimental Overview

Workflow

The experimental workflow for using the BioModule™ Transfection and Control Unit BLOCK-iT™ Technology is shown below.



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Experimental Overview, Continued

Materials Needed

You will need the following materials:

- Stealth™ RNAi or siRNA duplex of choice (supplied by the user, see page 10 for details)
 - Lipofectamine™ 2000 Reagent (supplied with the kit)
 - Opti-MEM® I Reduced Serum Medium (supplied with the kit)
 - BLOCK-iT™ Fluorescent Oligo (supplied with the kit)
 - Stealth™ RNAi Negative Control Duplex, medium GC (supplied with the kit)
 - Mammalian cell line of choice (supplied by the user)
 - Cell culture media and cell culture plates (supplied by the user)
-

Methods

General Guidelines

Introduction

General guidelines for using the BioModule™ Transfection and Control Unit are described in this section. Review the information in this section prior to performing the transfection and screening experiments to obtain the best results.



Note

To use the BioModule™ Transfection and Control Unit effectively, we recommend that you have a working knowledge of the RNAi pathway, designing appropriate screening assays, performing transfections and screening assays, and analyzing screening assay data to identify significant hits.

RNAi Molecules

To use the BioModule™ Transfection and Control Unit, you need to synthesize or purchase Stealth™ RNAi or siRNA molecules to your target gene of interest for performing RNAi analysis.

To obtain highly specific, effective gene knockdown and eliminate non-specific effects, we recommend that you use Stealth™ RNAi. For more information on Stealth™ RNAi, see page 3.

To obtain RNAi molecules, you may:

- Purchase pre-designed Stealth™ RNAi (see next page)
Choose from a large selection of pre-designed Stealth™ RNAi from Invitrogen.
- Synthesize Stealth™ RNAi or siRNA duplexes

If a Stealth™ RNAi molecule of choice is not available from our collection of pre-designed Stealth™ RNAi, use the BLOCK-iT™ RNAi Designer, an online tool from Invitrogen (www.invitrogen.com/rnaidesigner), to help you design and order custom Stealth™ RNAi or traditional, unmodified siRNA molecules for any target gene of interest. The RNAi Designer incorporates published rules on RNAi design into a proprietary algorithm to design most effective RNAi sequences to obtain high level gene knockdown.

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General Guidelines, Continued

Stealth™ RNAi Products

A large variety of ready-to-order, pre-designed Stealth™ RNAi products are available from Invitrogen to facilitate RNAi analysis.

Use the BLOCK-iT™ RNAi Express Search Engine (www.invitrogen.com/RNAiExpress) to search for a Stealth™ RNAi duplex of choice by gene name, GenBank accession number, or keyword. The search results are linked directly to the ordering page, allowing you to easily order the RNAi molecule of choice.

Validated Stealth™ RNAi

The Validated Stealth™ RNAi DuoPak includes two highly effective, **functionally tested** Stealth™ RNAi molecules designed for *in vitro* and *in vivo* RNAi analysis of a particular human target gene. Each Validated Stealth™ RNAi duplex is supplied in a ready-to-use format and targets a different region of the human gene of interest, demonstrating ≥ 80% target gene knockdown.

Stealth™ Select RNAi

The Stealth™ Select RNAi are highly effective pre-designed Stealth™ RNAi molecules available individually or as a set of three non-overlapping sequences to thousands of human, mouse, or rat genes. For each set of three Stealth™ Select RNAi, 2 out of 3 RNAi sequences are guaranteed to knockdown transcript levels by at least 70%.

Stealth™ RNAi Collections

The Stealth™ RNAi Collections combine the power of chemical modification with advanced *in silico* design algorithms to provide you with an advanced RNAi collection suited for RNAi studies. A large variety of Stealth™ RNAi Collections are available from Invitrogen.

Each Stealth™ RNAi Collection is composed of three, non-overlapping Stealth™ RNAi duplexes designed to target human or mouse genes that are arrayed in 96-well format for high-throughput gene silencing screening experiments.

Cell Lines

Stealth™ RNAi is designed for use with **species specific cell lines** only. Using non-species specific cell lines will not produce the desired level of target gene knockdown.

For example, use the Stealth™ RNAi designed for human targets with human cell lines only. Using mouse or rat cell lines will not produce the desired level of target gene knockdown.

Transfection

The BioModule™ Transfection and Control Unit includes Lipofectamine™ 2000 Reagent for delivery of dsRNA oligomers to mammalian cells for transfection.

An optimized transfection protocol for mammalian cells is described on page 17. However, depending on your cell type, you may need to optimize the transfection conditions such as cell number, amount of the transfection reagent and RNAi, and the time period to assay for target gene knockdown to obtain the best results. Cell-type specific RNAi transfection protocols are also available at www.invitrogen.com/rnai.

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General Guidelines, Continued

Factors Affecting Gene Knockdown Levels

A number of factors can influence the degree to which expression of your gene of interest is reduced (*i.e.* gene knockdown) in an RNAi experiment including:

- Transfection efficiency
- Transcription rate of the target gene of interest
- Stability of the target protein
- Growth characteristics of your mammalian cell line

Take these factors into account when designing your RNAi experiments.

Performing an Assay for Target Gene Knockdown

To validate your Stealth™ RNAi or siRNA, you must measure the effect of the RNAi molecule on the target mRNA using a screening assay. A screening assay usually measures the target mRNA expression or target gene expression depending on the assay.

Examples of screening assays include:

- Cell proliferation assays using the CyQUANT® Cell Proliferation Assay Kit (page 26)
- Quantitative RT-PCR (qRT-PCR)
- Signal transduction pathway analysis using CellSensor™ Cell Lines (page 27)
- Reporter based target screening system such as the BLOCK-iT™ RNAi Target Screening System with *lacZ* reporter (page 26)
- Protein expression assay using enzyme activity, immunocytochemistry, immunohistochemistry, western blotting, or ELISA

Prior to performing RNAi experiments, select a suitable screening assay to measure gene knockdown and optimize the assay to obtain the best results. Choose a screening assay with the following criteria:

- High sensitivity
- Low background
- Signal stability
- Fast, efficient, easy to perform

If you are measuring protein levels to analyze the Stealth™ RNAi or siRNA-mediated inhibition, any pre-existing pool of the protein must be degraded. If the protein of interest has a long half-life, you may need to perform long-term transfection experiments (*i.e.* perform multiple cycles of transfection) to observe effects at the protein level.

Note: The Stealth™ RNAi or siRNA may be effective at decreasing mRNA levels of the target gene; however, may not affect protein levels if the target protein has a long half-life. If possible, measure mRNA and protein level to confirm the effect of Stealth™ RNAi or siRNA on gene knockdown.

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General Guidelines, Continued

Appropriate Controls

When performing RNAi analysis, it is important to include proper positive and negative controls to help evaluate your results.

The BLOCK-iT[™] Fluorescent Oligo and Stealth[™] RNAi Negative Control Duplex are included in the BioModule[™] Transfection and Control Unit for your convenience. Use the BLOCK-iT[™] Fluorescent Oligo as an indicator of transfection efficiency with Stealth[™] RNAi or siRNA using any fluorescence microscope with a standard FITC filter set.

Be sure to include your assay specific controls and controls for mock or untransfected cells.

Transfecting Cells

Introduction



This section provides guidelines to transfect the Stealth™ RNAi or siRNA into the mammalian cell line of interest to perform RNAi analysis.

- We recommend Opti-MEM® I Reduced Serum Medium to dilute Lipofectamine™ 2000 Reagent and RNAi duplexes before complexing.
 - **Do not** add antibiotics to media during transfection as this causes cell death.
 - Use low-passage cells, and make sure that cells are healthy and greater than 90% viable before transfection.
 - Maintain the same seeding conditions between experiments.
 - **Transfect cells at 30-50% confluence.** 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.
 - To increase accuracy and reduce assay variability, we recommend performing **triplicate** transfections for each sample condition.
 - We recommend assessing BLOCK-iT™ Fluorescent Oligo uptake at 6-24 hours post-transfection; however, assay target gene knockdown levels (following Stealth™ RNAi or siRNA delivery) at a minimum of 24-72 hours following transfection. You may also assay for target gene knockdown at various time points such as 24, 48, 72, 96 hours post-transfection.
 - Determine the appropriate amount of BLOCK-iT™ Fluorescent Oligo to use such that fluorescence signal is readily detectable. For recommended reagent amounts to use, see page 17.
- Note:** Once you have determined optimal transfection conditions using the BLOCK-iT™ Fluorescent Oligo, you may use these conditions as a starting point to transfect Stealth™ RNAi or siRNA, with optimization as necessary.
- Test serum-free media for compatibility with Lipofectamine™ 2000 Reagent since some serum-free formulations (e.g. CD293, SFM II, VP-SFM) may inhibit cationic lipid-mediated transfection.

Handling the Stealth™ RNAi Negative Control Duplex

- Thaw Stealth™ RNAi Negative Control Duplex stock solution on ice or at room temperature. After use, return to -20°C storage.
- Multiple freeze/thaw cycles are permitted without loss of activity if stock solution is handled properly.
- Ensure that the stock solution does not become contaminated with RNase.

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Transfecting Cells, Continued

Handling the BLOCK-iT™ Fluorescent Oligo

The BLOCK-iT™ Fluorescent Oligo is supplied as a 20 μ M stock solution in an annealing buffer. Follow the guidelines below when handling the BLOCK-iT™ Fluorescent Oligo stock solution.

- The BLOCK-iT™ Fluorescent Oligo is **light sensitive**. Store the stock solution at -20°C, protected from light. The stock solution is stable for at least 6 months if stored properly.
 - 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.
 - The stock solution may be frozen and thawed multiple times without loss of fluorescence signal if handled properly.
 - 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.
-

Using the BLOCK-iT™ Fluorescent Oligo

Use the BLOCK-iT™ Fluorescent Oligo with Lipofectamine™ 2000 Reagent for delivery of Stealth™ RNAi or siRNA to mammalian cells. Follow the guidelines below when transfecting the BLOCK-iT™ Fluorescent Oligo.

- The amount of BLOCK-iT™ Fluorescent Oligo to use depends on the growth rate and transfection efficiency of the mammalian cells. If you are transfecting your mammalian cell line for the first time, we recommend evaluating several concentrations of lipid and varying the final concentration of the BLOCK-iT™ Fluorescent Oligo from **10 to 200 nM** to determine the optimal amount of BLOCK-iT™ Fluorescent Oligo to use to obtain a strong fluorescence signal.

Note: For most cell lines tested (*e.g.* HEK293, A549, HeLa), we obtain a readily detectable fluorescence signal when using 100 nM BLOCK-iT™ Fluorescent Oligo for transfection.
 - Prepare lipid-BLOCK-iT™ Fluorescent Oligo complexes as directed on page 17. Always dilute the BLOCK-iT™ Fluorescent Oligo immediately before transfection (*i.e.* do not store diluted Oligo) into Opti-MEM® I Reduced Serum Medium.
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Transfecting Cells, Continued

Amount of RNAi to Transfect

The amount of Stealth™ RNAi or siRNA duplex, BLOCK-iT™ Fluorescent Oligo, or the Stealth™ RNAi Negative Control Duplex required to achieve optimal target gene knockdown or minimal knockdown, as appropriate, should be determined experimentally for each mammalian cell line.

The recommended starting amount of each duplex used for transfection is listed in the table below:

<u>Duplex</u>	<u>Recommended Conc.</u>
Stealth™ RNAi or siRNA	40 nM
Stealth™ RNAi Negative Control Duplex	40 nM
BLOCK-iT™ Fluorescent Oligo	100 nM

Based on the initial results, you may need to optimize transfection conditions as described on page 18.

Materials Needed

You will need the following materials:

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

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Transfecting Cells, Continued



Note

- Use the transfection procedure described below as a starting point; optimize transfections as described in **Optimizing Transfection** (page 18), especially if you are transfecting a mammalian cell line for the first time.
- To reduce well-to-well variability when transfecting multiple replicates (e.g. triplicates), proportionally scale up the reagent volumes to form complexes (Step 2, below), then aliquot an equal volume of complexes into each well.
- Prepare Stealth™ RNAi or siRNA duplex at a concentration of 20 µM in 1X RNA Annealing Buffer. 20 µM Stealth™ RNAi or siRNA = 20 pmol/µl.

Transfection Protocol

Use this procedure to transfect Stealth™ RNAi or siRNA into mammalian cells in a **24-well format**. All amounts and volumes are given on a per well basis.

For other formats, see **Scaling Up or Down Transfections** (page 18).

1. One day before transfection, plate cells in 500 µl of growth medium without antibiotics such that they will be **30-50% confluent** at the time of transfection.
Note: Transfecting cells at a lower density allows a longer interval between transfection and assay time, and minimizes the loss of cell viability due to cell overgrowth.
2. **For each transfection sample**, prepare oligomer-Lipofectamine™ 2000 complexes as follows:
 - a. Dilute 20 pmol Stealth™ RNAi or siRNA oligomer (*i.e.* 1 µl of 20 µM Stealth™ RNAi or siRNA oligomer) in 50 µl Opti-MEM® I Reduced Serum Medium without serum (resulting concentration of RNA is 40 nM). Mix gently.
 - b. Dilute 60 pmol BLOCK-iT™ Fluorescent Oligo (*i.e.* 3 µl of 20 µM Fluorescent Oligo) in 50 µl Opti-MEM® I Reduced Serum Medium without serum (resulting concentration of the Fluorescent Oligo is 100 nM). Mix gently.
 - c. Mix Lipofectamine™ 2000 gently before use, then dilute 1 µl in 50 µl Opti-MEM® I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature. **Note:** Combine diluted Lipofectamine™ 2000 with diluted RNA within 30 minutes.
 - d. After the 5-minute incubation, combine the diluted oligomer with the diluted Lipofectamine™ 2000 (total volume is ~100 µl). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
3. Add the 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 CO₂ incubator for 24-96 hours until you are ready to assay for target gene knockdown. Assess BLOCK-iT™ Fluorescent Oligo uptake at 6-24 hours post-transfection. Medium may be changed after 4-6 hours.

An example of gene knockdown results are shown on page 20.

Continued on next page

Transfecting Cells, Continued

Optimizing Transfection

To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying RNA and Lipofectamine™ 2000 Reagent concentrations.

- 1 µl Lipofectamine™ 2000 Reagent per well is recommended as a starting point but optimization of transfection conditions may be required. A range of 0.5 µl to 1.5 µl Lipofectamine™ 2000 Reagent is recommended for 24-well format.
- 40 nM Stealth™ RNAi is recommended as a starting point but you can use a range from 20-100 nM Stealth™ RNAi for 24-well format.
- Depending on the nature of the target gene, transfecting cells at higher densities may also be considered when optimizing conditions.
- Note that for RNAi molecules inducing >90% target knockdown, the amount of dsRNA required to obtain effective knockdown may be less than the amount specified. This needs to be determined empirically for each cell line.

Scaling Up or Down Transfections

To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine™ 2000, nucleic acid, cells, and medium used in proportion to the relative surface area, as shown in the table.

With automated, high-throughput systems, a complexing volume of 50 µl is recommended for transfections in 96-well plates.

Tip: 20 µM Stealth™ RNAi or siRNA = 20 pmol/µl.

Note: You may perform rapid 96-well plate transfections by plating cells directly into the transfection mix. Prepare complexes in the plate and directly add cells at twice the cell density as in the basic protocol in a 100 µl volume. Cells will adhere as usual in the presence of complexes.

Culture vessel	Surface Area per well ¹	Volume of plating medium	RNAi (pmol) in media volume (µl)	Lipofectamine™ 2000 (µl) in media volume (µl)
96-well	0.3 cm ²	100 µl	5 pmol in 25 µl	0.25 µl in 25 µl
24-well	2 cm ²	500 µl	20 pmol in 50 µl	1.0 µl in 50 µl
12-well	4 cm ²	1 ml	40 pmol in 100 µl	2.0 µl in 100 µl
6-well	10 cm ²	2 ml	100 pmol in 250 µl	5 µl in 250 µl
60-mm	20 cm ²	5 ml	200 pmol in 0.5 ml	10 µl in 0.5 ml
10-cm	60 cm ²	15 ml	600 pmol in 1.5 ml	30 µl in 1.5 ml

¹Surface areas may vary depending on the manufacturer.

Continued on next page

Transfecting Cells, Continued

Detecting Fluorescence

If you have transfected your mammalian cells with the BLOCK-iT[™] Fluorescent Oligo, you may qualitatively assess Oligo uptake in live cells 6-24 hours post-transfection using fluorescence microscopy. You may use any type of fluorescence microscope and a standard FITC filter set ($\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 519 \text{ nm}$ green) for detection.

An example of expected results is shown on page 20.

The Next Step

Once you have assessed the gene knockdown, we recommend that you validate the results using additional methods such as qPCR, Western analysis, immunohistochemistry, or any other functional assay.

A variety of BioModule[™] Units that include qualified reagents and validated protocols are available from Invitrogen to perform further validation experiments (page 26).

Expected Results

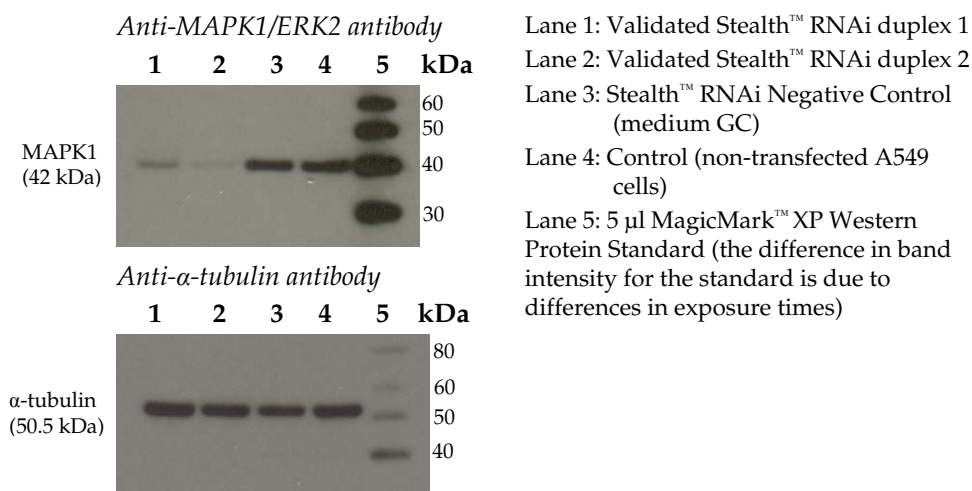
Example of Results

An example of results obtained after performing an RNAi experiment using the BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology and validation using western immunodetection with the BioModule™ Western Analysis are shown below.

In this experiment, A549 cells were transfected with MAPK1 Validated Stealth™ RNAi Duopak (2 different duplexes) targeting MAP Kinase 1 (MAPK1) available from Invitrogen (cat. no. 12935-025) or with Stealth™ RNAi Negative Control (medium GC) using Lipofectamine™ 2000 Reagent as described in this manual. Control transfection experiments were performed with BLOCK-iT™ Fluorescent Oligo (see next page for results).

48 hours post-transfection, cells were harvested and lysed with Cell Extraction Buffer containing the Protease Inhibitor Cocktail. The lysate (8 µg) was analyzed on a NuPAGE® Novex Bis-Tris Gel and proteins were transferred onto an Invitrolon™ PVDF membrane. The blots were analyzed using the WesternBreeze® Chemiluminescent Kit using a polyclonal rabbit anti-MAPK1/ERK2 antibody (cat. no. 71-1800) at 1:1500 dilution or a monoclonal mouse anti-α-tubulin antibody (cat. no. 32-2500) at a 1:2000 dilution.

The results indicate effective knockdown of MAPK1 by each Validated Stealth™ RNAi targeting MAPK1.



Continued on next page

Expected Results, Continued

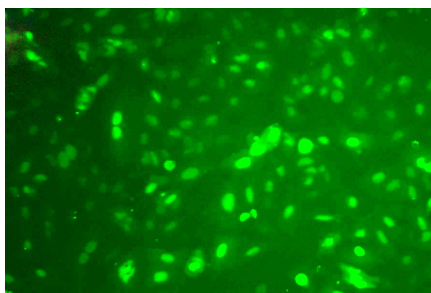
BLOCK-iT™ Fluorescent Oligo Results

An example of results obtained after transfection of BLOCK-iT™ Fluorescent Oligo into A549 cells are shown below.

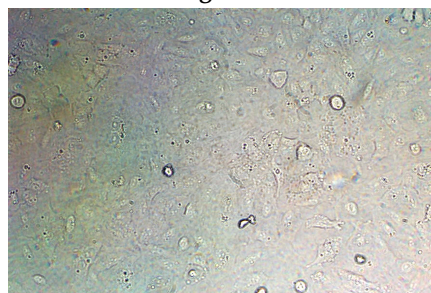
A549 cells were plated at 8×10^4 cells/well in a 12-well plate. The cells were transfected with 100 nM BLOCK-iT™ Fluorescent Oligo using 2 μ l Lipofectamine™ 2000 Reagent as described in this manual. Cells were visualized by fluorescence microscopy at 24 hours post-transfection using the appropriate filters as described in this manual.

Fluorescence microscopy results are shown below and demonstrate the presence of BLOCK-iT™ Fluorescent Oligo in virtually all cells, indicating good transfection efficiency.

Fluorescent



Brightfield



Troubleshooting

Introduction

Review the information in this section to troubleshoot your transfection and knockdown experiments with the BioModule™ Transfection and Control Unit.

Problem	Reason	Solution
Low knockdown of the target gene observed	<ul style="list-style-type: none"> Low transfection efficiency Expression assay not performed correctly 	<ul style="list-style-type: none"> See below Be sure the expression assay was performed correctly. See page 12 for guidelines.
Low levels of gene knockdown observed due to low transfection efficiency	<ul style="list-style-type: none"> Antibiotics added to the media during transfection Cells too sparse at the time of transfection Not enough RNAi duplex transfected Not enough transfection reagent used 	<ul style="list-style-type: none"> 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 RNAi duplex transfected (page 18). Optimize the transfection conditions for your cell line by varying the amount of transfection reagent used. Be sure to assess the transfection efficiency using BLOCK-iT™ Fluorescent Oligo.
Low levels of gene knockdown observed (other causes)	Did not wait long enough after transfection before assaying for gene knockdown	<p>Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown.</p> <p>Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs.</p>
	Cells not healthy or used non-species specific cell lines	Be sure to use healthy, >90% viable cells. Use species specific cell lines to obtain the best results.
	Lipofectamine™ 2000 Reagent handled incorrectly	Store the reagent at 4°C. Do not freeze. Mix gently by inversion before use. Do not vortex.
	Target protein is stable (<i>i.e.</i> has a long half-life)	Perform qRT-PCR analysis using primers to assay for target gene knockdown at the mRNA level.

Continued on next page

Troubleshooting, Continued

Problem	Reason	Solution
No gene knockdown observed	Used a cell line that does not express the target gene	Be sure to use a cell line that expresses the target gene.
	siRNA was not active	Design a siRNA to a different target region or convert the siRNA to a Stealth™ RNAi molecule and repeat the RNAi analysis.
Cytotoxic effects observed after transfection	Transfection of siRNA activates toxic pathways that result in cell death	<ul style="list-style-type: none"> Convert the siRNA to a Stealth™ RNAi molecule and repeat the RNAi analysis. Test another target sequence to the same gene to confirm the toxicity response.
	Lipofectamine™ 2000 Reagent may have cytotoxic effects	Optimize the transfection conditions for your cell line by decreasing or varying the amount of Lipofectamine™ 2000 Reagent used. Use the BLOCK-iT™ Fluorescent Oligo to help you optimize transfection conditions for your cell line.
	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.
Non-specific effects	siRNA targets regions with homology to other genes	Convert the siRNA to a Stealth™ RNAi molecule and repeat the RNAi analysis.
No fluorescence signal detected with BLOCK-iT™ Fluorescent Oligo	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter sets for detection of fluorescence (page 19) and use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for additional days before assaying for fluorescence.
	Low transfection efficiency	See previous page for details.

Appendix

Technical Service

Web Resources



Visit the Invitrogen Web site at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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Material Data Safety Sheets (MSDSs)

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Product Qualification

Introduction

Invitrogen qualifies the BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology components as described below.

Lipofectamine™ 2000 Reagent

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

Stealth™ RNAi Negative Control Duplex

The Stealth RNAi Negative Control Duplex is qualified as follows:

- The identity and concentration of each corresponding single-stranded RNA oligo is verified by mass spectrometry and optical density reading, respectively.
 - After annealing, the Stealth™ RNAi Negative Control Duplex is analyzed by gel electrophoresis to verify its integrity and to confirm the absence of RNA degradation.
-

BLOCK-iT™ Fluorescent Oligo

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

- The identity and concentration of each corresponding single-stranded RNA oligo is verified by mass spectrometry and optical density reading, respectively.
 - After annealing, the BLOCK-iT™ Fluorescent Oligo is analyzed by gel electrophoresis to verify its integrity and to confirm the absence of RNA degradation.
-

Opti-MEM® I Reduced Serum Medium

Opti-MEM® I Reduced Serum Medium is subjected to pH, osmolality, endotoxin, bacterial, fungal, and mycoplasma testing. The endotoxin level must be less than 1.0 EU/ml.

Each lot of Opti-MEM® I is evaluated utilizing sensitive quantitative assays for its ability to support cloning efficiency of a murine myeloma cell line, and growth over multiple subcultures of an adherent cell line. Test lots of Opti-MEM® I Reduced Serum Medium at 2% (CHO growth) and 4% (Sp2 cloning) serum supplementation are compared to a previously approved Opti-MEM® I Reduced Serum Medium control.

GIBCO® cell culture liquid products are prepared by an aseptic process for which each step has been validated to ensure that all products meet the industry standard sterility assurance level of 10^{-3} ; i.e., product that demonstrates a contamination level of no more than 1 of 1000 units during the manufacturing process. The highest level of sterility assurance (equal to or greater than 10^{-6}) cannot be achieved without terminal sterilization which is harmful to the performance of cell culture products.

Accessory Products

BioModule™ Units

Additional BioModule™ Units that can be used for validation experiments are available separately from Invitrogen. Ordering information is provided below. For more information, visit our web site at www.invitrogen.com or call Technical Service (page 24).

Product	Amount	Catalog no.
BioModule™ Western Analysis Unit		
<i>for chromogenic detection</i>	20 transfers	WFGE09
<i>for chemiluminescent detection</i>	20 transfers	WFGE10
BioModule™ qRT-PCR Unit		
	100 reactions	WFGE01
	1000 reactions	WFGE02
BioModule™ Immunohistochemical Staining (IHC) Unit for Tissue	150 slides	WFGE11
BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector	20 reactions	WFGE07
BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System	20 reactions	WFGE08
BioModule™ Microarray Unit		
<i>with indirect labeling</i>	15 arrays	WFGE03
<i>with direct labeling</i>	15 arrays	WFGE04

Additional Products

Additional reagents that may be used with the BioModule™ Transfection and Control Unit are available separately from Invitrogen. Ordering information is provided below. For more information, visit our web site at www.invitrogen.com or call Technical Service (page 24).

Product	Amount	Catalog no.
BLOCK-iT™ RNAi Target Screening System (w/ <i>lacZ</i> reporter)	20 reactions	K4916-00
Lipofectamine™ 2000 Reagent	0.75 ml	11668-027
	1.5 ml	11668-019
Stealth™ RNAi Negative Control Kit	1 kit	12935-100
Stealth™ RNAi Negative Control, Low GC	250 µl	12935-200
Stealth™ RNAi Negative Control, High GC	250 µl	12935-400
Opti-MEM® I Reduced Serum Medium	100 ml	31985-062
	500 ml	31985-070
CyQUANT® Cell Proliferation Assay Kit	1 kit	C7026

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Accessory Products, Continued

BLOCK-iT™ RNAi Designer

The BLOCK-iT™ RNAi Designer is an online tool (www.invitrogen.com/rnaidesigner) to help you design and order Stealth™ RNAi or siRNA molecules for any target gene of interest. The RNAi Designer incorporates published rules on RNAi design into a proprietary algorithm to design most effective RNAi sequences to obtain high level gene knockdown.

BLOCK-iT™ RNAi Products

A large variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis including Stealth™ RNAi, the Validated Stealth™ RNAi, Stealth™ RNAi Collection, and a large selection of RNAi vectors.

For details, visit the RNAi Central portal at www.invitrogen.com/rnai or contact Technical Service (see page 24).

CellSensor™ Cell Lines

CellSensor™ Cell Lines use the GeneBLAzer® Technology to provide you with a reliable, rapid, and sensitive method of analyzing the intracellular status of signal transduction pathways upon exposure to drug candidates or other stimuli. Each CellSensor™ Cell Line stably expresses a response element coupled to the beta-lactamase reporter. When pathways leading to the response element are activated or inhibited, beta-lactamase reporter activity is modulated and is measured with the GeneBLAzer® Loading Substrates.

Antibodies

A large variety of high-quality antibodies including the Zymed® Antibodies is available from Invitrogen for use in Western immunodetection, immunohistochemistry, or ELISA assays. For details, visit www.invitrogen.com or contact Technical Service (page 24).

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