invitrogen™

BLOCK-iT™ U6 RNAi Entry Vector Kit

A Gateway®-adapted entry vector for the expression of short hairpin RNA (shRNA) in mammalian cells under the control of a human U6 promoter

Catalog nos. K4944-00 and K4945-00

Version E
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User Manual

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U6 Entry Clone Generation Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the U6 entry clone generation procedure. If you are performing the annealing, cloning, or transformation procedures for the first time, follow the detailed protocols provided in the manual.

Step	Action
Design single-stranded DNA oligos	Follow the guidelines on pages 9-13 to design single-stranded DNA oligos encoding the shRNA of interest.
Anneal the single-stranded oligos to generate a ds oligo	1. Set up the following annealing reaction. 200 µM top strand oligo — 5 µl 200 µM bottom strand oligo — 5 µl 10X Oligo Annealing Buffer — 2 µl DNase/RNase-free water — 8 µl Total volume — 20 µl 2. Heat the reaction mixture to 95°C for 4 minutes. 3. Remove the sample and set on the laboratory bench. Allow the reaction to cool to room temperature for 5-10 minutes. 4. Spin down the sample in a microcentrifuge for 5 seconds. Mix gently. 5. Dilute the ds oligo mixture 10,000-fold by performing two serial 100-fold dilutions: the first into DNase/RNase-free water and the second into 1X Oligo Annealing Buffer. Final concentration is 5 nM.
Clone the ds oligo into pENTR™/U6	1. Set up the following ligation reaction. 5X Ligation Buffer — 4 µl pENTR™/U6 (0.5 ng/µl) — 2 µl ds oligo (5 nM; 1:10,000 dilution) — 1 µl DNase/RNase-Free water — 12 µl T4 DNA Ligase (1 U/µl) — 1 µl Total volume — 20 µl 2. Mix reaction well and incubate for 5 minutes at room temperature. 3. Place reaction on ice and proceed to transform *E. coli*, below.
Transform One Shot® TOP10 Chemically Competent *E. coli*	1. Add 2 µl of the ligation reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature S.O.C. Medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 20-100 µl of bacterial culture on a pre-warmed LB agar plate containing 50 µg/ml kanamycin and incubate overnight at 37°C.

Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Note: The BLOCK-iT™ Lentiviral RNAi Expression System is also supplied with the BLOCK-iT™ Lentiviral RNAi Expression System components and the BLOCK-iT™ Lentiviral RNAi Expression System manual.

Product	Catalog no.
BLOCK-iT™ U6 RNAi Entry Vector Kit	K4945-00
BLOCK-iT™ Lentiviral RNAi Expression System	K4944-00

Kit Components

The BLOCK-iT™ U6 RNAi Entry Vector Kit and the BLOCK-iT™ Lentiviral RNAi Expression System include the following components. For a detailed description of the contents of the BLOCK-iT™ U6 RNAi Entry Vector Kit, see pages viii-ix. For a detailed description of the contents of the BLOCK-iT™ Lentiviral RNAi Expression reagents, see the BLOCK-iT™ Lentiviral RNAi Expression System manual.

Component	Catalog no.	
	K4945-00	K4944-00
BLOCK-iT™ U6 RNAi Entry Vector Kit	√	√
BLOCK-iT™ Lentiviral RNAi Expression Reagents		√

Shipping/Storage

The BLOCK-iT™ U6 RNAi Entry Vector Kit and the BLOCK-iT™ Lentiviral RNAi Expression System are shipped as described below. Upon receipt, store each item as detailed below. For more detailed information about the BLOCK-iT™ Lentiviral RNAi Expression reagents supplied with the kit, refer to the BLOCK-iT™ Lentiviral RNAi Expression System manual.

Box	Component	Shipping	Storage
1	U6 RNAi Entry Vector Reagents	Dry ice	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	-80°C
3-7	BLOCK-iT™ Lentiviral RNAi Expression Reagents	Various	Various (refer to the BLOCK-iT™ Lentiviral RNAi Expression System manual for details)

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U6 RNAi Entry Vector Reagents

The following reagents are included with the U6 RNAi Entry Vector (Box 1). **Store the reagents at -20°C.**

Reagent	Composition	Amount
pENTR™/U6 vector, linearized	0.5 ng/µl in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0	4 x 10 µl
10X Oligo Annealing Buffer	100 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 1 M NaCl	250 µl
DNase/RNase-Free Water	--	3 x 1.5 ml
5X Ligation Buffer	250 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP 5 mM DTT 25% (w/v) polyethylene glycol-8000	80 µl
T4 DNA Ligase	1 (Weiss) U/µl in 10 mM Tris-HCl, pH 7.5 50 mM KCl 1 mM DTT 50% (v/v) glycerol	20 µl
U6 Forward Sequencing Primer	100 ng/µl in TE Buffer, pH 8.0	20 µl
M13 Reverse Primer	100 ng/µl in TE Buffer, pH 8.0	20 µl
LacZ double-stranded (ds) Control Oligo	50 µM in 1X Oligo Annealing Buffer	4 µl
pcDNA™1.2/V5-GW/*lacZ* control plasmid	500 ng/µl in TE Buffer, pH 8.0	20 µl

Unit Definition of T4 DNA Ligase

One (Weiss) unit of T4 DNA Ligase catalyzes the exchange of 1 nmol ^{32}P-labeled pyrophosphate into [γ/β-^{32}P]ATP in 20 minutes at 37°C (Weiss *et al.*, 1968). One unit is equal to approximately 300 cohesive-end ligation units.

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Kit Contents and Storage, continued

Primer Sequences

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

Primer	Sequence	Amount
U6 Forward	5′-GGACTATCATATGCTTACCG-3′	329 pmoles
M13 Reverse	5′-CAGGAAACAGCTATGAC -3′	385 pmoles

LacZ Control Oligo Sequences

The sequences of the lacZ control oligos are listed below. The lacZ control DNA oligos are annealed and are supplied in the kit as a 50 µM double-stranded oligo. The lacZ ds control oligo needs to be re-annealed and diluted 10,000-fold to 5 nM (see page 15) before use in the ligation reaction (page 19).

LacZ DNA Oligo	Sequence
Top strand	5′-CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTGTGTAG-3′
Bottom strand	5′-AAAACTACACAAATCAGCGATTTTTCGAAATCGCTGATTTGTGTAGC -3′

One Shot® TOP10 Reagents

The following reagents are included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10 Cells

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

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BLOCK-iT™ Lentiviral RNAi Expression Reagents

In addition to the BLOCK-iT™ U6 RNAi Entry Vector Kit, the BLOCK-iT™ Lentiviral RNAi Expression System (Catalog no. K4944-00) also includes the following components to facilitate production of a replication-incompetent lentivirus that expresses your short hairpin RNA (shRNA) of interest.

- BLOCK-iT™ Lentiviral RNAi Gateway® Vector Kit
- ViraPower™ Bsd Lentiviral Support Kit
- Gateway® LR Clonase™ II Enzyme Mix
- 293FT Cell Line

Refer to the BLOCK-iT™ Lentiviral RNAi Expression System manual for a detailed description of the lentiviral expression reagents provided with the kit and instructions to produce lentivirus. For instructions to grow and maintain the 293FT Cell Line, refer to the 293FT Cell Line manual. The BLOCK-iT™ Lentiviral RNAi Expression System and the 293FT Cell Line manuals are supplied with Catalog no. K4944-00, but are also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 38).

Accessory Products

Introduction

The products listed in this section may be used with the BLOCK-iT™ U6 RNAi Entry Vector Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 38).

Accessory Products

Some of the reagents supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below.

Item	Amount	Catalog no.
T4 DNA Ligase	100 units	15224-017
	500 units	15224-025
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
M13 Reverse Primer	2 µg	N530-02
Lipofectamine™ 2000 Reagent	0.75 ml	11668-027
	1.5 ml	11668-019
Lipofectamine™ LTX Reagent	1.0 ml	15338-100
Opti-MEM® I Reduced Serum Medium	100 ml	31985-062
	500 ml	31985-070
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml	10010-023
4% E-Gel® Starter Pak	9 gels and Base	G5000-04
10 bp DNA Ladder	50 µg	10821-015
BLOCK-iT™ Lentiviral RNAi Gateway® Vector Kit	20 constructions	K4943-00
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
293FT Cell Line	3 x 10^6 cells, frozen	R700-07
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100

Introduction

Overview

Introduction

The BLOCK-iT™ U6 RNAi Entry Vector Kit facilitates the generation of a vector to express short hairpin RNA (shRNA) for use in RNA interference (RNAi) analysis of a target gene in mammalian cells. The kit provides a Gateway®-adapted entry vector designed to allow efficient transient expression of shRNA or stable expression of shRNA following recombination with a suitable destination vector. For more information about the Gateway® Technology, see below.

Note: The BLOCK-iT™ Lentiviral RNAi Expression System includes the BLOCK-iT™ U6 RNAi Entry Vector Kit as well as the pLenti6/BLOCK-iT™-DEST destination vector and other reagents required to generate a lentiviral RNAi construct. For more information about the pLenti6/BLOCK-iT™-DEST vector and how to generate lentivirus, refer to the BLOCK-iT™ Lentiviral RNAi Expression System manual. This manual is supplied with the BLOCK-iT™ Lentiviral RNAi Expression System, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 38).

Advantages of the BLOCK-iT™ U6 RNAi Entry Vector Kit

Using the BLOCK-iT™ U6 RNAi Entry Vector Kit for vector-based expression of shRNA provides the following advantages:

- Provides a rapid and efficient way to clone double-stranded oligonucleotide (ds oligo) duplexes encoding a desired shRNA target sequence into an entry vector containing an RNA Polymerase III (Pol III)-driven expression cassette (*i.e.* U6 RNAi cassette) for use in RNAi analysis.
- The entry construct containing the U6 RNAi expression cassette may be directly transfected into mammalian cells to enable rapid screening of shRNA target sequences.
- The vector is Gateway®-adapted to allow easy transfer of the U6 RNAi cassette into any appropriate expression system (*e.g.* lentiviral system for stable delivery of shRNA into dividing or non-dividing mammalian cells).

Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest (*e.g.* U6 RNAi cassette) into multiple vector systems. To express your shRNA of interest using the pENTR™/U6 vector, simply:

1. Clone your ds oligo encoding the shRNA of interest into the pENTR™/U6 vector to generate an entry clone.
2. Transfect your entry construct into mammalian cells to transiently assay for the RNAi response **OR**
3. Perform an LR recombination reaction between the entry construct and a suitable Gateway® destination vector to generate an expression clone for use in other RNAi applications.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 38)

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Overview, continued

Purpose of this Manual

This manual provides the following information:

- An overview of the pathway by which shRNA facilitates gene knockdown in mammalian cells.
- Guidelines to design the appropriate single-stranded oligonucleotides representing the target gene.
- Instructions to anneal the single-stranded oligonucleotides to generate double-stranded oligonucleotides (ds oligo).
- Guidelines and instructions to clone the ds oligo into the pENTR™/U6 vector, and transform the ligation reaction into competent *E. coli*.
- Guidelines to transfect your pENTR™/U6 construct into mammalian cells.
- Guidelines to perform an LR recombination reaction with a suitable Gateway® destination vector to generate an expression clone. For detailed instructions to perform the LR recombination reaction, refer to the manual supplied with the destination vector that you are using.

Important

The BLOCK-iT™ U6 RNAi Entry Vector Kit is designed to help you generate a U6 promoter-based vector to express shRNA in mammalian cell lines for RNAi analysis. Although the kit has been designed to help you express shRNA representing a particular target sequence in the simplest, most direct fashion, use of the kit for RNAi analysis assumes that users are familiar with the principles of gene silencing, vector-based production of shRNA, 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 and expression of shRNA in mammalian cells, refer to published references (Brummelkamp *et al.*, 2002; McManus and Sharp, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002).

Where to Go For More Information

A large variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate your RNAi analysis. If you want to express an shRNA in mammalian cell lines in a regulated manner, use the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit (Catalog no. K4920-00). If you want to perform RNAi analysis with synthetic RNAi reagents, use Stealth™ RNAi or short interfering RNA (siRNA) duplexes (see the RNAi Designer at www.invitrogen.com/rnai to design your duplexes). In addition, the BLOCK-iT™ Dicer RNAi Kits are available to facilitate production of diced siRNA (d-siRNA). For more information about these or any other BLOCK-iT™ RNAi products, visit the RNAi Central application portal at www.invitrogen.com/rnai.

BLOCK-iT™ U6 RNAi Entry Vector Kit

Description of the System

The BLOCK-iT™ U6 RNAi Entry Vector Kit facilitates the generation of an entry construct that permits high-level expression of an shRNA of interest in mammalian cells for RNAi analysis of a target gene. The kit contains the following major components:

- The pENTR™/U6 entry vector into which a ds oligo encoding the shRNA of interest will be cloned to generate an entry clone that contains the elements required for expression of the shRNA in mammalian cells. The pENTR™/U6 vector is supplied linearized with 4-nucleotide 5′ overhangs on each strand to facilitate directional cloning of the ds oligo insert. The resulting entry clone containing the U6 RNAi cassette (*i.e.* human U6 promoter + ds oligo + Pol III terminator) may be transfected into mammalian cells for transient RNAi analysis or used to transfer the U6 RNAi cassette into a suitable destination vector using Gateway® Technology. For more information about the features of the pENTR™/U6 vector, see pages 4 and 35. For more information about the U6 RNAi cassette, see page 7.
- T4 DNA Ligase and an optimized ligation buffer to allow 5-minute room temperature ligation of the ds oligo insert into pENTR™/U6.
- One Shot® TOP10 Chemically Competent *E. coli* for high efficiency transformation of the ligation reaction.

Note: The kit also includes a lacZ ds control oligo that may be cloned into pENTR™/U6 to generate an entry construct expressing shRNA targeting the *lacZ* gene. Co-transfecting the entry clone and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into mammalian cells provide a means to assess the RNAi response in your cell line by assaying for knockdown of β-galactosidase.

Generating shRNA Using the Kit

Using the reagents supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit, you will perform the following steps to generate an entry clone in pENTR™/U6.

1. Design and synthesize two complementary single-stranded DNA oligonucleotides, with one encoding the shRNA of interest.
2. Anneal the single-stranded oligonucleotides to generate a double-stranded oligo (ds oligo).
3. Clone the ds oligo into the linearized pENTR™/U6 vector.
4. Transform the ligation reaction into One Shot® TOP10 chemically competent *E. coli* and select for kanamycin-resistant transformants.
5. Use the pENTR™/U6 entry construct for transient RNAi analysis in mammalian cells or perform an LR recombination reaction with a suitable Gateway® destination vector to generate an expression clone.

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Features of the pENTR™/U6 Vector

The pENTR™/U6 vector contains the following features:

- U6 cassette containing elements required to allow RNA Polymerase III (Pol III)-controlled expression of the shRNA of interest in mammalian cells (see page 7 for more information)
- Cloning site containing 4-nucleotide 5′ overhangs on each DNA strand for directional cloning of the ds oligo encoding the shRNA of interest

  Note: The 4-nucleotide 5′ overhangs on each DNA strand encode the last 4 nucleotides of the U6 promoter and the first 4 nucleotides of the Pol III terminator. Transcription initiates at the first duplexed nucleotide after the promoter overhang (see the diagram on page 13 for more information).
- Two recombination sites, *att*L1 and *att*L2 sites, flanking the U6 RNAi cassette for recombinational cloning of the U6 RNAi cassette into a Gateway® destination vector (Landy, 1989)
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy maintenance of the plasmid in *E. coli*

Important

If you have previously used other Gateway® entry vectors, note that not all entry vectors may be used to generate entry clones for use in RNAi applications. You **must** use an entry vector (*e.g.* pENTR™/U6) that contains elements necessary for RNA Polymerase III-dependent expression of your shRNA (*i.e.* Pol III promoter and terminator).

Using shRNA for RNAi Analysis

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). In addition to dsRNA, other endogenous RNA molecules including short temporal RNA (stRNA; see below) and microRNA (miRNA) (Ambros, 2001; Carrington and Ambros, 2003) have been identified and shown to be capable of triggering gene silencing.

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

stRNA and shRNA

Small temporal RNA (stRNA), a subclass of micro RNA (miRNA), were originally identified and shown to be endogenous triggers of gene silencing in *C. elegans* (Grishok *et al.*, 2001; Lee *et al.*, 1993). Short temporal RNA including *let-7* (Grishok *et al.*, 2001) and *lin-4* (Lee *et al.*, 1993) encode hairpin precursors that are processed by the Dicer enzyme into 21-23 nucleotide siRNA duplexes (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001) that then enter the RNAi pathway and result in gene silencing by blocking translation.

Short hairpin RNA (shRNA) are an artificially designed class of RNA molecules that can trigger gene silencing through interaction with cellular components common to the RNAi and miRNA pathways. Although shRNA are a structurally simplified form of miRNA, these RNA molecules behave similarly to siRNA in that they trigger the RNAi response by inducing cleavage and degradation of target transcripts (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002).

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Using shRNA for RNAi Analysis, continued

Structural Features of shRNA

Exogenous short hairpin RNA can be transcribed by RNA Polymerase III (Paule and White, 2000) and generally contain the following structural features:

- A short nucleotide sequence ranging from 19-29 nucleotides derived from the target gene, followed by
- A short spacer of 4-15 nucleotides (*i.e.* loop) and
- A 19-29 nucleotide sequence that is the reverse complement of the initial target sequence.

The resulting RNA molecule forms an intramolecular stem-loop structure that is then processed into an siRNA duplex by the Dicer enzyme.

Hallmarks of RNA Polymerase III-Based Expression

RNA Polymerase III transcribes a limited number of genes including 5S rRNA, tRNA, 7SL RNA, U6 snRNA, and a number of other small stable RNAs that are involved in RNA processing (Paule and White, 2000). Some of the hallmarks of RNA Polymerase III-based transcription are that:

- Transcription initiates and terminates at fairly precise points
- There is little addition of unwanted 5′ and 3′ sequences to the RNA molecule

For more information about RNA Polymerase III transcription, refer to published reviews or reference sources (Paule and White, 2000; White, 1998).

Using a Vector-Based System to Express shRNA

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address this limitation, a number of groups have developed vector-based systems to facilitate expression of siRNA and shRNA in mammalian cells (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). At Invitrogen, we have developed the Gateway®-adapted pENTR™/U6 vector to facilitate generation of an entry clone containing a ds oligo encoding an shRNA of interest within the context of an RNA Polymerase III-driven expression cassette (*i.e.* U6 RNAi cassette; see the next page). The resulting pENTR™/U6 entry construct may be introduced into dividing mammalian cells for transient expression of the shRNA of interest and initial RNAi screening, if desired. Once initial screening is complete, the U6 RNAi cassette may then be easily and efficiently transferred into a suitable destination vector by LR recombination for use in other RNAi applications (*e.g.* stable, constitutive expression of shRNA).

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Using shRNA for RNAi Analysis, continued

Features of the U6 RNAi Cassette

The U6 RNAi cassette in pENTR™/U6 contains all of the elements required to facilitate RNA Polymerase III-controlled expression of your shRNA of interest including a:

- Human U6 promoter (see below for more information)
- Double-stranded oligo encoding an shRNA to your target gene of interest
- Polymerase III (Pol III) terminator consisting of a cluster of six thymidine (T) residues (Bogenhagen and Brown, 1981)

See the diagram below for an illustration of the U6 RNAi cassette.

Human U6 Promoter

Expression of the shRNA of interest from pENTR™/U6 (or a suitable destination vector following LR recombination) is controlled by the human U6 promoter. The endogenous U6 promoter normally controls expression of the U6 RNA, a small nuclear RNA (snRNA) involved in splicing, and has been well-characterized (Kunkel *et al.*, 1986; Kunkel and Pederson, 1988; Paule and White, 2000). We and other groups have chosen this particular promoter to control vector-based expression of shRNA molecules in mammalian cells (Paddison *et al.*, 2002; Paul *et al.*, 2002) for the following reasons:

- The promoter is recognized by RNA Polymerase III and controls high-level, constitutive expression of shRNA
- The promoter is active in most mammalian cell types
- The promoter is a type III Pol III promoter in that all elements required to control expression of the shRNA are located upstream of the transcription start site (Paule and White, 2000)

Structure of the shRNA

The shRNA molecule expressed from the U6 RNAi cassette (in pENTR™/U6 or in a suitable destination vector) forms an intramolecular stem-loop structure similar to the structure of miRNA. This hairpin is then processed by the endogenous Dicer enzyme into a 21-23 nt siRNA duplex.

Example: The figure below illustrates the structure of the shRNA generated from the pENTR™/U6-GW/lacZ^{shRNA} construct. You may generate this construct by cloning the lacZ ds control oligo supplied with the kit into pENTR™/U6 following the protocols in this manual. The 19 bp lacZ target sequence is indicated in bold. The underlined bases are derived from the Pol III terminator.

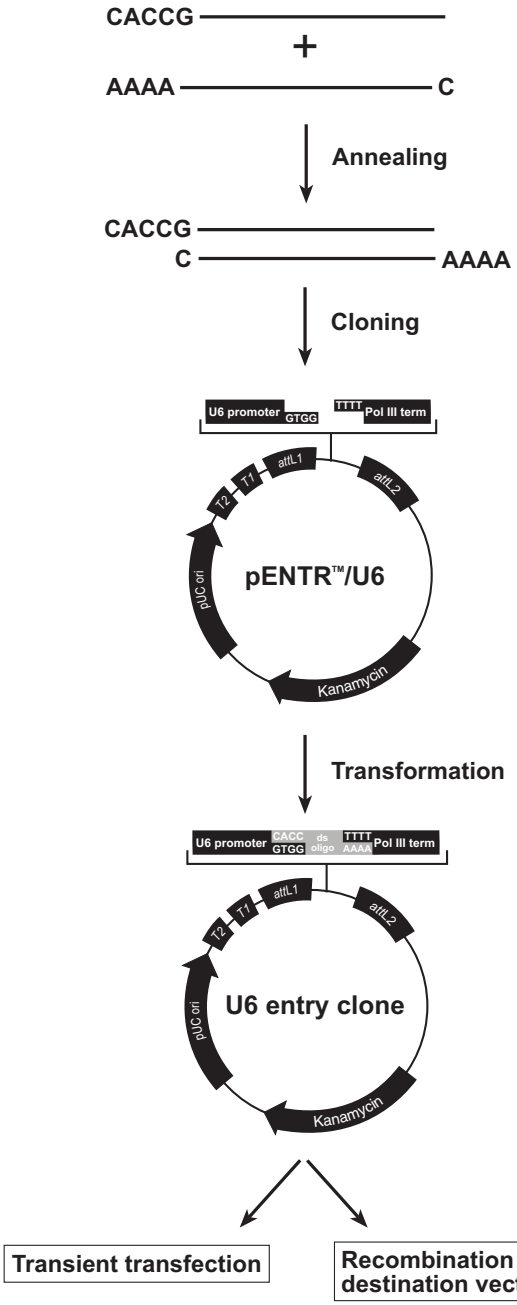
```
   5′-G CUACACAAAUCAGCGAUUU C G
3′-..UU GAUGUGUUUAGUCGCUAAA A A
```

Note: The length of the stem and loop may differ depending on how you design the oligonucleotides encoding your target sequence. For guidelines to design the oligonucleotides, refer to pages 9-13.

Experimental Outline

Flow Chart

The figure below illustrates the major steps necessary to produce a pENTR™/U6 entry clone using the BLOCK-iT™ U6 Entry Vector Kit.



1. Design and synthesize complementary DNA oligos, with each containing 4 nucleotide overhangs necessary for directional cloning.
2. Anneal DNA oligos to generate a ds oligo.
3. Clone the ds oligo into pENTR™/U6 using T4 DNA Ligase.
4. Transform *E. coli* and analyze colonies for the desired entry clone.
5. Transfect the entry clone for transient RNAi analysis or recombine with a suitable destination vector to generate an expression clone.

Methods

Designing the Single-Stranded DNA Oligos

Introduction

To use the BLOCK-iT™ U6 RNAi Entry Vector Kit, you will first need to design two single-stranded DNA oligonucleotides; one encoding the target shRNA ("top strand" oligo) and the other its complement ("bottom strand" oligo). You will then anneal the top and bottom strand oligos to generate a double-stranded oligonucleotide (ds oligo) suitable for cloning into the pENTR™/U6 vector.

The design of the single-stranded oligonucleotides (ss oligos) is critical to the success of both the cloning procedure and ultimately, the RNAi analysis. General guidelines are provided in this section to help you choose the target sequence and to design the ss oligos. Note however, that simply following these guidelines does not guarantee that the shRNA will be effective in knocking down the target gene. For a given target gene, you may need to generate and screen multiple shRNA sequences to identify one that is active in gene knockdown studies.

We recommend using Invitrogen's RNAi Designer, an online tool to help you design and order shRNA sequences for any target gene of interest. The RNAi Designer incorporates the guidelines provided in this manual as well as other design rules into a proprietary algorithm to design shRNA sequences that are compatible for use in cloning into the pENTR™/U6 or other appropriate RNAi entry vectors (e.g. pENTR™/H1/TO). Alternatively, if you have identified a synthetic siRNA that is active in triggering knockdown of your target gene, the RNAi Designer will convert the siRNA into a suitable shRNA. To use the RNAi Designer, see www.invitrogen.com/rnai.

Factors to Consider

When designing the top and bottom strand single-stranded oligos, consider the following factors:

Top strand oligo

- Sequences required to facilitate directional cloning
- Transcription initiation site
- Sequences encoding the shRNA of interest (*i.e.* stem and loop sequences)

Bottom strand oligo

- Sequences required to facilitate directional cloning
- Sequences complementary to the top strand oligo

For more information about the sequence requirements for directional cloning, see below. For guidelines to choose the target, loop, and transcription initiation sequences, see pages 10-11. For an example of ss oligo design, see page 12.

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Designing the Single-Stranded DNA Oligos, continued

Sequences Required for Directional Cloning

To enable directional cloning of the ds oligo into pENTR™/U6, you **must** add the following 4 nucleotides to the 5′ end of the corresponding ss oligo. See the diagram of the cloning site on page 13 to help you design your ss oligos.

- **Top strand oligo:** Add CACC to the 5′ end of the oligo. The CACC is complementary to the overhang sequence, GTGG, in the pENTR™/U6 vector and constitutes the last 4 bases of the U6 promoter.
- **Bottom strand oligo:** Add AAAA to the 5′ end of the oligo. The AAAA is complementary to the overhang sequence, TTTT, in the pENTR™/U6 vector and constitutes the first 4 bases of the Pol III terminator .

Structural Features of the shRNA

Reminder: When designing the top strand oligo encoding the shRNA, remember that an shRNA generally contains the following structural features:

- A short nucleotide sequence **derived from the target gene** (*i.e.* target sequence), followed by
- A short loop and
- A short nucleotide sequence that is the reverse complement of the initial target sequence

Upon transcription, the target sequence and its complement base pair to form the stem of the shRNA. For guidelines to choose the target and loop sequences, see below and the next page.

Choosing the Target Sequence

When performing RNAi analysis on a particular gene, your choice of target sequence can significantly affect the degree of gene knockdown observed. We recommend following the guidelines below when choosing your target sequence. These are general recommendations only; exceptions may occur.

Length: Choose a target sequence ranging from 19 to 29 nucleotides in length. Longer sequences may induce non-specific responses in mammalian cells.

Complexity:

- Make sure that the target sequence does **not** contain runs of more than three of the same nucleotide. Specifically, avoid choosing a target sequence with a run of four thymidines (T's) as this can lead to early transcription termination.
- Choose a sequence with low GC content (~30-50% GC content is suggested).
- Do not choose a target sequence that is a known site for RNA-protein interaction.

Homology: Make sure that the target sequence does **not** contain significant homology to other genes as this can increase off-target RNAi effects.

Orientation: You may choose a target sequence encoding the **sense** sequence of the target mRNA or the **antisense** sequence. Thus, you can generate an shRNA in two possible orientations: sense sequence-loop-antisense sequence **or** antisense sequence-loop-sense sequence.

siRNA: If you have identified a synthetic siRNA that is active in knocking down your target gene, try generating an shRNA using this same target sequence.

continued on next page

Designing the Single-Stranded DNA Oligos, continued

Loop Sequence

You may use a loop sequence of any length ranging from 4 to 11 nucleotides, although short loops (*i.e.* 4-7 nucleotides) are generally preferred. Avoid using a loop sequence containing thymidines (T's) as they may cause early termination. This is particularly true if the target sequence (see the previous page) ends in a T residue.

Note: We have included the following loop sequences in active shRNA molecules:

- 5′-CGAA-3′
- 5′-AACG-3′
- 5′-GAGA-3′

Transcription Initiation

Transcription of the shRNA initiates at the first base following the end of the U6 promoter sequence. In the top strand oligo, the transcription initiation site corresponds to the first nucleotide following the four base pair CACC sequence added to permit directional cloning. **We recommend initiating the shRNA sequence at a guanosine (G) because transcription of the native U6 snRNA initiates at a G.** Note the following:

- If G is part of the target sequence, then incorporate the G into the stem sequence in the top strand oligo and add a complementary C to the 3′ end of the top strand oligo.
- If G is not the first base of the target sequence, we recommend adding a G to the 5′ end of the top strand oligo directly following the CACC overhang sequence. In this case, **do not** add the complementary C to the 3′ end of the top strand oligo. For an example, see the next page.

  Note: We have found that adding the complementary C in this situation can result in reduced activity of the shRNA.

Alternative: If use of a G to initiate transcription is not desired, use an adenosine (A) rather than C or T. Note however, that use of any nucleotide other than G may affect initiation efficiency and position.

Note

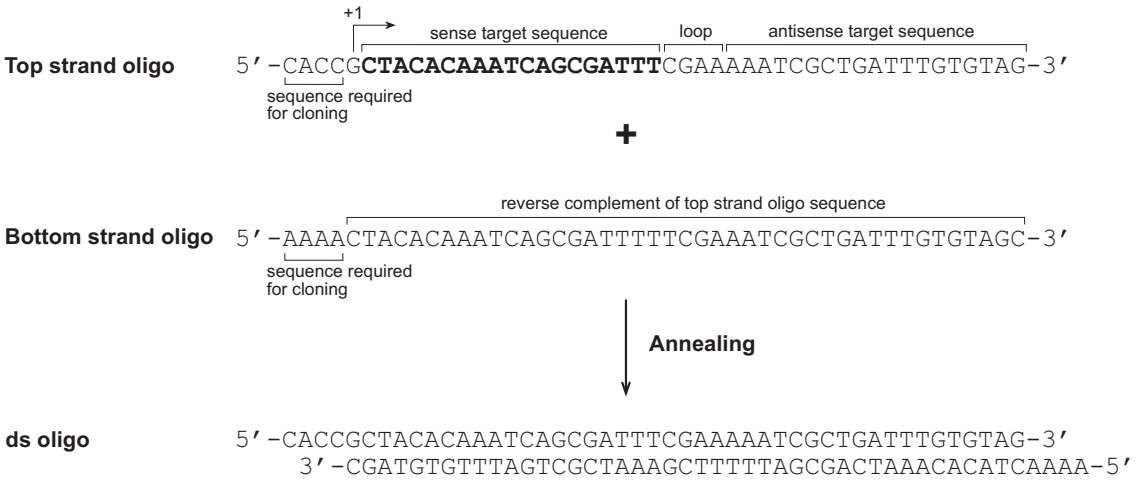
Do not add 5′ phosphates to your ss oligos during synthesis. The phosphate groups necessary for ligation are present in the linearized pENTR™/U6 vector.

continued on next page

Designing the Single-Stranded DNA Oligos, continued

Example of ss Oligo Design

The diagram below illustrates the required features of the top strand and bottom strand single-stranded oligos as discussed in this section. This particular example lists the sequences of top and bottom strand oligos encoding a shRNA targeting the *lacZ* gene. These particular ss oligos were annealed to generate the lacZ ds control oligo supplied in the kit.



We generally order unpurified, desalted single-stranded oligos using Invitrogen's custom primer synthesis service (see www.invitrogen.com for more information) The ss oligos obtained anneal efficiently and provide optimal cloning results. Note however, that depending on which supplier you use, the purity and quality of the ss oligos may vary. If you obtain variable annealing and cloning results using unpurified, desalted oligos, you may want to order oligos that are HPLC or PAGE-purified.

continued on next page

Designing the Single-Stranded DNA Oligos, continued

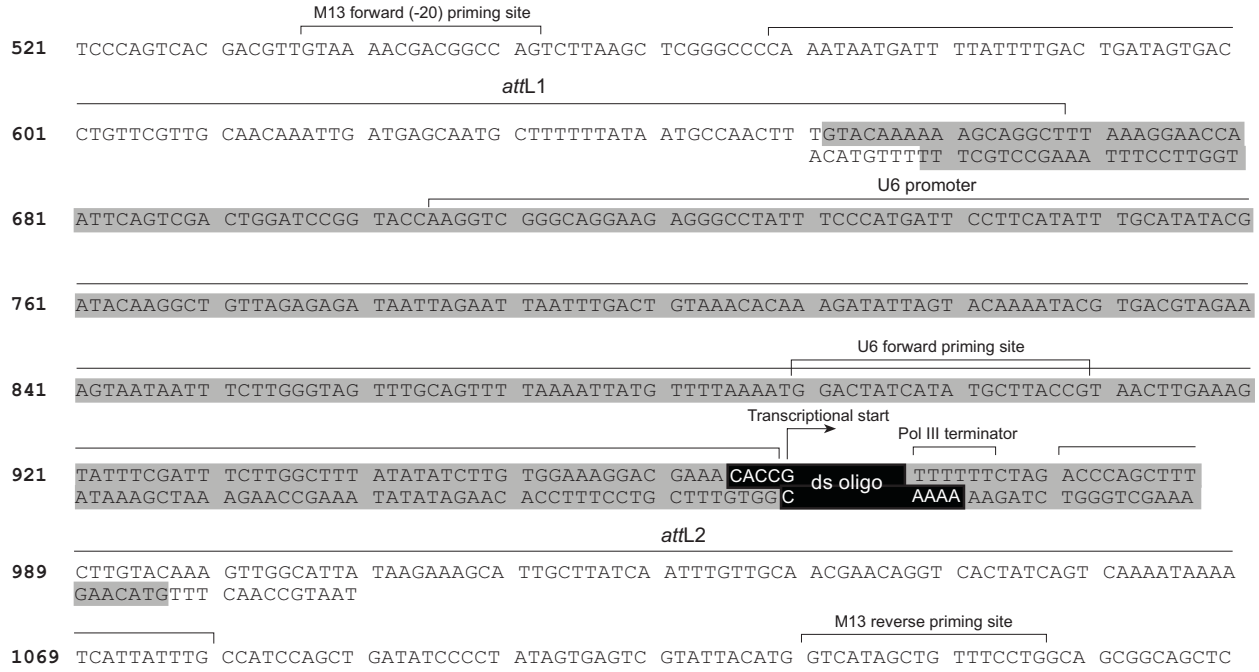
Cloning Site and Recombination Region of pENTR™/U6

Use the diagram below to help you design suitable DNA oligonucleotides to clone into pENTR™/U6 after annealing. Note the following features in the diagram below:

- The pENTR™/U6 vector is supplied linearized between nucleotides 968 and 969. The linearized vector contains 4 nucleotide overhangs on each strand encoding the last 4 nucleotides of the U6 promoter and the first 4 nucleotides of the Pol III terminator. Note that the annealed double-stranded (ds) oligo **must** contain specific 4 nucleotide 5′ overhangs on each strand as indicated.
- The shaded region corresponds to those DNA sequences that will be transferred from the entry clone into the Gateway® destination vector (*e.g.* pLenti6/BLOCK-iT™-DEST) following recombination.

  Note: Following recombination with a Gateway® destination vector, the resulting expression clone will contain an RNAi cassette consisting of the U6 promoter, shRNA sequence, and the Pol III terminator.

The sequence of pENTR™/U6 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 38). For a map of pENTR™/U6, see the **Appendix**, page 35.



Generating the Double-Stranded Oligo (ds oligo)

Introduction

Once you have synthesized the appropriate complementary single-stranded DNA oligos, you will anneal equal amounts of each single-stranded oligo to generate a double-stranded oligo (ds oligo). Guidelines and instructions are provided in this section.

Single-Stranded Oligos

Before beginning, make sure that you have synthesized the single-stranded oligos with the appropriate sequences required for cloning into the pENTR™/U6 vector and for annealing. See the figure below for an illustration.

- **"Top strand" oligo:** Make sure that this oligo contains the sequence, CACC, at the 5′ end.
- **"Bottom strand" oligo:** Make sure that this oligo contains the sequence, AAAA, at the 5′ end and is complementary to the top strand oligo.

Amount of DNA Oligo to Anneal

You will anneal equal amounts of the top and bottom strand oligos to generate the ds oligos. We generally perform the annealing reaction at a final single-stranded oligo concentration of 50 µM. Annealing at concentrations lower than 50 µM can significantly reduce the efficiency. Note that the annealing step is not 100% efficient; approximately half of the single-stranded oligos remain unannealed even at a concentration of 50 µM.

Resuspending the Oligos

If your single-stranded oligos are supplied lyophilized, resuspend them in water or TE Buffer to a final concentration of 200 µM before use.

Re-annealing LacZ ds Control Oligo

If you plan to use the lacZ ds control oligo in the ligation reaction, make sure to re-anneal it along with the other oligos as described on the next page. Since the lacZ ds control oligo already comes at a concentration of 50 µM in 1 x Oligo Annealing Buffer, re-anneal the lacZ ds control oligo without further dilution.

continued on next page

Generating the Double-Stranded Oligo (ds oligo), continued

Materials Needed

Have the following materials on hand before beginning:

- Your "top strand" single-stranded oligo (200 µM in water or TE Buffer)
- Your "bottom strand" single-stranded oligo (200 µM in water or TE Buffer)
- 50 µM stock of lacZ ds control oligo (thaw on ice)
- 10X Oligo Annealing Buffer (supplied with the kit, Box 1)
- DNase/RNase-Free Water (supplied with the kit, Box 1)
- 0.5 ml sterile microcentrifuge tubes
- 95°C water bath or heat block

Annealing Procedure

Follow this procedure to anneal your single-stranded oligos to generate the ds oligo. Note that the final concentration of the oligo mixture is 50 µM.

1. In a 0.5 ml sterile microcentrifuge tube, set up the following annealing reaction at room temperature.

Reagent	Amount
"Top strand" DNA oligo (200 µM)	5 µl
"Bottom strand" DNA oligo (200 µM)	5 µl
10X Oligo Annealing Buffer	2 µl
DNase/RNase-Free Water	8 µl
Total volume	20 µl

2. If re-annealing the lacZ ds control oligo, centrifuge its tube briefly (~5 seconds), and transfer contents to a separate 0.5 ml sterile microcentrifuge tube.
3. Incubate the reaction at 95°C for 4 minutes.
4. Remove the tube containing the annealing reaction from the water bath or the heat block and set on your laboratory bench.
5. Allow the reaction mixture to cool to room temperature for 5-10 minutes. The single-stranded oligos will anneal during this time.
6. Place the sample in a microcentrifuge and centrifuge briefly (~5 seconds). Mix gently.
7. Remove 1 µl of the annealing mixture and dilute the ds oligo as directed in **Diluting the ds Oligo**, next page.
8. Store the remainder of the 50 µM ds oligo mixture at -20°C.

continued on next page

Generating the Double-Stranded Oligo (ds oligo), continued

Diluting the ds Oligo

To clone your ds oligo or lacZ ds control oligo into pENTR™/U6, you **must** dilute the 50 µM stock to a final concentration of 5 nM (*i.e.* 10,000-fold dilution). We generally perform two 100-fold serial dilutions, the first into DNase/RNase-free water and the second into the 1X Oligo Annealing Buffer supplied with the kit. Follow the procedure below to dilute the ds oligo.

1. Dilute the 50 µM ds oligo mixture (from **Annealing Procedure**, Step 5, previous page) 100-fold into DNase/RNase-free water to obtain a final concentration of 500 nM. Vortex to mix thoroughly.

50 µM ds oligo	1 µl
DNase/RNase-free water	99 µl
Total volume	100 µl

2. Dilute the 500 nM ds oligo mixture (from Step 1) 100-fold into 1X Oligo Annealing Buffer as follows to obtain a final concentration of 5 nM. Vortex to mix thoroughly. Store the remaining 500 nM ds oligo stock at -20°C.

500 nM ds oligo	1 µl
10X Oligo Annealing Buffer	10 µl
DNase/RNase-free water	89 µl
Total volume	100 µl

3. Aliquot the 5 nM ds oligo stock and store at -20°C.

Important

The undiluted ds oligos are 10,000-fold more concentrated than the working concentration. **When performing the dilutions, be careful not to cross-contaminate the different ds oligo stocks.** Remember to wear gloves and change pipette tips after every manipulation.

Storing the ds Oligo

Once you have diluted your ds oligo, you should have three stocks of annealed ds oligo. Use each stock as follows:

- **50 µM ds oligo (undiluted):** Use this stock for long-term storage and to prepare new diluted ds oligo stocks if existing stocks become denatured or cross-contaminated.
- **500 nM ds oligo (100-fold dilution):** Use this stock for gel analysis (see **Checking the Integrity of the ds Oligo**, next page).
- **5 nM ds oligo (10,000-fold dilution):** Use this stock for cloning (see **Ligation Procedure**, page 20). This stock is not suitable for long-term storage.

Store the three ds oligo stocks at -20°C.

continued on next page

Generating the Double-Stranded Oligo (ds oligo), continued

Important

When using the diluted ds oligo stock solutions (*i.e.* 100-fold or 10,000-fold diluted stocks), thaw the solutions on ice. **Do not** heat or allow the ds oligo solutions to reach greater than room temperature as this causes the ds oligos to melt. The concentration of the oligos in the diluted solutions is not high enough to permit re-annealing and instead favors the formation of intramolecular hairpin structures. These intramolecular hairpin structures **will not clone** into pENTR™/U6.

If your diluted ds oligo stock solution(s) is heated, discard the ds oligo solution and prepare new diluted stocks using the procedure on the previous page.

Note: If the 50 µM ds oligo solution (undiluted stock) becomes heated, the oligos are sufficiently concentrated and may be re-annealed following the annealing procedure on page 15.

Checking the Integrity of the ds Oligo

You may verify the integrity of your annealed ds oligo using agarose gel electrophoresis, if desired. We suggest running an aliquot of the annealed ds oligo (5 µl of the 500 nM stock) and comparing it to an aliquot of each starting single-stranded oligo (dilute the 200 µM stock 400-fold to 500 nM; use 5 µl for gel analysis). Be sure to include an appropriate molecular weight standard. We generally use the following gel and molecular weight standard:

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

What You Should See

When analyzing an aliquot of the annealed ds oligo reaction by agarose gel electrophoresis, we generally see the following:

- A detectable higher molecular weight band representing annealed ds oligo.
- A detectable lower molecular weight band representing unannealed single-stranded oligos. Note that this band is detected since a significant amount of the single-stranded oligo remains unannealed.

For an example of expected results obtained from agarose gel analysis, see the next page. If the band representing ds oligo is weak or if you do not see a band, see **Troubleshooting**, page 30 for tips to troubleshoot your annealing reaction.

continued on next page

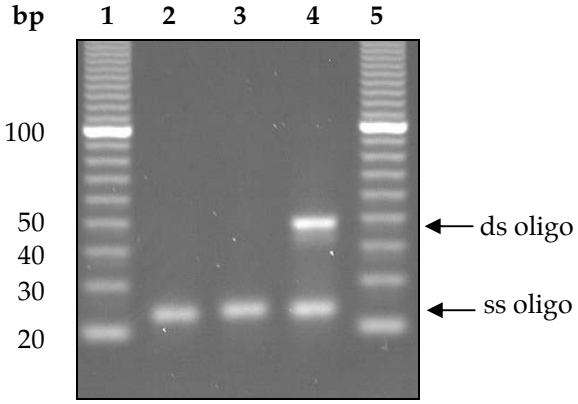
Generating the Double-Stranded Oligo (ds oligo), continued

Example of Expected Results

In this experiment, the lacZ control oligos (see page ix for the sequence of each DNA oligo) were annealed (50 µM final concentration) using the reagents supplied in the kit and following the procedure on page 15 to generate the lacZ ds control oligo. The annealing reaction was diluted 100-fold in water to a concentration of 500 nM. Aliquots of the diluted ds oligo (5 µl) and each corresponding single-stranded oligo (5 µl of a 500 nM stock) were analyzed on a 4% E-Gel®.

Results: The lacZ oligo annealing reaction shows a clearly detectable, higher molecular weight band that differs in size from each component single-stranded oligo. Remaining unannealed ss oligo is also detectable.

Note: The agarose gel is non-denaturing; therefore, the single-stranded oligos do not resolve at the expected size due to formation of secondary structure.



Lane 1. 10 bp DNA Ladder
Lane 2. lacZ top strand oligo
Lane 3. lacZ bottom strand oligo
Lane 4. lacZ ds oligo annealing reaction
Lane 5. 10 bp DNA Ladder

Performing the Ligation Reaction

Introduction

Once you have generated your ds oligo and have diluted it to the appropriate concentration, you will clone the ds oligo into the pENTR™/U6 vector and transform your ligation reaction into competent TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best results. We suggest that you read the sections entitled **Performing the Ligation Reaction** (pages 19-20) and **Transforming One Shot® TOP10 Competent *E. coli*** (page 21) before beginning.

Important

You will use T4 DNA Ligase and a 5X Ligation Buffer supplied with the kit to facilitate ligation of your ds oligo with the linearized pENTR™/U6 vector. When performing the ligation reaction, note the following:

- The T4 DNA Ligase and the 5X Ligation Buffer supplied with the kit have been optimized to permit ligation of the ds oligo into the pENTR™/U6 vector in **5 minutes at room temperature**. T4 DNA Ligase preparations and reaction buffers available from other manufacturers may not be appropriate for use in this application.

  Note: The T4 DNA Ligase and reaction buffer supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit is available separately from Invitrogen (Catalog no. 15224-017).

- Traditional ligation reactions are performed at 16°C overnight. **This is not recommended for this application.** Follow the ligation procedure on page 20.

Amount of ds Oligo to Use

For optimal results, use a 10:1 molar ratio of ds oligo insert:vector for ligation. Note that if you follow the recommended ligation procedure on the next page, you will be using a 10:1 molar ratio of insert:vector.

Positive Control

We recommend including the lacZ ds control oligo supplied with the kit as a positive control in your ligation experiment. The lacZ ds control oligo is supplied as a 50 µM stock in 1X Oligo Annealing Buffer, and needs to be re-annealed and diluted 10,000-fold before use in a ligation reaction (see page 15). See page ix for the sequence of each strand of the lacZ ds control oligo.

Note: Once you have cloned the lacZ ds control oligo into pENTR™/U6, you may use the resulting entry clone as a positive control for the RNAi response in your mammalian cell line. Simply co-transfect the entry clone and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your mammalian cell line and assay for knockdown of β-galactosidase expression.

Important

Reminder: When using the 5 nM ds oligo stock solution for cloning, thaw the solution on ice. **Do not thaw the ds oligo by heating or the ds oligo duplexes may melt and form intramolecular hairpin structures.** After use, return the tube to -20°C storage.

continued on next page

Performing the Ligation Reaction, continued

Materials Needed

Have the following reagents on hand before beginning:

- Double-stranded oligo of interest (5 nM in 1X Oligo Annealing Buffer; thaw on ice before use)
- lacZ ds control oligo (5 nM in 1X Oligo Annealing Buffer; thaw on ice before use)
- pENTR™/U6, linearized (0.5 ng/µl, supplied with the kit, Box 1; thaw on ice before use)
- 5X Ligation Buffer (supplied with the kit, Box 1)
- DNase/RNase-Free Water (supplied with the kit, Box 1)
- T4 DNA Ligase (1 U/µl, supplied with the kit, Box 1)

Ligation Procedure

Follow the procedure below to perform the ligation reaction. If you wish to include a negative control, set up a separate ligation reaction but omit the ds oligo.

1. Set up a 20 µl ligation reaction at room temperature using the following reagents **in the order** shown.

Reagent	Amount
5X Ligation Buffer	4 µl
pENTR™/U6 (0.5 ng/µl)	2 µl
ds oligo (5 nM; *i.e.* 1:10,000 dilution)	1 µl
DNase/RNase-Free Water	12 µl
T4 DNA Ligase (1 U/µl)	1 µl
Total volume	20 µl

2. Mix reaction well by pipetting up and down.

   Note: The presence of PEG and glycerol (supplied by the Ligation Buffer and the T4 DNA Ligase) will make the reaction mixture viscous. Be sure to mix the reaction thoroughly by pipetting up and down. **Do not vortex.**

3. Incubate for 5 minutes at room temperature.

   Note: The incubation time may be extended up to 2 hours and may result in a higher yield of colonies.

4. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent *E. coli***, next page.

   Note: You may store the ligation reaction at -20°C overnight.

Transforming One Shot® TOP10 Competent *E. coli*

Introduction

Once you have performed the ligation reaction, you will transform your ligation mixture into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation. Follow the guidelines and instructions provided in this section.

Note: One Shot® TOP10 *E. coli* possess a transformation efficiency of 1 x 10^9 cfu/µg DNA.

Materials to Have on Hand

You will need to have the following materials on hand before beginning:

- Ligation reaction (from Step 3, previous page)
- One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit, Box 2; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (supplied with the kit, Box 2; warm to room temperature)
- pUC19 positive control (supplied with the kit, Box 2; if desired)
- 42°C water bath
- LB plates containing 50 µg/ml kanamycin (two for each transformation; warm at 37°C for 30 minutes before use)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

One Shot® TOP10 Transformation Procedure

Use this procedure to transform your ligation reaction into One Shot® TOP10 Chemically Competent *E. coli*. For a positive control, transform 10 pg (1 µl) of pUC19 plasmid into a vial of One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µl of the ligation reaction (from Step 3, previous page) into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 20-100 µl from each transformation on a pre-warmed LB agar plate containing 50 µg/ml kanamycin and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. If you are transforming the pUC19 control, plate 20-100 µl of the transformation reaction on pre-warmed LB plates containing 100 µg/ml ampicillin.
8. An efficient ligation reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, next page).

Analyzing Transformants

Analyzing Transformants

To analyze positive clones, we recommend that you:

1. Pick 5-10 kanamycin-resistant colonies and culture them overnight in LB or SOB medium containing 50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. To obtain pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or S.N.A.P.™ MidiPrep Kit available from Invitrogen (Catalog no. K1910-01).
3. Sequence each pENTR™/U6 entry construct (see below) to confirm the following:
   a. The presence and correct orientation of the ds oligo insert.
   b. The sequence of the ds oligo insert.

   Note: Because of the small size of the ds oligo insert, we do not recommend using restriction enzyme analysis to screen transformants.

Important

We highly recommend sequencing positive transformants to confirm the sequence of the ds oligo insert. When screening transformants, we find that up to 20% of the clones may contain mutated inserts (generally 1 or 2 bp deletions within the ds oligo). The reason for this is not known, but may be due to triggering of repair mechanisms within *E. coli* as a result of the inverted repeat sequence within the ds oligo insert.

Note: Entry clones containing mutated ds oligo inserts generally elicit a poor RNAi response in mammalian cells. Identify entry clones with the correct ds oligo sequence and use these clones for your RNAi analysis.

Sequencing

To facilitate sequencing of your pENTR™/U6 entry clones, use the U6 Forward and M13 Reverse Primers supplied with the kit (Box 1). See the diagram on page 13 for the location of the priming sites.

Note

If you download the sequence for pENTR™/U6 from our Web site, note that the overhang sequences will be shown already hybridized to their complementary sequences (*e.g.* GTGG will be shown hybridized to CACC and TTTT will be shown hybridized to AAAA).

continued on next page

Analyzing Transformants, continued

In some cases, you may have difficulty sequencing the ds oligo insert in your pENTR™/U6 construct. This is because the hairpin sequence is an inverted repeat that can form secondary structure during sequencing, resulting in a drop in the sequencing signal when entering the hairpin. If you have difficulty sequencing your entry constructs, we suggest trying the following to improve your sequencing results:

- Use high-quality, purified plasmid DNA for sequencing. We recommend preparing DNA using Invitrogen's PureLink HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
- Add DMSO to the sequencing reaction to a final concentration of 5%.
- Increase the amount of template used in the reaction (up to twice the normal concentration).
- Standard sequencing kits typically use dITP in place of dGTP to reduce G:C compression. Other kits containing dGTP are available for sequencing G-rich and GT-rich templates. If you are using a standard commercial sequencing kit containing dITP, obtain a sequencing kit containing dGTP (*e.g.* dGTP BigDye® Terminator v3.0 Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Catalog no. 4390229) and use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction.

Long-Term Storage

Once you have identified the correct entry clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for a single colony on an LB plate containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until the culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store the glycerol stock at -80°C.

What to Do Next

Once you have obtained your pENTR™/U6 entry clone, you have the following options:

- Transfect the entry clone directly into the mammalian cell line of interest to perform transient RNAi analysis (see **Transfecting Cells**, next page).
- Perform an LR recombination reaction with your pENTR™/U6 construct and a suitable Gateway® destination vector to generate an expression clone (see **Guidelines to Perform the LR Recombination Reaction**, page 28).

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Transfecting Cells

Introduction

This section provides general guidelines to transfect your pENTR™/U6 construct into the mammalian cell line of interest to perform transient RNAi analysis. Performing transient RNAi analysis is useful to:

- Quickly test multiple shRNA sequences to a particular target gene
- Quickly screen for an RNAi response in your mammalian cell line

Once you have tested various shRNA target sequences using transient transfection, you may transfer the optimal shRNA cassettes into suitable destination vectors for use in other RNAi applications (*e.g.* stable expression in mammalian cells).

Important

You may express the shRNA and assay for knockdown of the target gene by transfecting your pENTR™/U6 construct directly into any mammalian cell line of choice. However, because the pENTR™/U6 vector **does not** contain a selection marker; only **transient** RNAi analysis may be performed. If you wish to generate stable cell lines, see page 28.

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
- Efficacy of the shRNA of interest

Take these factors into account when designing your RNAi experiments.

Plasmid Preparation

Once you have obtained your entry clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol or sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01), S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01), or CsCl gradient centrifugation.

continued on next page

Transfecting Cells, continued

Methods of Transfection

For established cell lines (*e.g.* COS, HEK-293), consult original references or the supplier of your cell line for the optimal method of transfection. Pay particular attention to media requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Choose the method and reagent that provides the highest efficiency transfection in your mammalian cell line. For a recommendation, see below.

For high-efficiency transfection in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen (Ciccarone *et al.*, 1999). Using Lipofectamine™ 2000 to transfect plasmid DNA into eukaryotic cells offers the following advantages:

- Provides the highest transfection efficiency in many mammalian cell types.
- DNA-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.

For more information on Lipofectamine™ 2000 Reagent, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 38).

Positive Control

If you have performed the positive control reaction and have cloned the lacZ ds oligo supplied with the kit into pENTR™/U6, we recommend using the resulting pENTR™/U6-GW/lacZ^{shRNA} entry construct as a positive control to assess the RNAi response in your cell line. Simply co-transfect the pENTR™/U6-GW/lacZ^{shRNA} entry construct and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your mammalian cells and assay for knockdown of β-galactosidase expression 24-48 hours post-transfection using Western blot analysis or activity assay. For more information about the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid, recommendations for transfection, and methods to assay for β-galactosidase activity, see the next page.

continued on next page

pcDNA™1.2/V5-GW/*lacZ* Reporter Plasmid

The pcDNA™1.2/V5-GW/*lacZ* reporter plasmid is supplied with the kit for use as a positive control to assay for the RNAi response in your mammalian cell line. In this vector, β-galactosidase is expressed as a C-terminally tagged fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). See page 37 for more information.

The pcDNA™1.2/V5-GW/*lacZ* vector is supplied as 500 ng/µl of plasmid DNA in TE Buffer, pH 8.0. Dilute the stock as necessary for use in transfection (see below). If you wish to propagate the plasmid, transform a *rec*A, *end*A *E. coli* strain such as TOP10. Use 10 ng of plasmid for transformation and select on LB agar plates containing 100 µg/ml ampicillin.

Transfecting the *LacZ*-Containing Reagents

To perform RNAi analysis using the lacZ control reagents, you will co-transfect the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and the pENTR™/U6-GW/lacZ^{shRNA} entry construct that you have generated into your mammalian cell line. For optimal results, we recommend using 6-fold more entry construct DNA than reporter plasmid DNA in the co-transfection. For example, use 600 ng of pENTR™/U6-GW/lacZ^{shRNA} DNA and 100 ng of pcDNA™1.2/V5-GW/*lacZ* DNA when transfecting cells plated in a 24-well format.

For an example of results obtained from such an RNAi experiment, see the next page.

Assaying for β-galactosidase Expression

If you perform RNAi analysis using the control entry clone containing the lacZ ds oligo (*i.e.* pENTR™/U6-GW/lacZ^{shRNA}), 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 38).

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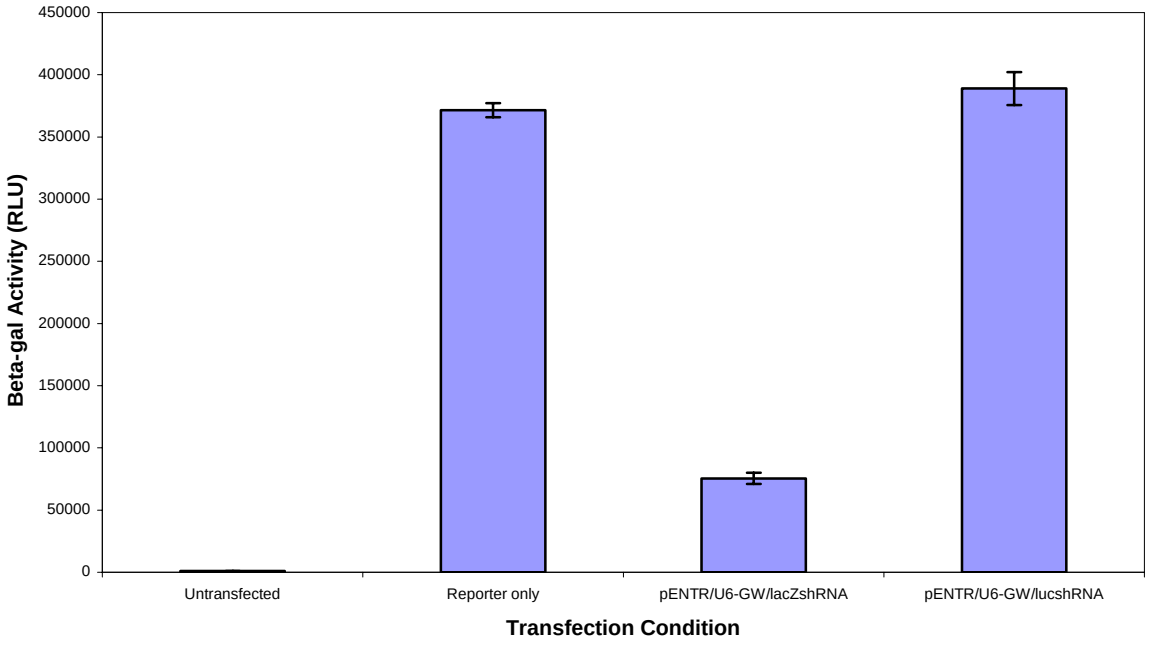
Example of Expected Results: Knockdown of a Reporter Gene

In this experiment, pENTR™/U6 entry constructs containing ds oligo encoding shRNA targeting the *lacZ* (*i.e.* pENTR™/U6-GW/lacZ^{shRNA}) or luciferase (*i.e.* pENTR™/U6-GW/luc^{shRNA}) reporter genes were generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ U6 Entry Vector Kit. Note that the lacZ ds oligo used in this experiment is the same as the lacZ ds control oligo supplied with the 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 700 ng of plasmid DNA (100 ng of the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and 600 ng of non-specific plasmid DNA). In some wells, the reporter plasmid was co-transfected with 600 ng of the pENTR™/U6-GW/lacZ^{shRNA} or pENTR™/U6-GW/luc^{shRNA} constructs. Cell lysates were prepared 48 hours after transfection and assayed for β-galactosidase activity.

Results: Potent and specific inhibition of β-galactosidase activity is evident from the lacZ-derived shRNA and not from the luciferase-derived shRNA.



Guidelines to Perform the LR Recombination Reaction

Introduction

The pENTR™/U6 vector contains *att*L sites to facilitate transfer of your U6 RNAi cassette (U6 promoter + ds oligo of interest + Pol III terminator) into an appropriate Gateway® destination vector to generate an expression clone. We recommend generating an expression clone if you wish to perform RNAi applications including:

- Delivery of the shRNA of interest to "hard-to-transfect" or non-dividing mammalian cells
- Generation of stable cell lines for long-term RNAi studies

To transfer your U6 RNAi cassette into the destination vector, you will perform an LR recombination reaction using Gateway® LR Clonase™ II Enzyme Mix. Guidelines are provided in this section.

Appropriate Destination Vectors

Because the U6 RNAi cassette contains its own promoter (*i.e.* U6 promoter), we do not recommend transferring the U6 RNAi cassette into a destination vector that contains a promoter (*e.g.* pcDNA™6.2/V5-DEST). We suggest performing LR recombination with a promoterless destination vector (*e.g.* pLenti6/BLOCK-iT™-DEST, pBLOCK-iT™6-DEST). Other promoterless destination vectors are available. For more information about suitable destination vectors to use for this application, see the RNAi Central application portal at www.invitrogen.com/rnai or call Technical Service (see page 38).

E. coli Host

Once you have performed the LR recombination reaction, you will transform the recombination reaction into competent *E. coli* and select for the appropriate transformants. You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation. **DO NOT** transform the LR recombination reaction into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Note: When performing the LR recombination reaction with the pLenti6/BLOCK-iT™-DEST RNAi vector, transformation into the Stbl3™ *E. coli* strain is recommended for optimal results (see ordering information below).

Product	Amount	Catalog no.
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl 40 x 50 µl	C4040-03 C4040-06
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03

continued on next page

Guidelines to Perform the LR Recombination Reaction, continued

Important

We recommend performing the LR recombination reaction using a:

- Supercoiled *att*L-containing pENTR™/U6 entry clone
- Supercoiled *att*R-containing destination vector

Materials Needed

You will need the following reagents to perform the LR recombination reaction:

- Purified plasmid DNA of your pENTR™/U6 entry clone (50-150 ng/µl in TE Buffer, pH 8.0)
- Destination vector of choice (150 ng/µl in TE Buffer, pH 8.0)
- LR Clonase™ II enzyme mix (Invitrogen, Catalog no. 11791-020)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- Appropriate chemically competent *E. coli* host and growth media for expression
- S.O.C. Medium
- Appropriate selective plates

Performing the LR Recombination Reaction

For detailed guidelines and instructions to perform the LR recombination reaction with an appropriate destination vector and transform competent *E. coli*, refer to the manual for the destination vector you are using.

Troubleshooting

Introduction

Use the information in this section to troubleshoot the annealing, cloning, transformation, and transfection procedures.

Annealing Reaction

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

Problem	Reason	Solution
Weak band representing ds oligo observed on an agarose gel	Single-stranded oligos designed incorrectly	Verify that the sequence of the bottom strand oligo is complementary to the sequence of the top strand oligo. If not, re-synthesize the bottom strand oligo.
	Allowed oligos to cool at +4°C instead of room temperature during annealing procedure	After heating to 95°C, anneal the oligos by setting the microcentrifuge tube at room temperature for 5-10 minutes (see the procedure on page 15).
	Did not anneal equal amounts of top and bottom strand oligo	Anneal equal amounts of the top and bottom strand oligo using the procedure on page 15.
No band representing ds oligo observed on an agarose gel	Single-stranded oligos designed incorrectly	Verify that the sequence of the bottom strand oligo is complementary to the sequence of the top strand oligo. If not, re-synthesize the bottom strand oligo.
	Used the wrong single-stranded oligos	Make sure that you mix single-stranded oligos with complementary sequence.

Ligation and Transformation Reactions

The table below lists some potential problems and possible solutions that may help you troubleshoot the ligation and transformation procedures.

Problem	Reason	Solution
Few kanamycin-resistant colonies obtained on the selective plate	Single-stranded oligos designed incorrectly	Make sure that each single-stranded oligo contains the 4 nucleotides on the 5′ end required for cloning into pENTR™/U6: • Top strand oligo: include CACC on the 5′ end. • Bottom strand oligo: include AAAA on the 5′ end.
	ds oligos were degraded	• Store the 5 nM ds oligo stock in 1X Oligo Annealing Buffer. • Avoid repeated freeze/thaw cycles. Aliquot the 5 nM ds oligo stock and store at -20°C.

continued on next page

Troubleshooting, continued

Ligation and Transformation Reactions, continued

Problem	Reason	Solution
Few kanamycin-resistant colonies obtained on the selective plate, continued	ds oligos stored incorrectly	Store the ds oligo stocks at -20°C.
	500 nM ds oligo stock solution diluted into water instead of 1X Oligo Annealing Buffer	To dilute the 50 µM ds oligo reaction: 1. Dilute the 50 µM stock 100-fold into DNase/RNase-free water to generate a 500 nM stock. 2. Dilute the 500 nM stock 100-fold into 1X Oligo Annealing Buffer to generate a 5 nM stock. Use the 5 nM stock for cloning.
	5 nM ds oligo stock solution heated above room temperature prior to use	Thaw ds oligo stock solution on ice or at +4°C prior to use. **Important:** Dilute ds oligos will melt and form intramolecular hairpins if heated above room temperature. These hairpins **will not** clone into pENTR™/U6.
	Incorrect vector:insert ratio used in ligation reaction • Forgot to dilute annealed ds oligo or LacZ ds Control Oligo 1:10,000 before use • Annealed ds oligo diluted incorrectly	Dilute the 50 µM ds oligo mixture as instructed on page 16 to generate a 5 nM stock. Use the 5 nM ds oligo stock for cloning.
	Ligation reaction not adequately mixed or incorrectly mixed prior to incubation	• Mix the ligation reaction well by pipetting up and down. **Note:** Flicking the tube is not adequate to mix the reagents. • Do not vortex the ligation reaction.
	Did not use the 5X Ligation Buffer supplied with the kit	Use the T4 DNA Ligase and 5X Ligation Buffer supplied with the kit for ligation as these reagents have been optimized to facilitate 5-minute ligation at room temperature. **Important:** Other T4 DNA Ligase and ligation buffers may not support 5-minute, room temperature ligation.
	Ligation reaction not incubated for long enough	Extend the incubation time of the ligation reaction up to 2 hours at room temperature.
	Ligation reaction incubated overnight at 16°C	The ligation conditions used to clone the ds oligo into pENTR™/U6 differ from traditional ligation conditions. **Incubate the ligation reaction at room temperature for 5 minutes.**

continued on next page

Troubleshooting, continued

Ligation and Transformation Reactions, continued

Problem	Reason	Solution
Few kanamycin-resistant colonies obtained on the selective plate, continued	Not enough transformation mixture plated	Increase the amount of the transformation mixture plated.
	Selective plates contained too much kanamycin	Use LB agar plates containing 50 µg/ml kanamycin for selection.
	Did not use the competent cells supplied with the kit	Use the One Shot® TOP10 Chemically Competent *E. coli* supplied with the kit; transformation efficiency is > 1 x 10^9 cfu/µg DNA.
	Not enough of the ligation reaction transformed	Increase the amount of ligation reaction transformed.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the bacterial culture for 1 hour at 37°C with shaking before plating.
Many clones contain inserts with sequence mutations	Poor quality single-stranded oligos used • Oligo preparation contains mutated sequences • Oligo preparation contains contaminants	• Use mass spectrometry to check for peaks of the wrong mass. • Order HPLC or polyacrylamide gel (PAGE)-purified oligos. • Order oligos from Invitrogen's custom primer synthesis service (see our Web site for more information).
	Did not use the competent cells supplied with the kit	Use the One Shot® TOP10 Chemically Competent *E. coli* supplied with the kit; transformation efficiency is > 1 x 10^9 cfu/µg DNA.
Poor sequencing results	Loss of sequencing signal in the hairpin region due to secondary structure formation	• Use high-quality, purified plasmid DNA for sequencing. • Add DMSO to the sequencing reaction to a final concentration of 5%. • Increase the amount of template used for sequencing (up to twice the normal amount). • Use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction.
No colonies obtained on the selective plate	Used the wrong antibiotic for selection	Select for transformants on LB agar plates containing 50 µg/ml kanamycin.

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.

Problem	Reason	Solution
Low levels of gene knockdown observed	Low transfection efficiency (if using Lipofectamine™ 2000 Reagent)	
	• Antibiotics added to the media during transfection	• Do not add antibiotics to the media during transfection.
	• Cells too sparse at the time of transfection	• Plate cells such that they will be 90-95% confluent at the time of transfection.
	• Not enough plasmid DNA transfected	• Increase the amount of plasmid DNA transfected.
	• Not enough Lipofectamine™ 2000 used	• 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.
	ds oligo insert in your pENTR™/U6 construct contains mutations	When analyzing kanamycin-resistant transformants, sequence the ds oligo insert to verify its sequence. Select constructs containing the correct ds oligo insert for use in RNAi analysis.
	shRNA sequence not optimal due to: • Target region selected • Length of the shRNA sequence (*i.e.* stem length) • Loop sequence • Orientation of shRNA sequence	• Verify that the shRNA sequence does not contain > 3 tandem T's which can cause premature transcription termination. • Select a different target region. • Vary the length of the shRNA sequence (*e.g.* if the target sequence is 19 bp, try increasing the stem length 3 nucleotides) • Select a different loop sequence. • Vary the length of the loop. • Reverse the orientation of the shRNA hairpin sequence (*e.g.* change oligo sequence from sense-loop-antisense to antisense-loop-sense orientation).

continued on next page

Troubleshooting, continued

Transfection and RNAi Analysis, continued

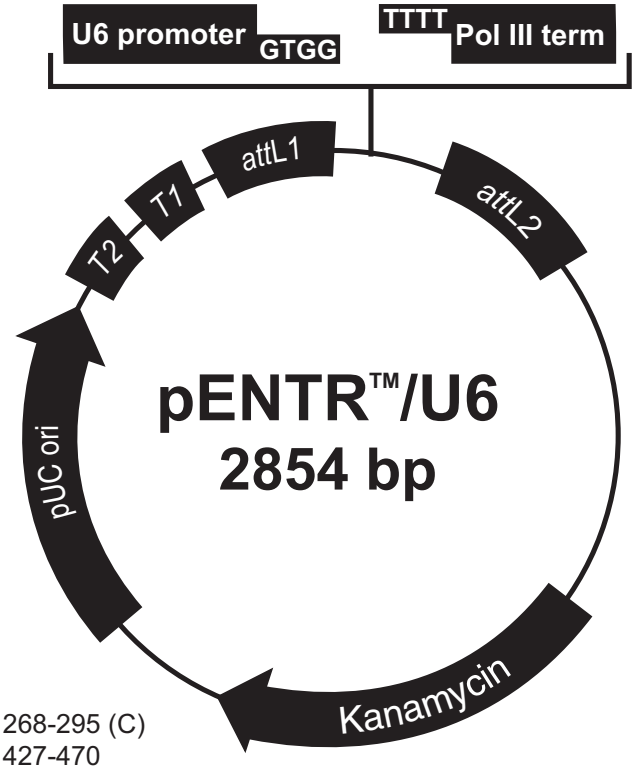
Problem	Reason	Solution
Cytotoxic effects observed after transfection	Too much Lipofectamine™ 2000 Reagent used	Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used.
	Plasmid DNA not pure	Prepare purified plasmid DNA for transfection. We recommend using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) to prepare purified plasmid DNA.
	Targeted an essential gene	Make sure that your target gene is not essential for cell viability or growth.
No gene knockdown observed	shRNA with no activity chosen	• Verify that the shRNA sequence does not contain > 3 tandem T's which can cause premature transcription termination. • Select a different target region.
	Hairpin designed incorrectly	Follow the guidelines on pages 9-13 to select the target sequence and design the single-stranded oligos.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a new target sequence.

Appendix

Map and Features of pENTR™/U6

pENTR™/U6 Map

The figure below shows the features of the pENTR™/U6 vector. The vector is supplied linearized between nucleotides 968 and 969 with 4 base pair 5′ overhangs on each strand as indicated. **The complete sequence of pENTR/U6 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 38).**



Comments for pENTR™/U6
2854 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (C)
*rrn*B T1 transcription terminator: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (C)
U6 promoter: bases 705-968
U6 forward priming site: bases 890-909
5′ overhang: bases 965-968 (C)
5′ overhang: bases 969-972
Pol III transcription terminator: bases 969-974
*att*L2: bases 979-1078
M13 reverse priming site: bases 1119-1135
Kanamycin resistance gene: bases 1248-2057
pUC origin: bases 2178-2851

(C) = complementary strand

continued on next page

Map and Features of pENTR™/U6, continued

Features of pENTR™/U6

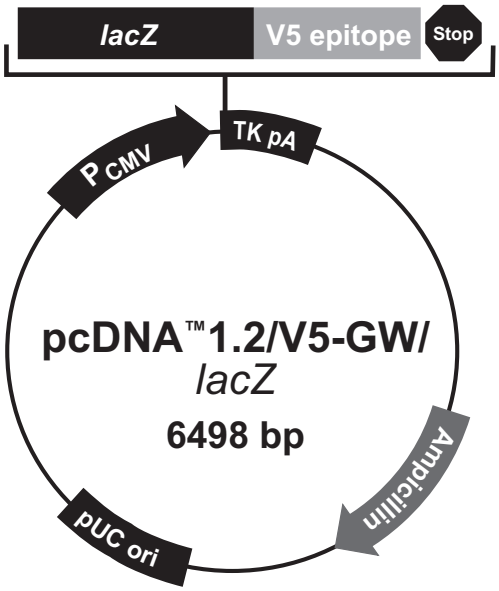
pENTR™/U6 (2854 bp) contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Reduces potential toxicity in *E. coli* by preventing basal expression of the double-stranded oligonucleotide of interest.
M13 forward (-20) priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
Human U6 promoter	Allows RNA Polymerase III-dependent expression of the short hairpin RNA (shRNA) (Kunkel *et al.*, 1986; Kunkel and Pederson, 1988).
U6 forward priming site	Allows sequencing of the insert.
5′ overhangs	Allows ligase-mediated directional cloning of the double-stranded oligonucleotide of interest.
Pol III terminator	Allows efficient termination of RNA Polymerase III-dependent transcription.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

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 Invitrogen (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 (Cole and Stacy, 1985) 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 Web site (www.invitrogen.com) or by contacting Technical Service (see page 38).**



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

Technical Service

Web Resources

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

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

Contact Us

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

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Certificate of Analysis

Product qualification is described in the Certificate of Analysis (CofA), available on our website by product lot number at www.invitrogen.com/cofa.

Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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Technical Service, continued

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Purchaser Notification

Introduction

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 42.

continued on next page

Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

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References

Ambros, V. (2001). MicroRNAs: Tiny Regulators with Great Potential. Cell *107*, 823-826.

Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H., and Vance, V. B. (1998). A Viral Suppressor of Gene Silencing in Plants. Proc. Natl. Acad. Sci. USA *95*, 13079-13084.

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference. Nature *409*, 363-366.

Bogenhagen, D. F., and Brown, D. D. (1981). Nucleotide Sequences in Xenopus 5S DNA Required for Transcription Termination. Cell *24*, 261-270.

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Bosher, J. M., and Labouesse, M. (2000). RNA Interference: Genetic Wand and Genetic Watchdog. Nature Cell Biol. *2*, E31-E36.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science *296*, 550-553.

Carrington, J. C., and Ambros, V. (2003). Role of MicroRNAs in Plant and Animal Development. Science *301*, 336-338.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999). Lipofectamine™ 2000 Reagent for Rapid, Efficient Transfection of Eukaryotic Cells. Focus *21*, 54-55.

Cogoni, C., and Macino, G. (1999). Gene Silencing in *Neurospora crassa* Requires a Protein Homologous to RNA-Dependent RNA Polymerase. Nature *399*, 166-169.

Cogoni, C., and Macino, G. (1997). Isolation of Quelling-Defective (qde) Mutants Impaired in Posttranscriptional Transgene-Induced Gene Silencing in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA *94*, 10233-10238.

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References, continued

Cogoni, C., Romano, N., and Macino, G. (1994). Suppression of Gene Expression by Homologous Transgenes. Antonie Van Leeuwenhoek *65*, 205-209.

Cole, C. N., and Stacy, T. P. (1985). Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003). Killing the Messenger: Short RNAs that Silence Gene Expression. Nat. Rev. Mol. Cell Biol. *4*, 457-467.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G., and Mello, C. C. (2001). Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs That Control *C. elegans* Developmental Timing. Cell *106*, 23-34.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-Directed Nuclease Mediates Genetic Interference in *Caenorhabditis elegans*. Nature *404*, 293-296.

Hannon, G. J. (2002). RNA Interference. Nature *418*, 244-251.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. Science *293*, 811-813.

Jones, A. L., Thomas, C. L., and Maule, A. J. (1998). *De novo* Methylation and Co-Suppression Induced by a Cytoplasmically Replicating Plant RNA Virus. EMBO J. *17*, 6385-6393.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001). Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in *C. elegans*. Genes Dev. *15*, 2654-2659.

Kunkel, G. R., Maser, R. L., Calvet, J. P., and Pederson, T. (1986). U6 Small Nuclear RNA is Transcribed by RNA Polymerase III. Proc. Natl. Acad. Sci. USA *83*, 8575-8579.

Kunkel, G. R., and Pederson, T. (1988). Upstream Elements Required for Efficient Transcription of a Human U6 RNA Gene Resemble Those of U1 and U2 Genes Even Though a Different Polymerase is Used. Genes Dev. *2*, 196-204.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The *C. elegans* Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*. Cell *75*, 843-854.

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References, continued

Li, W. X., and Ding, S. W. (2001). Viral Suppressors of RNA Silencing. Curr. Opin. Biotechnol. *12*, 150-154.

McManus, M. T., and Sharp, P. A. (2002). Gene Silencing in Mammals by Small Interfering RNAs. Nature Rev. Genet. *3*, 737-747.

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chalcone Synthase Gene into *Petunia* Results in Reversible Co-Suppression of Homologous Genes *in trans*. Plant Cell *2*, 279-289.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

Nykanen, A., Haley, B., and Zamore, P. D. (2001). ATP Requirements and Small Interfering RNA Structure in the RNA Interference Pathway. Cell *107*, 309-321.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002). Short Hairpin RNAs (shRNAs) Induce Sequence-Specific Silencing in Mammalian Cells. Genes Dev. *16*, 948-958.

Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002). Effective Expression of Small Interfering RNA in Human Cells. Nat. Biotechnol. *20*, 505-508.

Paule, M. R., and White, R. J. (2000). Transcription by RNA Polymerases I and III. Nuc. Acids Res. *28*, 1283-1298.

Plasterk, R. H. A., and Ketting, R. F. (2000). The Silence of the Genes. Curr. Opin. Genet. Dev. *10*, 562-567.

Romano, N., and Macino, G. (1992). Quelling: Transient Inactivation of Gene Expression in *Neurospora crassa* by Transformation with Homologous Sequences. Mol. Microbiol. *6*, 3343-3353.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Smith, C. J., Watson, C. F., Bird, C. R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a Truncated Tomato Polygalacturonase Gene Inhibits Expression of the Endogenous Gene in Transgenic Plants. Mol. Gen. Genet. *224*, 477-481.

Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., Forrester, W. C., and Shi, Y. (2002). A DNA Vector-Based RNAi Technology to Suppress Gene Expression in Mammalian Cells. Proc. Natl. Acad. Sci. USA *99*, 5515-5520.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N., and Stuitje, A. R. (1990). Flavonoid Genes in *Petunia*: Addition of a Limited Number of Gene Copies May Lead to a Suppression of Gene Expression. Plant Cell *2*, 291-299.

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References, continued

Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. (1999). Suppression of Gene Silencing: A General Strategy Used by Diverse DNA and RNA Viruses of Plants. Proc. Natl. Acad. Sci. USA *96*, 14147-14152.

Weiss, B., Jacquemin-Sablon, A., Live, T. R., Fareed, G. C., and Richardson, C. C. (1968). Enzymatic Breakage and Joining of Deoxyribonucleic Acid. VI. Further Purification and Properties of Polynucleotide Ligase from *Escherichia coli* Infected with Bacteriophage T4. J. Biol. Chem. *243*, 4543-4555.

White, R. J. (1998). RNA Polymerase III Transcription (New York, NY: Springer-Verlag).

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Yu, J. Y., DeRuiter, S. L., and Turner, D. L. (2002). RNA Interference by Expression of Short-interfering RNAs and Hairpin RNAs in Mammalian Cells. Proc. Natl. Acad. Sci. USA *99*, 6047-6052.

Zamore, P. D. (2001). RNA Interference: Listening to the Sound of Silence. Nat. Struct. Biol. *8*, 746-750.

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