USER GUIDE

invitrogen™
by life technologies™

BLOCK-iT™ RNAi TOPO® Transcription Kit

For TOPO®-mediated generation of templates and production of double-stranded RNA (dsRNA) for use in RNA interference (RNAi) analysis

Catalog numbers K3500-01 and K3650-01

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dsRNA Generation Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the dsRNA generation procedure. If you are performing the TOPO® Linking, secondary amplification, transcription, purification, or annealing steps for the first time, follow the detailed protocols provided in the manual. We recommend using the pcDNA™1.2/V5-GW/*lacZ* plasmid and the control PCR primers (LacZ Forward 2 and LacZ Reverse 2 primers) included with the kit to generate dsRNA. See pages 38-40 for instructions.

Step	Action
Produce the PCR product	1. Amplify your sequence of interest using Platinum® *Taq* DNA polymerase and your own protocol. End the PCR reaction with a final 7 minute extension step. 2. Use agarose gel electrophoresis to check the integrity and yield of your PCR product.
Perform the TOPO® Linking reaction	1. Set up the following TOPO® Linking reaction. Your PCR product (≥ 20 ng/µl) 1 µl Salt Solution 1 µl Sterile water 3 µl BLOCK-iT™ T7-TOPO® Linker 1 µl Total volume 6 µl 2. Mix reaction gently and incubate for 15 minutes at 37°C. 3. Place the reaction on ice and proceed directly to perform secondary amplification.
Perform secondary amplification reactions to generate sense and antisense DNA templates	1. Set up 2 PCR reactions – in each reaction, amplify 1 µl of the TOPO® Linking reaction using Platinum® *Taq* DNA polymerase and your own protocol. End the PCR reaction with a final 7 minute extension step. For PCR primers, use the following: • **Sense template:** use the BLOCK-iT™ T7 Primer and your gene-specific reverse primer • **Antisense template:** use the BLOCK-iT™ T7 Primer and your gene-specific forward primer 2. Use agarose gel electrophoresis to check the integrity and yield of your PCR products. 3. Proceed to perform the RNA transcription reactions, see next step.

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dsRNA Generation Procedure for Experienced Users, continued

Step	Action
Perform the RNA transcription reaction to generate sense and antisense ssRNA	1. Set up two separate transcription reactions using either the sense or antisense linear DNA template. RNase-free water up to 21 µl 75 mM NTPs 8 µl DNA template (250 ng-1 µg) 1-10 µl 10X Transcription buffer 4 µl BLOCK-iT™ T7 Enzyme Mix 6 µl Total volume 40 µl 2. Incubate the reaction at 37°C for 2 hours. 3. Add 2 µl of DNase I to each reaction. Incubate at 37°C for 15 minutes.
Purify the sense and antisense transcripts	1. To each RNA transcription reaction, add 160 µl of RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol followed by 100 µl of 100% ethanol. Mix well by pipetting up and down 5 times. 2. Apply the sample to the RNA Spin Cartridge, and centrifuge at 14,000 × g for 15 seconds at room temperature. Discard the flow-through. 3. Add 500 µl of 1X RNA Wash Buffer to the RNA Spin Cartridge, and centrifuge at 14,000 × g for 15 seconds at room temperature. Discard the flow-through. 4. Repeat Step 3. 5. Centrifuge the RNA Spin Cartridge at 14,000 × g for 1 minute at room temperature. 6. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube. Add 40 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 × g for 2 minutes at room temperature to elute the ssRNA. 7. Add 40 µl of RNase-Free Water to the RNA Spin Cartridge and repeat Step 7, eluting the ssRNA into the same RNA Recovery Tube. Add 1.4 µl of 50X RNA Annealing Buffer to the eluted ssRNA. 8. Quantitate the yield of ssRNA by spectrophotometry.
Anneal the sense and antisense transcripts to produce dsRNA	1. In a microcentrifuge tube, mix equal amounts of purified sense and antisense ssRNA. 2. Heat 250 ml of water to boiling in a 500 ml glass beaker, remove from the heat, and set the beaker on the laboratory bench. 3. Place the tube containing the ssRNA mixture (in a tube float) in the glass beaker and allow the water to cool to room temperature for 1-1.5 hours. 4. Aliquot and store the dsRNA at -80°C.

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™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Dicer RNAi Kits manual.

Product	Catalog no.
BLOCK-iT™ RNAi TOPO® Transcription Kit	K3500-01
BLOCK-iT™ Complete Dicer RNAi Kit	K3650-01

Kit Components

The BLOCK-iT™ RNAi Kits include the following components. For a detailed description of the contents of the BLOCK-iT™ RNAi TOPO® Transcription Kit, see pages 7-8.

Note: The BLOCK-iT™ Complete Dicer RNAi Kit also includes the BLOCK-iT™ Dicer RNAi Transfection Kit. For a detailed description of the reagents supplied in the BLOCK-iT™ Dicer RNAi Transfection Kit, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

Component	Catalog no.	
	K3500-01	K3650-01
BLOCK-iT™ RNAi TOPO® Transcription Kit	√	√
BLOCK-iT™ Dicer RNAi Transfection Kit		√

Shipping/Storage

The BLOCK-iT™ RNAi TOPO® Transcription Kit is shipped as described below. Upon receipt, store each item as detailed below.

Box	Component	Shipping	Storage
1	BLOCK-iT™ TOPO® Linker Kit	Dry ice	-20°C
2	BLOCK-iT™ RNAi Transcription Kit	Dry ice	-20°C
3	BLOCK-iT™ RNAi Purification Kit	Room temperature	Room temperature

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BLOCK-iT™ TOPO® Linker Kit Reagents

The following reagents are supplied with the BLOCK-iT™ TOPO® Linker Kit (Box 1). **Note that the user must supply *Taq* polymerase. Store the reagents at -20°C.**

Reagent	Composition	Amount
BLOCK-iT™ T7-TOPO® Linker	0.1-1 ng/µl double-stranded DNA in: 50 mM Tris-HCl, pH 7.3 100 mM NaCl 0.2 mM EDTA 0.9 mM DTT 45 µg/ml BSA 0.05% (v/v) Triton X-100 40% (v/v) glycerol	5 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	75 µl
40 mM dNTPs	10 mM dATP 10 mM dTTP 10 mM dGTP 10 mM dCTP neutralized at pH 8.0 in water	15 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	10 µl
Sterile Water	--	750 µl
BLOCK-iT™ T7 Primer	75 ng/µl in TE Buffer, pH 8.0	10 µl
LacZ Forward 2 Primer	65 ng/µl in TE Buffer, pH 8.0	10 µl
LacZ Reverse 2 Primer	65 ng/µl in TE Buffer, pH 8.0	10 µl
pcDNA™1.2/V5-GW/*lacZ* control plasmid	500 ng/µl in TE Buffer, pH 8.0	20 µl

Primer Sequences

The table below provides the sequence and the amount supplied of the primers included in the kit.

Primer	Sequence	Amount
BLOCK-iT™ T7	5′-GATGACTCGTAATACGACTCACTA-3′	103 pmoles
LacZ Forward 2	5′-ACCAGAAGCGGTGCCGGAAA-3′	105 pmoles
LacZ Reverse 2	5′-CCACAGCGGATGGTTCGGAT-3′	106 pmoles

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BLOCK-iT™ RNAi Transcription Kit Reagents

The following reagents are included with the BLOCK-iT™ RNAi Transcription Kit. **Store reagents at -20°C**.

Reagent	Composition	Amount
BLOCK-iT™ T7 Enzyme Mix	Proprietary	60 µl
10X Transcription Buffer	Proprietary	40 µl
75 mM NTPs	18.75 mM ATP 18.75 mM UTP 18.75 mM CTP 18.75 mM GTP neutralized at pH 8.0 in water	80 µl
RNase-Free Water	--	800 µl
DNase I	1 U/µl in 20 mM sodium acetate, pH 6.5 5 mM $CaCl_2$ 0.1 mM PMSF 50% (v/v) glycerol	20 µl

BLOCK-iT™ RNAi Purification Kit

The following reagents are included with the BLOCK-iT™ RNAi Purification Kit. **Store reagents at room temperature**. Use caution when handling the RNA Binding Buffer (see page 9 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 the single-stranded RNA (ssRNA). The second box is supplied with the BLOCK-iT™ Dicer RNAi Transfection Kit for purification of diced siRNA (d-siRNA).

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

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

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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.

Accessory Products

The table below provides ordering information for products available from Life Technologies that are suitable for use with the BLOCK-iT™ RNAi TOPO® Transcription Kit.

Item	Amount	Catalog no.
BLOCK-iT™ Dicer RNAi Transfection Kit	5 genes × 150 transfections each*	K3600-01
Taq DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
6% Novex® TBE Gel	1 box	EC6265BOX
0.16-1.77 kb RNA Ladder	75 µg	15623-010

*Based on transfection in 24-well plates

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Introduction

Overview

Introduction

The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates rapid generation of T7 promoter-based DNA templates. Using the DNA templates and reagents supplied with the kit, RNA transcripts are produced, purified, and annealed to generate double-stranded RNA (dsRNA). The resulting dsRNA may be used directly for RNA interference (RNAi) analysis in invertebrate systems and other systems lacking the interferon response (see page 16 for more information) or as a substrate to produce short interfering RNA (siRNA) for RNAi analysis in mammalian cells.

Advantages of the BLOCK-iT™ RNAi TOPO® Transcription Kit

Use of the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate production of dsRNA provides the following advantages:

- The BLOCK-iT™ T7-TOPO® Linker provides a method to quickly and easily add a T7 promoter to any existing *Taq*-amplified PCR product without the need for new primers or subcloning.
- Use of the TOPO® Linking Technology and secondary amplification enables simultaneous production of linear DNA templates that may be used directly for *in vitro* transcription to generate sense and antisense transcripts. Creation of a T7 expression plasmid, bacterial transformation, and plasmid purification are not required.
- Separate transcription reactions using sense and antisense templates allow precise quantitation of ssRNA concentration prior to annealing.
- Provides optimized purification reagents to obtain highly pure sense and antisense transcripts that can be annealed to generate an optimal yield of dsRNA. Double-stranded RNA can be used directly for RNAi analysis in invertebrate systems or as a substrate for the Dicer enzyme to generate siRNA.

Purpose of this Manual

This manual provides instructions and guidelines to:

1. Amplify your sequence of interest and use TOPO® Linking to join the primary PCR product to the BLOCK-iT™ T7-TOPO® Linker.
2. Use the appropriate primers to amplify the TOPO® Linked PCR product to generate linear sense and antisense DNA templates.
3. Use the linear sense and antisense DNA templates in transcription reactions to generate sense and antisense single-stranded RNA (ssRNA) transcripts of the sequence of interest.
4. Purify the sense and antisense ssRNA transcripts and anneal them to generate dsRNA. The resulting dsRNA may then be used in the application of choice (*e.g.* RNAi analysis in invertebrate organisms or as a substrate for "dicing" to produce d-siRNA for RNAi analysis in mammalian cells).

For details and instructions to generate d-siRNA using Dicer, refer to the BLOCK-iT™ Dicer RNAi Kits manual. This manual is supplied with the BLOCK-iT™ Dicer RNAi Transfection and Complete Dicer RNAi Kits but is also available for downloading from our website (**www.lifetechnologies.com**) or by contacting Technical Support (see page 45).

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Important

The BLOCK-iT™ RNAi TOPO® Transcription Kit is designed to help you generate dsRNA for direct use in RNAi analysis in invertebrate systems or as a substrate in a dicing reaction to produce d-siRNA for RNAi analysis in mammalian cells. Although the kit has been designed to help you generate dsRNA representing a particular target sequence in the simplest, most direct fashion, use of the resulting dsRNA for RNAi analysis assumes that users are familiar with the mechanism of gene silencing and the techniques that exist to introduce dsRNA into the organism or cell type of choice. We highly recommend that users possess a working knowledge of the RNAi pathway and the methodologies required to perform RNAi analysis in the organism or cell type of choice.

For more information about these topics, refer to published reviews (Bosher and Labouesse, 2000; Hannon, 2002; Plasterk and Ketting, 2000; Zamore, 2001).

Where to Go For More Information

A variety of BLOCK-iT™ RNAi products are available from Life Technologies to facilitate your RNAi analysis. For more information about these products and other reference material, refer to the RNAi product page on our website at **www.lifetechnologies.com/rnai**.

The BLOCK-iT™ RNAi TOPO® Transcription Kit

Description of the System

The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates generation of T7 promoter-based DNA templates for *in vitro* transcription and production of dsRNA, and consists of three major components:

- The BLOCK-iT™ T7-TOPO® Linker for quick and easy creation of T7 promoter-based DNA templates for *in vitro* transcription. Using TOPO® Linking Technology, the BLOCK-iT™ T7-TOPO® Linker may be linked to any *Taq*-amplified PCR product. The linked PCR product is then amplified to generate a linear DNA template. For more information about how TOPO® Linking works, see page 14.
- BLOCK-iT™ RNAi Transcription reagents for generation of sense and antisense ssRNA transcripts from your T7-based, linear DNA template. The reagents include an optimized T7 Enzyme Mix for highly efficient production of ssRNA.
- The BLOCK-iT™ RNAi Purification reagents for silica-based column purification of sense and antisense ssRNA transcripts, and an RNA Annealing Buffer to stabilize dsRNA duplexes for long-term storage.

Important

The BLOCK-iT™ RNAi TOPO® Transcription Kit also includes a control expression plasmid containing the *lacZ* gene and PCR primers that may be used as controls to generate dsRNA. Once generated, the lacZ dsRNA may be used for the following types of RNAi analysis:

Invertebrate Systems

- As a negative control for non-specific gene knockdown in any invertebrate system.

  Note: The lacZ dsRNA is not suitable for use as a positive control to knock down β-galactosidase expression from the control pcDNA™1.2/V5-GW/*lacZ* plasmid in any invertebrate system. This is because expression of the *lacZ* gene from the control plasmid is controlled by the human cytomegalovirus (CMV) promoter, and this promoter is **not** active in most invertebrate systems.

Mammalian Systems

- As a negative control for non-specific gene knockdown **or** as a positive control for knockdown of β-galactosidase expression from the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid. Note that to perform RNAi analysis in mammalian cells, the lacZ dsRNA must first be "diced" to generate d-siRNA. For details, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

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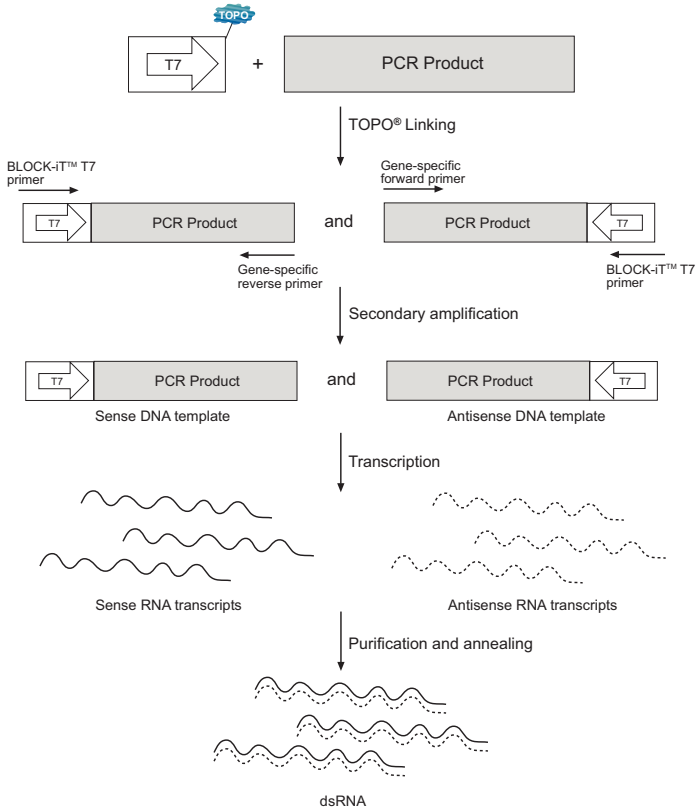
Generating dsRNA Using the BLOCK-iT™ RNAi TOPO® Transcription Kit

You will perform the following steps to generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit. For a diagram, see below.

1. Amplify your sequence of interest using *Taq* polymerase.
2. Perform a TOPO® Linking reaction to link your PCR product to the BLOCK-iT™ T7-TOPO® Linker containing the T7 promoter.
3. Using a combination of the BLOCK-iT™ T7 Primer (supplied with the kit) and your gene-specific forward or reverse primer, amplify the TOPO® Linked PCR product with *Taq* polymerase to produce linear sense and antisense DNA templates.
4. Use the sense and antisense DNA templates and the reagents supplied in the kit in an *in vitro* transcription reaction to produce sense and antisense RNA transcripts, respectively.
5. Purify the sense and antisense RNA transcripts using the RNAi Purification reagents supplied in the kit.
6. Quantitate the yield of purified sense and antisense ssRNA transcripts, and anneal equal amounts of each single-stranded transcript to form dsRNA.

Diagram of the System

The figure below illustrates the major steps necessary to generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription System.



How TOPO® Linking Works

How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Linking exploits this reaction to efficiently join PCR products to the BLOCK-iT™ T7-TOPO® Linker.

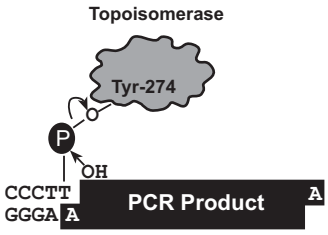
TOPO® Linking

The BLOCK-iT™ T7-TOPO® Linker is supplied linearized with:

- A single 3´ thymidine (T) overhang for TA Cloning®
- Topoisomerase I covalently bound to the linker (this is referred to as "activated linker")

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linear BLOCK-iT™ T7-TOPO® linker supplied in this kit has a single, overhanging 3´ deoxythymidine (T) residue. This allows PCR products to ligate efficiently with the linker.

TOPO® Linking exploits the ligation activity of topoisomerase I by providing an "activated" linearized TA linker using proprietary technology (Shuman, 1994). Ligation of the linker with a PCR product containing 3´ A-overhangs is very efficient and occurs spontaneously with maximum efficiency at 37°C within 15 minutes.



The RNAi Pathway

The RNAi Pathway

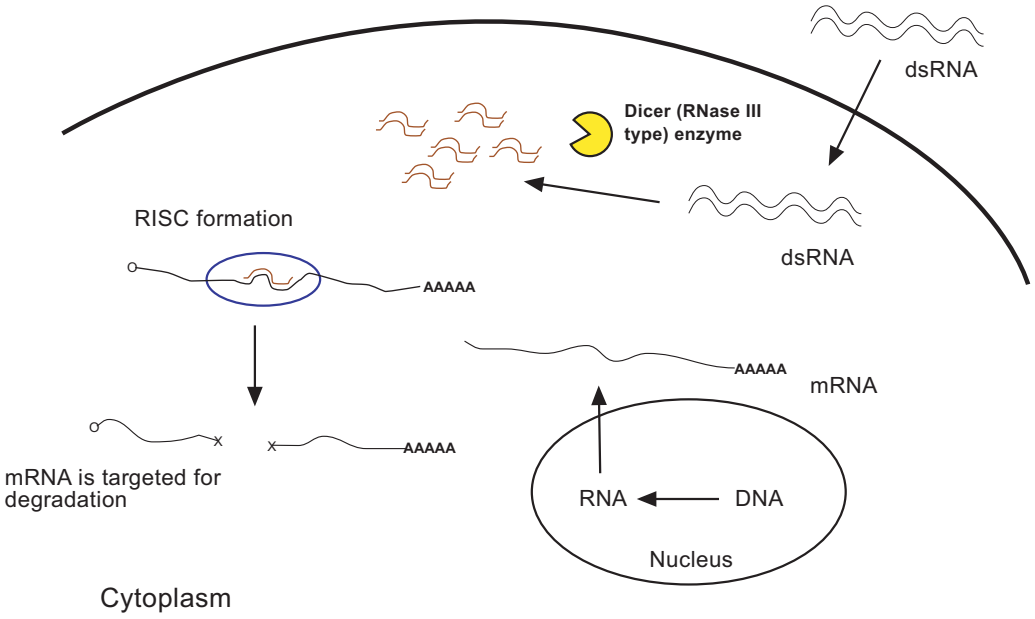
RNAi describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et 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). The siRNA then incorporate into the RNA-induced silencing complex (RISC), a second 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).

Using the Kit for RNAi Analysis

The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates *in vitro* production of dsRNA that is targeted to a particular gene of interest. The long dsRNA is introduced into the appropriate organism or cells (see page 16), where the endogenous Dicer enzyme processes the dsRNA into siRNA. The resulting siRNA can then inhibit expression of the target gene. For a diagram of the process, see the figure below.



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Use of dsRNA for RNAi Analysis

Long dsRNA duplexes can be used directly for RNAi analysis in organisms and systems lacking the interferon response, including insects (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), insect cell lines (Caplen *et al.*, 2000), *C. elegans* (Fire *et al.*, 1998), trypanosomes (Ngo *et al.*, 1998), some mammalian embryonic cell lines (Billy *et al.*, 2001; Yang *et al.*, 2001), and mouse oocytes and preimplantation embryos (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000).

Important

Long dsRNA duplexes **cannot** be used directly for RNAi analysis in most somatic mammalian cell lines. This is because introduction of 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 perform RNAi analysis in mammalian cell lines, long dsRNA must first be cleaved into 21-23 nucleotide siRNA duplexes. This cleavage process may be performed *in vitro* using recombinant Dicer enzyme such as is provided in the BLOCK-iT™ Dicer RNAi Transfection Kit or the BLOCK-iT™ Complete Dicer RNAi Kit. For more information, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

Experimental Outline

Experimental Outline

The table below describes the major steps required to generate a dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit. Refer to the specified pages for details to perform each step.

Step	Action	Page
1	Produce your PCR product using *Taq* polymerase or Platinum® *Taq* DNA polymerase.	20-21
2	Verify the integrity and concentration of your PCR product.	21
3	Perform the TOPO® Linking reaction to link your PCR product to the BLOCK-iT™ T7-TOPO® Linker.	22
4	Amplify the TOPO® Linked PCR product using the appropriate primers to produce sense and antisense linear DNA templates.	23-24
5	Use **each** linear DNA template in an RNA transcription reaction to produce sense and antisense RNA transcripts.	25-27
6	Purify sense and antisense RNA transcripts.	28-29
7	Quantitate the yield of each purified ssRNA obtained, and anneal equal amounts of sense and antisense ssRNA to generate dsRNA.	30-32

Designing PCR Primers

Introduction

To use the BLOCK-iT™ RNAi TOPO® Transcription Kit, you will first need to design PCR primers to amplify your sequence of interest. Guidelines to choose the target sequence and to design PCR primers are provided below.

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 transcription efficiency and thus the yield of dsRNA 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. If you plan to "dice" the dsRNA to produce d-siRNA for use in mammalian RNAi analysis, note that dsRNA that are under 1 kb in size are efficiently diced. Larger dsRNA can be used but yields may decline as the size increases.

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

Factors to Consider When Designing PCR Primers

Once you have selected an appropriate target sequence, you will need to design gene-specific primers to amplify your target sequence of interest. Consider the following factors when designing gene-specific primers.

- Make sure that your primers do not contain sequence that is homologous to other genes.
- Once you have linked your primary PCR product to the BLOCK-iT™ T7-TOPO® Linker, you will amplify the resulting linked product using the BLOCK-iT™ T7 Primer and either your gene-specific forward primer or gene-specific reverse primer. When designing your gene-specific PCR primers, make sure that the T_m of each primer is compatible with the T_m of the BLOCK-iT™ T7 primer (*i.e.* T_m = 62°C).

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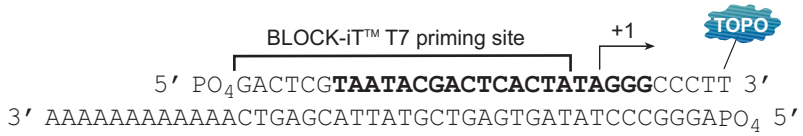
Designing PCR Primers, continued

Diagram of the BLOCK-iT™ T7-TOPO® Linker

Use the diagram below to help you design appropriate PCR primers to join your sequence of interest with the BLOCK-iT™ T7-TOPO® Linker. The BLOCK-iT™ T7-TOPO® Linker is supplied as a double-stranded DNA fragment adapted with topoisomerase I.

Features of the BLOCK-iT™ T7-TOPO® Linker:

- The sequence of the T7 promoter is indicated in bold.
- The transcription start site is indicated by +1.



Important

- **To obtain consistent and efficient results in the TOPO® Linking reaction, we recommend using HPLC-purified oligonucleotides to produce your PCR products.** Using a mixture of full-length and non full-length primers to produce your PCR products can reduce the efficiency of TOPO® Linking and result in poor yield of the linear DNA templates after secondary amplification.
- **Do not add 5′ phosphates to your primers for PCR.** This will prevent TOPO® Linking.

Amplifying Your Sequence of Interest

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your PCR product.

Choosing a Thermostable DNA Polymerase

To amplify your sequence of interest, use a thermostable DNA polymerase that generates PCR products with 3′ A-overhangs. **We recommend using Platinum® *Taq* polymerase available from Life Technologies (see page 9 for ordering information).** *Taq* polymerase is also suitable.

Note: You may use *Taq* polymerase and proofreading polymerase mixtures to generate PCR products, however, a certain proportion of your PCR products will be blunt-ended. You can add 3′ A-overhangs to your PCR products using the method on page 42.

Control Plasmid

We recommend amplifying the control template included with the kit in parallel with your sample. Use the LacZ Forward 2 and the LacZ Reverse 2 primers included with the kit and the protocol on page 38 to amplify the pcDNA™1.2/V5-GW/*lacZ* plasmid. The resulting control PCR product (representing a 1 kb fragment of the *lacZ* gene) may then be used as a positive control for subsequent procedures including TOPO® Linking, transcription, and production of dsRNA. For a map of pcDNA™1.2/V5-GW/*lacZ*, refer to the **Appendix**, page 44.

To use the pcDNA™1.2/V5-GW/*lacZ* plasmid as a template for amplification, dilute as appropriate and use 1-10 ng of plasmid DNA in the PCR reaction.

Materials Needed

You should have the following materials on hand before beginning:

- Thermocycler
- Thermostable DNA polymerase (*e.g.* Platinum® *Taq* DNA Polymerase)
- DNA template
- Gene-specific forward and reverse PCR primers (10 µM each)
- 10X PCR Buffer (supplied with the kit, Box 1)
- 40 mM dNTPs (supplied with the kit, Box 1)
- Sterile water (supplied with the kit, Box 1)

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Setting Up the PCR Reaction

Use the procedure below to amplify your sequence of interest using Platinum® *Taq* DNA polymerase. Use less DNA if you are using plasmid DNA as a template (1-10 ng) and more DNA if you are using genomic DNA as a template (10-100 ng).

Note: If you are using a different thermostable DNA polymerase, reaction conditions may vary.

1. Set up the following 50 µl PCR reaction.

DNA Template	1-100 ng
10X PCR Buffer	5 µl
40 mM dNTPs	1 µl
PCR Primers (10 µM **each**)	1 µl each
Sterile water	add to a final volume of 49.5 µl
Platinum® *Taq* polymerase (5 U/µl)	0.5 µl
Total volume	50 µl

2. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length and 3′ adenylated.
3. After cycling, place the tube on ice. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

Analyze 1-5 µl of the PCR reaction using agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following:

- A single discrete band of the expected size corresponding to your sequence of interest. If you do not obtain a single, discrete band from your PCR, follow the manufacturer's recommendations or use the PCR Optimizer™ Kit (Catalog no. K1220-01) from Life Technologies to optimize your PCR conditions using your DNA polymerase. Other tips may be found on page 34 or in published reference sources (Innis *et al.*, 1990). Alternatively, you may gel-purify your fragment before proceeding to TOPO® Linking (see page 41 for protocols).
- Estimate the concentration of your PCR product. For optimal TOPO® Linking, the concentration of your PCR should be ≥ 20 ng/µl. If your PCR product is too dilute, see **Concentrating Dilute PCR Products**, below.

Once you have verified that your PCR product is of the appropriate quality and concentration, proceed to **Performing the TOPO® Linking Reaction**, next page.

For optimal results, use fresh PCR product in the TOPO® Linking reaction.

Note: You may store the PCR product at -20°C for up to 1 week.

Concentrating Dilute PCR Products

If you obtain a single band from PCR, but your PCR product is too dilute, you may purify and concentrate the PCR product before proceeding to the TOPO® Linking reaction. A procedure to purify and concentrate PCR products is provided in the **Appendix**, page 43.

Performing the TOPO® Linking Reaction

Introduction

Once you have produced your PCR product, you will use TOPO® Linking to join the PCR product to the BLOCK-iT™ T7-TOPO® Linker. Before performing the TOPO® Linking reaction, you should have everything you need set up and ready to use to ensure that you obtain the best results. If you have produced the control PCR product and this is the first time you have performed TOPO® Linking, we recommend performing the control TOPO® Linking reaction (see page 39) in parallel with your samples.

Materials Needed

Have the following reagents on hand before beginning:

- Your primary PCR product (≥ 20 ng/µl)
- BLOCK-iT™ T7-TOPO® Linker (supplied with the kit, Box 1; keep at -20°C until use)
- Salt Solution (supplied with the kit; Box 1)
- Sterile Water (supplied with the kit; Box 1)
- 37°C water bath

TOPO® Linking Procedure

Follow the procedure below to perform the TOPO® Linking reaction.

1. Set up a 6 µl TOPO® Linking reaction using the following reagents in the order given.

Your PCR product (≥ 20 ng/µl)	1 µl
Salt Solution	1 µl
Sterile water	3 µl
BLOCK-iT™ T7-TOPO® Linker	1 µl
Total volume	6 µl

2. Mix reaction gently and incubate for 15 minutes at 37°C.

   Note: Do not incubate the reaction for longer than 15 minutes as this may negatively affect TOPO® Linking.

3. Place the reaction on ice and proceed directly to **Performing Secondary Amplification**, see page 23.

   Note: You may store the TOPO® Linking reaction at -20°C overnight, if desired.

Performing Secondary Amplification Reactions

Introduction

Once you have performed the TOPO® Linking reaction, you will use this reaction mixture in **two** PCR reactions with the appropriate PCR primers to produce sense and antisense linear DNA templates. Guidelines to perform secondary amplification are provided in this section.

Thermostable DNA Polymerase

You may use any thermostable DNA polymerase to produce sense and antisense linear DNA templates. We generally use the same thermostable DNA polymerase to perform secondary amplification as we use to generate the primary PCR product (i.e. Platinum® *Taq* DNA Polymerase; see page 9 for ordering information).

PCR Primers

To produce sense and antisense linear DNA templates, you will perform two amplification reactions using the TOPO® Linking reaction and the appropriate primers (see table below). For gene-specific PCR primers, use the primers that you used to produce your primary PCR product. The BLOCK-iT™ T7 Primer is supplied with the kit.

Sense Template	Antisense Template
BLOCK-iT™ T7 Primer	BLOCK-iT™ T7 Primer
Gene-specific reverse primer	Gene-specific forward primer

General Guidelines

When amplifying the TOPO® Linked PCR product, we recommend the following:

- Perform the PCR reaction in a total volume of 50 µl.
- Use 1 µl of the TOPO® Linking reaction as the DNA template.
- If you use the same thermostable DNA polymerase to perform secondary amplification as was used to generate the primary PCR product, you may generally use similar cycling conditions. However, because you are using different PCR primers, you may need to adjust the cycling conditions.

Materials Needed

You should have the following materials on hand before beginning:

- Thermocycler
- Thermostable DNA polymerase (*e.g.* Platinum® *Taq* DNA Polymerase)
- TOPO® Linking reaction (from Step 3, page 22)
- Gene-specific forward and reverse primers (10 µM each)
- BLOCK-iT™ T7 Primer (supplied with the kit, Box 1)
- 10X PCR Buffer (supplied with the kit, Box 1)
- 40 mM dNTPs (supplied with the kit, Box 1)
- Sterile water (supplied with the kit, Box 1)

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Setting Up the Secondary PCR Reactions

Use the procedure below to amplify the TOPO® Linked PCR product using Platinum® *Taq* DNA polymerase. If you are using a different thermostable DNA polymerase, reaction conditions may vary.

1. Set up the following 50 µl PCR reactions:

Reagent	Sense Template	Antisense Template
10X PCR Buffer	5 µl	5 µl
40 mM dNTPs	1 µl	1 µl
BLOCK-iT™ T7 Primer (75 ng/µl)	1 µl	1 µl
Gene-specific forward primer (10 µM)	--	1 µl
Gene-specific reverse primer (10 µM)	1 µl	--
Sterile water	40.5 µl	40.5 µl
TOPO® Linking reaction	1 µl	1 µl
Platinum® *Taq* Polymerase (5 U/µl)	0.5 µl	0.5 µl
Total volume	50 µl	50 µl

2. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length.
3. After cycling, place the tube on ice. Proceed to **Checking the PCR Products**, below.

Checking the PCR Products

Analyze 1-5 µl of each PCR reaction using agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following:

- A single discrete band of the expected size corresponding to your linked linear DNA template.

  Note: You may see some minor background bands. These are generally due to smaller PCR products that were in the primary PCR reaction and should not affect the efficiency of the transcription reaction.
- Estimate the concentration of each PCR product. For optimal transcription efficiency, the concentration of each PCR product should be ≥ 25 ng/µl. If your PCR product(s) is too dilute, you may increase the number of cycles of the amplification reaction or use the procedure provided in the **Appendix**, page 43 to purify and concentrate your PCR product.

Once you have verified that your PCR products are of the appropriate quality and concentration, proceed to **Performing the RNA Transcription Reaction**, see page 25.

Storing the PCR Products

For optimal results, use fresh PCR products in the RNA transcription reaction.

Note: You may store the PCR products at -20°C for up to 1 month, if desired.

Performing the RNA Transcription Reactions

Introduction

Once you have produced the sense and antisense DNA templates of your target sequence, you will perform two transcription reactions using the reagents supplied in the RNA Transcription Kit (Box 2) to generate sense and antisense transcripts.

Amount of DNA Template to Use

For each RNA transcription reaction, you will need 250 ng to 1 µg of your DNA template. For best results, make sure that the concentration of your sense and antisense DNA templates is ≥ 25 ng/µl.

Positive Control

If you have performed the control reactions described on pages 38-39, we recommend using the resulting sense and antisense lacZ templates as controls in the RNA transcription, purification, and annealing procedures. Once you have produced control lacZ dsRNA, you may:

- Use this dsRNA as a negative control for non-specific, off-target effects in your RNAi studies.
- Include the lacZ dsRNA in a dicing reaction (refer to the BLOCK-iT™ Dicer RNAi Kits manual for instructions), then use the resulting lacZ d-siRNA as a positive control for RNAi in mammalian cells. Co-transfect the lacZ d-siRNA and the pcDNA™1.2/V5-GW/*lacZ* plasmid into mammalian cells and assay for knockdown of β-galactosidase expression.

Important

When performing the RNA transcription reaction and all subsequent procedures, 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 setting up the transcription reaction.

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Performing the RNA Transcription Reactions, continued

Materials Needed

You should have the following materials on hand before beginning:

- Sense and antisense DNA templates (from the Secondary Amplification reactions, Step 3, page 24; ≥ 25 ng/µl each)
- RNase-Free Water (supplied with the kit, Box 2)
- 75 mM NTPs (supplied with the kit, Box 2)
- 10X Transcription Buffer (supplied with the kit, Box 2; keep on ice until use)
- BLOCK-iT™ T7 Enzyme Mix (supplied with the kit, Box 2; keep at -20°C until use)
- DNase I (supplied with the kit, Box 2)
- RNase-free supplies (e.g. microcentrifuge tubes and pipette tips)
- 37°C water bath

Guidelines to Set Up the Transcription Reactions

Follow the guidelines below when setting up the transcription rections.

- Set up the transcription reaction at room temperature. **Do not** set up the reaction on ice as components in the transcription buffer may precipitate the DNA template.
- Keep the 10X Transcription Buffer on ice; do not thaw until immediately before use.

  Note: Upon thawing the 10X Transcription Buffer, you may notice some precipitate in the bottom of the tube. Warm the buffer to 37°C and vortex briefly to allow the precipitate to go back into solution.
- When setting up the transcription reaction, add the components to the microcentrifuge tube **exactly** in the order stated. Add the 10X Transcription Buffer to the mixture directly before adding the BLOCK-iT™ T7 Enzyme Mix, and mix immediately to avoid precipitation of the template. After use, return the 10X Transcription Buffer and the BLOCK-iT™ T7 Enzyme Mix to -20°C.

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RNA Transcription Procedure

Use the procedure below to synthesize transcripts from your DNA template. Remember that for each gene, you will generate sense and antisense transcripts using the sense and antisense DNA templates, respectively. Be sure to use RNase-free supplies and wear gloves to prevent RNase contamination.

Note: If you wish to include a negative control, set up the transcription reaction as described below, except omit the DNA template.

1. For each sample, add the following components **exactly** in the order stated to a 0.5 ml sterile, microcentrifuge tube at room temperature and mix. Note that the amount of RNase-free water added will depend on the concentration of your DNA template.

Reagents	Amount
RNase-Free Water	up to 21 µl
75 mM NTPs	8 µl
DNA template (250 ng-1 µg)	1-10 µl
10X Transcription Buffer	4 µl
BLOCK-iT™ T7 Enzyme Mix	6 µl
Total volume	40 µl

2. Incubate the reaction at 37°C for 2 hours.

   Note: The length of the RNA transcription reaction can be extended up to 6 hours. Most of the transcripts are produced within the first 2 hours, but yields can be increased with longer incubation.

3. Add 2 µl of DNase I to each reaction. Incubate for 15 minutes at 37°C.
4. Proceed to **Purifying RNA Transcripts**, next page.

   Note: You may store the RNA transcription reactions at -20°C overnight before purification, if desired.

Purifying RNA Transcripts

Introduction

This section provides guidelines and instructions to purify the single-stranded RNA transcripts (ssRNA) produced in the RNA transcription reaction. Use the BLOCK-iT™ RNA Purification reagents (Box 3) supplied with the kit. Remember that for each gene, you will perform 2 purification reactions to purify sense and antisense RNA transcripts.

Experimental Outline

To purify RNA transcripts, you will:

1. Add RNA Binding Buffer and ethanol to the transcription reaction to denature the proteins and to enable the ssRNA to bind to the column.
2. Add the sample to an RNA spin cartridge. The ssRNA binds to the silica-based membrane in the cartridge, and the digested DNA, free NTPs, and denatured proteins flow through the cartridge.
3. Wash the membrane-bound ssRNA to eliminate residual RNA Binding Buffer and any remaining impurities.
4. Elute the ssRNA from the RNA spin cartridge with water.

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 generate 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:

- RNA transcription reactions (from Step 4, page 27; for each gene, you should have a sense transcription reaction and an antisense transcription reaction)
- RNA Binding Buffer (supplied with the kit, Box 3)
- β-mercaptoethanol
- 100% ethanol
- RNA spin cartridges (supplied with the kit, Box 3; one for each sample)
- 1X RNA Wash Buffer (see **Advance Preparation**, above)
- RNase-Free Water (supplied with the kit, Box 3)
- RNA Recovery Tubes (supplied with the kit, Box 3; one for each sample)
- 50X RNA Annealing Buffer (supplied with the kit, Box 3)

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ssRNA Purification Procedure

Use this procedure to purify ssRNA produced in the transcription reaction, Step 4, page 27.

Important: Immediately 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 RNA transcription reaction (~40 µl volume), add 160 µl of RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol followed by 100 µl of 100% ethanol to obtain a final volume of 300 µl. Mix well by pipetting up and down 5 times.
2. Apply the sample (~300 µl) to the RNA Spin Cartridge. Centrifuge at 14,000 × g for 15 seconds at room temperature. Discard the flow-through.
3. Add 500 µl of 1X RNA Wash Buffer to the RNA Spin Cartridge containing bound ssRNA. Centrifuge at 14,000 × g for 15 seconds at room temperature. Discard the flow-through.
4. Repeat the wash step (Step 3, above).
5. Centrifuge the RNA Spin Cartridge at 14,000 × g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer from the cartridge and to dry the membrane.
6. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube.
7. Add 40 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 × g for 2 minutes at room temperature to elute the ssRNA.
8. Add 40 µl of RNase-Free Water to the RNA Spin Cartridge and repeat Step 7, eluting the ssRNA into the same RNA Recovery Tube. The total volume of eluted ssRNA is 80 µl.
9. Depending on your downstream application, perform the following:
   - If you plan to use the purified ssRNA to generate dsRNA for use in RNAi studies, add 1.6 µl of 50X RNA Annealing Buffer to the eluate to obtain a final concentration of 1X RNA Annealing Buffer. Proceed to **Determining the RNA Concentration**, next page or to Step 10.
   - If you plan to use the purified ssRNA for applications such as Northern analysis, proceed to Step 10.
10. Store the purified ssRNA at -80°C.

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Determining the ssRNA Purity and Concentration

Follow the guidelines below to determine the purity and concentration of your purified ssRNA.

1. Dilute an aliquot of the purified ssRNA 100-fold into 1X RNA Annealing Buffer in a total volume appropriate for your quartz cuvette and spectrophotometer.
2. Measure OD at A260 and A280 in a spectrophotometer. Blank the sample against 1X RNA Annealing Buffer.
3. Calculate the concentration of the ssRNA by using the following equation:

   ssRNA concentration (µg/ml) = A260 × Dilution factor (100) × 40 µg/ml
4. Calculate the yield of the ssRNA by using the following equation:

   ssRNA yield (µg) = ssRNA concentration (µg/ml) × volume of ssRNA (ml)
5. Evaluate the purity of the purified ssRNA by determining the A260/A280 ratio. For optimal purity, the A260/A280 ratio should range from 1.9-2.2.

How Much ssRNA to Expect

The typical yield of purified ssRNA obtained from a 1 kb DNA template ranges from 50-80 µg in a 40 µl transcription reaction. However, yields may vary depending on the size of the DNA template and its sequence. Generally, ssRNA yields are lower for DNA templates smaller than 500 bp or larger than 1 kb.

After purification, we recommend saving an aliquot of your sense and antisense ssRNA samples for gel analysis. We generally verify the integrity of the dsRNA sample (after annealing) and compare it to the sense and antisense ssRNA samples using agarose or polyacrylamide gel electrophoresis (see page 32).

If you wish to verify the integrity of your sense and antisense ssRNA samples before annealing, we suggest running a small aliquot of each sample on a 6% Novex® TBE-Urea Gel (Life Technologies, Catalog no. EC68652BOX), and including the 0.16-1.77 kb RNA Ladder (Life Technologies, Catalog no. 15623-010) as a molecular weight standard.

Generating dsRNA

Introduction

To generate dsRNA, you will anneal equal amounts of the purified sense and antisense transcripts of your gene of interest (from **ssRNA Purification Procedure**, Step 8, page 29). Guidelines and instructions are provided below.

Amount of ssRNA to Anneal

You may anneal any amount of sense and antisense transcripts to generate dsRNA; however, use equal amounts of **each** transcript for optimal results. We generally anneal 50-80 µg of ssRNA to generate 100-160 µg of dsRNA, respectively (*e.g.* annealing 50 µg of sense transcripts and 50 µg of antisense transcripts results in 100 µg of dsRNA). You may assume that the annealing step is nearly 100% efficient.

Note: You will need to know the concentration of each ssRNA before beginning.

Materials Needed

Have the following materials on hand before beginning.

- Purified sense transcripts of your gene of interest
- Purified antisense transcripts of your gene of interest
- 0.5 ml sterile, RNase-free microcentrifuge tube
- 500 ml glass beaker

Annealing Procedure

Use the procedure below to anneal sense and antisense transcripts to generate dsRNA. Remember to use RNase-free supplies and wear gloves to prevent RNase contamination.

1. In a sterile, RNase-free microcentrifuge tube, mix equal amounts of purified sense and antisense transcripts. Place the tube on ice.
2. Heat approximately 250 ml of water to boiling in a 500 ml glass beaker.
3. Remove the beaker of water from the hot plate or microwave and set on your laboratory bench.
4. Place the tube containing the mixture of sense and antisense transcripts in a tube float or a rack in the glass beaker.
5. Allow the water to cool to room temperature for 1-1.5 hours. The ssRNAs will anneal during this time.
6. Remove a small aliquot of dsRNA and analyze by agarose or polyacrylamide gel electrophoresis to check the quality of your dsRNA (see the next page for more information).
7. Store the dsRNA at -80°C. Depending on the amount of dsRNA produced and your downstream application, you may want to aliquot the dsRNA before storage at -80°C.

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

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Alternative Annealing Procedure

If you want to generate dsRNA more quickly, use the alternative annealing procedure below. Note however, that this method is less efficient and will result in lower yields of dsRNA than the slow-annealing method described on the previous page.

1. In a sterile, RNase-free microcentrifuge tube, mix equal amounts of purified sense and antisense transcripts.
2. Place the tube in a 75°C heat block for 5 minutes.
3. Remove the tube from the heat block and place in a rack at room temperature for 5 minutes. The ssRNAs will anneal during this time.
4. Remove a small aliquot of dsRNA and analyze by agarose or polyacrylamide gel electrophoresis to check the integrity of your dsRNA (see the following section).
5. Store the dsRNA at -80°C. Depending on the amount of dsRNA produced and your downstream application, you may want to aliquot the dsRNA before storage at -80°C.

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

Checking the Integrity of dsRNA

You may verify the integrity of your dsRNA using agarose or polyacrylamide gel electrophoresis, if desired. We suggest running a small aliquot of your annealing reaction (equivalent to 100-200 ng of dsRNA) on the appropriate gel and comparing it to an aliquot (100-200 ng) of your starting sense and antisense ssRNA. Be sure to include an appropriate molecular weight standard. We generally use the following gels and molecular weight standard:

- **Agarose gel:** 1.2% agarose-TAE gel
- **Polyacrylamide gel:** 6% Novex® TBE Gel (Life Technologies, Catalog no. EC6265BOX)
- **Molecular weight standard:** 0.16-1.77 kb RNA Ladder (Life Technologies, Catalog no. 15623-010)

What You Should See

When analyzing the annealing reaction (from Step 6, page 31 **or** Step 4, above) using gel electrophoresis, we generally observe a predominant band corresponding to the dsRNA (see example on page 33). If you have used one of the recommended annealing procedures (page 31 or page 32), no ssRNA molecules should be detected.

Note: A high molecular weight smear is often visible in the annealed samples. This is generally due to branched annealing that occurs when multiple overlapping ssRNA anneal to each other. These products can be diced *in vitro* or *in vivo* to generate siRNA.

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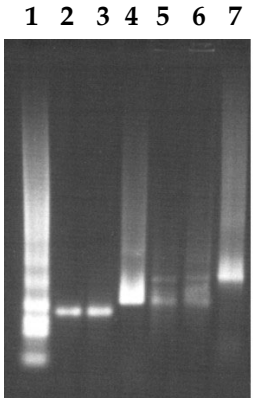
Example of Expected Results

In this experiment, dsRNA representing a 730 bp region of the green fluorescent protein (GFP) gene and a 1 kb region of the luciferase gene were generated using the reagents supplied in the kit and following the recommended protocols in the manual. One microgram of each dsRNA was analyzed on a 1.2% agarose-TAE gel and compared to 0.5 µg of each corresponding purified sense and antisense ssRNA (non-denatured).

Results: The annealed GFP (lane 4) and luciferase (lane 7) dsRNA samples both show a predominant band that differs in size from each component sense and antisense ssRNA. No ssRNA is visible in the annealed sample. A high molecular weight smear due to branched annealing products is also visible in the annealed samples (lanes 4 and 7).

Note: In some cases, multiple bands due to secondary structure are observed in the ssRNA samples (*e.g.* lanes 5 and 6). This is a result of analysis on non-denaturing agarose gels.



Lane 1. 0.16-1.77 kb RNA Ladder (denatured; Life Technologies, Catalog no. 15623-010)

Lane 2. GFP sense transcript

Lane 3. GFP antisense transcript

Lane 4. GFP annealed dsRNA

Lane 5. Luciferase sense transcript

Lane 6. Luciferase antisense transcript

Lane 7. Luciferase annealed dsRNA

What to Do Next

Once you have obtained dsRNA, you have the following options:

- Use the dsRNA directly to perform RNAi studies in invertebrate systems. Depending on the invertebrate system chosen (*e.g. C. elegans*, *Drosophila*, trypanosomes), multiple methods may exist to introduce the dsRNA into the organism or cell line of choice including injection, soaking in media containing dsRNA, or transfection. Choose the method best suited for your invertebrate system.
- Use the dsRNA in an *in vitro* reaction with the Dicer enzyme to generate d-siRNA. The resulting d-siRNA may then be transfected into mammalian cells for RNAi studies. For optimized reagents and protocols to generate highly pure d-siRNA from a dsRNA substrate using recombinant human Dicer enzyme, and to efficiently transfect the d-siRNA into a mammalian cell line of interest using Lipofectamine® 2000 Reagent, we recommend using the BLOCK-iT™ Dicer RNAi Transfection Kit (Catalog no. K3600-01) or the BLOCK-iT™ Complete Dicer RNAi Kit (Catalog no. K3650-01) available from Life Technologies. For detailed instructions to perform the dicing and transfection reactions, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

Troubleshooting

Introduction

Review the information in this section to troubleshoot the amplification, TOPO® Linking, transcription, and purification procedures.

Amplifying the Gene of Interest

The table below lists some potential problems and possible solutions that may help you troubleshoot your amplification reactions.

Problem	Reason	Solution
No PCR product	Poor quality of DNA template	Prepare new template DNA and verify the integrity of the DNA before amplification.
	Poor quality PCR reagents or inactive thermostable DNA polymerase	Amplify the control vector using the primers supplied with the kit and the protocol on page 38. If no PCR product is produced, use fresh PCR reagents and thermostable DNA polymerase.
	Suboptimal PCR conditions	• Check the T_m of the PCR primers and adjust your cycling conditions. • Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.
Low yield of PCR product	Suboptimal PCR conditions	Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.
	Used old DNA polymerase	Use fresh thermostable DNA polymerase.
	Not enough PCR cycles performed	Increase the number of PCR cycles.
Multiple non-specific bands or smearing observed on agarose gel	Suboptimal cycling conditions	Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.
	DNA template contaminated with other DNA	Prepare new template DNA and verify the integrity of the DNA before amplification.
	Poor quality PCR primers	Use HPLC-purified primers to produce your PCR product.

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TOPO® Linking and Secondary Amplification

The table below lists some potential problems and possible solutions that you may use to help you troubleshoot the TOPO® Linking reaction and the secondary amplification reactions.

Problem	Reason	Solution
No linear DNA template(s) of the expected size obtained	Inefficient TOPO® Linking • Incubated the TOPO® Linking reaction at 37°C for too long • Used a proofreading polymerase to generate the primary PCR product	• Do not incubate the TOPO® Linking reaction at 37°C for longer than 15 minutes. • Use *Taq* polymerase (*e.g.* Platinum® *Taq*) to generate the primary PCR product. Alternatively, add 3′ A-overhangs to the PCR product (see procedure on page 42).
	Poor quality PCR reagents or inactive thermostable DNA polymerase	Use fresh PCR reagents and thermostable DNA polymerase for the secondary amplification reactions.
	Primers used to produce the primary PCR product contained 5′ phosphates	Do not add 5′ phosphates to the primers used to produce the primary PCR product.
	TOPO® Linking reaction stored incorrectly	For optimal results, perform secondary amplification reactions directly after TOPO® Linking. If desired, store the TOPO® Linking reaction at -20°C overnight.
Low yield of linear DNA template obtained	Inefficient TOPO® Linking • Primary PCR product was too dilute • Primary PCR product was not fresh • *Taq* polymerase and proofreading polymerase mixture used to generate primary PCR product	• Purify and concentrate the PCR product using the procedure on page 43. • For optimal results, use fresh PCR product in the TOPO® Linking reaction. • Use *Taq* polymerase to generate the primary PCR product or use the procedure on page 42 to add 3′ A-over-hangs to the PCR product prior to TOPO® Linking.
	Annealing temperature was too high	Check the T_ms of your PCR primers. Reduce the annealing temperature.
	T_m of the gene-specific primer(s) not compatible with the T_m of the BLOCK-iT™ T7 Primer	Re-design the gene-specific primer(s), making sure that the T_m of each primer is compatible with the T_m of the BLOCK-iT™ T7 Primer.
	Not enough PCR cycles performed	Increase the number of PCR cycles.

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Transcribing and Purifying ssRNA

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

Problem	Reason	Solution
Low ssRNA yield	No ethanol or RNA Binding Buffer added to the sample	Add RNA Binding Buffer containing 1% (v/v) β-mercaptoethanol followed by 100% ethanol to the sample (see **ssRNA Purification Procedure**, Step 1, page 29).
	Linear DNA template too dilute	• Purify and concentrate the linear DNA template using the procedure on page 43. • Extend the incubation time of the transcription reaction up to 6 hours at 37°C.
	Transcription reaction not incubated long enough	Extend the incubation time of the transcription reaction up to 6 hours at 37°C.
	Eluted ssRNA from the RNA Spin Cartridge using buffer, not water	Elute ssRNA from the RNA Spin Cartridge using RNase-free water.
	Concentration of ssRNA incorrectly determined • Sample diluted into water for spectrophotometry • Sample blanked against water	• Dilute sample in 1X RNA Annealing Buffer for spectrophotometry. • Blank sample against 1X RNA Annealing Buffer.
No ssRNA obtained	Sample contaminated with RNase	• Use RNase-free reagents and supplies. • Wear gloves when handling RNA-containing samples.
	Gene-specific primers used to amplify TOPO® Linked products, not the BLOCK-iT™ T7 Primer	Use the BLOCK-iT™ T7 Primer and the gene-specific forward or reverse primer in the secondary amplification reaction to generate sense and antisense DNA templates, respectively.
	Forgot to add ethanol to the 5X RNA Wash Buffer	Add 10 ml of ethanol to the 5X RNA Wash Buffer (2.5 ml) to obtain a 1X RNA Wash Buffer.
Volume of eluted ssRNA is > 80 µl	RNA Spin Cartridge containing bound ssRNA not centrifuged to remove residual 1X RNA Wash Buffer	Centrifuge RNA Spin Cartridge at 14,000 × g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 5, page 29). **Important:** Contamination of eluted ssRNA with 1X RNA Wash Buffer or other impurities can result in inaccurate quantitation of ssRNA, potential toxic effects on invertebrate cells, or reduced dicing efficiency.

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Transcribing and Purifying ssRNA, continued

Problem	Reason	Solution
A260/A280 ratio not in the 1.9-2.2 range	Sample was not washed with 1X RNA Wash Buffer	Wash the RNA Spin Cartridge containing bound ssRNA twice with 1X RNA Wash Buffer (see Steps 3 and 4, page 29).
	RNA Spin Cartridge containing bound ssRNA not centrifuged to remove residual 1X RNA Wash Buffer	Centrifuge RNA Spin Cartridge at 14,000 × g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 5, page 29).

RNAi Analysis

The table below lists some potential problems and possible solutions that may help you troubleshoot your RNAi analysis using dsRNA.

Problem	Reason	Solution
Low levels of gene knockdown observed	dsRNA was degraded • dsRNA was not stored in 1X RNA Annealing Buffer • dsRNA was frozen and thawed multiple times	• Be sure to store the dsRNA in 1X RNA Annealing Buffer. • Aliquot dsRNA and avoid repeated freeze/thaw cycles.
No gene knockdown observed	Target sequence contains no active siRNA	Select a larger target region or a different target sequence.
	dsRNA contaminated with RNase	• Use RNase-free reagents and supplies. • Wear gloves when handling RNA-containing samples.
Non-specific gene knockdown effects 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.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves the following steps:

1. Producing a control PCR product using the pcDNA™1.2/V5-GW/*lacZ* control plasmid and the LacZ Forward 2 and LacZ Reverse 2 primers supplied with the kit.
2. Performing a TOPO® Linking reaction with the control PCR product and the BLOCK-iT™ T7-TOPO® Linker.
3. Performing two secondary amplification reactions with the TOPO® Linked PCR product to produce sense and antisense control DNA templates.
4. Using the control DNA templates in transcription reactions to generate sense and antisense RNA transcripts.
5. Purifying the sense and antisense RNA transcripts, and annealing the ssRNAs to produce control dsRNA.

Producing the Control PCR Product

Use this procedure to amplify the pcDNA™1.2/V5-GW/*lacZ* control plasmid using Platinum® *Taq* polymerase. If you are using another thermostable DNA polymerase, follow the manufacturer's instructions to set up the PCR reaction.

1. To produce the 1 kb control PCR product, set up the following 50 µl PCR:

pcDNA™1.2/V5-GW/*lacZ* (10 ng/µl)	1 µl
10X PCR Buffer	5 µl
40 mM dNTPs	1 µl
LacZ forward 2 primer (65 ng/µl)	1 µl
LacZ reverse 2 primer (65 ng/µl)	1 µl
Sterile Water	40.5 µl
Platinum® *Taq* Polymerase (5 U/µl)	0.5 µl
Total Volume	50 µl

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	15 seconds	94°C	30X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 1-5 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 1 kb band should be visible.

continued on next page

Control TOPO® Linking Reaction

Using the control PCR product produced in Step 3, previous page and the BLOCK-iT™ T7-TOPO® Linker, set up the TOPO® Linking reaction as described below.

1. Set up the following control TOPO® Linking reaction:

Control PCR product	1 µl
Salt Solution	1 µl
Sterile water	3 µl
BLOCK-iT™ T7-TOPO® Linker	1 µl
Total volume	6 µl

2. Incubate at 37°C for 15 minutes and place on ice.
3. Proceed directly to the **Secondary Control PCR Reactions**, below.

Secondary Control PCR Reactions

Use this procedure to amplify the TOPO® Linked control PCR product using Platinum® *Taq* polymerase to generate sense and antisense control DNA templates. If you are using another thermostable DNA polymerase, follow the manufacturer's instructions to set up the PCR reaction.

1. Set up the following 50 µl PCR reactions:

Reagent	Sense Template	Antisense Template
Control TOPO® Linking Reaction	1 µl	1 µl
10X PCR Buffer	5 µl	5 µl
40 mM dNTPs	1 µl	1 µl
BLOCK-iT™ T7 Primer (75 ng/µl)	1 µl	1 µl
LacZ Forward 2 Primer (65 ng/µl)	--	1 µl
LacZ Reverse 2 Primer (65 ng/µl)	1 µl	--
Sterile Water	40.5 µl	40.5 µl
Platinum® *Taq* Polymerase (5 U/µl)	0.5 µl	0.5 µl
Total volume	50 µl	50 µl

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	15 seconds	94°C	30X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 1-5 µl from the reaction and analyze by agarose gel electrophoresis. A discrete band of approximately 1 kb should be visible.

continued on next page

Generating Control dsRNA

Once you have generated the sense and antisense control DNA templates, you may use these templates in transcription reactions to produce sense and antisense control transcripts. After purification, these transcripts may then be annealed to produce control dsRNA. Follow the protocols on pages 25-32 to produce and purify sense and antisense transcripts, and to anneal the purified transcripts to produce dsRNA.

What To Do With the Control dsRNA

The lacZ dsRNA may be used as a control for RNAi analysis in the following ways:

Invertebrate Systems

- Use as a negative control for non-specific activity in any invertebrate system.

Mammalian Systems

- For some embryonic stem cell (ES) cell lines in which the CMV promoter is active (*e.g.* AB2.2), you may use the lacZ dsRNA as a positive control for gene knockdown (Yang *et al.*, 2001). Simply introduce the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and the lacZ dsRNA into cells and assay for inhibition of β-galactosidase expression.
- Alternatively, you may use the lacZ dsRNA in Life Technologies' BLOCK-iT™ Dicer RNAi Transfection Kit as a substrate to produce diced short interfering RNA (d-siRNA). The lacZ d-siRNA may then be used as a negative control for non-specific activity in the mammalian cell line of interest or as a positive control for knockdown of β-galactosidase expression from the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid. For detailed instructions to produce d-siRNA, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (> 1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are provided below.

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate will interfere with the sodium iodide in step 2.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M sodium iodide solution.
3. Add 1.5 volumes of Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 × g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 30 µl of sterile water. Use 1 µl for the TOPO® Linking reaction and proceed as described on page 22.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Linking reaction (page 22). Be sure to make the gel slice as small as possible for best results.

Adding 3´ A-Overhangs Post-Amplification

Introduction

Direct TOPO® Linking of DNA amplified by proofreading polymerases with the BLOCK-iT™ T7-TOPO® Linker is difficult because of very low TOPO® Linking efficiencies. These low efficiencies are caused by the 3´ to 5´ exonuclease activity associated with proofreading polymerases which removes the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. Proceed to TOPO® Linking (see page 22).

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Linking, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Purifying and Concentrating PCR Products

Introduction

If your gene of interest has not amplified efficiently and the yield of your PCR product is low, you may use the S.N.A.P.™ MiniPrep Kit available from Life Technologies (Catalog no. K1900-25) to rapidly purify and concentrate the PCR product. Other resin-based purification kits are suitable.

Materials Needed

You should have the following reagents on hand before beginning:

- Isopropanol
- Binding Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Wash Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Final Wash Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Sterile water
- S.N.A.P.™ MiniPrep columns (supplied with the S.N.A.P.™ MiniPrep Kit)

Purification Protocol

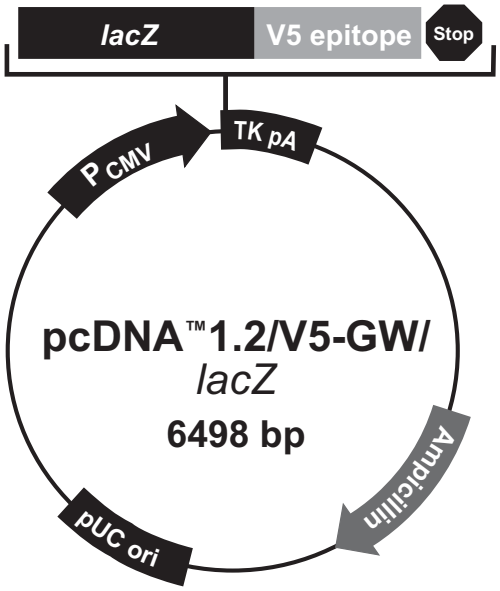
Follow the protocol below to purify your PCR product using the S.N.A.P.™ MiniPrep Kit. The protocol provides instructions to purify PCR products from a 50 µl reaction volume. To purify PCR products from larger reaction volumes (*e.g.* several PCR reactions pooled together), scale up the volumes of each buffer accordingly. **Note:** Details about the components of the S.N.A.P.™ MiniPrep Kit can be found in the S.N.A.P.™ MiniPrep Kit manual. The manual is available for downloading from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 45).

1. Add 150 µl of Binding Buffer to the 50 µl PCR reaction. Mix well by pipetting up and down.
2. Add 350 µl of isopropanol. Mix well by vortexing.
3. Immediately load solution from Step 2 onto a S.N.A.P.™ MiniPrep column. Centrifuge for 30 seconds at 1000 × g in a microcentrifuge and discard the flow-through.
4. Add 250 µl of the Wash Buffer and centrifuge for 30 seconds at 1000 × g in a microcentrifuge. Discard the flow-through.
5. Add 450 µl of the Final Wash Buffer and centrifuge for 30 seconds at 1000 × g in a microcentrifuge. Discard the flow-through.
6. Centrifuge for an additional 30 seconds at full-speed in a microcentrifuge to dry the column.
7. Transfer the column to a new collection tube. Add 30 µl of sterile water to the column. Incubate at room temperature for 1 minute.
8. Centrifuge for 30 seconds at full-speed in a microcentrifuge to elute the DNA. Collect the flow-through. Use 1 µl in the TOPO® Linking reaction (see page 22.

Map of pcDNA™1.2/V5-GW/*lacZ*

Description

pcDNA™1.2/V5-GW/*lacZ* (6498 bp) is a control vector expressing a C-terminally-tagged β-galactosidase fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987), and was generated using the MultiSite Gateway® Three-Fragment Vector Construction Kit available from Life Technologies (Catalog no. 12537-023). Briefly, a MultiSite Gateway® LR recombination reaction was performed with pDEST™R4-R3 and entry clones containing the CMV promoter, *lacZ* gene, and V5 epitope and TK polyadenylation signal to generate the pcDNA™1.2/V5-GW/*lacZ* vector. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 119 kDa. **The complete sequence of pcDNA™1.2/V5-GW/*lacZ* is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 45).**



Comments for pcDNA™1.2/V5-GW/*lacZ*
6498 nucleotides

*att*B4: bases 5-25
CMV promoter: bases 137-724
*att*B1: bases 614-637
LacZ fusion protein: bases 643-3798
  LacZ ORF: bases 643-3714
  *att*B2: bases 3716-3739
  V5 epitope: bases 3739-3780
lacZ forward 2 priming site: 840-859
lacZ reverse 2 priming site: 1820-1839 (C)
TK polyadenylation signal: bases 3807-4078
*att*B3: bases 4079-4099
bla promoter: bases 4603-4701
Ampicillin (*bla*) resistance gene: bases 4702-5562
pUC origin: bases 5707-6380

(C) = complementary strand

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