invitrogen™

BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit

A Gateway®-adapted entry vector for regulated expression of short hairpin RNA (shRNA) in mammalian cells

Catalog nos. K4920-00 and K4925-00

Version F
23 June 2010
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User Manual

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H1/TO Entry Clone Generation Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the H1/TO 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 14-19 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™/H1/TO	1. Set up the following ligation reaction. 5X Ligation Buffer — 4 µl pENTR™/H1/TO (0.75 ng/µl) — 2 µl ds oligo (5 nM; 1:10,000 dilution) — 1-5 µl DNase/RNase-Free water — to a final volume of 19 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 40-200 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™ Inducible H1 Lentiviral RNAi System also contains the BLOCK-iT™ Inducible H1 Lentiviral RNAi System components and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

Product	Catalog no.
BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit	K4920-00
BLOCK-iT™ Inducible H1 Lentiviral RNAi System	K4925-00

Kit Components

The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System include the following components. For a detailed description of the contents of the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, see pages viii-ix. For a detailed description of the contents of the BLOCK-iT™ Inducible H1 Lentiviral RNAi reagents, see the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

Component	Catalog no.	
	K4920-00	K4925-00
BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit	√	√
BLOCK-iT™ Inducible H1 Lentiviral RNAi Reagents		√

Shipping/Storage

The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System are shipped as described below. Upon receipt, store each item as detailed below. For more detailed information about the BLOCK-iT™ Inducible H1 Lentiviral RNAi reagents supplied with the kit, refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

Box	Component	Shipping	Storage
1	Inducible H1 RNAi Entry Vector Reagents and Tetracycline	Dry ice	Tetracycline: -20°C, protected from light All other reagents: -20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	-80°C
3-9	BLOCK-iT™ Inducible H1 Lentiviral RNAi Reagents	Various	Various (refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual for details)

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Kit Contents and Storage, continued

Inducible H1 RNAi Entry Vector Reagents and Tetracycline

The following reagents are included with the Inducible H1 RNAi Entry Vector and Tetracycline box (Box 1). **Store the tetracycline at -20°C, protected from light. Store the other reagents at -20°C.**

Reagent	Composition	Amount
pENTR™/H1/TO vector, linearized	0.75 ng/µl plasmid DNA 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
H1 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
LacZ2.1 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
Tetracycline	10 mg/ml in water	1 ml

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
H1 Forward	5′-TGTTCTGGGAAATCACCATA-3′	328 pmoles
M13 Reverse	5′-CAGGAAACAGCTATGAC -3′	385 pmoles

LacZ2.1 Control Oligo Sequences

The sequences of the lacZ2.1 control oligos are listed below. The lacZ2.1 control DNA oligos are annealed and are supplied in the kit as a 50 µM double-stranded oligo. The LacZ2.1 ds Control Oligo needs to be re-annealed and diluted 10,000-fold to 5 nM (see page 21) before use in the ligation reaction (see page 25).

LacZ2.1 DNA Oligo	Sequence
Top strand	5′-CACCAAATCGCTGATTTGTGTAGTCGGAGACGACTACACAAATCAGCGA-3′
Bottom strand	5′-AAAATCGCTGATTTGTGTAGTCGTCTCCGACTACACAAATCAGCGATTT-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 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™ Inducible H1 Lentiviral RNAi Reagents

In addition to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Catalog no. K4925-00) also includes the following components to facilitate production of a replication-incompetent lentivirus that expresses your short hairpin RNA (shRNA) of interest in a regulated manner.

- pLenti4/BLOCK-iT™-DEST Gateway® Vector Kit (Box 3)
- pLenti6/TR Vector Kit (Box 4)
- One Shot® Stbl3™ Chemically Competent *E. coli* (Box 5)
- ViraPower™ Zeo Lentiviral Support Kit (Boxes 6 and 7)
- Gateway® LR Clonase™ II Enzyme Mix (Box 8)
- 293FT Cell Line (Box 9)

Refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi 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™ Inducible H1 Lentiviral RNAi System and the 293FT Cell Line manuals are supplied with Catalog no. K4925-00, but are also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 59).

Accessory Products

Introduction

The products listed in this section may be used with the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 59).

Accessory Products

Some of the reagents supplied in the BLOCK-iT™ Inducible H1 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.

Product	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
M13 Reverse Primer	2 µg	N530-02
PureLink™ HQ Mini Plasmid Purification Kit	100 purifications	K2100-01
S.N.A.P.™ MidiPrep Kit	20 purifications	K1910-01
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
Zeocin™	1 g	R250-01
	5 g	R250-05
Kanamycin Sulfate	5 g	11815-024
Tetracycline	5 g	Q100-19
4% E-Gel® Starter Pak	9 gels and Base	G5000-04
10 bp DNA Ladder	50 µg	10821-015
β-gal Antiserum	50 µl*	R901-25
FluoReporter® *lacZ*/Galactosidase Quantitation Kit	1000 reactions	F-2905
pBLOCK-iT™3-DEST Gateway® Vector	6 µg	V486-20

*The amount of antibody supplied is sufficient for 25 western blots.

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Accessory Products, continued

T-REx™ Products

Invitrogen has available a number of cell lines that stably express the Tet repressor from pcDNA™6/TR (TetR expressing plasmid from the T-REx™ System). The cell lines should be maintained in medium containing Blasticidin. In addition, the pcDNA™6/TR plasmid is available to facilitate generation of your own T-REx™ cell lines. For more information about the T-REx™ Cell Lines, pcDNA™6/TR, or the T-REx™ System, see our Web site (www.invitrogen.com) or contact Technical Service (see page 59).

Product	Amount	Catalog no.
T-REx™-293 Cell Line	3 x 10^6 cells, frozen	R710-07
T-REx™-HeLa Cell Line	3 x 10^6 cells, frozen	R714-07
T-REx™-CHO Cell Line	3 x 10^6 cells, frozen	R718-07
T-REx™-Jurkat Cell Line	3 x 10^6 cells, frozen	R722-07
Flp-In™ T-REx™ 293 Cell Line	3 x 10^6 cells, frozen	R780-07
pcDNA™6/TR	20 µg	V1025-20

BLOCK-iT™ Inducible Lentiviral RNAi Reagents

The reagents supplied in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit	20 constructions	V488-20
pLenti6/TR Vector Kit	20 µg	V480-20
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03
ViraPower™ Zeo Lentiviral Support Kit	20 reactions	K4985-00
293FT Cell Line	3 x 10^6 cells, frozen	R700-07
Blasticidin	50 mg	R210-01

Introduction

Overview

Introduction

The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit combines Invitrogen's BLOCK-iT™ RNAi and T-REx™ technologies to facilitate tetracycline-regulated expression of a short hairpin RNA (shRNA) of interest from an H1/TO RNAi cassette for use in RNA interference (RNAi) analysis in mammalian cells. The kit provides a Gateway®-adapted entry vector designed to allow efficient transient or stable, regulated expression of shRNA in dividing mammalian cells or easy transfer of the H1/TO RNAi cassette into other suitable Gateway® destination vectors for other RNAi applications. For more information about the BLOCK-iT™ RNAi, T-REx™, and Gateway® technologies, see below and the next page.

Advantages of the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit

Using the BLOCK-iT™ Inducible H1 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.* H1/TO RNAi cassette) for use in RNAi analysis.
- The entry construct containing the H1/TO RNAi expression cassette may be directly transfected into mammalian cells expressing the Tet repressor to enable rapid, tetracycline-regulated screening of shRNA target sequences.
- The entry construct contains a Zeocin™ resistance marker to allow generation of stable cell lines that express the shRNA of interest upon tetracycline addition.
- The vector is Gateway®-adapted to allow easy transfer of the H1/TO RNAi cassette into any appropriate expression system for other RNAi applications (*e.g.* lentiviral system for stable delivery of regulated shRNA in hard-to-transfect or non-dividing mammalian cells).

BLOCK-iT™ RNAi Technology

A variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis in mammalian and invertebrate systems. The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit and the BLOCK-iT™ U6 RNAi Entry Vector Kit (Catalog nos. K4920-00 and K4945-00, respectively) use a vector-based approach to allow efficient generation of RNAi cassettes for constitutive or regulated expression of shRNA molecules in mammalian cells. Other BLOCK-iT™ RNAi products are available to facilitate production and delivery of synthetic short interfering RNA (siRNA), diced siRNA (d-siRNA) or double-stranded RNA (dsRNA) for RNAi analysis in mammalian cells or invertebrate organisms, as appropriate. For more information about any of the BLOCK-iT™ RNAi products, see the RNAi Central application portal at www.invitrogen.com/rnai or contact Technical Service (see page 59).

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Overview, continued

The T-REx™ Technology

The T-REx™ Technology facilitates tetracycline-regulated expression of a gene of interest in mammalian cells through the use of regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen *et al.*, 1983). Tetracycline regulation in the T-REx™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao *et al.*, 1998). The main components of the T-REx™ System include:

- An inducible expression construct to facilitate tetracycline-regulated expression of your gene of interest under the control of a hybrid promoter containing two tetracycline operator 2 ($TetO_2$) sites.
- A regulatory expression construct that facilitates high-level, constitutive expression of the Tet repressor (TetR). In the T-REx™ System, expression of the *TetR* gene is controlled by the CMV promoter.
- Tetracycline for inducing expression.

When the inducible expression construct and the regulatory expression construct are present in the same mammalian cell, expression of your gene of interest is repressed in the absence of tetracycline and induced in its presence (Yao *et al.*, 1998).

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.* H1/TO RNAi cassette) into multiple vector systems. To express your shRNA of interest using the pENTR™/H1/TO vector, simply:

1. Clone your ds oligo encoding the shRNA of interest into the pENTR™/H1/TO vector to generate an entry clone.
2. Choose one of the following options:
   a. Transfect your entry construct into Tet repressor (TetR)-expressing mammalian cells. Add tetracycline to transiently assay for target gene knockdown.
   b. Transfect the entry construct into TetR-expressing mammalian cells and use Zeocin™ selection to generate a stable cell line. Add tetracycline to assay for target gene knockdown.
   c. 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 59).

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Overview, continued

Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit and provides instructions and guidelines to:

- Design the appropriate single-stranded oligonucleotides representing the target gene.
- Anneal the single-stranded oligonucleotides to generate a double-stranded oligonucleotide (ds oligo).
- Clone the ds oligo into the pENTR™/H1/TO vector, and transform the ligation reaction into competent *E. coli*.
- Generate stable, mammalian TetR-expressing cell lines (provides guidelines only).
- Transfect your pENTR™/H1/TO construct into a mammalian TetR-expressing cell line to perform transient, tetracycline-regulated RNAi analysis.
- Transfect your pENTR™/H1/TO construct into a mammalian TetR-expressing cell line and perform Zeocin™ selection to generate a stable cell line for tetracycline-regulated RNAi analysis.
- Perform an LR recombination reaction with a suitable Gateway® destination vector to generate an expression clone (provides guidelines only). 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™ Inducible H1 RNAi Entry Vector Kit is designed to help you generate a construct to express shRNA in a regulated fashion in mammalian cell lines for RNAi analysis. Although the kit has been designed to help you express an shRNA 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 *et al.*, 2002; McManus & Sharp, 2002; Paul *et al.*, 2002; Yu *et al.*, 2002).

BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit

Description of the System

The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit facilitates the generation of an entry construct that permits high-level, tetracycline-regulated 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™/H1/TO 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 tetracycline-inducible expression of the shRNA in mammalian cells. The pENTR™/H1/TO 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 H1/TO RNAi cassette (*i.e.* H1/TO promoter + ds oligo + Pol III terminator) may be used in a number of RNAi applications (see the next page). For more information about the features of the pENTR™/H1/TO vector, see below and page 56. For more information about the H1/TO RNAi cassette, see page 9. For more information about how tetracycline regulation works, see page 11.
- T4 DNA Ligase and an optimized ligation buffer to allow 5-minute room temperature ligation of the ds oligo insert into pENTR™/H1/TO.
- One Shot® TOP10 Chemically Competent *E. coli* for high efficiency transformation of the ligation reaction.
- Tetracycline, the inducing agent for regulated expression of the shRNA of interest.

Note: The kit also includes a lacZ2.1 ds control oligo that may be cloned into pENTR™/H1/TO to generate an entry construct expressing an 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 tetracycline-regulated knockdown of β-galactosidase.

Features of the pENTR™/H1/TO Vector

The pENTR™/H1/TO vector contains the following features:

- H1/TO RNAi cassette containing elements required to allow RNA Polymerase III-dependent, tetracycline-regulated expression of the shRNA of interest in mammalian cells (see page 9 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 H1/TO 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 19 for more information).
- Two recombination sites, *att*L1 and *att*L2, for recombinational cloning of the H1/TO RNAi cassette into a suitable Gateway® destination vector (Landy, 1989)
- Kanamycin resistance gene for selection in *E. coli*
- Zeocin™ resistance gene for selection in mammalian cells and *E. coli*
- pUC origin for high-copy maintenance of the plasmid in *E. coli*

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BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, continued

Important

If you have previously used other Gateway® entry vectors, note that **specific entry vectors** are required to generate entry clones for use in RNAi applications. Specifically, use:

- pENTR™/H1/TO (Catalog no. K4920-00) for regulated expression of shRNA for RNAi analysis
- pENTR™/U6 (Catalog no. K4945-00) for constitutive expression of shRNA for RNAi analysis

Note: Other Gateway® entry vectors (*e.g.* pENTR™/D-TOPO®) do not contain the RNAi cassette required for Pol III-dependent expression of shRNA.

Generating shRNA Using the Kit

Using the reagents supplied in the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, you will perform the following steps to generate an entry clone in pENTR™/H1/TO.

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™/H1/TO vector.
4. Transform the ligation reaction into One Shot® TOP10 chemically competent *E. coli* and select for kanamycin- (or Zeocin-) resistant transformants.
5. Use the pENTR™/H1/TO entry construct for the desired RNAi application (see below).

RNAi Applications

Once you have cloned the ds oligo insert into pENTR™/H1/TO, you may use the resulting entry clone in the following RNAi applications:

- Transfect the entry clone into a tetracycline repressor (TetR)-expressing mammalian cell line for transient, tetracycline-regulated RNAi analysis.
- Transfect the entry clone into a TetR-expressing mammalian cell line and select for a stable cell line(s) expressing the shRNA of interest. Use the stable cell line for tetracycline-regulated RNAi analysis.
- Transfect the entry clone into a non TetR-expressing mammalian cell line for transient, non tetracycline-regulated RNAi analysis. Select for a stable cell line to constitutively express the shRNA of interest, if desired.
- Transfer the H1/TO RNAi cassette into a suitable destination vector using Gateway® Technology (*e.g.* pLenti4/BLOCK-iT™-DEST).

For more information about TetR-expressing mammalian cell lines available from Invitrogen (*i.e.* T-REx™ Cell Lines) or how to generate your own TetR-expressing cell line, see the next page.

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Tet Repressor-Expressing Cell Lines

To express your shRNA of interest in a tetracycline-regulated manner, you will need a mammalian host cell line that stably expresses the tetracycline repressor (TetR) to prevent basal target gene knockdown. You may obtain or generate this TetR-expressing cell line in the following ways:

- Use one of the T-REx™ Cell Lines available from Invitrogen. These cell lines express the Tet repressor from the pcDNA™6/TR plasmid, the regulatory vector available with Invitrogen's T-REx™ System. For more information about the T-REx™ Cell Lines, see page xii.
- Use the pcDNA™6/TR plasmid available from Invitrogen (Catalog no. V1025-20) to generate your own TetR-expressing cell line. This is recommended if you plan to perform your RNAi experiments using the pENTR™/H1/TO construct **only**.
- Use the pLenti6/TR lentiviral construct available from Invitrogen to generate a TetR-expressing cell line. pLenti6/TR is a lentiviral-based vector that expresses the Tet repressor and is the regulatory vector available with Invitrogen's BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Catalog no. K4925-00) or the ViraPower™ T-REx™ System (Catalog no. K4965-00). If you plan to perform RNAi analysis using both the pENTR™/H1/TO construct and the pLenti4/BLOCK-iT™-DEST construct, we recommend using pLenti6/TR to generate your TetR-expressing cell Line.

  Note: pLenti6/TR is also available separately from Invitrogen (Catalog no. V480-20).

For guidelines to generate your own TetR-expressing cell line, see pages 52-53.

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 & Macino, 1997; Cogoni & Macino, 1999; Romano & Macino, 1992). In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li & Ding, 2001; Voinnet *et al.*, 1999).

In eukaryotic organisms, dsRNA produced *in vivo* or introduced by pathogens is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001). 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 & Ambros, 2003) have been identified and shown to be able to trigger gene silencing.

For more information about the RNAi pathway and the mechanism of gene silencing, refer to recent reviews (Bosher & Labouesse, 2000; Dykxhoorn *et al.*, 2003; Hannon, 2002; Plasterk & 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 & 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, H1 RNA, and a number of other small stable RNAs that are involved in RNA processing (Paule & 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 & 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; McManus *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™/H1/TO 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.* H1/TO RNAi cassette; see the next page). The resulting pENTR™/H1/TO entry construct may be introduced into dividing mammalian cells for transient or stable tetracycline-regulated expression of the shRNA of interest and initial RNAi screening, if desired. Once initial screening is complete, the H1/TO 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.* expression of shRNA in hard-to-transfect or non-dividing cells).

continued on next page

Using shRNA for RNAi Analysis, continued

Features of the H1/TO RNAi Cassette

The H1/TO RNAi cassette in pENTR™/H1/TO contains all of the elements required to facilitate RNA Polymerase III-controlled expression of your shRNA of interest including a:

- Modified human H1 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 & Brown, 1981)

See the diagram below for an illustration of the H1/TO RNAi cassette.

Note: The H1/TO RNAi cassette in pENTR™/H1/TO is flanked by *att*L sites to allow easy transfer of the cassette into other suitable Gateway® destination vectors.

Human H1 Promoter

Expression of the shRNA of interest from pENTR™/H1/TO (or a suitable destination vector following LR recombination) is controlled by the human H1 promoter, which has been modified to include two prokaryotic *tet* operator 2 ($TetO_2$) sequences. The endogenous human H1 promoter normally controls expression of H1 RNA, the RNA component of human RNase P involved in tRNA processing (Baer *et al.*, 1990), and has been well-characterized(Hannon *et al.*, 1991; Myslinksi *et al.*, 2001). We and other groups have chosen this particular promoter to control vector-based expression of shRNA molecules in mammalian cells (Brummelkamp *et al.*, 2002; McManus *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 & White, 2000)

For more information about the *tet* operator sequences and how tetracycline regulation works, see pages 11.

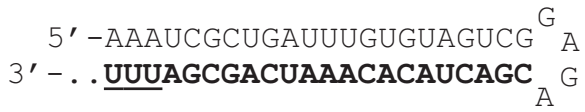
continued on next page

Using shRNA for RNAi Analysis, continued

Structure of the shRNA

The shRNA molecule expressed from the H1/TO RNAi cassette (in pENTR™/H1/TO or in a suitable destination vector) forms an intramolecular stem-loop structure similar to the structure of miRNA. The endogenous Dicer enzyme then processes this hairpin into a 21-23 nt siRNA duplex.

Example: The figure below illustrates the structure of the shRNA generated from the pENTR™-GW/H1/TO-lacZ2.1^{shRNA} construct. You may generate this construct by cloning the lacZ2.1 ds control oligo supplied with the kit into pENTR™/H1/TO following the protocols in this manual. The 22 nt lacZ target sequence is indicated in bold. The underlined bases are derived from the Pol III terminator.



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 14-19.

How Tetracycline Regulation Works

Introduction

As described previously, the H1 promoter in the pENTR™/H1/TO entry vector has been modified to include two prokaryotic *tet* operator sequences. The presence of the *tet* operator sequences enables the shRNA of interest to be expressed in a tetracycline-dependent manner. This section describes the *tet* operator sequences in the pENTR™/H1/TO vector, and the mechanism of tetracycline regulation in the BLOCK-iT™ Inducible H1 RNAi System.

Tetracycline Regulation and *tet* Operator Sequences

The BLOCK-iT™ Inducible H1 RNAi System uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen *et al.*, 1983) to allow tetracycline-regulated expression of your shRNA of interest from the pENTR™/H1/TO vector. The mechanism of tetracycline regulation in the system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the shRNA of interest. In the system, expression of your shRNA of interest is repressed in the absence of tetracycline and induced in its presence.

In the BLOCK-iT™ Inducible H1 RNAi Entry Vector System, expression of the shRNA of interest from the pENTR™/H1/TO is controlled by a human H1 promoter into which 2 copies of the 19 nt *tet* operator 2 ($TetO_2$) sequence have been incorporated (*i.e.* H1/TO promoter). Each 19 nt $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor. Refer to the diagram on page 19 for the location and sequences of the $TetO_2$ sites in the H1/TO promoter.

Mechanism of Repression/ Derepression

In the absence of tetracycline, the Tet repressor (expressed from the pcDNA™6/TR plasmid or pLenti6/TR lentiviral construct, as desired) forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence (Hillen & Berens, 1994) in the H1/TO promoter of the pENTR™/H1/TO. The 2 $TetO_2$ sites in the H1/TO promoter serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor (see figure on the next page). Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of your shRNA of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind the Tet operator. The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription of the shRNA of interest, resulting in target gene knockdown (see figure on the next page).

Note: The affinity of the Tet repressor for the *tet* operator is $K_B = 2 \times 10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen & Berens, 1994). The association constant, K_A, of tetracycline for the Tet repressor is $3 \times 10^9\ M^{-1}$ (Takahashi *et al.*, 1991).

continued on next page

How Tetracycline Regulation Works, continued

Diagram of Tetracycline Regulation

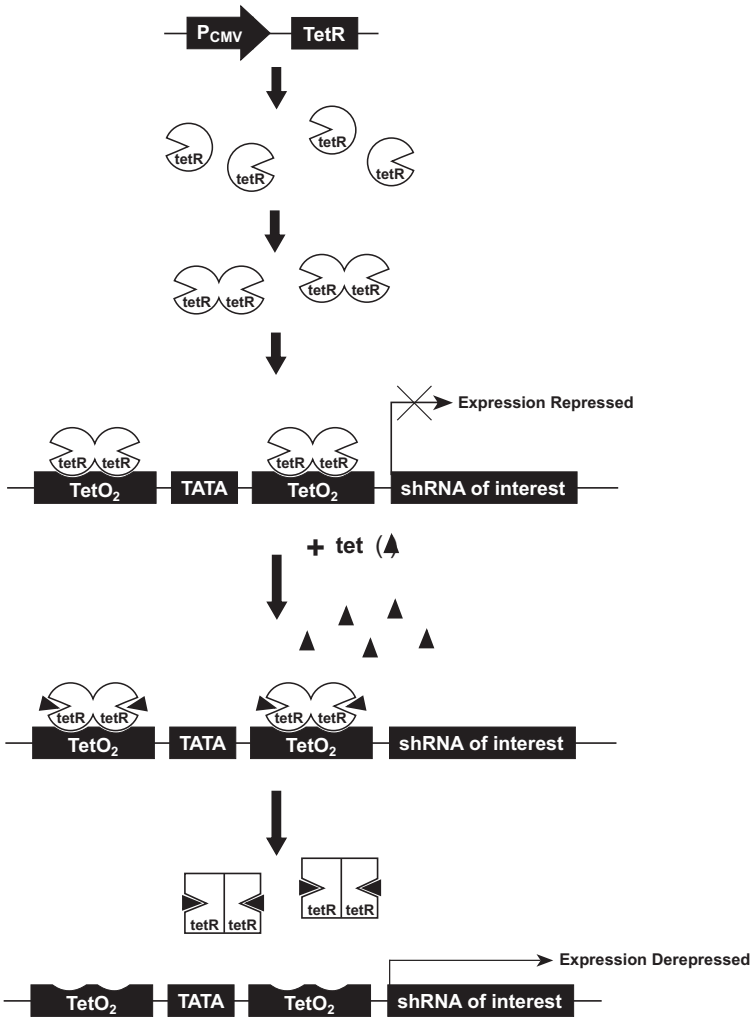
The figure below illustrates the mechanism of tetracycline-regulated expression and derepression of the shRNA of interest in the BLOCK-iT™ Inducible H1 RNAi Entry Vector System.

1. Introduce the pcDNA®6/TR plasmid or the Lenti6/TR lentiviral construct into the mammalian cells of interest. Add Blasticidin to select for a stable Tet repressor (TetR)-expressing cell line.

2. Transfect the pENTR™/H1/TO entry construct into TetR-expressing cells. Upon transfection, TetR homodimers bind to Tet operator 2 ($TetO_2$) sequences in the pENTR™/H1/TO construct, repressing transcription of the shRNA of interest.

3. Added tetracycline (tet) binds to tetR homodimers.

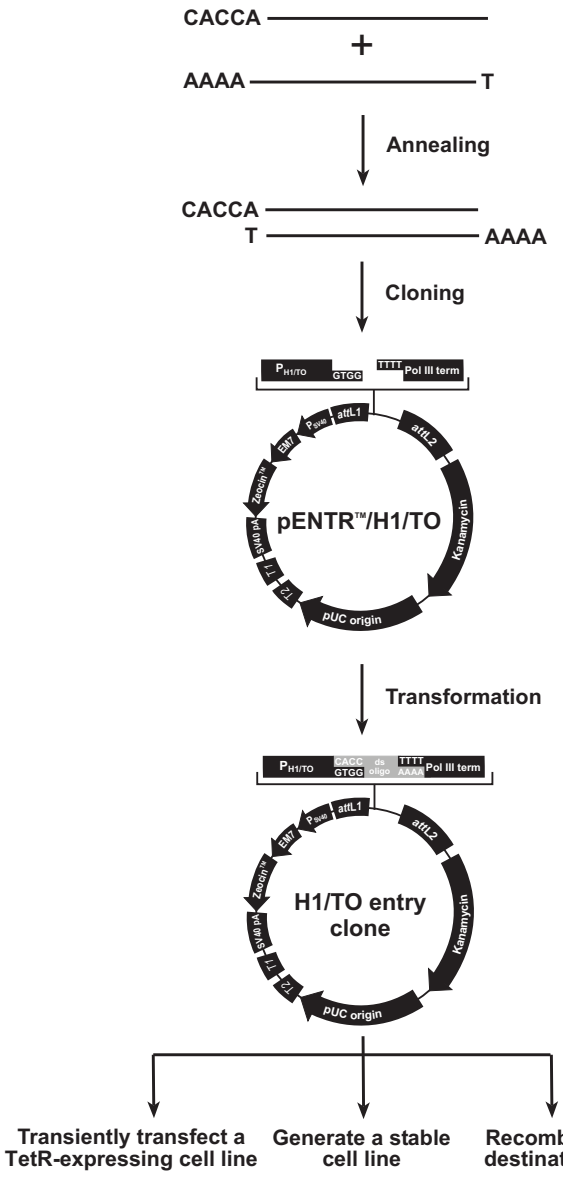
4. Binding of tet to tetR homodimers causes a conformational change in tetR, release from the Tet operator sequences, and induction of shRNA transcription.



Experimental Outline

Flow Chart

The figure below illustrates the major steps necessary to produce a pENTR™/H1/TO entry clone using the BLOCK-iT™ Inducible H1 RNAi 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 the pENTR™/H1/TO vector using T4 DNA Ligase.
4. Transform *E. coli* and analyze colonies for the desired entry clone.
5. Transfect the entry clone into a TetR-expressing cell line to transiently express the shRNA **OR** generate a stable cell line **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™ Inducible H1 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™/H1/TO 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 from a target sequence that are compatible for use in cloning into the pENTR™/H1/TO or pENTR™/U6 vectors. 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 16-16. For an example of ss oligo design, see page 18.

continued on next page

Designing the Single-Stranded DNA Oligos, continued

Sequences Required for Directional Cloning

To enable directional cloning of the ds oligo into pENTR™/H1/TO, you **must** add the following 4 nucleotides to the 5′ end of the corresponding ss oligo:

- **Top strand oligo:** Add CACC to the 5′ end of the oligo. The CACC is complementary to the overhang sequence, GTGG, in the pENTR™/H1/TO vector and constitutes the last 4 bases of the H1/TO 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™/H1/TO vector and constitutes the first 4 bases of the Pol III terminator.

Refer to page 19 for a diagram of the cloning site for pENTR™/H1/TO.

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.

Note that 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 the next page.

continued on next page

Designing the Single-Stranded DNA Oligos, continued

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. Note that these are general recommendations only, and that exceptions may occur.

Length: We recommend choosing 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. In particular, avoid choosing a target sequence that contains runs of four thymidines (T's) as this will result in early transcription termination.
- Choose a sequence with low GC content (~30-50% GC content is recommended).
- Avoid choosing 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 triggering knockdown of your target gene, try generating an shRNA using this same target sequence.

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) itself ends in one or more T nucleotides.

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

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

continued on next page

Designing the Single-Stranded DNA Oligos, continued

Transcription Initiation

Transcription of the shRNA initiates at the first base following the end of the H1/TO 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 an adenosine (A) or a guanosine (G). Note that transcription of the native H1 RNA initiates at an A.** Initiating transcription at a C or T is generally not recommended as this may affect initiation efficiency and position. When choosing the transcription initiation site, you should also keep the following in mind:

Initiation at an A

- If A is the first base of the target sequence, you do not need to add the complementary T to the 3′ end of the top strand oligo because the T will be supplied by the first base of the Pol III terminator. Similarly, if the first 2 or 3 bases of the target sequence are A's, you may omit adding the complementary T's to the 3′ end of the top strand oligo. For an example, see **Example 2** on the next page.
- If A is not the first base of the target sequence, add an A to the 5′ end of the top strand oligo. You may omit adding a complementary T to the 3′ end of the top strand oligo as the T will be supplied by the first base of the Pol III terminator.

Initiation at a G

- If G is the first base of the target sequence, then 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 **Example 1** on the next page.

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

If you plan to express the same shRNA from both the pENTR™/H1/TO vector and Invitrogen's pENTR™/U6 vector (*i.e.* BLOCK-iT™ U6 RNAi Entry Vector Kit, Catalog no. K4945-00), we recommend initiating the shRNA sequence at a G as this is the preferred initiation site for the U6 promoter. Generating shRNA sequence that initiates at a G allows the shRNA to be compatible for cloning and expression from either pENTR™/H1/TO or pENTR™U6.

Do not add 5′ phosphates to your ss oligos during synthesis. The phosphate groups necessary for ligation are present in the linearized pENTR™/H1/TO vector.

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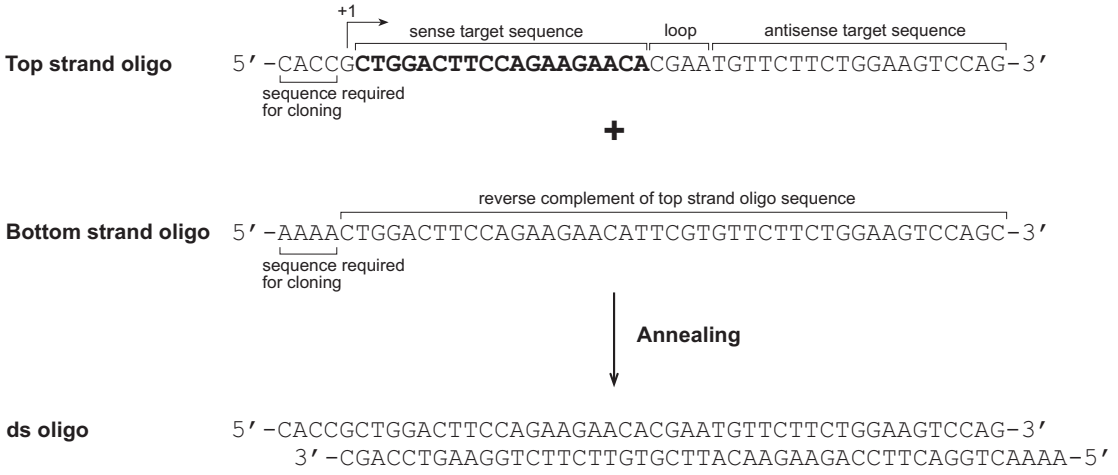
Designing the Single-Stranded DNA Oligos, continued

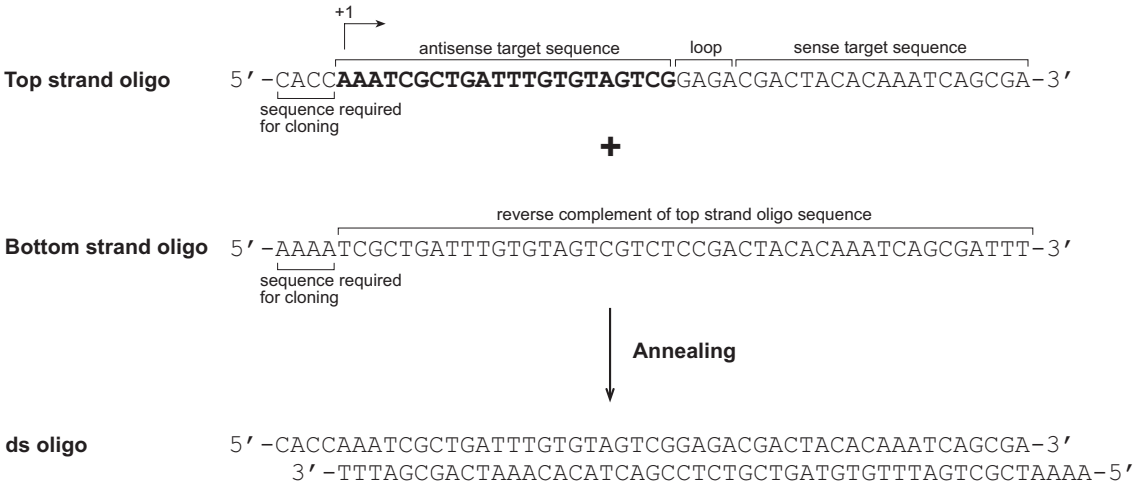
Example 1: ss Oligo Design

This example lists the sequences of top and bottom strand oligos encoding an shRNA targeting the lamin A/C gene. These particular ss oligos were annealed to generate a lamin ds oligo that was cloned into pENTR™/H1/TO. The resulting lamin H1/TO RNAi cassette was transferred into the pLenti4/BLOCK-iT™-DEST vector in an LR recombination reaction to generate the pLenti4-GW/H1/TO-lamin^{shRNA} construct supplied in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Catalog no. K4925-00).



Example 2: ss Oligo Design

This example lists the sequences of top and bottom strand oligos encoding an shRNA targeting the *lacZ* gene. These particular ss oligos were annealed to generate the lacZ2.1 ds control oligo supplied in the kit. Note that in this shRNA sequence, the first 3 bases of the target sequence are A's. Thus, the 3 corresponding T's were omitted from the 3′ end of the top strand oligo.



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Designing the Single-Stranded DNA Oligos, continued

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.

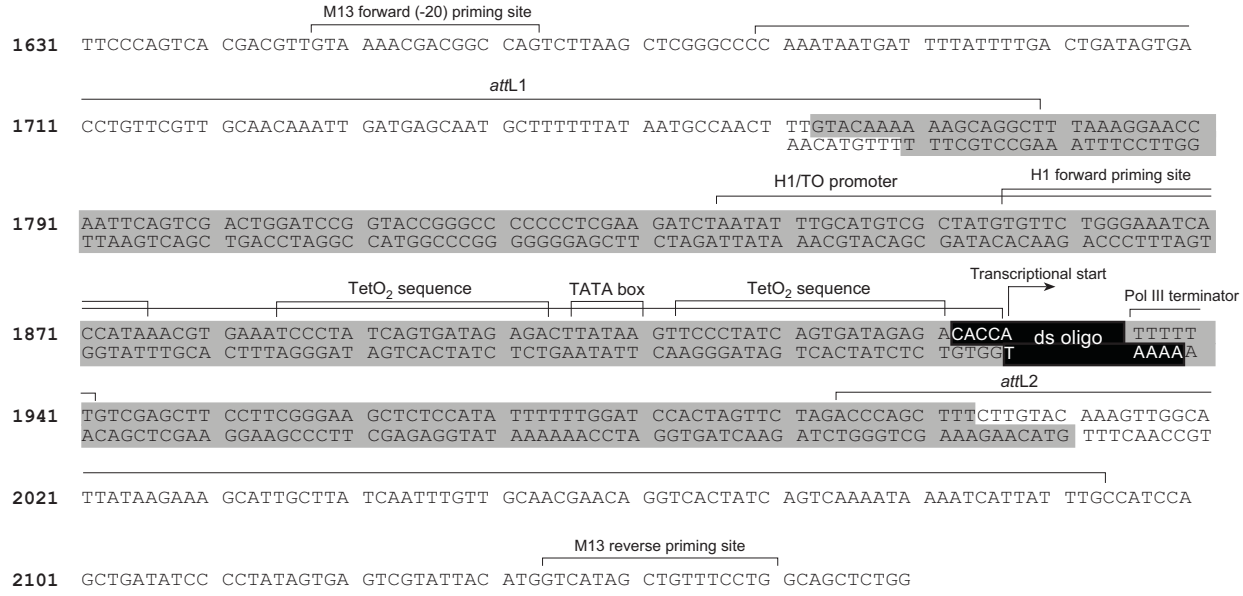
Cloning Site and Recombination Region of pENTR™/H1/TO

Use the diagram below to help you design suitable DNA oligonucleotides to clone into pENTR™/H1/TO after annealing. Note the following features in the diagram below:

- The pENTR™/H1/TO vector is supplied linearized between nucleotides 1935 and 1936. The linearized vector contains 4 nucleotide overhangs on each strand encoding the last 4 nucleotides of the H1/TO 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.* pLenti4/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 H1/TO promoter, shRNA sequence, and the Pol III terminator.

The sequence of pENTR™/H1/TO is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 59). For a map of pENTR™/H1/TO, see the **Appendix**, page 51.



Generating the Double-Stranded Oligo (ds oligo)

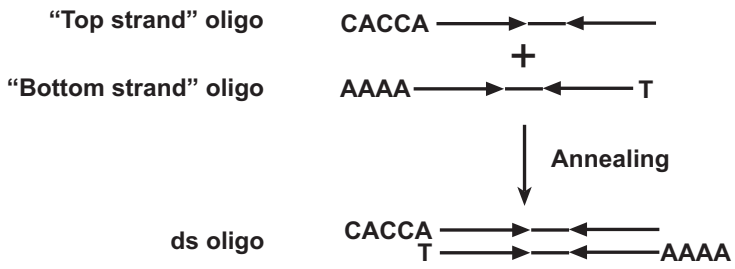
Introduction

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™/H1/TO 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; at least 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 LacZ2.1 Control Oligo

If you plan to use the lacZ2.1 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 lacZ2.1 control oligo already comes at a concentration of 50 μM in 1 x Oligo Annealing Buffer, re-anneal the lacZ2.1 control oligo without further dilution.

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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 LacZ2.1 ds Control Oligo (if desired; 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 lacZ2.1 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.

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

Diluting the ds Oligo

To clone your ds oligo or LacZ2.1 ds Control Oligo into pENTR™/H1/TO, 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 27). 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™/H1/TO.

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 21.

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 45 for tips to troubleshoot your annealing reaction.

The efficiency at which ss oligos anneal may vary depending on their sequence and length. When analyzing the annealed ds oligo reaction by agarose gel electrophoresis, evaluate the annealing efficiency and roughly estimate the percentage of annealed ds oligo produced by comparing the intensity of the higher molecular weight band (annealed ds oligo) to the lower molecular band (unannealed ss oligos). You will use this information when setting up your ligation reaction (see **Amount of ds Oligo to Use**, page 25 for details).

continued on next page

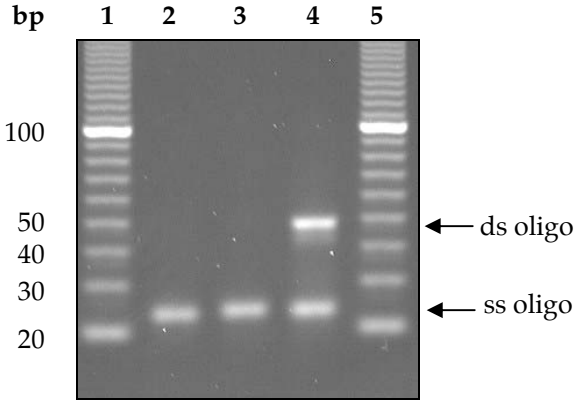
Generating the Double-Stranded Oligo (ds oligo), continued

Example of Expected Results

In this experiment, two 47 bp oligos (top and bottom strand) were annealed (50 µM final concentration) using the reagents supplied in the kit and following the procedure on page 21 to generate a 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 ds 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. In this reaction, we estimate that the efficiency of the annealing reaction was greater than 50%.

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. Top strand oligo
Lane 3. Bottom strand oligo
Lane 4. 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™/H1/TO 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 25-27) and **Transforming One Shot® TOP10 Competent *E. coli*** (page 28) 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™/H1/TO 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™/H1/TO 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™ Inducible H1 RNAi Kits 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 27.

Amount of ds Oligo to Use

For optimal results, use a 10:1 to 50:1 molar ratio of ds oligo insert:vector in the ligation reaction. This ratio is achieved when 1-5 µl of the 5 nM ds oligo stock solution is used for ligation. Note the following:

- If your ss oligos have annealed efficiently (*i.e.* the intensity of the higher molecular weight band is greater than the intensity of the lower molecular weight band on an agarose gel), then use 1-2 µl of the 5 nM ds oligo stock in the ligation reaction.
- If your ss oligos anneal less efficiently (*i.e.* the intensity of the higher molecular is equivalent to or less than the intensity of the lower molecular weight band on an agarose gel), then increase the amount of the 5 nM ds oligo stock used in the ligation reaction from 1 µl up to 5 µl.

continued on next page

Performing the Ligation Reaction, continued

Positive Control

We recommend including the lacZ2.1 ds control oligo supplied with the kit as a positive control in your ligation experiment. The lacZ2.1 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 21). See page ix for the sequence of each strand of the lacZ2.1 ds control oligo. Note that the lacZ2.1 ss control oligos anneal less efficiently than other ss oligos; therefore, we recommend using 5 µl of the 5 nM ds oligo stock in the ligation reaction.

Tip: Once you have cloned the lacZ2.1 ds control oligo into pENTR™/H1/TO, 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.

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)
- pENTR™/H1/TO, linearized (0.75 ng/µl, supplied with the kit, Box 1; thaw on ice before use)
- lacZ2.1 ds control oligo (if desired; 5 nM in 1X Oligo Annealing Buffer; 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)

continued on next page

Performing the Ligation Reaction, continued

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	Sample	Positive Control
5X Ligation Buffer	4 µl	4 µl
pENTR™/H1/TO (0.75 ng/µl)	2 µl	2 µl
ds oligo (5 nM; *i.e.* 1:10,000 dilution)	1-5 µl	--
lacZ2.1 ds control oligo (5 nM *i.e.* 1:10,000 dilution)	--	5 µl
DNase/RNase-Free Water	to a final volume of 19 µl	8 µl
T4 DNA Ligase (1 U/µl)	1 µl	1 µl
Total volume	20 µl	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. One Shot® TOP10 *E. coli* have a transformation efficiency of ≥ 1 x 10^9 cfu/µg plasmid 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)

  Alternative: You may use Low Salt LB plates containing 50 µg/ml Zeocin™ to select for transformants, if desired. Note that for Zeocin™ to be active, the salt concentration of the bacterial medium **must** be < 90 mM and the pH **must** be 7.5. For a recipe to prepare Low Salt LB agar plates containing Zeocin™, see page 51. For more information about Zeocin™, see page 54.
- LB plates containing 100 µg/ml ampicillin (if transforming the pUC19 control)
- 37°C shaking and non-shaking incubator

continued on next page

Transforming One Shot® TOP10 Competent *E. coli*, continued

One Shot® TOP10 Transformation Procedure

Use this procedure to transform your ligation reaction into One Shot® TOP10 Chemically Competent *E. coli*. To include a positive control for transformation, transform 10 pg (1 µl) of pUC19 plasmid into a separate vial of One Shot® TOP10 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 40-200 µl from each transformation on a pre-warmed selective plate 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 or Low Salt LB medium containing 50 µg/ml Zeocin™.
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 the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen.
3. Sequence each pENTR™/H1/TO 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™/H1/TO entry clones, use the H1 Forward and M13 Reverse Primers supplied with the kit (Box 1). See the diagram on page 19 for the location of the priming sites.

Note

If you download the sequence for pENTR™/H1/TO 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).

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Analyzing Transformants, continued

In some cases, you may have difficulty sequencing the ds oligo insert in your pENTR™/H1/TO 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 or a Low Salt LB plate containing 50 µg/ml Zeocin™.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin or Low Salt LB containing 50 µg/ml Zeocin™.
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™/H1/TO entry clone, you have a number of options to express your shRNA of interest to perform RNAi analysis. See **General Considerations for Transfection and Regulated Expression**, next section for a discussion of your expression options.

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General Considerations for Transfection and Regulated Expression

Introduction

Once you have generated your pENTR™/H1/TO entry construct, you are ready to express your shRNA of interest and to perform RNAi analysis of your target gene. This section provides general guidelines to help you design your transfection and RNAi experiment. We recommend that you read through this section before beginning.

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 (see page 34 for more information))
- 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.

shRNA Expression Options

A number of options exist to express your shRNA of interest in the mammalian cell line of choice for RNAi analysis. Choose the option that best fits your needs.

Option	Procedure	Benefit
1	Co-transfect the pENTR™/H1/TO construct and a TetR-expressing plasmid (*e.g.* pcDNA™6/TR or pLenti6/TR) into mammalian cells	Perform regulated shRNA expression experiments with a single construct for quick screening purposes. **Note:** Significant basal expression of the shRNA may be observed with this option.
2	Obtain or generate a mammalian cell line that stably expresses the Tet repressor. Use this cell line as the host for the pENTR™/H1/TO construct. Select for a double stable cell line, if desired.	Perform transient or stable, regulated shRNA expression experiments with multiple shRNA constructs using a cell line that consistently expresses the same amount of Tet repressor.
3	Transfer the H1/TO RNAi cassette from pENTR™/H1/TO into a suitable Gateway® destination vector (*e.g.* pLenti4/BLOCK-iT™-DEST) by LR recombination to generate an expression clone.	Perform other RNAi applications (*e.g.* regulated shRNA expression in non-dividing mammalian cells using the pLenti4/BLOCK-iT™-DEST construct).
4	Transfect the pENTR™/H1/TO construct into any non-TetR-expressing, dividing mammalian cell line. Select for a stable cell line, if desired.	Constitutively express the shRNA of interest.

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General Considerations for Transfection and Regulated Expression, continued

Expression of Tet Repressor (TetR)

Because tetracycline-regulated shRNA expression in the BLOCK-iT™ Inducible H1 RNAi System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line will determine the level of transcriptional repression of the Tet operator sequences in your pENTR™/H1/TO construct. **Tet repressor levels need to be sufficiently high to suitably repress basal level transcription of the shRNA, thus suppressing target gene knockdown in uninduced cells.** In addition, the most effective repression of basal shRNA expression is achieved when Tet repressor is present in mammalian cells prior to introduction of the pENTR™/H1/TO construct. For these reasons, we recommend first generating a stable cell line expressing the Tet repressor, then using this cell line as the host for your pENTR™/H1/TO entry construct (Option 2, previous page) or other suitable inducible expression construct (Option 3, previous page). This option is particularly recommended if you want to:

- Perform regulated RNAi knockdown experiments with several shRNA expression constructs in the same mammalian cell line.
- Obtain the lowest levels of basal shRNA expression (*i.e.* lowest levels of target gene knockdown in the absence of tetracycline)

To obtain a TetR-expressing stable cell line from Invitrogen, see the **Recommendation** below. For guidelines to generate your own stable TetR-expressing cell line, see **Generating a TetR-Expressing Host Cell Line**, **Appendix**, page 52.

Several T-REx™ cell lines that stably express the Tet repressor are available from Invitrogen (see page xii for ordering information). If you wish to assay for tetracycline-regulated expression of your gene of interest in 293, HeLa, CHO, or Jurkat cells, you may want to use one of the T-REx™ cell lines as the host for your pENTR™/H1/TO entry construct.

Note: The T-REx™ cell lines stably express the Tet repressor from the pcDNA™6/TR expression plasmid. This plasmid is used to generate stable TetR-expressing cell lines in Invitrogen's T-REx™ System. Both pLenti6/TR and pcDNA™6/TR contain the same *TetR* gene. For more information about the T-REx™ cell lines or pcDNA™6/TR, see our Web site (www.invitrogen.com) or contact Technical Service (see page 59).

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General Considerations for Transfection and Regulated Expression, continued

Methods of Transfection

For established cell lines (*e.g.* COS, A549), 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 & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner & Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa & 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 is 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 59).

Transient vs. Stable Expression of Your shRNA

When designing your RNAi experiment, you should consider how to assay for knockdown of the target gene. After you have transfected your pENTR™/H1/TO construct into TetR-expressing mammalian cells, you may:

- Pool a heterogeneous population of cells and test for target gene knockdown after induction with tetracycline (*i.e.* transient knockdown). We recommend waiting for a minimum of 24-48 hours after induction before assaying for target gene knockdown to allow time for the shRNA to be expressed and processed.
- Select for stably transfected cells using Zeocin™. Selection requires a minimum of 10-14 days after transfection, but allows generation of clonal cell lines that stably express the shRNA of interest. shRNA expression will be tetracycline-regulated (expression Options 2, page 32) or constitutive (expression Option 4, page 32). For more information about Zeocin™ selection, see **Generating a Stable Cell Line**, page 40.

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General Considerations for Transfection and Regulated Expression, continued

Tetracycline

Tetracycline (MW = 444.4) is commonly used as a broad spectrum antibiotic and acts to inhibit translation by blocking polypeptide chain elongation in bacteria. In the BLOCK-iT™ Inducible H1 RNAi System, tetracycline functions as an inducing agent to regulate transcription of the shRNA of interest from the H1/TO RNAi cassette. Tetracycline is supplied with the BLOCK-iT™ Inducible H1 RNAi Kits as a 10 mg/ml stock solution that is ready-to-use, but is also available separately from Invitrogen in powdered form (Catalog no. Q100-19). For a recipe to prepare a 10 mg/ml stock solution from the powdered form, see page 51.

Using Tetracycline

To induce transcription of the shRNA of interest in mammalian cells, we generally add tetracycline to a final concentration of 1 µg/ml in complete growth medium. If desired, you may vary the concentration of tetracycline used for induction from 0.001 to 1 µg/ml to modulate expression of the shRNA of interest.

Note: The concentrations of tetracycline used for induction in the BLOCK-iT™ Inducible H1 RNAi System are generally not high enough to be toxic to mammalian cells.

Follow the guidelines below when handling tetracycline.

- Tetracycline is light sensitive. Store the stock solution at -20°C, protected from light. Prepare medium containing tetracycline immediately before use.
- Tetracycline is toxic. Do not ingest solutions containing the drug. If handling the powdered form, do not inhale.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling tetracycline and tetracycline-containing solutions.

Tetracycline in Fetal Bovine Serum

When culturing cells in medium containing fetal bovine serum (FBS), note that many lots of FBS contain tetracycline as FBS is often isolated from cows that have been fed a diet containing tetracycline. If you culture your mammalian cells in medium containing FBS that is not reduced in tetracycline, you may observe some basal expression of your shRNA of interest in the absence of tetracycline. We generally culture our mammalian cells in medium containing FBS that may not be reduced in tetracycline, and have observed low basal expression of shRNA (as assayed by % target gene knockdown) in the absence of tetracycline. Depending on your application (*e.g.* if targeting a protein involved in cell viability), you may wish to culture your cells in tetracycline-tested FBS. You may obtain tetracycline-tested GIBCO® FBS from Invitrogen. Contact Technical Service (see page 59) for more information.

Transfecting Cells

Introduction

This section provides general guidelines to transfect your pENTR™/H1/TO construct into a TetR-expressing mammalian cell line of interest to perform transient, regulated 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

If you want to generate a stable cell line expressing the shRNA of interest, see the next section.

Important

Reminder: For optimal results, we recommend that you transfect your pENTR™/H1/TO construct into a mammalian cell line that stably expresses high levels of the Tet repressor (*i.e.* use one of Invitrogen's T-REx™ Cell Lines or a cell line that you have generated). If you have not generated a stable TetR-expressing cell line, you may co-transfect the pENTR™/H1/TO plasmid with a suitable TetR-expressing plasmid (*i.e.* pcDNA™6/TR or pLenti6/TR) into your mammalian cell line. If you wish to use this method, **we recommend using 6-fold more TetR expression plasmid DNA than pENTR™/H1/TO plasmid DNA in the co-transfection.** For example, use 600 ng of pcDNA™6/TR plasmid and 100 ng of pENTR™/H1/TO entry construct DNA when transfecting cells plated in a 24-well format. Note that you may need to optimize repression and inducibility by varying the ratio of TetR expression plasmid:pENTR™/H1/TO used for transfection.

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.

Positive Control

If you have performed the positive control reaction and have cloned the lacZ2.1 ds oligo supplied with the kit into pENTR™/H1/TO, we recommend using the resulting pENTR™-GW/H1/TO-lacZ2.1^{shRNA} entry construct as a positive control to assess the RNAi response in your cell line. Simply co-transfect the pENTR™-GW/H1/TO-lacZ2.1^{shRNA} entry construct and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your TetR-expressing mammalian cells and assay for knockdown of β-galactosidase expression 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.

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Transfecting Cells, continued

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.

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 the next page). 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™-GW/H1/TO-lacZ2.1^{shRNA} entry construct that you have generated into your TetR-expressing 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™-GW/H1/TO-lacZ2.1^{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 page 39.

Materials Needed

Have the following materials on hand before beginning:

- TetR-expressing mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)

  Note: If your cell line expresses TetR from pcDNA™6/TR or pLenti6/TR, maintain the cells in medium containing the appropriate concentration of Blasticidin.
- pENTR™/H1/TO entry construct
- pcDNA™1.2/V5-GW/lacZ plasmid (if performing the positive control transfection; supplied with the kit, Box 1)
- pENTR™-GW/H1/TO-lacZ2.1^{shRNA} plasmid (if you have performed the positive control ligation reaction and are performing the positive control transfection)
- Transfection reagent of choice (*e.g.* Lipofectamine™ 2000)
- Tetracycline (supplied with the kit, Box 1; 10 mg/ml stock solution)
- Appropriate tissue culture dishes and supplies

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Transfecting Cells, continued

Guidelines for Transfection and Induction

Guidelines are provided below to transfect your pENTR™/H1/TO entry construct into the TetR-expressing mammalian cell line of choice and to induce expression of the shRNA of interest with tetracycline.

1. One day before transfection, plate cells at a density recommended by the manufacturer of the transfection reagent you are using.
2. On the day of transfection (Day 1), transfect your pENTR™/H1/TO construct into cells following the recommendations of the manufacturer of your transfection reagent. If you are co-transfecting the pENTR™/H1/TO construct and a TetR expression plasmid or the pcDNA™1.2/V5-GW/lacZ and pENTR™-GW/H1/TO-lacZ2.1^{shRNA} plasmids, use the appropriate amounts of each plasmid as recommended on page 36 and 37, respectively.
3. At an appropriate time (generally 3 to 24 hours) after transfection, remove medium and replace with fresh growth medium containing 1 µg/ml tetracycline to induce shRNA expression. Note the following:
   - If you have transfected your cells using Lipofectamine™ 2000, you may add tetracycline to induce expression of your shRNA as early as 3 hours following transfection.
   - If you have included the *lacZ* positive control plasmids in your experiment, add tetracycline to cells 3 hours after transfection. This induces expression of the lacZ2.1 shRNA and prevents accumulation of β-galactosidase, enabling detectable measurement of *lacZ* knockdown that might otherwise be masked by the long half-life of β-galactosidase.
   - If you have transfected your cells using another transfection reagent, you may need to replace the medium and allow cells to recover for 24 hours before induction.
4. Incubate cells in medium containing tetracycline for 24 to 96 hours, as appropriate before assaying for target gene knockdown.

Assaying for β-galactosidase Expression

If you perform RNAi analysis using the control entry clone containing the lacZ2.1 ds oligo (*i.e.* pENTR™-GW/H1/TO-lacZ2.1^{shRNA}), you may assay for β-galacto-sidase 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), the β-Gal Assay Kit (Catalog no. K1455-01), and the FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Catalog no. F-2905) for detection of β-galactosidase expression. For an example of results obtained from a β-galacto-sidase 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 59).

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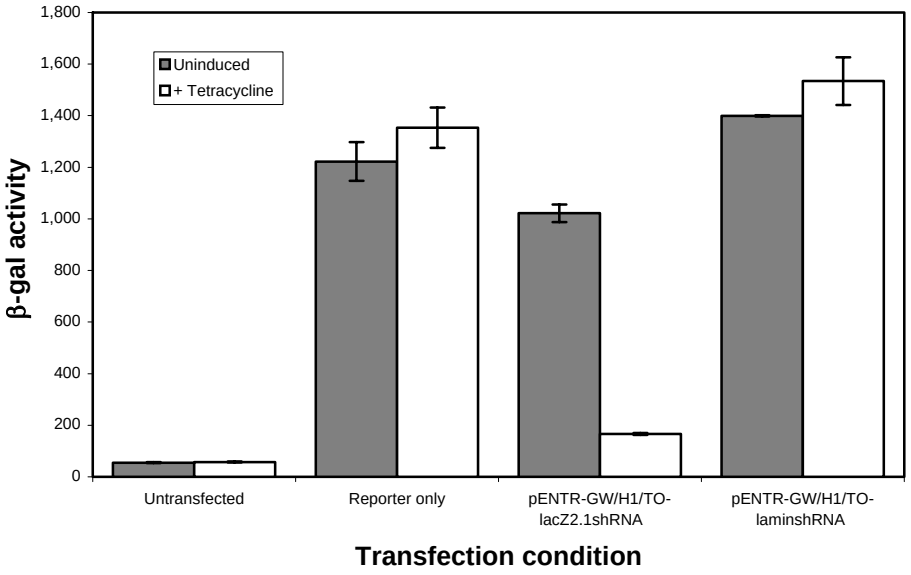
Example of Expected Results: Transient, Regulated Knockdown of a *lacZ* Reporter Gene

In this experiment, pENTR™/H1/TO entry constructs containing ds oligo encoding shRNA targeting the *lacZ* (*i.e.* pENTR™-GW/H1/TO-lacZ2.1^{shRNA}) reporter gene or the endogenous lamin (*i.e.* pENTR™-GW/H1/TO-lamin^{shRNA}) gene were generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit. Note that the lacZ ds oligo used in this experiment is the same as the lacZ2.1 ds control oligo supplied with the kit.

T-REx™-293 cells (Invitrogen, Catalog no. R710-07) 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™-GW/H1/TO-lacZ2.1^{shRNA} or pENTR™-GW/H1/TO-lamin^{shRNA} constructs. Three hours after transfection, the medium was replaced with medium containing 1 µg/ml tetracycline. Cell lysates were prepared 48 hours after induction and assayed for β-galactosidase activity.

Results: Potent and specific inhibition of β-galactosidase activity is evident from the lacZ-derived shRNA but not from the lamin-derived shRNA after cells have been treated with tetracycline.

Note: In this experiment, some basal expression of the lacZ-derived shRNA occurs as evidenced by the ~ 15% inhibition of β-galactosidase activity in cells cultured in the absence of tetracycline.



Generating a Stable Cell Line

Introduction

Once you have established that your shRNA can be inducibly expressed from pENTR™/H1/TO, you may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your shRNA. As with transient transfection, we recommend using a cell line that stably expresses the Tet repressor as a host for your pENTR™/H1/TO construct. Use a T-REx™ Cell Line available from Invitrogen or your own TetR-expressing cell line (see page 52 for guidelines to generate the cell line).

Zeocin™ Selection

The pENTR™/H1/TO plasmid contains the Zeocin™ resistance gene (Calmels *et al.*, 1991; Drocourt *et al.*, 1990) to facilitate generation of cell lines (Mulsant *et al.*, 1988) that inducibly express the shRNA of interest. For a brief description of Zeocin™ and guidelines to prepare and handle the antibiotic, refer to the **Appendix**, page 52.

Note: If you are using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, Zeocin™ is supplied with the kit. Otherwise, Zeocin™ is available separately from Invitrogen (see pages xi-xii for ordering information).

Determining Zeocin™ Sensitivity for Your Cell Line

If you plan to select for stable cell lines expressing the pENTR™/H1/TO construct, you must first determine the minimum concentration of Zeocin™ that is required to kill your untransfected mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 50-1000 µg/ml Zeocin™ are sufficient to kill most untransfected mammalian cell lines. We recommend testing a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 6-7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Zeocin™.
3. Replenish the selective media every 3-4 days and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Zeocin™ that kills the cells within 10-14 days after addition of antibiotic.

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Generating a Stable Cell Line, continued

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from that of other common antibiotics such as Blasticidin, Geneticin®, and hygromycin. **Zeocin™-sensitive cells do not round up and detach from the plate, but may exhibit the following morphological changes:**

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and Golgi apparatus or scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)

Eventually, these "cells" will completely break down and only "strings" of protein will remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to non-selected cells.

Materials Needed

Have the following materials on hand before beginning:

- TetR-expressing mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)

  Note: If your cell line expresses TetR from pcDNA™6/TR or pLenti6/TR, maintain the cells in medium containing the appropriate concentration of Blasticidin.
- pENTR™/H1/TO entry construct
- Transfection reagent of choice (*e.g.* Lipofectamine™ 2000)
- Zeocin™ (100 mg/ml in sterile water)
- Blasticidin (to maintain the pcDNA™6/TR or pLenti6/TR construct) in the TetR-expressing cell line
- Tetracycline (supplied with the kit, Box 1; 10 mg/ml stock solution)
- Appropriate tissue culture dishes and supplies

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Generating a Stable Cell Line, continued

Guidelines for Transfection and Selection

Guidelines are provided below to transfect your pENTR™/H1/TO entry construct into the TetR-expressing mammalian cell line of choice and to select for stable cell lines using Zeocin™.

1. One day before transfection, plate cells at a density recommended by the manufacturer of the transfection reagent you are using.
2. On the day of transfection (Day 1), transfect your pENTR™/H1/TO construct into cells following the recommendations of the manufacturer of your transfection reagent.
3. Four to six hours after transfection, remove the medium and replace with fresh growth medium. Incubate the cells overnight at 37°C.
4. The following day (Day 2), trypsinize and replate cells into a larger-sized tissue culture format in fresh complete medium containing the appropriate concentrations of Blasticidin and Zeocin™. **Note:** Blasticidin is required to maintain the pcDNA™6/TR or pLenti6/TR construct in the TetR-expressing cells.

   Example: If transfecting cells in a 6-well format, trypsinize and replate cells into a 10 cm tissue culture plate in medium containing Blasticidin and Zeocin™.
5. Replace medium with fresh medium containing Blasticidin and Zeocin™ every 3-4 days until Blasticidin- and Zeocin™-resistant colonies can be identified (generally 10-14 days after selection).
6. Pick at least 10 Blasticidin- and Zeocin™-resistant colonies and expand each clone.
7. Induce expression of the shRNA of interest by adding tetracycline to a final concentration of 1 μg/ml. Wait for the appropriate length of time (*e.g.* 24-48 hours) before assaying for target gene knockdown. Compare to uninduced cells.

Guidelines to Perform the LR Recombination Reaction

Introduction

The pENTR™/H1/TO vector contains *att*L sites to facilitate transfer of your H1/TO RNAi cassette (H1/TO promoter + ds oligo of interest + Pol III terminator) into an appropriate Gateway® destination vector to generate an expression clone. To transfer your H1/TO 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

We recommend transferring the H1/TO RNAi cassette into a promoterless Gateway® destination vector for the following RNAi applications:

- Perform delivery of the regulated shRNA of interest to "hard-to-transfect" or non-dividing mammalian cells. Use the pLenti4/BLOCK-iT™-DEST vector (see **Note** below).
- Generate stable cell lines expressing the regulated shRNA using a selection marker other than Zeocin™. Use the pBLOCK-iT™3-DEST vector containing the neomycin selection marker (Catalog no. V486-20).

Important: Because the H1/TO RNAi cassette contains its own promoter (*i.e.* H1/TO promoter), we do not recommend transferring the H1/TO RNAi cassette into destination vectors that contain a promoter (*e.g.* pcDNA™3.2/V5-DEST).

Note

If you plan to perform regulated RNAi analysis in a lentiviral-based system, transfer your H1/TO RNAi cassette into Invitrogen's pLenti4/BLOCK-iT™-DEST destination vector (Catalog nos. V488-20 or K4925-00). **Do not** transfer the H1/TO RNAi cassette into the pLenti6/BLOCK-iT™-DEST vector. The pLenti6/BLOCK-iT™-DEST vector contains the Blasticidin resistance marker for selection, making it incompatible for use with Blasticidin-resistant T-REx™ cell lines (both commercially available and those generated using the pcDNA™6/TR or pLenti6/TR constructs).

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.

Important: When performing the LR recombination reaction with the pLenti4/BLOCK-iT™-DEST vector, use the Stbl3™ *E. coli* strain for transformation to obtain 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

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Guidelines to Perform the LR Recombination Reaction, continued

Important

We recommend performing the LR recombination reaction using a:

- Supercoiled *att*L-containing pENTR™/H1/TO 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™/H1/TO 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 pLenti4/BLOCK-iT™-DEST and transform competent *E. coli*, refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual. If you are using another destination vector, 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 21).
	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 21.
	Oligos strongly favor formation of intramolecular hairpins	Proceed to ligation reaction, but increase the amount of ds oligo used from 1 µl up to 5 µl.
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™/H1/TO: • Top strand oligo: include CACC on the 5′ end. • Bottom strand oligo: include AAAA on the 5′ end.
	ds oligos stored incorrectly	Store the ds oligo stocks at -20°C.
	Ligation reaction not incubated for long enough	Extend the incubation time of the ligation reaction up to 2 hours at room temperature.

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 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.
	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™/H1/TO.
	Incorrect vector:insert ratio used in ligation reaction • Forgot to dilute annealed target ds oligo or LacZ2.1 ds Control Oligo 1:10,000 before use • Annealed ds oligo diluted incorrectly	Dilute the 50 µM ds oligo mixture as instructed on page 22 to generate a 5 nM stock. Use the 5 nM ds oligo stock for cloning.
	ds oligo mixture had a lower percentage of annealed ds oligo	Increase the amount of ds oligo used in the ligation reaction (*e.g.* from 1 µl to 5 µl).
	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. These reagents are optimized to facilitate 5-minute ligation at room temperature. **Important:** Other T4 DNA Ligase preparations may not support 5-minute, room temperature ligation.
	Not enough transformation mixture plated	Increase the amount of the transformation mixture plated.

continued on next page

Troubleshooting, continued

Ligation and Transformation Reactions, continued

Problem	Reason	Solution
Few kanamycin-resistant colonies obtained on the selective plate, continued	Ligation reaction incubated overnight at 16°C	The ligation conditions used to clone the ds oligo into pENTR™/H1/TO differ from traditional ligation conditions. **Incubate the ligation reaction at room temperature for 5 minutes.**
	Selective plates contained too much kanamycin	Use LB agar plates containing 50 µg/ml kanamycin for selection.
	Used LB agar to make selective plates containing Zeocin™	Use **Low Salt** LB agar to make selective plates containing Zeocin™.
	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. • Add DMSO to the sequencing reaction to a final concentration of 5%. • Increase the amount of template used for sequencing (up to 2X the normal amount). • Use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction (if using commercial kits).
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.

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Troubleshooting, continued

Transient Transfection and RNAi Analysis

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

Problem	Reason	Solution
Low levels of tetracycline-regulated gene knockdown observed	Low transfection efficiency (if using Lipofectamine™ 2000 Reagent) • Antibiotics added to the media during transfection • Cells too sparse at the time of transfection • Not enough plasmid DNA transfected • Not enough Lipofectamine™ 2000 used	 • Do not add antibiotics to the media during transfection. • Plate cells such that they will be 90-95% confluent at the time of transfection. • Increase the amount of plasmid DNA transfected. • Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 used. • Select for a stable cell line.
	Did not wait long enough after induction before assaying for gene knockdown	• Repeat the transfection and wait for a longer period of time after induction 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™/H1/TO 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

Transient Transfection and RNAi Analysis, continued

Problem	Reason	Solution
Low levels of tetracycline-regulated gene knockdown observed, continued	Did not add enough tetracycline	Increase the amount of tetracycline used for induction.
	Targeted an essential gene	Generate a stable cell line, then add tetracycline to induce shRNA expression.
Gene knockdown observed, but not tetracycline-regulated	Did not transfect the pENTR™/H1/TO entry construct into a cell line expressing Tet repressor	Transfect the entry construct into a cell line that expresses Tet repressor: • Use one of Invitrogen's T-REx™ Cell Lines **OR** • Generate your own stable TetR-expressing cell line using pcDNA™6/TR or pLenti6/TR, as desired.
Significant target gene knockdown observed in uninduced cells	Insufficient amount of Tet repressor expressed (when transfecting a stable TetR-expressing cell line)	Screen other TetR-expressing clones. Choose the clone that exhibit the highest level of TetR expression for use as the host for your pENTR™/H1/TO construct.
	Co-transfected a TetR expression plasmid and the pENTR™/H1/TO construct	• Use 6-fold more TetR expression plasmid DNA than pENTR™/H1/TO plasmid DNA in the co-transfection. • Transfect the pENTR™/H1/TO construct into a cell line that stably expresses TetR.
	When generating the TetR-expressing cell line, pcDNA™6/TR or pLenti6/TR construct introduced into a mammalian cell line in which the CMV promoter is down-regulated	Use a mammalian cell line in which the CMV promoter is not down-regulated as the host for the pcDNA™6/TR or pLenti6/TR construct.
No gene knockdown observed, even after tetracycline induction	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 14-19 to select the target sequence and design the single-stranded oligos.
	Forgot to add tetracycline	Treat cells 3 to 24 hours after transfection with tetracycline to induce shRNA expression. Assay for target gene knockdown 24-96 hours following induction, as appropriate.

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Troubleshooting, continued

Transient 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 PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) to prepare purified plasmid DNA.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a new target sequence. Use the RNAi Designer at www.invitrogen.com/rnai to help you design your shRNA sequence.

Generating Stable Cell Lines

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

Problem	Reason	Solution
Few Zeocin™-resistant colonies obtained	Used too much Zeocin™ for selection	• Decrease the concentration of Zeocin™ used for selection. • Perform a kill curve experiment to determine the optimal concentration of Zeocin™ to use for selection in your mammalian cell line.
Cells not selected after Zeocin™ addition (*i.e.* cells form a monolayer)	Cells too confluent at the time of Zeocin™ addition	Zeocin™ selection is most effective when cells are less than 50% confluent at the time of selection. After transfection of the pENTR™/H1/TO construct, trypsinize and replate cells such that they are approximately 25%-50% confluent before adding medium containing Zeocin™.
	Insufficient amount of Zeocin™ used for selection	• Increase the concentration of Zeocin™ used for selection. • Perform a kill curve experiment to determine the optimal concentration of Zeocin™ to use for selection in your mammalian cell line.

Appendix

Recipes

LB (Luria-Bertani) Medium

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if desired.
4. Store at +4°C.

Low Salt LB Medium or Plates Containing Zeocin™

1.0% Tryptone
0.5% Yeast Extract
0.5% NaCl
pH 7.5

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter. If preparing plates, add 15 g/L agar.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add Zeocin™ to a final concentration of 50 µg/ml. If preparing plates, pour into 10 cm plates.
4. Store at +4°C. Plates containing Zeocin™ may be stored at +4°C for up to 2 weeks.

Tetracycline

Use this procedure to prepare a 10 mg/ml stock solution from the tetracycline salt available separately from Invitrogen (Catalog no. Q100-19). Note that the tetracycline provided with the BLOCK-iT™ Inducible H1 RNAi Kits is supplied as a 10 mg/ml solution that is ready-to-use.

Important: If you are using a different form of tetracycline (*i.e.* free base form), prepare the stock solution in 100% ethanol rather than water.

1. Weigh out 10 mg of tetracycline and transfer to a sterile microcentrifuge tube.
2. Resuspend the tetracycline in 1 ml of sterile water to produce a 10 mg/ml stock solution that is yellow in color.
3. Wrap the tube in foil and store the stock solution at -20°C, protected from exposure to light.

Generating a TetR-Expressing Host Cell Line

Introduction

Guidelines are provided in this section to generate your own stable TetR-expressing host cell line. For detailed instructions, refer to the manual for the TetR expression plasmid that you are using.

Options to Generate Your Own TetR-Expressing Cell Lines

Two options exist to generate a stable TetR-expressing mammalian cell line using reagents available separately from Invitrogen. Choose the option that best fits your needs.

- Transfect the pcDNA™6/TR plasmid (*i.e.* TetR expression plasmid from the T-REx™ System) into your mammalian cells of interest. Use Blasticidin to select for a stable cell line.
- Transfect the pLenti6/TR plasmid (*i.e.* TetR expression plasmid from the ViraPower™ T-REx™ and BLOCK-iT™ Inducible H1 Lentiviral RNAi System) into your mammalian cells of interest. Alternatively, produce a Lenti6/TR lentiviral stock, and use this stock to transduce the mammalian cells of interest. Use Blasticidin to select for a stable cell line.

For more information about pcDNA™6/TR, pLenti6/TR, and Blasticidin, see the manual for each product. All manuals are available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 59). To obtain the pcDNA™6/TR or pLenti6/TR plasmids or Blasticidin, see page xii.

Note

Both pcDNA™6/TR and pLenti6/TR contain the same *TetR* gene (Postle *et al.*, 1984). Similarly, expression of TetR from both plasmids is controlled by the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Although highly active in most mammalian cell lines, activity of the viral CMV promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both. When generating your own TetR-expressing cell line, be sure to use a mammalian cell line in which activity of the CMV promoter is not down-regulated.

Determining Blasticidin Sensitivity for Your Cell Line

After transfecting or transducing the pcDNA™6/TR or pLenti6/TR construct into your mammalian cells, as appropriate, you will use Blasticidin to select for a stable cell line. Before beginning, remember to determine the minimum concentration of Blasticidin that is required to kill your untransfected or untransduced mammalian cell line, as appropriate (*i.e.* perform a kill curve experiment).

Generating a TetR-Expressing Cell Line

For detailed instructions to generate a TetR-expressing cell line using pcDNA™6/TR or pLenti6/TR, refer to the manual for the expression plasmid you are using. If you wish to produce a lentiviral stock from pLenti6/TR and transduce mammalian cells to generate your TetR-expressing cell line, refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi System or the ViraPower™ T-REx™ manual. All manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 59).

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Generating a TetR-Expressing Host Cell Line, continued

Important

After you have introduced the TetR expression construct into your mammalian cell line and have performed Blasticidin selection, screen individual clones to determine the amount of Tet repressor expressed (see below). Select for clones that express the highest levels of Tet repressor to use as hosts for your inducible pENTR™/H1/TO entry construct. Those clones that express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your shRNA of interest.

Detecting TetR Expression

To detect Tet repressor expression, we recommend performing Western blot analysis using an Anti-Tet repressor antibody (MoBiTec, Göttingen, Germany, Catalog no. TET01).

Maintaining TetR-Expressing Cell Lines

Once you have generated your stable TetR-expressing cell line and have verified that the cells express suitable levels of Tet repressor, we recommend the following:

- Maintain the cell line in medium containing Blasticidin
- Remember to freeze and store vials of early passage cells

Zeocin™

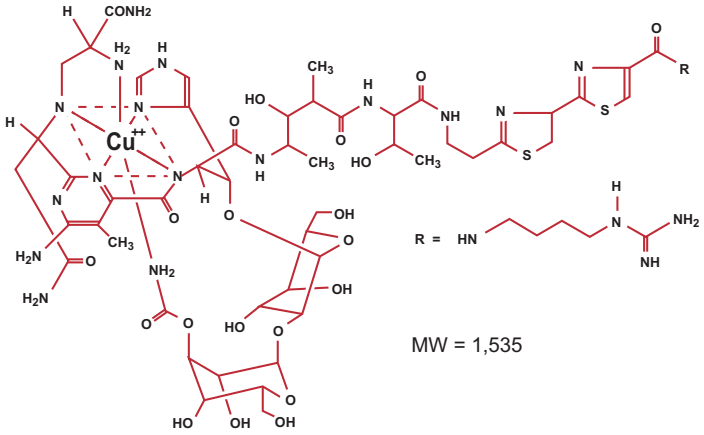
Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25-50 µg/ml in **Low Salt LB** medium* (see page 51 for recipe)
Mammalian Cells	50-1000 µg/ml (varies with cell line)

*Efficient selection requires that the concentration of NaCl be no more than 5 g/L (< 90 mM).

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Zeocin™, continued

Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see the recipe for **Low Salt LB Medium,** page 51). Note that the pH and salt concentration do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark at +4°C. Culture medium containing Zeocin™ may be stored at +4°C protected from exposure to light for up to 1 month.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

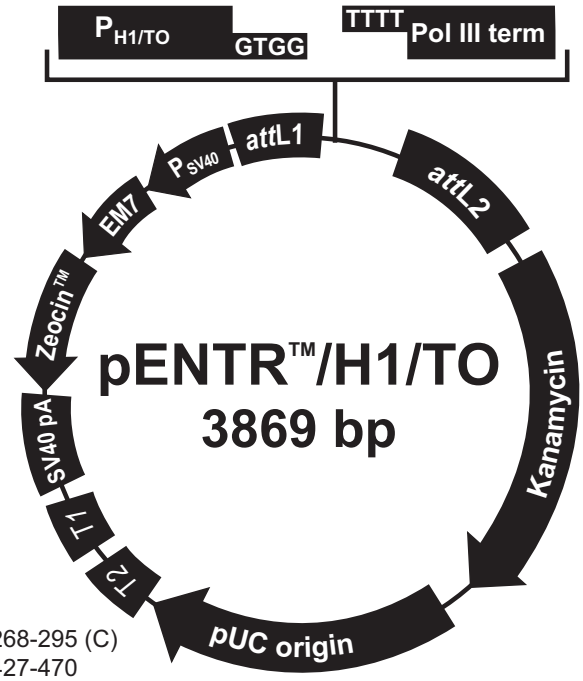
Preparing and Storing Zeocin™

Zeocin™ is supplied in autoclaved, deionized water in 1.25 ml aliquots at a concentration of 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C protected from exposure to light.

Map and Features of pENTR™/H1/TO

pENTR™/H1/TO Map

The figure below shows the features of the pENTR™/H1/TO vector. The vector is supplied linearized between nucleotides 1935 and 1936 with 4 base pair 5′ overhangs on each strand as indicated. **The complete sequence of pENTR/H1/TO is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 59).**



Comments for pENTR™/H1/TO 3869 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (C)
*rrn*B T1 transcription terminator: bases 427-470
SV40 polyadenylation signal: bases 513-642 (C)
Zeocin™ resistance gene: bases 772-1146 (C)
EM7 promoter: bases 1147-1213 (C)
SV40 early promoter and origin: bases 1268-1576 (C)
M13 forward (-20) priming site: bases 1648-1663
*att*L1: bases 1680-1779 (C)
H1/TO promoter: bases 1836-1935
    $tetO_2$ site: bases 1885-1903
    TATA box: bases 1906-1910
    $tetO_2$ site: bases 1913-1931
H1 forward priming site: bases 1856-1875
5′ overhang: bases 1932-1935 (C)
5′ overhang: bases 1936-1939
Pol III transcription terminator: bases 1936-1941
*att*L2: bases 1994-2093
M13 reverse priming site: bases 2134-2150
Kanamycin resistance gene: bases 2263-3072
pUC origin: bases 3193-3866

(C) = complementary strand

continued on next page

Map and Features of pENTR™/H1/TO, continued

Features of pENTR™/H1/TO

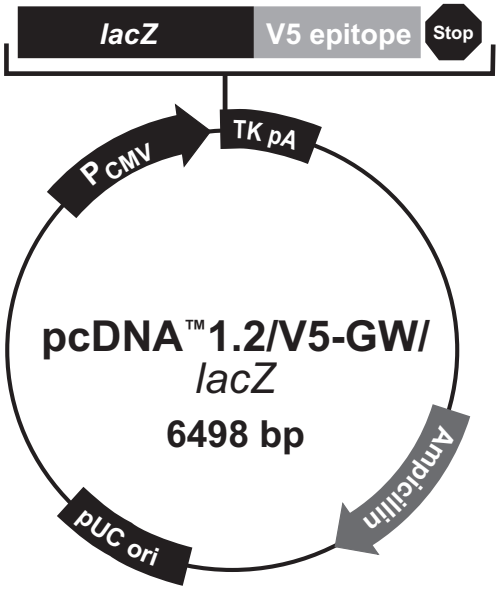
pENTR™/H1/TO (3869 bp) contains the following elements. All features have been functionally tested and the vector fully sequenced.

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.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
Zeocin™ resistance (*Sh ble*) gene	Allows stable selection in mammalian cells and prokaryotes (Drocourt *et al.*, 1990; Mulsant *et al.*, 1988).
EM7 promoter	Synthetic prokaryotic promoter for expression of the Zeocin™ resistance marker in *E. coli.*
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
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 the H1/TO RNAi cassette in the entry construct with a Gateway® destination vector (Landy, 1989).
H1 forward priming site	Allows sequencing of the insert.
Human H1/TO promoter	Hybrid promoter consisting of the human H1 promoter (Hannon *et al.*, 1991; Myslinksi *et al.*, 2001) and two tetracycline operator ($tetO_2$) sequences for RNA Polymerase III-dependent, regulated expression of the short hairpin RNA (shRNA). The $tetO_2$ sequences serve as binding sites for Tet repressor homodimers (Hillen & Berens, 1994).
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 & 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 59).**



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

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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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continued on next page

Purchaser Notification, continued

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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 63.

continued on next page

Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

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Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

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Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

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