BLOCK-iT™ Dicer RNAi Kits

For the generation, purification, and transfection of gene-specific d-siRNA for use in RNA interference (RNAi) analysis

Catalog nos. K3600-01 and K3650-01

Version B
18 November 2004
25-0662

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## d-siRNA Generation and Transfection Procedure for Experienced Users

### Introduction

This quick reference sheet is provided for experienced users of the d-siRNA generation and transfection procedure. If you are performing the dicing, purification, or transfection procedures for the first time, follow the detailed protocols provided in the manual.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Produce dsRNA</td>
<td>Follow the guidelines on page 7 to generate dsRNA. If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for instructions to generate dsRNA.</td>
</tr>
</tbody>
</table>
| Perform the dicing reaction   | 1. Set up the following dicing reaction.  
                                   | 10X Dicer Buffer 30 µl  
                                   | RNase-Free Water up to 210 µl  
                                   | Purified dsRNA (60 µg) 1-150 µl  
                                   | BLOCK-iT™ Dicer Enzyme (1U/µl) 60 µl  
                                   | Total volume 300 µl  
                                   | 2. Mix reaction gently and incubate for 14-18 hours at 37°C.  
                                   | 3. Add 6 µl of 50X Dicer Stop Solution.  
                                   | 4. Check integrity of the d-siRNA, if desired. Proceed to purify d-siRNA. |
| Purify d-siRNA                | 1. To each 300 µl dicing reaction, add 300 µl of RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol followed by 300 µl of isopropanol. Mix well by pipetting up and down 5 times.  
                                   | 2. Apply half the sample (~450 µl) to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. **Save the flow-through.**  
                                   | 3. Transfer the RNA Spin Cartridge to an siRNA Collection Tube and repeat Step 2, using the other half of the dicing reaction sample (~450 µl). **Save the flow-through.**  
                                   | 4. Transfer the flow-through from Step 2 to the siRNA Collection Tube containing the flow-through from Step 3. Add 600 µl of isopropanol and mix well by pipetting up and down 5 times.  
                                   | 5. Apply one-third of the sample (~500 µl) to a new RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.  
                                   | 6. Repeat Step 5 twice, applying one-third of the remaining sample (~500 µl) to the RNA Spin Cartridge each time.  
                                   | 7. Add 500 µl of 1X RNA Wash Buffer to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.  
                                   | 8. Repeat Step 7. Proceed to the next page. |

*continued on next page*
## d-siRNA Generation and Transfection Procedure for Experienced Users, continued

### Purify d-siRNA

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature.</td>
</tr>
<tr>
<td>2.</td>
<td>Remove the RNA Spin Cartridge from the Wash Tube and place it in an RNA Recovery Tube.</td>
</tr>
<tr>
<td>3.</td>
<td>Add 30 µl of RNase-Free Water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the d-siRNA.</td>
</tr>
<tr>
<td>4.</td>
<td>Repeat Step 1, eluting the d-siRNA into the same RNA Recovery Tube.</td>
</tr>
<tr>
<td>5.</td>
<td>Add 1.2 µl of 50X RNA Annealing Buffer to the eluted d-siRNA.</td>
</tr>
<tr>
<td>6.</td>
<td>Quantitate the yield of d-siRNA by spectrophotometry. Aliquot and store the d-siRNA at -80°C.</td>
</tr>
</tbody>
</table>

### Transfect d-siRNA

Follow the procedure below to transfect cells using Lipofectamine™ 2000. Refer to the table on page 21 for the appropriate reagent amounts and volumes to add for different tissue culture formats.

1. One day before transfection, plate cells in growth medium without antibiotics such that they will be 30-50% confluent at the time of transfection.
2. For each transfection sample, prepare d-siRNA:Lipofectamine™ 2000 complexes as follows:
   a. Dilute d-siRNA 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. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5-minute incubation, combine the diluted d-siRNA with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature.
3. Add the d-siRNA: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 until they are ready to assay for gene knockdown.

### Control Reaction

If you have purchased the BLOCK-iT™ Complete Dicer RNAi Kit, we recommend using the control template and control PCR primers included with the kit to produce dsRNA (see the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for details). Once you have produced dsRNA, use this dsRNA as a control in your dicing, purification, and transfection experiments.
Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Note: The BLOCK-iT™ Complete Dicer RNAi Kit is also supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit and the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

<table>
<thead>
<tr>
<th>Product</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOCK-iT™ Dicer RNAi Transfection Kit</td>
<td>K3600-01</td>
</tr>
<tr>
<td>BLOCK-iT™ Complete Dicer RNAi Kit</td>
<td>K3650-01</td>
</tr>
</tbody>
</table>

Kit Components

The BLOCK-iT™ Dicer RNAi Kits include the following components. For a detailed description of the contents of each component, see pages viii-ix. For a detailed description of the contents of the BLOCK-iT™ RNAi TOPO® Transcription Kit, see the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

<table>
<thead>
<tr>
<th>Component</th>
<th>Catalog no.</th>
<th>K3600-01</th>
<th>K3650-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOCK-iT™ Dicer Enzyme Kit</td>
<td></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>BLOCK-iT™ RNAi Purification Kit</td>
<td></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Lipofectamine™ 2000 Reagent</td>
<td></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>BLOCK-iT™ RNAi TOPO® Transcription Kit</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shipping/Storage

The BLOCK-iT™ Dicer RNAi Kits are shipped as described below. Upon receipt, store each item as detailed below. For more detailed information about the reagents supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

<table>
<thead>
<tr>
<th>Box</th>
<th>Component</th>
<th>Shipping</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BLOCK-iT™ Dicer Enzyme Kit</td>
<td>Dry ice</td>
<td>-20°C</td>
</tr>
<tr>
<td>2</td>
<td>BLOCK-iT™ RNAi Purification Kit</td>
<td>Room temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>3</td>
<td>Lipofectamine™ 2000 Reagent</td>
<td>Blue ice</td>
<td>+4°C (do not freeze)</td>
</tr>
<tr>
<td>4-6</td>
<td>BLOCK-iT™ RNAi TOPO® Transcription Kit</td>
<td>BLOCK-iT™ TOPO® Linker Kit and BLOCK-iT™ RNAi Transcription Kit: Dry ice</td>
<td>BLOCK-iT™ TOPO® Linker Kit and BLOCK-iT™ RNAi Transcription Kit: -20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLOCK-iT™ RNAi Purification Kit: Room temperature</td>
<td>BLOCK-iT™ RNAi Purification Kit: Room temperature</td>
</tr>
</tbody>
</table>

continued on next page
### BLOCK-iT™ Dicer Enzyme Kit

The following reagents are included with the BLOCK-iT™ Dicer Enzyme Kit (Box 1). **Store the reagents at -20°C.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOCK-iT™ Dicer Enzyme</td>
<td>1 U/µl in a proprietary buffer</td>
<td>300 µl</td>
</tr>
<tr>
<td>10X Dicer Buffer</td>
<td>Proprietary</td>
<td>150 µl</td>
</tr>
<tr>
<td>50X Dicer Stop Buffer</td>
<td>0.5 mM EDTA, pH 8.0</td>
<td>30 µl</td>
</tr>
<tr>
<td>RNase-Free Water</td>
<td>--</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

#### Unit Definition

One unit of BLOCK-iT™ Dicer enzyme cleaves 1 µg of double-stranded RNA (dsRNA) in 16 hours at 37°C.

### BLOCK-iT™ RNAi Purification Kit

The following reagents are included with the BLOCK-iT™ RNAi Purification Kit (Box 2). **Store reagents at room temperature.** Use caution when handling the RNA Binding Buffer (see the next page for more information).

**Note:** Catalog no. K3650-01 includes two boxes of BLOCK-iT™ RNAi Purification reagents. One box is supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit for purification of sense and antisense single-stranded RNA (ssRNA). The second box is supplied for purification of diced siRNA (d-siRNA).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Binding Buffer</td>
<td>Proprietary</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>5X RNA Wash Buffer</td>
<td>Proprietary</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>RNase-Free Water</td>
<td>--</td>
<td>800 µl</td>
</tr>
<tr>
<td>RNA Spin Cartridges</td>
<td>--</td>
<td>10</td>
</tr>
<tr>
<td>RNA Recovery Tubes</td>
<td>--</td>
<td>10</td>
</tr>
<tr>
<td>siRNA Collection Tubes*</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>50X RNA Annealing Buffer</td>
<td>500 mM Tris-HCl, pH 8.0</td>
<td>50 µl</td>
</tr>
<tr>
<td></td>
<td>1 M NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA, pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>

*siRNA Collection Tubes are used for purification of d-siRNA only, and are not required for the purification of the ssRNA.

*continued on next page*
The RNA Binding Buffer supplied in the BLOCK-iT™ RNAi Purification Kit contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical. Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

**Lipofectamine™ 2000 Reagent**

Each BLOCK-iT™ Dicer RNAi Kit includes Lipofectamine™ 2000 Reagent (Box 3) for high efficiency transfection of d-siRNA into mammalian cells. Lipofectamine™ 2000 Reagent is supplied as follows:

- **Size:** 0.75 ml
- **Concentration:** 1 mg/ml
- **Storage:** +4°C; do not freeze

**BLOCK-iT™ RNAi TOPO® Transcription Kit**

The BLOCK-iT™ Complete Dicer RNAi Kit (Catalog no. K3650-01) includes the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate production of double-stranded RNA (dsRNA) from your gene of interest. Refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for a detailed description of the reagents provided with the kit and instructions to produce dsRNA.
# Accessory Products

## Introduction

The products listed in this section may be used with the BLOCK-iT™ Dicer RNAi Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 30).

## Accessory Products

Some of the reagents supplied in the BLOCK-iT™ Dicer RNAi Kits as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below.

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOCK-iT™ RNAi TOPO® Transcription Kit</td>
<td>5 genes</td>
<td>K3500-01</td>
</tr>
<tr>
<td>Lipofectamine® 2000 Reagent</td>
<td>0.75 ml</td>
<td>11668-027</td>
</tr>
<tr>
<td></td>
<td>1.5 ml</td>
<td>11668-019</td>
</tr>
<tr>
<td>Opti-MEM® I Reduced Serum Medium</td>
<td>100 ml</td>
<td>31985-062</td>
</tr>
<tr>
<td></td>
<td>500 ml</td>
<td>31985-070</td>
</tr>
<tr>
<td>Phosphate-Buffered Saline (PBS), pH 7.4</td>
<td>500 ml</td>
<td>10010-023</td>
</tr>
<tr>
<td>4% E-Gel® Starter Pak</td>
<td>9 gels and Base</td>
<td>G5000-04</td>
</tr>
<tr>
<td>20% Novex® TBE Gel</td>
<td>1 box</td>
<td>EC63152BOX</td>
</tr>
<tr>
<td>10 bp DNA Ladder</td>
<td>50 µg</td>
<td>10821-015</td>
</tr>
<tr>
<td>β-Gal Assay Kit</td>
<td>100 reactions</td>
<td>K1455-01</td>
</tr>
</tbody>
</table>
Introduction

Overview

Introduction

The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate generation of purified diced siRNA duplexes (d-siRNA) that are suitable for use in RNAi analysis of a target gene in mammalian cells. The kits contain the BLOCK-iT™ Dicer Enzyme for dicing dsRNA, reagents to purify the d-siRNA, and an optimized transfection reagent for highly efficient delivery of d-siRNA to mammalian cells.

Note: The BLOCK-iT™ Complete Dicer RNAi Kit also includes the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate high-yield generation of purified dsRNA. For more information, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual. This manual is supplied with the BLOCK-iT™ Complete Dicer RNAi Kit, but is also available for downloading from www.invitrogen.com or by contacting Technical Service (page 30).

Advantages of the BLOCK-iT™ Dicer RNAi Transfection Kit

Using the BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit to generate d-siRNA for RNAi analysis in mammalian provides the following advantages:

- Provides a cost-effective means to enzymatically generate a pool of d-siRNA that cover a larger portion of the target gene (e.g., 500 bp to 1 kb) without the need for expensive chemical synthesis of siRNA.
- Provides the BLOCK-iT™ Dicer Enzyme and an optimized protocol to facilitate generation of the highest yields of d-siRNA from a dsRNA substrate.
- Includes BLOCK-iT™ RNAi Purification reagents for efficient purification of d-siRNA. Purified d-siRNA can be quantitated, enabling highly reproducible RNAi analysis.
- Includes the Lipofectamine™ 2000 Reagent for the highest efficiency transfection in a wide variety of mammalian cell lines.

Purpose of this Manual

This manual provides the following information:

- A description of the components in the BLOCK-iT™ Dicer RNAi Transfection Kit and an overview of the pathway by which d-siRNA facilitates gene knockdown in mammalian cells.
- Guidelines to produce dsRNA corresponding to the target gene. For detailed instructions to produce dsRNA, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.
- Guidelines and instructions to use the BLOCK-iT™ Dicer Enzyme to cleave dsRNA to generate a complex pool of d-siRNA.
- Instructions to purify d-siRNA.
- Guidelines and instructions to transfect purified d-siRNA into mammalian cells using Lipofectamine™ 2000 Reagent for RNAi studies.

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The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit are designed to help you generate d-siRNA for use in RNAi analysis in mammalian cell lines. Although the kits have been designed to help you generate d-siRNA representing a particular target sequence in the simplest, most direct fashion, use of the resulting d-siRNA for RNAi analysis assumes that users are familiar with the principles of gene silencing and transfection in mammalian systems. We highly recommend that users possess a working knowledge of the RNAi pathway and lipid-mediated transfection.

For more information about the RNAi pathway in mammalian cells, refer to published reviews (Elbashir et al., 2002; McManus and Sharp, 2002).

A large selection of BLOCK-iT™ RNAi products is available from Invitrogen to facilitate RNAi analysis in mammalian and invertebrate systems. For more information about these products and other RNAi resources, visit the RNAi Central application portal at www.invitrogen.com/rnai.
**BLOCK-iT™ Dicer RNAi Kit**

**Components of the BLOCK-iT™ Dicer RNAi Kit**

The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate generation and delivery of purified d-siRNA duplexes into mammalian cells for RNAi analysis. The kits contain three major components:

- The BLOCK-iT™ Dicer Enzyme and optimized reagents for production of high yields of d-siRNA from a dsRNA substrate. For more information about how the BLOCK-iT™ Dicer Enzyme works, see page 5.

- The BLOCK-iT™ RNAi Purification reagents for silica-based column purification of d-siRNA, and an RNA Annealing Buffer to stabilize d-siRNA duplexes for long-term storage.

- Lipofectamine™ 2000 Reagent for high-efficiency transfection of d-siRNA into a wide range of mammalian cell types and cell lines for RNAi analysis.

**Note:** If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, note that the kit also includes a control expression plasmid containing the lacZ gene and PCR primers that may be used to generate control lacZ dsRNA. The control lacZ dsRNA may be used in a dicing and purification reaction to generate purified lacZ d-siRNA. Co-transfecting the purified lacZ d-siRNA and the control expression plasmid into mammalian cells provide a means to assess the RNAi response in your cell line by assaying for knockdown of β-galactosidase. In addition, the lacZ d-siRNA can be used as a negative control for non-specific off-target effects in your RNAi studies.

**Important**

If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, note that the kit includes 2 boxes of BLOCK-iT™ RNAi Purification reagents. One box is intended for purification of dsRNA, while the second box is intended for purification of d-siRNA. The protocols to purify dsRNA and d-siRNA differ significantly from one another. **When purifying d-siRNA, be sure to use the purification procedure provided in this manual (page 16).** To purify dsRNA, use the purification procedure provided in the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

**Generating d-siRNA Using the Kit**

Using the reagents supplied in the kit, you will perform the following steps to generate pure d-siRNA that is ready for transfection into the mammalian cell line of interest.

1. Use dsRNA representing your target sequence (generated with the BLOCK-iT™ RNAi TOPO® Transcription Kit) in a reaction with the BLOCK-iT™ Dicer enzyme to generate d-siRNA.

2. Purify the d-siRNA using the purification reagents supplied in the kit. Quantitate the yield of purified d-siRNA obtained.

3. Transfect d-siRNA into the mammalian cell line of interest using Lipofectamine™ 2000 Reagent.
The RNAi Pathway and How Dicer Works

The 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 and Macino, 1999; Cogoni and Macino, 1997; Romano and 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 and 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). Each siRNA then incorporates 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).

For more information about the RNAi pathway and the mechanism of gene silencing, refer to recent reviews (Bosher and Labouesse, 2000; Hannon, 2002; Plasterk and Ketting, 2000; Zamore, 2001).

Performing RNAi Analysis in Mammalian Cells

A number of kits including the BLOCK-iT™ RNAi TOPO® Transcription Kit now exist to facilitate in vitro production of dsRNA that is targeted to a particular gene of interest. The dsRNA may be introduced directly into some invertebrate organisms or cell lines, where it functions to trigger the endogenous RNAi pathway resulting in inhibition of the target gene. Long dsRNA duplexes cannot be used directly for RNAi analysis in most somatic mammalian cell lines because introduction of long dsRNA into these cell lines induces a non-specific, interferon-mediated response, resulting in shutdown of translation and initiation of cellular apoptosis (Kaufman, 1999). To avoid triggering the interferon-mediated host cell response, dsRNA duplexes of less than 30 nucleotides must be introduced into cells (Stark et al., 1998). For optimal results in gene knockdown studies, the size of the dsRNA duplexes (i.e. siRNA) introduced into mammalian cells is further limited to 21-23 nucleotides.

continued on next page
The RNAi Pathway and How Dicer Works, continued

Using the Kit for RNAi Analysis

The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate \textit{in vitro} production of a complex pool of 21-23 nucleotide siRNA duplexes that is targeted to a particular gene of interest. The kits use a recombinant human Dicer enzyme (see below for more information) to cleave a long dsRNA substrate (produced with the BLOCK-iT™ RNAi TOPO® Transcription Kit) into a pool of 21-23 nucleotide d-siRNA that may be transfected into mammalian cells. Introduction of d-siRNA into the cells then triggers the endogenous RNAi pathway, resulting in inhibition of the target gene. For a diagram of the process, see the figure below.

![Diagram of RNAi pathway](image)

BLOCK-iT™ Dicer Enzyme

BLOCK-iT™ Dicer is a recombinant human enzyme (Myers \textit{et al.}, 2003; Provost \textit{et al.}, 2002) that cleaves long dsRNA processively into 21-23 nucleotide d-siRNA duplexes with 2 nucleotide 3' overhangs. The Dicer enzyme is a member of the RNase III family of double-stranded RNA-specific endonucleases, and consists of an ATP-dependent RNA helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain, two RNase III domains, and a dsRNA-binding domain (Bernstein \textit{et al.}, 2001; Zamore, 2001). In addition to its role in the generation of siRNA, Dicer is also involved in the processing of short temporal RNA (stRNA) (Hutvagner \textit{et al.}, 2001; Ketting \textit{et al.}, 2001) and microRNA (miRNA) (Carrington and Ambros, 2003) from stable hairpin or stem-loop precursors.
# Experimental Outline

The table below outlines the steps required when using the BLOCK-iT™ Dicer RNAi Kits to generate, purify, and transfect your d-siRNA of interest.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Produce dsRNA from your target gene.</td>
<td>7-8 and the BLOCK-iT™ RNAi TOPO® Transcription Kit manual</td>
</tr>
<tr>
<td>2</td>
<td>Use the dsRNA in a reaction with the BLOCK-iT™ Dicer enzyme to generate d-siRNA.</td>
<td>9-11</td>
</tr>
<tr>
<td>3</td>
<td>Purify d-siRNA using the BLOCK-iT™ RNAi Purification Reagents.</td>
<td>13-18</td>
</tr>
<tr>
<td>4</td>
<td>Transfect purified d-siRNA into your mammalian cell line of interest using Lipofectamine™ 2000 Reagent.</td>
<td>19-22</td>
</tr>
<tr>
<td>5</td>
<td>Assay for inhibition of target gene expression using your method of choice.</td>
<td>22</td>
</tr>
</tbody>
</table>
Methods

Generating Double-Stranded RNA (dsRNA)

Introduction

Before you can use the BLOCK-iT™ Dicer Enzyme to produce short interfering RNA (siRNA), you must generate double-stranded RNA (dsRNA) substrate representing your target sequence of interest. Guidelines and recommendations to generate dsRNA are provided below.

For optimal, high-yield production of dsRNA, we recommend using the BLOCK-iT™ RNAi TOPO® Transcription Kit available from Invitrogen (Catalog no. K3500-01). The BLOCK-iT™ RNAi TOPO® Transcription Kit supplies the reagents necessary to generate T7 promoter-based DNA templates from any Taq-amplified PCR product, then use these templates in in vitro transcription reactions to generate sense and antisense RNA transcripts. The kit also includes reagents to enable purification and annealing of the RNA transcripts to produce high yields of dsRNA that are ready-to-use in the dicing reaction.

For detailed protocols and guidelines to generate dsRNA from your target gene sequence, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual. This manual is supplied with the BLOCK-iT™ Complete Dicer RNAi Kit, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 30).

Choosing the Target Sequence

When performing RNAi analysis, your choice of target sequence can significantly affect the degree of gene knockdown observed. In addition, the size of the target sequence and the resulting dsRNA can affect the yields of d-siRNA produced. Consider the following factors when choosing your target sequence.

- Select a target sequence that covers a reasonable portion of the gene of interest and that does not contain regions of strong homology with other genes.

- Limit the size of the target sequence. Although smaller or larger target sequences are possible, we recommend limiting the initial target sequence to a size range of 500 bp to 1 kb for the following reasons.
  a. This balances the risk of including regions of strong homology between the target gene and other genes that could result in non-specific off-target effects during RNAi analysis with the benefits of using a more complex pool of siRNA.
  b. When producing sense and antisense transcripts of the target template, the highest transcription efficiencies are obtained with transcripts in the 500 bp to 1 kb size range. Target templates outside this size range transcribe less efficiently, resulting in lower yields of dsRNA.
  c. Double-stranded RNA that is under 1 kb in size is efficiently diced. Larger dsRNA substrates can be used but yields may decline as the size increases.

Note: The BLOCK-iT™ Dicer RNAi Kits have been used successfully to knock down gene activity with dsRNA substrates ranging from 150 bp to 1.3 kb in size.

continued on next page
Factors to Consider When Generating dsRNA

If you are using your own method or another kit to produce dsRNA, consider the following factors when generating your dsRNA. These factors will influence the yields of d-siRNA produced from the dicing reaction.

- **Amount of dsRNA required for dicing:** We use 60 µg of dsRNA in a typical 300 µl dicing reaction to recover 12-18 µg of d-siRNA after purification. This amount of dsRNA is generally sufficient to transfect approximately 150 wells of cells plated in a 24-well format. You should have an idea of the scale and scope of your RNAi experiment to determine how much dsRNA you will need to dice.

  **Note:** If you wish to dice less than 60 µg of dsRNA, you will need to scale down the dicing reaction proportionally (see page 9 for more information).

- **Concentration of dsRNA:** The amount of dsRNA in a dicing reaction should not exceed half the reaction volume; therefore, the concentration of your dsRNA should be ≥ 400 ng/µl if you wish to dice 60 µg of dsRNA.

- **Buffering of dsRNA:** We recommend storing your dsRNA sample in a buffered solution containing 1 mM EDTA and no more than 100 mM salt (i.e. TE Buffer at pH 7-8 or 1X RNA Annealing Buffer). This helps to stabilize the dsRNA and provides the optimal environment for efficient cleavage by the Dicer Enzyme.

  **Note:** If you have used the BLOCK-iT™ RNAi TOPO® Transcription Kit to produce dsRNA, your dsRNA sample will be in 1X RNA Annealing Buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, pH 8.0).

- **The quality of your dsRNA:** To obtain the highest yields of d-siRNA, we recommend using purified dsRNA in the dicing reaction.

Once you have generated your purified dsRNA, we recommend saving an aliquot of the dsRNA for future gel analysis. We generally use agarose or polyacrylamide gel electrophoresis to assess the success of the dicing reaction by comparing an aliquot of the dicing reaction to an aliquot of the dsRNA substrate. For an example, see page 12.
Performing the Dicing Reaction

Introduction

Once you have produced your target dsRNA, you will perform an *in vitro* dicing reaction using the reagents supplied in the BLOCK-iT™ Dicer Enzyme Kit (Box 1) to generate d-siRNA duplexes of 21-23 nucleotides in size.

BLOCK-iT™ Dicer Enzyme Activity

One unit of BLOCK-iT™ Dicer Enzyme cleaves 1 µg of dsRNA in 16 hours at 37°C. Note that the Dicer enzyme does not cleave dsRNA to d-siRNA with 100% efficiency, *i.e.* dicing 1 µg of dsRNA does not generate 1 µg of d-siRNA. Under these optimal reaction conditions, the Dicer enzyme cleaves dsRNA to d-siRNA with an efficiency of approximately 25-35%. For example, dicing 60 µg of dsRNA in a 300 µl dicing reaction typically yields 12-18 µg of d-siRNA following purification.

Note

For best results, we recommend following the dicing procedure exactly as described on page 10 as the reaction conditions have been optimized to provide the highest mass yield of d-siRNA under the most efficient dicing conditions. Note the following:

- It is possible to use more than 60 µg of dsRNA in a 300 µl dicing reaction; however, the BLOCK-iT™ Dicer Enzyme becomes less efficient under these conditions. Although you may generate a higher mass yield of d-siRNA, the % yield of d-siRNA will decrease.
- Do not increase the amount of BLOCK-iT™ Dicer Enzyme used in the dicing reaction (to greater than 60 units in a 300 µl reaction) or increase the length of the dicing reaction (to greater than 18 hours). Under either of these conditions, the BLOCK-iT™ Dicer Enzyme can bind to d-siRNA and cleave the 21-23 nt duplexes into smaller products, resulting in lower yields of d-siRNA.

Amount of dsRNA to Use

For a typical 300 µl dicing reaction, you will need 60 µg of target dsRNA. If you want to dice less than 60 µg of dsRNA, scale down the entire reaction proportionally.

The total volume of dsRNA added should not exceed half the volume of the reaction. Thus, for best results, make sure that the starting concentration of your dsRNA is ≥ 400 ng/µl.

Positive Control

If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, and have performed all of the recommended control reactions using the control reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription portion of the kit, you should have purified dsRNA representing a 1 kb portion of the *lacZ* gene. We recommend setting up a separate dicing and purification reaction using the control *lacZ* dsRNA. You can then co-transfect the resulting purified *lacZ* d-siRNA and the pcDNA™1.2/V5-GW/*lacZ* control plasmid supplied with the kit into your mammalian cell line as a positive control for the RNAi response in that cell line. Alternatively, you may use the *lacZ* d-siRNA as a negative control for non-specific, off-target effects in your cell line.

*continued on next page*
Performing the Dicing Reaction, continued

When performing the dicing reaction and subsequent purification of d-siRNA, take precautions to avoid RNase contamination.

- Use RNase-free sterile pipette tips and supplies for all manipulations.
- Use DEPC-treated solutions as necessary.
- Wear gloves when handling reagents and solutions, and when performing reactions.

Materials Needed

Have the following reagents on hand before beginning:

- Purified dsRNA (> 400 ng/µl in 1X RNA Annealing Buffer or TE Buffer, pH 7-8; see page 8 for more information)
- BLOCK-iT™ Dicer Enzyme (1 U/µl; supplied with the kit, Box 1; keep at -20°C until immediately before use)
- 10X Dicer Buffer (supplied with the kit, Box 1)
- RNase-Free Water (supplied with the kit, Box 1)
- 50X Dicer Stop Buffer (supplied with the kit, Box 1)

Dicing Procedure

Follow the procedure below to perform the dicing reaction. Make sure that the volume of dsRNA added does not exceed half the volume of the reaction (i.e., ≤ 150 µl).

1. Set up a 300 µl dicing reaction on ice using the following reagents in the order shown.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Dicer Buffer</td>
<td>30 µl</td>
</tr>
<tr>
<td>RNase-Free Water</td>
<td>up to 210 µl</td>
</tr>
<tr>
<td>Purified dsRNA (60 µg)</td>
<td>1-150 µl</td>
</tr>
<tr>
<td>BLOCK-iT™ Dicer Enzyme (1 U/µl)</td>
<td>60 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>300 µl</td>
</tr>
</tbody>
</table>

2. Mix reaction gently and incubate for 14-18 hours at 37°C.

   Note: Do not incubate the reaction for longer than 18 hours as this may result in a lower yield of d-siRNA due to cleavage of d-siRNA by the Dicer enzyme.

3. Add 6 µl of 50X Dicer Stop Solution to the reaction.

4. Check the integrity of your d-siRNA, if desired (see the next page).

5. Proceed to purify the d-siRNA (see Purifying Diced siRNA (d-siRNA), page 13) or store the dicing reaction overnight at -20°C.

continued on next page
Performing the Dicing Reaction, continued

Checking the Integrity of d-siRNA

You may verify the integrity of your d-siRNA using polyacrylamide or agarose gel electrophoresis, if desired. We suggest running an aliquot of your dicing reaction (0.5-1 µl of a 300 µl reaction; equivalent to 100-200 ng of dsRNA) on the appropriate gel and comparing it to an aliquot of your starting dsRNA. Be sure to include an appropriate molecular weight standard. We generally use the following gels and molecular weight standard:

- **Agarose gel**: 4% E-Gel® (Invitrogen, Catalog no. G5000-04)
- **Polyacrylamide gel**: 20% Novex® TBE Gel (Invitrogen, Catalog no. EC63152BOX)
- **Molecular weight standard**: 10 bp DNA Ladder (Invitrogen, Catalog no. 10821-015)

What You Should See

When analyzing an aliquot of the dicing reaction by gel electrophoresis, we generally see the following:

- A predominant band of approximately 21-23 nt representing the d-siRNA.
- 4% E-Gel®: A high molecular weight smear representing uncleaved dsRNA and partially cleaved products. Generally, this band does not resolve well on an agarose gel and runs close to the well.

  **Novex® 20% TBE Gel**: A high molecular weight band and a smear representing uncleaved dsRNA and partially cleaved products. The dsRNA band generally resolves better on a polyacrylamide gel.

For an example of expected results obtained from agarose gel electrophoresis, see the next page. If the band representing d-siRNA is weak or if you do not see a band, see Troubleshooting, page 26 for tips to troubleshoot your dicing reaction.

*continued on next page*
Performing the Dicing Reaction, continued

**Example of Expected Results**

In this experiment, purified dsRNA representing a 1 kb region of the *lacZ* gene was generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription Kit. The *lacZ* dsRNA was diced using the procedure on page 10. Aliquots of the dicing reaction (equivalent to 200 ng of dsRNA) and the initial dsRNA substrate were analyzed on a 4% E-Gel®.

**Results**: A prominent band representing d-siRNA of the expected size is clearly visible in the dicing reaction sample (lane 3). This band is not visible in the initial dsRNA substrate sample (lane 2).

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**Lane 1.** 10 bp DNA Ladder  
**Lane 2.** 200 ng purified *lacZ* dsRNA  
**Lane 3.** 200 ng *lacZ* dicing reaction
**Purifying Diced siRNA (d-siRNA)**

**Introduction**

This section provides guidelines and instructions to purify the d-siRNA produced in the dicing reaction. Use the BLOCK-iT® RNAi Purification reagents (Box 2) supplied with the kit.

**Important**

Before proceeding to transfection, note that you must purify the d-siRNA produced in the dicing reaction to remove contaminating long dsRNA duplexes. Transfection of unpurified d-siRNA can trigger the interferon-mediated response and cause host cell shutdown and cellular apoptosis. When purifying d-siRNA, follow the purification procedure provided on page 16 exactly as instructed. This procedure is optimized to allow removal of contaminating long dsRNA and recovery of high yields of d-siRNA.

**Experimental Outline**

To purify d-siRNA, you will:

1. Add RNA Binding Buffer and isopropanol to the dicing reaction to denature the proteins and to enable the contaminating dsRNA to bind to the column.

2. Add half the volume of the sample to an RNA spin cartridge. The dsRNA binds to the silica-based membrane in the cartridge, and the d-siRNA and denatured proteins flow through the cartridge. Save the flow-through.

3. Transfer the RNA spin cartridge to an siRNA Collection Tube and add the remaining sample to the RNA spin cartridge. Repeat Step 2. Save the flow-through.

4. Pool the flow-throughs from Step 2 and Step 3 in the siRNA Collection Tube and add isopropanol to the sample to enable the d-siRNA to bind to the column.

5. Add the sample to a second RNA spin cartridge. The d-siRNA bind to the membrane in the cartridge.

6. Wash the membrane-bound d-siRNA to eliminate residual RNA Binding Buffer, isopropanol, and any remaining impurities.

7. Elute the d-siRNA from the RNA spin cartridge with water.

8. Add 50X RNA Annealing Buffer to the eluted d-siRNA to stabilize the d-siRNA for storage.

For an illustration of the d-siRNA purification process, see the next page.
The figure below illustrates the d-siRNA purification process.

Add RNA Binding Buffer and isopropanol to the dicing reaction and mix thoroughly.

Add 1/2 of sample to RNA Spin Cartridge.

Transfer RNA Spin Cartridge to an siRNA Collection Tube, and add remaining sample to RNA Spin Cartridge.

Combine flow-throughs and add isopropanol, mix thoroughly.

Add sample (in 3 parts) to RNA Spin Cartridge.

Wash cartridge 2X.

Elute d-siRNA into RNA Recovery Tube.

continued on next page
Purifying Diced siRNA (d-siRNA), continued

 Advance Preparation  
Before using the BLOCK-iT™ RNA Purification reagents for the first time, add 10 ml of 100% ethanol to the entire amount of 5X RNA Wash Buffer to obtain a 1X RNA Wash Buffer (total volume = 12.5 ml). Place a check in the box on the 5X RNA Wash Buffer label to indicate that the ethanol was added. Store the 1X RNA Wash Buffer at room temperature.

 The RNA Binding Buffer contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical. Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

Materials Needed  
Have the following materials on hand before beginning:
- Dicing reaction (from Step 5, page 10)
- RNA Binding Buffer (supplied with the kit, Box 2)
- β-mercaptoethanol
- Isopropanol
- RNA Spin Cartridges (supplied with the kit, Box 2; two for each sample)
- siRNA Collection Tube (supplied with the kit, Box 2)
- 1X RNA Wash Buffer (see Advance Preparation, above)
- RNase-Free Water (supplied with the kit, Box 2)
- RNA Recovery Tube (supplied with the kit, Box 2)
- 50X RNA Annealing Buffer (supplied with the kit, Box 2)
- RNase-free supplies

continued on next page
Purifying Diced siRNA (d-siRNA), continued

Use this procedure to purify d-siRNA produced from dicing 60 µg of dsRNA in a 300 µl reaction volume (see Step 5, page 10). If you have digested < 60 µg of dsRNA and have scaled down the volume of your dicing reaction, scale down the volume of your purification reagents proportionally. For example, if you have digested 30 µg of dsRNA in a 150 µl dicing reaction, scale down the volume of purification reagents used by half.

**Important:** Before beginning, remove the amount of RNA Binding Buffer needed and add β-mercaptoethanol to a final concentration of 1% (v/v). Use fresh and discard any unused solution.

1. To each dicing reaction (~300 µl volume), add 300 µl of RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol followed by 300 µl of isopropanol to obtain a final volume of 900 µl. Mix well by pipetting up and down 5 times.

2. Apply half of the sample (~450 µl) to the RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature.

3. Transfer the RNA spin cartridge to an siRNA Collection Tube. Save the flow-through containing d-siRNA from Step 2.

4. Apply the remaining half of the sample (~450 µl) to the RNA Spin Cartridge. Centrifuge at 14,000 x g for 2 minutes at room temperature.

5. Remove the RNA Spin Cartridge from the siRNA Collection Tube and discard. Save the flow-through containing d-siRNA.

6. Transfer the flow-through from Step 2 (~450 µl) to the siRNA Collection Tube containing the flow-through from Step 4 (~450 µl) to obtain a final volume of ~900 µl. Add 600 µl of isopropanol to the sample to obtain a final volume of 1.5 ml. Mix well by pipetting up and down.

7. Apply one-third of the sample (~500 µl) to a new RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.

8. Repeat Step 7 twice, applying one-third of the remaining sample (~500 µl) to the RNA Spin Cartridge each time.

9. Add 500 µl of 1X RNA Wash Buffer to the RNA Spin Cartridge containing bound d-siRNA. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.

10. Repeat the wash step (Step 9).

11. Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer from the cartridge and to dry the membrane.

12. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube.

13. Add 30 µl of RNase-Free Water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the d-siRNA. Proceed to Step 14, next page.

*continued on next page*
Purifying Diced siRNA (d-siRNA), continued

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**d-siRNA Purification Procedure, continued**

14. Repeat Step 13, previous page, eluting the d-siRNA into the same RNA Recovery Tube. The total volume of eluted d-siRNA is 60 µl.

15. Add 1.2 µl of the 50X RNA Annealing Buffer to the eluted d-siRNA to obtain a final concentration of 1X RNA Annealing Buffer. Adding RNA Annealing Buffer to the sample increases the stability of the d-siRNA.

16. Proceed to quantitate the concentration of your purified d-siRNA (see **Determining the Purity and Concentration of d-siRNA**, below).

17. Store the purified d-siRNA at -80°C. Depending on the amount of d-siRNA produced and your downstream application, you may want to aliquot the d-siRNA before storage at -80°C.

**Important:** When using the d-siRNA, avoid repeated freezing and thawing as d-siRNA can degrade with each freeze/thaw cycle.

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**Determining the Purity and Concentration of d-siRNA**

Use the procedure below to determine the purity and concentration of your purified d-siRNA.

1. Dilute an aliquot of the purified d-siRNA 20-fold into 1X RNA Annealing Buffer in a total volume appropriate for your quartz cuvettes and spectrophotometer.


3. Calculate the concentration of the d-siRNA by using the following equation:

\[
\text{d-siRNA concentration (µg/ml)} = \text{A260} \times \text{Dilution factor (20)} \times 40 \, \text{µg/ml}
\]

4. Calculate the yield of the d-siRNA by using the following equation:

\[
\text{d-siRNA yield (µg)} = \text{d-siRNA concentration (µg/ml)} \times \text{vol. of d-siRNA (ml)}
\]

5. Evaluate the purity of the purified d-siRNA by determining the A260/A280 ratio. For optimal purity, the A260/A280 ratio should range from 1.9-2.2.

---

**Verifying the Quality of Your d-siRNA**

You may verify the quality of your purified d-siRNA using polyacrylamide or agarose gel electrophoresis, if desired. We suggest running a small aliquot of your purified d-siRNA (0.5-1 µl) on the appropriate gel and comparing it to an aliquot of your dicing reaction (equivalent to 100-200 ng of dsRNA). Be sure to include an appropriate molecular weight standard. For recommended gels and a molecular weight standard, we generally use the same gels and molecular weight standard that we use to analyze the quality of the dicing reaction (see page 11).

For an example of expected results obtained from agarose gel electrophoresis, see the next page. If the band representing purified d-siRNA is weak or if you do not see a band, see **Troubleshooting**, page 28 for tips to purify your d-siRNA.
Purifying Diced siRNA (d-siRNA), continued

Example of Expected Results
In this experiment, the lacZ d-siRNA generated in the dicing reaction depicted in the Example on page 12 were purified using the procedure on page 16-17. Aliquots of the purified lacZ d-siRNA (80 ng) and the lacZ dicing reaction (equivalent to 200 ng of dsRNA) were analyzed on a 4% E-Gel®.

Results: A prominent band representing purified d-siRNA of the expected size is clearly visible in lane 3. No contaminating dsRNA or other high molecular weight products remain in the purified d-siRNA sample.

How Much d-siRNA to Expect
The typical yield of d-siRNA obtained from dicing 60 µg of dsRNA (500 bp to 1 kb in size) in a 300 µl dicing reaction ranges from 12-18 µg, with a concentration of 200-300 ng/µl. Note that yields may vary depending on the size and quality of the dsRNA.
Transfecting Cells

Introduction

Once you have purified your d-siRNA, you may perform RNAi analysis by transfecting the d-siRNA into the mammalian cell line of interest, and assaying for inhibition of target gene expression. This section provides general guidelines and protocols to transfect your purified d-siRNA into mammalian cells using the Lipofectamine™ 2000 Reagent (Box 3) supplied with the kit. Suggested transfection conditions are provided as a starting point. You will need to optimize transfection conditions to obtain the best results for your target gene and mammalian cell line.

Reminder: You must transfect mammalian cells with purified d-siRNA. Note that transfecting cells with unpurified d-siRNA containing contaminating long dsRNA (i.e. with material directly taken from the dicing reaction) can trigger the interferon-mediated cellular response, resulting in host cell shutdown and cellular apoptosis.

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 consideration when designing your transfection and RNAi experiments.

Lipofectamine™ 2000 Reagent

The Lipofectamine™ 2000 Reagent supplied with the kit is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids including d-siRNA and siRNA into eukaryotic cells (Ciccarone et al., 1999; Gitlin et al., 2002; Yu et al., 2002). Using Lipofectamine™ 2000 to transfect d-siRNA into eukaryotic cells offers the following advantages:

- Provides the highest transfection efficiency in many cell types
- Is the most widely used transfection reagent for delivery of d-siRNA or siRNA into eukaryotic cells (Gitlin et al., 2002; Yu et al., 2002)
- d-siRNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes, medium change, or medium addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity.

Lipofectamine™ 2000 is also available separately from Invitrogen (see page x for ordering information).

continued on next page
Transfecting Cells, continued

**Important Guidelines**

Follow these guidelines when transfecting siRNA into mammalian cells using Lipofectamine™ 2000:

1. **Cell density:** For optimal results, we recommend plating cells such that they will be 30-50% confluent at the time of transfection. Gene knockdown levels are generally assayed 24-72 hours following transfection. 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. Depending on the nature of the target gene, higher or lower cell densities may be suitable with optimization of conditions.

2. For optimal results, use Opti-MEM® I Reduced Serum Medium (Invitrogen, Catalog no. 31985-062) to dilute Lipofectamine™ 2000 and d-siRNA prior to complex formation.

3. **Do not include antibiotics** in media used during transfection as this will reduce transfection efficiency and cause cell death.

**Materials to Have on Hand**

Have the following materials on hand before beginning:

- Mammalian cell line of interest (make sure that cells are healthy and greater than 90% viable before transfection)
- Purified d-siRNA of interest (≥ 40 ng/µl)

  **Note:** If you have diced 60 µg of dsRNA, the typical yield of d-siRNA obtained after purification is 12-18 µg at a concentration of 200-300 ng/µl
- Positive control, if desired (see below)
- Lipofectamine™ 2000 Reagent (supplied with the kit; store at +4°C until use)
- Opti-MEM® I Reduced Serum Medium (Invitrogen, Catalog no. 31985-062; pre-warmed)
- Sterile tissue culture plates and other tissue culture supplies

**Positive Control**

If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, and have diced the control lacZ dsRNA, two options exist to use the resulting purified lacZ d-siRNA for RNAi analysis:

1. Use the lacZ d-siRNA as a negative control for non-specific off-target effects.

2. Use the lacZ d-siRNA as a positive control to assess the RNAi response in your cell line by co-transfecting the lacZ d-siRNA and the pcDNA™1.2/V5-GW/lacZ reporter plasmid supplied with the kit into your mammalian cells using Lipofectamine™ 2000. Assay for knockdown of β-galactosidase expression 24 hours post-transfection using Western blot analysis or activity assay (see page 23).

  **Important:** Transfection conditions (i.e. cell density and reagent amounts) vary slightly when d-siRNA and plasmid DNA are co-transfected into mammalian cells. For details, see Co-transfecting d-siRNA and Plasmid DNA, page 22.

*continued on next page*
Transfecting Cells, continued

**Transfection Procedure**

Use this procedure to transfect mammalian cells using Lipofectamine™ 2000. Refer to the table in *Recommended Reagent Amounts and Volumes*, below for the appropriate reagent amounts and volumes to add for different tissue culture formats. Use the recommended Lipofectamine™ 2000 amounts as a starting point for your experiments, and optimize conditions for your cell line and d-siRNA.

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 d-siRNA:Lipofectamine™ 2000 complexes as follows:
   a. Dilute d-siRNA 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.
      
      **Note:** Combine the diluted Lipofectamine™ 2000 with the diluted d-siRNA within 30 minutes. Longer incubation times may decrease activity.
   c. After the 5 minute incubation, combine the diluted d-siRNA with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature to allow the d-siRNA:Lipofectamine™ 2000 complexes to form (solution may appear cloudy).

3. Add the d-siRNA: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 as appropriate until you are ready to assay for gene knockdown. It is not necessary to remove the complexes or change the medium; however, growth medium may be replaced after 4-6 hours without loss of transfection activity.

**Recommended 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 d-siRNA (see column 4) and Lipofectamine™ 2000 (see column 6) as a starting point for your experiments, and optimize conditions for your cell line and target gene.

**Note:** With automated, high-throughput systems, larger complexing volumes are recommended for transfections in 96-well plates.

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Relative Surface Area (vs. 24-well)</th>
<th>Volume of Plating Medium</th>
<th>d-siRNA (ng) and Dilution Volume (µl)</th>
<th>d-siRNA Amounts (ng) for Optimization</th>
<th>Lipofectamine™ 2000 (µl) and Dilution Volume (µl)</th>
<th>Lipofectamine™ 2000 Amounts (µl) for Optimization</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>0.2</td>
<td>100 µl</td>
<td>20 ng in 25 µl</td>
<td>5-50 ng</td>
<td>0.6 µl in 25 µl</td>
<td>0.2-1.0 µl</td>
</tr>
<tr>
<td>24-well</td>
<td>1</td>
<td>500 µl</td>
<td>50 ng in 50 µl</td>
<td>20-200 ng</td>
<td>1 µl in 50 µl</td>
<td>0.5-1.5 µl</td>
</tr>
<tr>
<td>6-well</td>
<td>5</td>
<td>2 ml</td>
<td>250 ng in 250 µl</td>
<td>100-1000 ng</td>
<td>5 µl in 250 µl</td>
<td>2.5-6 µl</td>
</tr>
</tbody>
</table>

*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 the cell density (from 30-50% confluence) and the amounts of d-siRNA (see column 5) and Lipofectamine™ 2000 (see column 7) as suggested in the table on the previous page. For cell lines that are particularly sensitive to transfection-mediated cytotoxicity (e.g. HeLa, HT1080), use the lower amounts of Lipofectamine™ 2000 suggested in the table on the previous page (see column 7).

What You Should See

When performing RNAi experiments using d-siRNA, we generally observe inhibition of the gene of interest within 24 to 96 hours after transfection. The degree of gene knockdown depends on the time of assay, stability of the protein of interest, and on the other factors listed on page 19. Note that 100% gene knockdown is generally not observed, but > 95% is possible with optimized conditions.

For examples of results obtained from RNAi experiments using d-siRNA, see the next page.

Co-transfecting d-siRNA and Plasmid DNA

If you are using the lacZ d-siRNA as a positive control to assess the RNAi response in your cell line, you will co-transfect the lacZ d-siRNA and the pcDNA™ 1.2/V5-GW/lacZ reporter plasmid into the mammalian cell line and assay for inhibition of β-galactosidase expression after 24 hours. When co-transfecting d-siRNA and plasmid DNA, follow the procedure on the previous page with the following exceptions:

- Plate cells such that they will be 90% confluent at the time of transfection.
- Refer to the table below for the recommended amount of d-siRNA (see column 3) and plasmid DNA (see column 4) to transfect in a particular tissue culture format.

  Note: We generally transfect twice the mass of plasmid DNA as d-siRNA.

- Use the recommended Lipofectamine™ 2000 amounts in the table below (see column 6) as a starting point, and optimize conditions for your cell line if desired. To optimize conditions, vary the amount of Lipofectamine™ 2000 as suggested in the table below (see column 7).

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Volume of Plating Medium</th>
<th>d-siRNA (ng)</th>
<th>Plasmid DNA (ng)</th>
<th>Nucleic Acid Dilution Volume</th>
<th>Lipofectamine™ 2000 (µl) and Dilution Volume (µl)</th>
<th>Lipofectamine™ 2000 Amounts (µl) for Optimization</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>100 µl</td>
<td>20 ng</td>
<td>40 ng</td>
<td>25 µl</td>
<td>0.6 µl in 25 µl</td>
<td>0.2-1.0 µl</td>
</tr>
<tr>
<td>24-well</td>
<td>500 µl</td>
<td>50 ng</td>
<td>100 ng</td>
<td>50 µl</td>
<td>2 µl in 50 µl</td>
<td>0.5-2.0 µl</td>
</tr>
<tr>
<td>6-well</td>
<td>2 ml</td>
<td>250 ng</td>
<td>500 ng</td>
<td>250 µl</td>
<td>10 µl in 250 µl</td>
<td>2.5-10 µl</td>
</tr>
</tbody>
</table>

continued on next page
Transfecting Cells, continued

**Assaying for β-galactosidase Expression**

If you perform RNAi analysis using the control lacZ d-siRNA, you may assay for β-galactosidase expression and knockdown by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-gal Antiserum (Catalog no. R901-25) and the β-Gal Assay Kit (Catalog no. K1455-01) for fast and easy detection of β-galactosidase expression. For an example of results obtained from a β-galactosidase knockdown experiment, see the next page.

**Note:** The β-galactosidase protein expressed from the pcDNA™1.2/V5-GW/lacZ control plasmid is fused to a V5 epitope and is approximately 119 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (e.g. Anti-V5-HRP Antibody; Catalog no. R961-25 or Anti-V5-AP Antibody, Catalog no. R962-25) for detection. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 30).
Examples of Expected Results

Introduction

This section provides some examples of results obtained from RNAi experiments performed with d-siRNA generated using the BLOCK-iT™ Complete Dicer RNAi Kit. The first example depicts knockdown of expression of a reporter gene, and the second example depicts knockdown of expression of the endogenous lamin A/C gene.

Example of Expected Results: Knockdown of a Reporter Gene

In this experiment, d-siRNA targeting two reporter genes (i.e. luciferase and lacZ) and an endogenous gene (i.e. lamin A/C) was generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Complete Dicer RNAi Kit.

GripTite™ 293 MSR cells (Invitrogen, Catalog no. R795-07) were grown to 90% confluence. Individual wells in a 24-well plate were transfected using Lipofectamine™ 2000 Reagent with 100 ng each of lacZ and luciferase-containing reporter plasmids. In some wells, the reporter plasmids were co-transfected with 50 ng of purified lacZ, luciferase, or lamin A/C d-siRNA. Cell lysates were prepared 24 hours after transfection and assayed for luciferase and β-galactosidase activity. Activities were normalized to those of the reporter plasmids alone.

Results: Potent and specific inhibition is evident from luciferase and lacZ-derived d-siRNA. Note that in this experiment, lamin A/C d-siRNA serves as a negative control and does not inhibit luciferase or β-galactosidase expression.

Note: Introduction of d-siRNA into mammalian cells can, in some cases lead to a slight induction of gene expression, as is observed with β-galactosidase and luciferase expression upon transfection of lamin d-siRNA.

continued on next page
**Example of Expected Results:**

**Knockdown of an Endogenous Gene**

In this experiment, dsRNA representing a 1 kb region of the lamin A/C gene and the luciferase gene were produced following the recommended protocols and using reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription Kit. The target sequences chosen for the lamin A/C and luciferase genes were as described by Elbashir *et al.*, 2001. The resulting dsRNA were used as substrates to generate lamin A/C and luciferase d-siRNA following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Complete Dicer RNAi Kit.

50 ng each of lamin A/C and luciferase d-siRNA as well as 4 pmoles each (about 50 ng) of synthetic lamin A/C and luciferase siRNA (21 nucleotide duplexes) were transfected into A549 (human lung carcinoma) cells plated in a 24-well plate using Lipofectamine™ 2000. Cell lysates were prepared 48 hours post-transfection and analyzed by Western blot using an Anti-Lamin A/C Antibody (1:1000 dilution, BD Biosciences, Catalog no. 612162) and an Anti-β-Actin Antibody (1:5000 dilution, Abcam, Catalog no. ab6276).

**Results:** Only the lamin A/C-specific d-siRNA (lane 2) and siRNA (lane 4) were able to inhibit expression of the lamin A/C gene, while no lamin A/C gene knockdown was observed with the luciferase d-siRNA (lane 3) or siRNA (lane 5). In addition, the degree of lamin A/C gene blocking achieved using the lamin A/C d-siRNA was similar to that achieved with the well-characterized, chemically-synthesized siRNA.

![Western blot image with lane labels](image.png)
Troubleshooting

**Introduction**

Use the information in this section to troubleshoot your dicing, purification, and transfection experiments.

**Dicing Reaction**

The table below lists some potential problems and possible solutions that may help you troubleshoot the dicing reaction.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
</table>
| Weak band representing d-siRNA observed on a polyacrylamide or agarose gel (*i.e.* low yield of d-siRNA) | Poor quality dsRNA | • Generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit (refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for instructions).  
• Verify the concentration of your dsRNA. |
| Didn’t use enough dsRNA in the dicing reaction | | • Use 60 µg of dsRNA in a 300 µl dicing reaction. If you are dicing less dsRNA, scale down the entire dicing reaction proportionally.  
• Make sure that the amount of dsRNA added does not exceed half the reaction volume (*i.e.* concentration of initial dsRNA substrate must be > 400 ng/µl). |
| dsRNA was degraded | | • Make sure that the dsRNA sample is in a buffer containing 1 mM EDTA (*i.e.* TE Buffer, pH 7-8 or 1X RNA Annealing Buffer).  
• Avoid repeated freeze/thaw cycles. Aliquot the dsRNA and store at -80°C. |
| Incubated the dicing reaction for longer than 18 hours | | Do not incubate the dicing reaction for longer than 18 hours. |
| Incubated the dicing reaction for less than 14 hours | | Incubate the dicing reaction at 37°C for 14-18 hours. |

*continued on next page*
## Troubleshooting, continued

### Dicing Reaction, continued

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear with molecular weight &lt; 21 nt observed on a polyacrylamide gel</td>
<td>Used too much BLOCK-iT™ Dicer Enzyme in the dicing reaction</td>
<td>Follow the recommended procedure to set up the dicing reaction. Do not use more than 60 units of BLOCK-iT™ Dicer Enzyme in a 300 µl reaction.</td>
</tr>
<tr>
<td>Incubated the dicing reaction for longer than 18 hours</td>
<td></td>
<td>Do not incubate the dicing reaction for longer than 18 hours.</td>
</tr>
</tbody>
</table>
| Sample contaminated with RNase | | • Use RNase-free supplies and solutions.  
• Wear gloves when handling reagents and setting up the dicing reaction. |
| No d-siRNA produced | dsRNA was degraded | • Make sure that the dsRNA sample is in a buffer containing 1 mM EDTA (i.e. TE Buffer, pH 7-8 or 1X RNA Annealing Buffer).  
• Avoid repeated freeze/thaw cycles. Aliquot the dsRNA and store at -80°C. |
| Sample was contaminated with RNase | | • Use RNase-free supplies and solutions.  
• Wear gloves when handling reagents and setting |
| ssRNA used as substrate | If you have used to the BLOCK-iT™ RNAi TOPO® Transcription Kit to generate sense and antisense ssRNA, you must anneal the ssRNA to generate dsRNA prior to dicing. | 

continued on next page
### Purifying d-siRNA

The table below lists some potential problems and possible solutions that may help you troubleshoot the purification procedure.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low yield of purified d-siRNA obtained</td>
<td>Eluted d-siRNA from the RNA Spin Cartridge using TE Buffer</td>
<td>Elute d-siRNA from the RNA Spin Cartridge using water.</td>
</tr>
<tr>
<td></td>
<td>Concentration of d-siRNA incorrectly determined</td>
<td>• Dilute sample in 1X RNA Annealing Buffer for spectrophotometry.</td>
</tr>
<tr>
<td></td>
<td>• Sample diluted into water for spectrophotometry</td>
<td>• Blank sample against 1X RNA Annealing Buffer.</td>
</tr>
<tr>
<td></td>
<td>• Sample blanked against water</td>
<td></td>
</tr>
<tr>
<td>No d-siRNA obtained</td>
<td>Forgot to add ethanol to the 5X RNA Wash Buffer</td>
<td>Add 10 ml of ethanol to the 5X RNA Wash Buffer (2.5 ml) to obtain a 1X RNA Wash Buffer.</td>
</tr>
<tr>
<td></td>
<td>Forgot to add isopropanol to the combined flow-throughs from the first RNA Spin Cartridge</td>
<td>You must add isopropanol to the combined flow-throughs from the first RNA Spin Cartridge to enable the d-siRNA to bind to the second RNA Spin Cartridge.</td>
</tr>
<tr>
<td></td>
<td>Forgot to keep flow-throughs from the first RNA Spin Cartridge</td>
<td>Keep the flow-throughs from the first RNA Spin Cartridge (Steps 3 and 5, page 16). The flow-throughs contain the d-siRNA.</td>
</tr>
<tr>
<td>dsRNA present in purified d-siRNA sample</td>
<td>Forgot to add isopropanol to the dicing reaction</td>
<td>You must add RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol and isopropanol to the dicing reaction to denature the proteins and enable the dsRNA to bind the first RNA Spin Cartridge.</td>
</tr>
<tr>
<td></td>
<td>Added the mixture containing the flow-through and isopropanol from the first RNA Spin Cartridge (Step 6, page 16) back onto the first RNA Spin Cartridge</td>
<td>You must add the mixture containing the flow-through and isopropanol from the first RNA Spin Cartridge (Step 6, page 16) to a second RNA Spin Cartridge as the first RNA Spin Cartridge contains bound dsRNA.</td>
</tr>
<tr>
<td>A260/A280 ratio not in the 1.9-2.2 range</td>
<td>Sample was not washed with 1X RNA Wash Buffer</td>
<td>Wash the RNA Spin Cartridge containing bound d-siRNA twice with 1X RNA Wash Buffer (see Steps 9 and 10, page 17).</td>
</tr>
<tr>
<td></td>
<td>RNA Spin Cartridge containing bound d-siRNA not centrifuged to remove residual 1X RNA Wash Buffer</td>
<td>Centrifuge RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 11, page 17).</td>
</tr>
</tbody>
</table>

*continued on next page*
## Troubleshooting, continued

### Transfection and RNAi Analysis

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

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
</table>
| Low levels of gene knockdown observed        | Low transfection efficiency                 | • Do not add antibiotics to the media during transfection.  
|                                               | • Antibiotics added to the media during transfection  
|                                               | • Cells were confluent at the time of transfection  
|                                               | • Not enough d-siRNA transfected              | • Plate cells such that they will be 30-50% confluent at the time of transfection.  
|                                               | • Not enough Lipofectamine™ 2000 used         | • Increase the amount of d-siRNA transfected.  
|                                               |                                             | • Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 used.  
| 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.  
| d-siRNA was degraded                          |                                             | • Make sure that the d-siRNA is stored in 1X RNA Annealing Buffer.  
|                                              |                                             | • Aliquot purified d-siRNA and avoid repeated freeze/thaw cycles.  
| Cytotoxic effects observed after transfection | Too much Lipofectamine™ 2000 Reagent used    | Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used.  
|                                              | Cells transfected with unpurified d-siRNA    | Purify d-siRNA using the RNAi Purification reagents supplied with the kit.  
| Important: Transfecting unpurified d-siRNA is not recommended as the contaminating dsRNA will cause host cell shutdown and apoptosis.  
| No gene knockdown observed                    | d-siRNA was degraded                         | • Make sure that the d-siRNA is stored in 1X RNA Annealing Buffer.  
|                                              | • d-siRNA was stored in water                | • Aliquot purified d-siRNA and avoid repeated freeze/thaw cycles.  
|                                              | • d-siRNA was repeatedly frozen and thawed   |                                              
|                                              | Target region contains no active siRNA       | Select a larger target region or a different region.  
| Non-specific off-target gene knockdown observed | Target sequence contains strong homology to other genes | • Select a new target sequence.  
|                                              |                                             | • Limit the size range of the target sequence to 1 kb.  

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...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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Product Qualification

Introduction

The components of the BLOCK-iT™ Dicer RNAi Kits are qualified as described below.

Functional Qualification

The BLOCK-iT™ Dicer enzyme and RNAi Purification reagents are functionally qualified as follows:

1. The BLOCK-iT™ Dicer enzyme is diluted to 1 U/µl and tested (in triplicate) in a dicing reaction following the procedure on page 10 using lacZ dsRNA produced using the BLOCK-iT™ RNAi TOPO® Transcription Kit. Each dicing reaction is assessed by analyzing an aliquot of the reaction on a 20% Novex® TBE gel (Catalog no. EC63152BOX). The 10 bp DNA Ladder (Catalog no. 10821-015) is included as a molecular weight standard. Polyacrylamide gel analysis must demonstrate a minimal amount of dsRNA remaining in the reaction and minimal to no degradation of siRNA apparent.

2. The dicing reactions are purified using the RNAi purification reagents supplied in the kit and following the procedure on page 16. Purified d-siRNA is quantitated using spectrophotometry. The amount of d-siRNA recovered must be at least 25%.

Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 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.
References


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