USER GUIDE

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BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP

Gateway®-adapted expression system for expression of microRNA (miRNA) in mammalian cells under control of a tetracycline-regulated Pol II promoter

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Contents

Expression Clone Generation for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit. 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 16 to design single-stranded DNA oligos encoding the pre-miRNA 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 at 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 5,000-fold by performing serial 100-fold and 50-fold dilutions: the first into DNase/RNase-free water and the second into 1X Oligo Annealing Buffer. Final concentration is 10 nM.
Clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR	1. Set up the following ligation reaction. 5X Ligation Buffer — 4 µL pcDNA™6.2-GW/EmGFP-miR (5 ng/µL), linearized — 2 µL ds oligo (10 nM; 1:5,000 dilution) — 4 µL DNase/RNase-Free water — 9 µL T4 DNA Ligase (1 U/µL) — 1 µL Total volume — 20 µL 2. Mix reaction well and incubate for 5 minutes at room temperature. 3. Place reaction on ice and proceed to transform *E. coli*, below.
Transform One Shot® TOP10 Chemically Competent *E. coli*	1. Add 2 µL of the ligation reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µL of room temperature S.O.C. Medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 20–100 µL of bacterial culture on a pre-warmed LB agar plate containing 50 µg/mL spectinomycin and incubate overnight at 37°C. 7. Isolate DNA using PureLink® HQ Mini Plasmid Purification Kit or equivalent and verify positive clones by sequence analysis.

Continued on next page

Expression Clone Generation for Experienced Users, Continued

Linearizing Expression Clones

1. Linearize 1–2 µg of the expression clone with a restriction enzyme (such as *Eag* I or *Bsr*D I) that does not digest within the region of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50–150 ng/µL.

Setting Up the Rapid BP/LR Recombination Reaction

Perform the Rapid BP/LR reaction between your linearized expression clone, suitable donor vector, and the pT-REx™-DEST30 Gateway® destination vector.

1. Add the following components to sterile 0.5-mL microcentrifuge tubes at room temperature and mix.

Component	Sample	Positive Control
Linearized *att*B expression clone, (60–150 ng)	1–7 µL	—
pcDNA™6.2-GW/miR-neg control (50 ng/µL)	—	2 µL
pDONR™221 (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the BP Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. To the sample above, add 2 µL of BP Clonase® II enzyme mix. Mix well by pipetting up and down.
5. Incubate the reaction at 25°C for 1 hour.

   Important: Unlike the standard BP reaction, **do not** add Proteinase K but proceed immediately to the next step.
6. Transfer 3 µL from each of the BP reaction from Step 5 to clean, sterile 0.5-mL microcentrifuge tubes.
7. Add the following components to the microcentrifuge tubes containing the 3 µL BP-reaction at room temperature and mix.

Component	Sample	Positive Control
pT-REx™-DEST30 (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	4 µL	4 µL

8. Remove the LR Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
9. Vortex the LR Clonase® II enzyme mix briefly twice (2 seconds each time).
10. To the samples above, add 2 µL LR Clonase® II enzyme mix. Mix well by pipetting.
11. Incubate the reaction at 25°C for 2–4 hours.
12. Add 1 µL Proteinase K solution to each reaction. Incubate for 10 minutes at 37ºC.
13. Transform an appropriate *E. coli* strain as recommended for your destination vector.
14. Isolate DNA using PureLink® HQ Mini Plasmid Purification Kit or equivlent and perform restriction analysis to find a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

Kit Contents and Storage

Shipping/Storage

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP Kit is shipped as described below. Upon receipt, store each item as detailed below. For more detailed information about the reagents supplied in the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.

Box	Component	Shipping	Storage
1	BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP	Dry ice	–20°C
2	pT-REx™-DEST30 Gateway® Vector • pT-REx™-DEST30 Gateway® vector • pT-REx/GW-30/*lac*Z control vector	Dry ice	 –20°C –20°C
3	T-REx™ Support Kit • pcDNA™6/TR • Tetracycline, 10 mg/mL	Dry ice	 –20°C –20°C
4–6	One Shot® Stbl™3 Chemically Competent *E. coli* (3 kits)	Dry ice	–80°C
7	pDONR™221 Vector	Room temperature	–20°C
8	Blasticidin	Room temperature	–20°C
9	Gateway® BP Clonase® II • Gateway® BP Clonase® II Plus Enzyme Mix • Proteinase K solution • 30% PEG8000/30 mM $MgCl_2$ Solution • pEXP7-tet Positive Control	Dry ice	–20°C
10	Gateway® LR Clonase® II Plus • Gateway® LR Clonase® II Plus Enzyme Mix • Proteinase K solution	Dry ice	–20°C

Kit Contents

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP Kit contains the following components listed in the table below. For a detailed description of the contents of the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP Kit, see next page..

Components	Quantity
BLOCK-iT™ Pol II MiR RNAi Expression Vector with EmGFP	1 kit
One Shot® TOP10 Chemically Competent *E. coli*	3 × 20 reactions
BP Clonase® II	20 reactions
LR Clonase® II	20 reactions
T-REx™ Regulatory Module	1 box
pT-REx™-DEST30 Gateway® Vector	1 box
pDONR™221 Gateway® Vector	6 µg
Blasticidin	50 mg

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Kit Contents and Storage, Continued

BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Reagents

The following reagents are included with the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP. **Store the reagents at –20°C.**

Reagent	Composition	Quantity
pcDNA™6.2-GW/EmGFP-miR, linearized	5 ng/µL in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0	4 × 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 × 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
EmGFP forward sequencing primer	100 ng/µL in TE Buffer, pH 8.0	20 µL
miRNA reverse sequencing primer	100 ng/µL in TE Buffer, pH 8.0	20 µL
miR-*lacZ* positive 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
pcDNA™6.2-GW/EmGFP-miR-neg control plasmid	500 ng/µL in TE Buffer, pH 8.0	20 µL

Unit Definition of T4 DNA Ligase

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

Product Use

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

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Kit Contents and Storage, Continued

Primer Sequences

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

Primer	Sequence	Quantity
EmGFP forward sequencing primer	5′-GGCATGGACGAGCTGTACAA-3′	2 µg (323 pmol)
miRNA reverse sequencing primer	5′-CTCTAGATCAACCACTTTGT-3′	2 µg (332 pmol)

*Lac*Z Control Oligo Sequences

The sequences of the miR-*lacZ* positive ds control oligo are listed below. The miR-*lacZ* positive ds control oligo are annealed and are supplied in the kit as a 50 µM double-stranded oligo. The miR-*lacZ* positive ds control oligo needs to be reannealed and diluted 5000-fold to 10 nM before use in the ligation reaction.

lacZ DNA Oligo	Sequence
Top strand	5′-TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCACTGACTGACGACTACACATCAGCGATTT-3′
Bottom strand	5′-CCTGAAATCGCTGATGTGTAGTCGTCAGTCAGTGGCCAAAACGACTACACAAATCAGCGATTTC-3′

One Shot® TOP10 Chemically Competent *E. coli*

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

Reagent	Composition	Quantity
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	3 × 6 mL
TOP10 cells	—	3 × 21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	3 × 50 µL

Genotype of TOP10 Cells

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 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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Kit Contents and Storage, Continued

BP Clonase® II

The following reagents are included with the BP Clonase® II. **Store at –20°C for up to 6 months.** For long-term storage, store at –80°C.

Reagent	Composition	Quantity
Gateway® BP Clonase® II Enzyme Mix	Proprietary	40 µL
Proteinase K Solution	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% Glycerol	40 µL
PEG Solution	30% PEG 8000 30 mM $MgCl_2$	1mL
pEXP7-tet Positive Control	50 ng/µL in TE Buffer, pH 8.0	20 µL

LR Clonase® II

The following reagents are included with the LR Clonase® II. **Store at –20°C for up to 6 months.** For long-term storage, store at –80°C.

Reagent	Composition	Quantity
Gateway® LR Clonase® II Enzyme Mix	Proprietary	40 µL
Proteinase K Solution	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% Glycerol	40 µL
pENTR™-gus Positive Control	50 ng/µL in TE Buffer, pH 8.0	20 µL

Note: The pENTR™-gus control included with the LR Clonase® II Enzyme Mix may be used as a positive control for the LR recombination reaction only.

Inducing and Selection Agents

In addition to the vector provided, the T-REx™ Regulatory Module also includes the following inducing and selection agents. Store the tetracycline at –20°C protected from exposure to light. Store the blasticidin powder at +4°C.

Reagent	Quantity and Concentration	Comments
Tetracycline	1 mL (10mg/mL)	Inducing agent
Blasticidin	50 mg, powder	Selection agent for pcDNA™6/TR plasmid

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Kit Contents and Storage, Continued

T-REx™ Regulatory Module

The following vector is included with the T-REx™ Regulatory Module. **Store vector at –20°C.**

Vector	Quantity and Concentration	Comments
pcDNA™6/TR©	20 µg, lyophilized in TE Buffer, pH 8.0	Regulatory vector that expresses the tetracycline (Tet) repressor

pT-REx™-DEST30 Gateway® Vector

The pT-REx™-DEST Gateway® vector components are listed below. **Store vectors at –20°C.**

Vector	Quantity and Concentration
pT-REx™-DEST30	6 µg, lyophilized in TE Buffer, pH 8.0
pT-REx/GW-30/*lacZ*	10 µg, lyophilized in TE Buffer, pH 8.0

pDONR™221 Gateway® Vector

The pDONR™221 Gateway® vector is shipped at room temperature. **Store vector at –20°C.**

Vector	Quantity and Concentration
pDONR™221	6 µg, lyophilized in TE, pH 8.0

Accessory Products

Accessory Products

Some of the reagents supplied in the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kits as well as other products suitable for use with the kit are available separately from Life Technologies. Ordering information is provided below.

Item	Quantity	Catalog no.
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP	20 reactions	K4936-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP	20 reactions	K4938-00
BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP	20 reactions	K4934-00
BLOCK-iT™ Pol II miR-XXXX Validated miRNA DuoPak (XXXX=gene symbol)	10 µg	V49300-01 through V49300-53
Gateway® pT-Rex™-DEST30 Vector	6 µg	12301-016
pcDNA™6/TR©	20 µg	V1025-20
T4 DNA Ligase	100 units 500 units	15224-017 15224-025
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions 40 reactions	C4040-10 C4040-03 C4040-06
Gateway® LR Clonase® II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Gateway® BP Clonase® II Enzyme Mix	20 reactions 100 reactions	11789-020 11789-100
pDONR™221	6 µg	12536-017
Blasticidin	50 mg	R210-01
Geneticin®	1 g 5 g 20 mL (50 mg/mL) 100 mL (50 mg/mL)	11811-023 11811-031 10131-035 10131-027
Tetracycline	5 g	Q100-19
PureLink® HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink® HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine® 2000 Transfection Reagent	0.75 mL 1.5 mL	11668-027 11668-019
Lipofectamine® LTX Reagent	1.0 mL	15338-100

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Accessory Products, Continued

Accessory Products Continued

Item	Quantity	Catalog no.
Opti-MEM® I Reduced Serum Medium	100 mL 500 mL	31985-062 31985-070
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023
4% E-Gel® Starter Pak	9 gels and Base	G6000-04
2% E-Gel® Starter Pak	9 gels and Base	G6000-02
10 bp DNA Ladder	50 µg	10821-015
293FT Cell Line	3×10^6 cells, frozen	R700-07
PureLink® Quick Gel Extraction Kit	50 preps	K2100-12

Spectinomycin

For selection of pcDNA™6.2-GW/EmGFP-miR transformants in *E. coli*, you will need to obtain spectinomycin. Spectinomycin dihydrochloride pentahydrate is available from Sigma (Catalog no. S4014). For a recipe to prepare spectinomycin for use, see **Appendix**, page 61.

RNAi Designer and RNAi Express

The BLOCK-iT™ RNAi Designer is an online tool to help you design and order microRNA 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 microRNA sequences that are compatible for use in cloning into the BLOCK-iT™ Pol II miR RNAi Expression Vectors.

BLOCK-iT™ miR RNAi Select

Life Technologies has predesigned miR RNAi sequences, called BLOCK-iT™ miR RNAi Select, targeting >70% of the human, mouse and rat RefSeq genes.

BLOCK-iT™ miR RNAi Select provides up to four miR sequences per gene that are supplied as 8 tubes containing four top oligos and four bottom DNA oligos. Upon annealing and cloning into the BLOCK-iT™ Pol II miR RNAi Expression vector pcDNA™6.2-GW/EmGFP-miR, these oligos generate up to four different miR RNAi expression vectors directed against your gene of interest.

The resulting miR RNAi expression vectors can be transfected into cells to knock down the gene of interest, or the hairpins can be transferred into the T-REx™ inducible vector or other compatible DEST vectors (see **Gateway® Destination Vectors**, next page) to knock down the gene of interest in hard-to-transfect or primary cells. We guarantee that at least two out of the four miR RNAi expression vectors will result in >70% knockdown of the target gene (provided that the transfection efficiency in your experiment is at least 80%).

To order BLOCK-iT™ miR RNAi Select online using the BLOCK-iT™ RNAi Express search engine (**www.lifetechnologies.com/rnaiexpress**). Just enter the gene name, accession number, or keyword, and choose your desired BLOCK-iT™ miR RNAi Select.

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Accessory Products, Continued

BLOCK-iT™ RNAi Products

A large variety of BLOCK-iT™ RNAi products are available from Life Technologies to facilitate RNAi analysis including Stealth RNAi®, Validated Stealth RNAi® Collection, Validated miRNA Vector Collection, and a large selection of RNAi vectors.

For details, visit the RNAi Central portal or contact **Technical Support** (page 71).

Gateway® Destination Vectors

Life Technologies provides a large selection of Gateway® destination vectors that facilitate the transfer of the pre-miRNA sequence into a suitable destination vector. These destination vectors allow miRNA expression in multiple systems, including viral expression systems, and tissue-specific expression. See below for a sample list of compatible destination vectors.

Destination Vector	Quantity	Catalog no.
pLenti6.3/V5-DEST™	6 µg	V496-10
pLenti6/UbC/V5-DEST™	6 µg	V499-10
pEF-DEST51	6 µg	12285-011
pEF5/FRT/V5-DEST™ (Flp-In™)	6 µg	V6020-20
pLenti6.4/R4R2/V5-DEST™	1 kit	K4934-00
N-terminal reporter tag vectors , e.g.:		
pcDNA™6.2/nGeneBLAzer®-DEST	1 kit	12578-068
pcDNA™6.2/N-YFP-DEST	6 µg	V358-20

Note: Transferring the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR to the pLenti6/BLOCK-iT™-DEST destination vector will not yield a functional miRNA expression vector. Expression of the pre-miRNA requires the destination vector to supply a Pol II promoter.

T-REx™ Cell Lines

Life Technologies offers four mammalian cell lines that stably express the Tet repressor from the pcDNA™6/TR plasmid and should be maintained in medium containing blasticidin. Ordering information is provided below.

Cell Line	Source	Catalog no.
T-REx™-293	Human embryonic kidney	R710-07
T-REx™-HeLa	Human cervical adenocarcinoma	R714-07
T-REx™-CHO	Chinese hamster ovary	R718-07
T-REx™-Jurkat	Human T-c ll leukemia	R722-07

Introduction

Overview

Introduction

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP combines Life Technologies' BLOCK-iT™ RNAi and T-REx™ technologies to facilitate tetracycline-regulated expression of a microRNA (miRNA) of interest from a Pol II/TO RNAi cassette for use in RNA interference (RNAi) analysis in mammalian cells. The Gateway®-adapted destination vector is designed to allow efficient, regulated expression (transient or stable) of miRNA in dividing mammalian cells. The pT-REx™-DEST30 vector is designed for use with the T-REx™ system and allows for high-level tetracycline-regulated expression of EmGFP and your miRNA of interest in mammalian cells expressing the Tet repressor. The T-REx™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and 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). For more information about the BLOCK-iT™ RNAi, T-REx™, and Gateway® technologies, see below.

Advantages of the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP

Using the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP for vector-based expression of miRNA provides the following advantages:

- Provides a rapid and efficient way to clone double-stranded oligonucleotide (ds oligo) duplexes encoding a desired miRNA target sequence into a vector containing a Pol II-driven expression cassette (i.e., CMV EmGFP-miR RNAi cassette) for use in RNAi analysis. The expression cassette can be easily transferred into pT-REx™-DEST30 using Gateway® recombination reaction.
- The expression construct containing the CMV/TO RNAi expression cassette may be directly transfected into mammalian cells expressing the Tet repressor to enable rapid, tetracycline-regulated screening of miRNA target sequences.
- The expression construct contains a neomycin resistance marker to allow generation of stable cell lines using Geneticin® that express the miRNA of interest upon tetracycline addition.
- Enables targeting of multiple genes or increasing knockdown of a single target gene with one construct (see **Chaining of miRNAs** on page 8).
- Permits visual or automated selection of cells expressing the pre-miRNA through co-cistronic expression of EmGFP
- Offers easy transfer of the pre-miRNA expression cassette into Gateway®-adapted viral expression systems or vectors driven by a variety of promoters, including tissue-specific and regulated promoters for *in vivo* experiments
- Permits design of predictable RNAi constructs with a high rate of success
- In conjunction with the pre-designed BLOCK-iT™ miR RNAi Select oligos, covers >70% of the human, mouse and rat RefSeq genes with a guaranteed rate of success

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Overview, Continued

The BLOCK-iT™ Inducible Pol II miR RNAi Technology

The BLOCK-iT™ Pol II miR RNAi Technology is a next generation RNAi technology employing miRNA expression vectors that allow constitutive or regulated expression of knockdown cassettes driven by RNA Polymerase II (Pol II) promoters in mammalian cells. The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vectors are specifically designed to allow expression of miRNA sequences and contain specific miR flanking sequences that allow proper processing of the miRNA. The expression vector design is based on the miRNA vector system developed in the laboratory of David Turner (U.S. Patent Publication No. 2004/0053876) and includes the use of endogenous murine miR-155 flanking sequences.

A variety of BLOCK-iT™ RNAi products are available from Life Technologies to facilitate RNAi analysis in mammalian and invertebrate systems. For more information about any of the BLOCK-iT™ RNAi products, see the RNAi Central application portal at **www.lifetechnologies.com/rnai** or contact **Technical Support** (see page 71).

Alternative Expression Systems

The pcDNA™6.2-GW/EmGFP-miR vector expresses EmGFP and the pre-miRNA in most mammalian cells at a high, constitutive level using the human cytomegalovirus (CMV) immediate early promoter. If different expression of the pre-miRNA is required, such as tissue-specific, regulated or lentiviral expression, the vector allows easy recombination with other suitable destination vectors using the Gateway® Technology.

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 (the miRNA sequence) into multiple vector systems.

To transfer your pre-miRNA expression cassette into the destination vector:

1. Generate an entry clone by performing a BP recombination reaction between the pcDNA™6.2-GW/EmGFP-miR expression clone and a suitable donor vector (pDONR™221).
2. Perform an LR recombination reaction between the resulting entry clone and a destination vector (pT-REx™-DEST30).
3. Co-transfect your expression clone and pcDNA™6/TR into the cell line of choice for tetracycline-regulated expression of EmGFP and your miRNA of interest.

For additional information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual which is available for downloading from **www.lifetechnologies.com** or by contacting **Technical Support** (see page 71).

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T-REx™ Technology

The T-REx™ Technology facilitates tetracycline-regulated expression of a gene and/or miRNA 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 or miRNA of interest (Yao et al., 1998). In the T-REx™ System, the Tet repressor is expressed from the pcDNA™6/TR plasmid. The pT-REx™-DEST30 vector is designed for use with the T-REx™ System. This vector contains two tetracycline operator 2 ($TetO_2$) sites within the human CMV promoter for tetracycline-regulated expression of EmGFP and your miRNA of interest (Yao et al., 1998). The $TetO_2$ sequences serve as binding sites for 4 Tet repressor molecules (comprising two Tet repressor homodimers) and confer tetracycline-responsiveness to EmGFP and your miRNA of interest. In the absence of tetracycline, expression is repressed by the binding of Tet repressor homodimers to the $TetO_2$ sequences. Addition of tetracycline to the cells derepresses the hybrid CMV/$TetO_2$ promoter and allows expression of EMGFP and your miRNA of interest. The main components of the T-REx™ System include:

- An inducible expression construct to facilitate tetracycline-regulated expression of EmGFP and your miRNA 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.

For more details about the $TetO_2$ sequences, see page 14. For more information about pcDNA™6/TR and the Tet repressor, refer to the T-REx™ System manual. This manual is available for downloading from **www.lifetechnologies.com** or by contacting **Technical Support** (see page 71).

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Purpose of this Manual

This manual provides the following information:

- An overview of the pathway by which miRNA facilitates gene knockdown in mammalian cells.
- Guidelines to design the appropriate single-stranded oligonucleotides representing the target gene.
- Instructions to anneal the single-stranded oligonucleotides to generate a double-stranded oligonucleotide (ds oligo).
- Instructions to clone the ds oligo into the pcDNA™6.2-GW/EmGFP-miR vector, and transform the ligation reaction into competent *E. coli*.
- Instructions to perform a Rapid BP/LR recombination reaction with pT-REx™-DEST30 to generate a tetracycline-regulated expression clone.
- Instructions to generate stable, mammalian TetR-expressing cell lines.
- Instructions to transfect your EmGFP-miR expression clone into a mammalian TetR-expressing cell line to perform transient, tetracycline-regulated RNAi analysis.
- Guidelines to transfect your pT-REx™ expression construct into a mammalian TetR-expressing cell line and perform Geneticin® selection to generate a stable cell line for tetracycline-regulated RNAi analysis.
- Guidelines to perform the chaining reaction to enable co-cistronic expression of multiple pre-miRNAs from one construct.
- Guidelines for detection of the EmGFP expressed pT-REx expression constructs.

Important

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP is designed to help you generate a CMV promoter-based vector to express miRNA in mammalian cell lines for RNAi analysis. Although the kit has been designed to help you express miRNA representing a particular target sequence in the simplest, most direct fashion, use of the kit for RNAi analysis assumes that users are familiar with the principles of gene silencing, vector-based production of miRNA, transfection in mammalian systems, and cloning. We highly recommend that users possess a working knowledge of the RNAi pathway and lipid-mediated transfection.

For more information about miRNA and the RNAi pathways and expression of miRNA in mammalian cells, refer to published references (Ambros, 2004; Bartel, 2004; Boden et al., 2004; Cullen, 2004; Kim, 2005; McManus & Sharp, 2002; Zeng et al., 2002).

Refer to Molecular Biology handbooks, such as *Current Protocols in Molecular Biology* (Ausubel et al., 1994), if you are not familiar with the cloning steps involved.

Using miRNA for RNAi Analysis

Introduction

RNA interference (RNAi) describes the phenomenon by which short, homologous RNA duplexes induce 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 experimental settings, RNAi is widely used to silence genes through transfection of RNA duplexes or introduction of vector-expressed short hairpin RNA (shRNA).

The RNAi Pathway

In eukaryotic organisms, dsRNA produced *in vivo*, introduced by pathogens, or through research, 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, or translational repression (Hammond et al., 2000; Nykanen et al., 2001). MicroRNAs (miRNAs) are endogenous RNAs that trigger gene silencing (Ambros, 2001; Carrington & Ambros, 2003).

miRNA Pathway

MicroRNAs (miRNAs) are endogenously expressed small ssRNA sequences of ~22 nucleotides in length which naturally direct gene silencing through components shared with the RNAi pathway (Bartel, 2004). Unlike shRNAs, however, the miRNAs are found embedded, sometimes in clusters, in long primary transcripts (pri-miRNAs) of several kilobases in length containing a hairpin structure and driven by RNA Polymerase II (Lee et al., 2004), the polymerase also responsible for mRNA expression.

Drosha, a nuclear RNase III, cleaves the stem-loop structure of the pri-miRNA to generate small hairpin precursor miRNAs (pre-miRNAs) which are ~70 nucleotides in length (Zeng et al., 2005). The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5, a nuclear transport receptor(Bohnsack et al., 2004; Yi et al., 2003). Following the nuclear export, the pre-miRNAs are processed by Dicer into a ~22 nucleotides miRNA (mature miRNA) molecule, and incorporated into an miRNA-containing RNA-induced silencing complex (miRISC)(Cullen, 2004).

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Using miRNA for RNAi Analysis, Continued

Translational Repression versus Target Cleavage

The mature miRNAs regulate gene expression by mRNA cleavage (mRNA is nearly complementary to the miRNA) or translational repression (mRNA is not sufficiently complementary to the miRNA). Target cleavage can be induced artificially by altering the target or the miRNA sequence to obtain complete hybridization (Zeng *et al.*, 2002).

In animals, most miRNAs imperfectly complement their targets and interfere with protein production without directly inducing mRNA degradation (Ambros, 2004). Nonetheless, these miRNAs are found associated with the RNAi nuclease AGO2 (Liu et al., 2004; Meister et al., 2004), and at least two miRNAs with close matches to their target sequences, particularly in their 5′ regions, have been shown to cleave cognate mRNAs (Yekta et al., 2004; Yu et al., 2005).

The engineered miRNAs produced by the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit (see below) fully complement their target site and cleave the target mRNA. Sequence analysis showed that the primary cleavage site at the phosphodiester bond in the mRNA found opposite the tenth and eleventh bases of the engineered miRNA as predicted for RNAi-mediated cleavage (Elbashir et al., 2001) similar to siRNA mediated cleavage.

Using a Vector-Based System to Express Engineered miRNA

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address this limitations, a number of groups have developed vector-based systems to facilitate expression of engineered short hairpin RNA (shRNA) sequences in mammalian cells using Pol III promoters (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). However, the use of shRNA vectors for RNAi analysis requires the screening of large number of sequences to identify active sequences and the use of Pol III promoters limits applications such as tissue-specific expression.

To overcome limitations with siRNA and shRNA, we have developed Gateway®-adapted expression vectors that enable the expression of engineered miRNA sequences from Pol II promoters. The pcDNA™6.2-GW/EmGFP-miR expression vector facilitates the generation of an expression clone containing a ds oligo encoding a pre-miRNA sequence. The resulting expression construct may be introduced into mammalian cells for transient expression of the miRNA sequence, or stable transfectants can be generated. The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP facilitates the easy and efficient transfer of the pre-miRNA sequences and EmGFP into the pT-REx™-DEST30 vector for tetracycline-regulated expression in the T-REx™ System. If desired, the EmGFP and pre-miRNA sequence may also be transferred into any other suitable destination vector by Gateway® recombination reactions (see page 37).

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Using miRNA for RNAi Analysis, Continued

miRNA Vector

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit is supplied with pcDNA™6.2-GW/EmGFP-miR vector that allows the expression of your engineered pre-miRNA under the control of the strong, Pol II human CMV (cytomegalovirus) promoter and Herpes Simplex virus (HSV) thymidine kinase (TK) polyadenylation signal. The coding sequence of EmGFP (Emerald Green Fluorescent Protein) is incorporated into the vector such that the pre-miRNA insertion site is in the 3′ untranslated (3′UTR) region of the fluorescent protein mRNA. Addition of EmGFP allows tracking of the miRNA expression and provides strong correlation of EmGFP expression with the knockdown of the target gene by your miRNA.

Human CMV Promoter

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector contain the human cytomegalovirus (CMV) immediate early promoter to allow high-level, constitutive miRNA expression in mammalian cells (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).

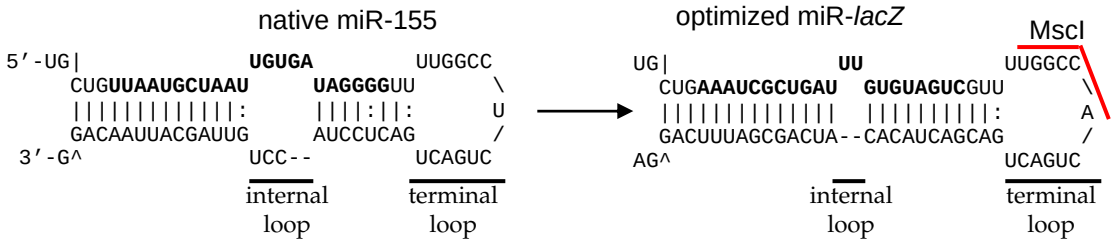
We have chosen the human CMV promoter to control vector-based expression of miRNA molecules in mammalian cells for the following reasons:

- The promoter is recognized by RNA Polymerase II and controls high-level, constitutive expression of miRNA and co-cistronic reporter genes
- The promoter is active in most mammalian cell types

Note: 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.

Design of the Engineered Pre-miRNA

The engineered pre-miRNA sequence structure is based on the murine miR-155 sequence (Lagos-Quintana et al., 2002). The 5′ and 3′ flanking regions derived from the miR-155 transcript were inserted in the vector to preserve as much as possible of the miR-155 structure. We optimized the stem-loop structure and a 2 nucleotide internal loop results in higher knockdown rate than the 5 nucleotide /3 nucleotide internal loop found in native miR-155 molecule. An *Msc* I site was incorporated in the terminal loop to aid in sequence analysis. Below the changes are shown made to the native miR-155 to form an engineered pre-miRNAs directed against *lacZ* (targeting sequence in bold).



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Using miRNA for RNAi Analysis, Continued

Structure of the Engineered Pre-miRNA

The pcDNA™6.2-GW/EmGFP-miR vectors are designed to accept engineered pre-miRNA sequences targeting your gene of interest. The engineered pre-miRNA sequence structure is based on the murine miR-155 sequence and the stem-loop structure was optimized to obtain a high knockdown rate as described on the previous page.

For optimized knockdown results, we recommend that the ds oligo encoding the engineered pre-miRNA have the following structural features:

- A 4 nucleotide, 5′ overhang (TGCT) complementary to the vector (required for directional cloning)
- A 5′G + short 21 nucleotide antisense sequence (mature miRNA) derived from the target gene, followed by
- A short spacer of 19 nucleotides to form the terminal loop and
- A short sense target sequence with 2 nucleotides removed (2) to create an internal loop
- A 4 nucleotide, 5′ overhang (CAGG) complementary to the vector (required for directional cloning)

The structural features are depicted in the figure below.

For guidelines to design the oligonucleotides, refer to page 16. We recommend using Life Technologies' RNAi Designer at **www.lifetechnologies.com/rnai**, an online tool to help you design and order pre-miRNA sequences for any target gene of interest.

Pre-miRNA Expression Cassette

The engineered pre-miRNA sequence is cloned into the cloning site of BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector that is flanked on either side with sequences from murine miR-155 to allow proper processing of the engineered pre-miRNA sequence.

The pre-miRNA sequence and adjacent miR-155 flanking regions are denoted as the pre-miRNA expression cassette and are shown below. This expression cassette is transferred between vectors during Gateway® recombination reactions.

Once the engineered pre-miRNA expression cassette is introduced into the mammalian cells for expression, the pre-miRNA forms an intramolecular stem-loop structure similar to the structure of endogenous pre-miRNA that is then processed by the endogenous Dicer enzyme into a 22 nucleotide mature miRNA.

Note: The 21 nucleotides are derived from the target sequence while the 3′ most nucleotide is derived from the native miR-155 sequence.

Chaining of miRNAs

miRNAs are sometimes expressed in clusters in long primary transcripts driven by RNA Pol II (Lee et al., 2004). Our vectors support chaining of miRNAs to express them in one primary transcript, thus ensuring co-cistronic expression of multiple miRNAs.

Note: Chaining should be done in the pcDNA™6/TR vector **before** transfer to pT-REx™-DEST30, as restriction sites present in the pT-REx™-DEST30 vector make it incompatible with the chaining protocol.

BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP Kit

Description of the System

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP facilitates the generation of an expression construct that permits tetracycline-regulated, high-level expression of a pre-miRNA in mammalian cells for RNAi analysis of a target gene. The kit contains the following major components:

- BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP (pcDNA™6.2-GW/EmGFP-miR), pDONR™221 and pT-REx™-DEST30 Gateway® Vector for the production of a tetracycline-regulated expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA (oligos have to be ordered separately). The pcDNA™6.2-GW/EmGFP-miR vector is supplied linearized with 4-nucleotide 5′ overhangs on each strand to facilitate directional cloning of the ds oligo insert.
- pT-REx™-DEST30 Gateway® vector for high-leveltetracycline-regulated expression of your gene of interest in mammalian cells expressing the Tet repressor and that are designed for use with the T-REx™ System.
- Gateway® Clonase® II Enzyme Mixes for transfer of miRNA expression cassette into an inducible expression vector.
- T-REx™ Regulatory Module for high-level tetracycline-regulated expression of the miRNA of interest in mammalian cells expressing the Tet repressor.
- Tetracycline for induction of miRNA expression and blasticidin for selection of Tet repressor-expressing cells.

Controls

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP also includes a negative control plasmid, a ds positive control oligo , and T-REx™ cell tranfection and expression positive control.

- The pcDNA™6.2-GW/EmGFP-miR-neg control plasmid contains an insert that can form a hairpin structure that is processed into mature miRNA, but is predicted not to target any known vertebrate gene. Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with pcDNA™6.2-GW/EmGFP-miR expression vectors. The neg control sequence without 5′ overhangs is shown below (for map, see page 62):

5′-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′

- The miR-*lacZ* positive double-stranded (ds) control oligo serves as a positive control during the miRNA expression vector generation. Use this oligo to generate a pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression clone.
- Co-transfecting the resulting pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression clone targeting the *lacZ* gene and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into mammalian cells provide a means to assess the RNAi response in your cell line by assaying for knockdown of β-galactosidase.
- pT-REx™/GW-30/*lacZ* is provided as a positive control vector for mammalian cell transfection and expression (see page 68 for map) and may be used to optimize recombinant protein expression levels in your cell line. This vector allows expression of β-galactosidase which may be detected by Western blot or functional assay.

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Generating an miRNA Expression Vector Using the Kit

Using the reagents supplied in the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kits, you will perform the following steps to generate an expression clone in pcDNA™6.2-GW/EmGFP-miR:

1. Design and synthesize two complementary single-stranded DNA oligonucleotides, with one encoding the miRNA of interest. Alternatively, order BLOCK-iT™ miR RNAi Select oligos targeting your gene(s) (see page xiv).
2. Anneal the single-stranded oligonucleotides to generate a double-stranded oligo (ds oligo).
3. Clone the ds oligo into the linearized pcDNA™6.2-GW/EmGFP-miR vector.
4. Transform the ligation reaction into One Shot® TOP10 chemically competent *E. coli* and select for spectinomycin-resistant transformants.
5. Use the pcDNA™6.2-GW/EmGFP-miR expression construct for transient RNAi analysis in mammalian cells and perform a Gateway® recombination reaction with pT-REx™-DEST30 Gateway® destination vector and pDONR™221 to generate a tetracycline-regulated EmGFP-miRNA expression vector.

Features of the pcDNA™6.2-GW/EmGFP-miR Vector

The pcDNA™6.2-GW/EmGFP-miR vectors contain the following features:

- Human CMV promoter for high-level, constitutive expression of the miRNA from a RNA Polymerase II-dependent promoter
- 5′ and 3′ miR flanking regions for formation of an engineered pre-miRNA
- Cloning site containing 4-nucleotide 5′ overhangs on each DNA strand for directional cloning of the ds oligo encoding the pre-miRNA of interest
- Two recombination sites, *att*B1 and *att*B2 sites, flanking the pre-miRNA expression cassette for recombinational cloning of the pre-miRNA expression cassette into a Gateway® destination vector
- Herpes Simplex virus (HSV) thymidine kinase (TK) polyadenylation signal for termination and polyadenylation of the transcript
- Spectinomycin resistance gene for selection in *E. coli*
- pUC origin for high-copy maintenance of the plasmid in *E. coli*
- Blasticidin resistance gene for selection in *E. coli* and mammalian cells to generate cell lines stably expressing the miRNA
- pcDNA™6.2-GW/EmGFP-miR also contains an EmGFP coding sequence for co-cistronic expression with the pre-miRNA.

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Green Fluorescent Protein (GFP)

The BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP contains the Emerald Green Fluorescent Protein (EmGFP) derived from *Aequorea victoria* GFP within the pre-miRNA expression cassette. Green Fluorescent Protein (GFP) is a naturally occurring bioluminescent protein derived from the jellyfish *Aequorea victoria* (Shimomura et al., 1962). GFP emits fluorescence upon excitation, and the gene encoding GFP contains all of the necessary information for posttranslational synthesis of the luminescent protein. GFP is often used as a molecular beacon because it requires no species-specific cofactors for function, and the fluorescence is easily detected using fluorescence microscopy and standard filter sets. GFP can function as a reporter gene downstream of a promoter of interest and upstream of one or more pre-miRNAs.

After transferring the pre-miRNA expression cassette into pT-REx™-DEST30, you may transfect tet repressor-expressing cells for tetracycline regulated expression of the EmGFP protein and miRNA, allowing you to visually track the cells in which knockdown is occurring.

GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include amino acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescence signal, resulting in "enhanced" GFP (Zhang et al., 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998). The Emerald GFP (EmGFP) is such a variant of enhanced GFP.

Note

We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable.

EmGFP

The EmGFP variant has been described in a published review (Tsien, 1998) and is summarized below. The amino acid mutations are represented by the single letter abbreviation for the amino acid in the consensus GFP sequence, followed by the codon number and the single letter amino acid abbreviation for the substituted amino acid.

Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T

*Mutations listed are as described in the literature. When examining the actual sequence, the vector codon numbering starts at the first amino acid **after** the initiation methionine of the fluorescent protein, so that mutations appear to be increased by one position. For example, the S65T mutation actually occurs in codon 66 of EmGFP.

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EmGFP Fluorescence

The EmGFP from the pcDNA™6.2-GW/EmGFP-miR expression vector has the following excitation and emission wavelengths, as published in the literature (Tsien, 1998):

Excitation (nm)	Emission (nm)
487	509

Filter Sets for Detecting EmGFP Fluorescence

The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein. The filter set for fluorescence microscopy and the manufacturer are listed below:

Filter Set	Manufacturer
Omega XF100	Omega (www.omegafilters.com)

The T-REx™ System

Description of the T-REx™ System

In the T-REx™ System, expression of your gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline (Yao et al., 1998). Unlike other tetracycline-regulated systems which use hybrid regulatory molecules and viral transactivation domains (Gossen and Bujard, 1992), the T-REx™ System uses only regulatory elements from the native Tet operon (Yao et al., 1998). Tetracycline-regulated gene expression in the T-REx™ System more closely resembles the regulation of the native bacterial *tet* operon (Hillen and Berens, 1994; Hillen et al., 1983) and avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.

The major component of the T-REx™ System that is provided in this kit, is the inducible pT-REx™-DEST30 expression plasmid. Expression of your miRNA of interest and EmGFP from pT-REx™-DEST30 is controlled by the strong CMV promoter (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987) into which 2 copies of the *tet* operator 2 ($TetO_2$) sequence have been inserted in tandem. The $TetO_2$ sequences consist of 2 copies of the 19 nucleotide sequence, 5´-TCCCTATCAGTGATAGAGA-3´ separated by a 2 base pair spacer (Hillen and Berens, 1994; Hillen et al., 1983). Each 19 nucleotide $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor. For details on the Tet operator sequences and the specific features of each inducible expression vector, please refer to the manual for this vector.

The second major component of the T-REx™ System is the pcDNA™6/TR regulatory vector which expresses high levels of the *Tet*R gene (Postle et al., 1984) under the control of the human CMV promoter (see **Appendix**, pages 69 for more information about pcDNA™6/TR© and the *Tet*R gene). Both T-REx™ vectors can be introduced into mammalian host cells by standard transfection methods.

Features of the pT-REx™-DEST30 Vector

The pT-REx™-DEST vector contains the following elements. For a map of pT-REx™-DEST30, see page 66.

- Hybrid promoter consisting of human cytomegalovirus immediate-early (CMV) promoter/enhancer and tetracycline operator 2 ($TetO_2$) sites for tetracycline-regulated expression in a wide range of mammalian cells
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- *ccd*B gene located between the two *att*R sites for negative selection
- SV40 polyadenylation sequence for proper termination and processing of the transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the neomycin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Neomycin resistance gene for selection of stable cell lines
- The ampicillin (*bla*) resistance gene for selection in *E. coli*
- The pUC origin for high copy replication and plasmid maintenance in *E. coli*

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Tet Operator Sequences

The promoters of bacterial *tet* genes contain two types of operator sequences, O_1 and O_2, that serve as high affinity binding sites for the Tet repressor (Hillen and Berens, 1994; Hillen et al., 1983). Each O_1 and O_2 site binds to one Tet repressor homodimer. While Tet repressor homodimers bind to both *tet* operators with high affinity, studies have shown that the affinity of the Tet repressor homodimer for O_2 is three- to five-fold higher than for O_1 (Hillen and Berens, 1994).

Tet operators have been incorporated into heterologous eukaryotic promoters to allow tetracycline-regulated gene expression in mammalian cells (Gossen and Bujard, 1992; Yao et al., 1998). In the T-REx™ System, two copies of the O_2 operator sequence ($TetO_2$) are inserted into the strong CMV promoter of the pT-REx™-DEST vectors to allow regulated expression of your gene of interest by tetracycline. For more information about *tet* operators, refer to Hillen and Berens (1994).

Yao *et al.* (1998) have recently demonstrated that the location of *tet* operator sequences in relation to the TATA box of a heterologous promoter is critical to the function of the *tet* operator. Regulation by tetracycline is only conferred upon a heterologous promoter by proper spacing of the $TetO_2$ sequences from the TATA box (Yao et al., 1998). For this reason, the first nucleotide of the $TetO_2$ operator sequence has been placed 10 nucleotides after the last nucleotide of the TATA element in the CMV promoter for the pT-REx™-DEST vectors.

In other tetracycline-regulated systems, the $TetO_2$ sequences are located upstream of the TATA element in the promoter of the inducible expression vector (Gossen and Bujard, 1992). These systems differ substantially from the T-REx™ System in that they use regulatory molecules composed of the Tet repressor fused to a viral transactivation domain. The presence of viral transactivation domains appears to overcome the requirement for specific positioning of the $TetO_2$ sequences in relation to the TATA box of the heterologous promoter. However, the presence of viral transactivation domains has been found to have deleterious effects in some mammalian cell lines.

Mechanism of Repression

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence in the promoter of the inducible expression vector (Hillen and Berens, 1994). The 2 $TetO_2$ sites in the promoter of the inducible expression vector serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor. 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 and Berens, 1994). Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of your gene 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 to the Tet operator. The association constant, K_A, of tetracycline for the Tet repressor is $3 \times 10^9\ M^{-1}$ (Hillen and Berens, 1994). The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest.

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The T-REx™ System, Continued

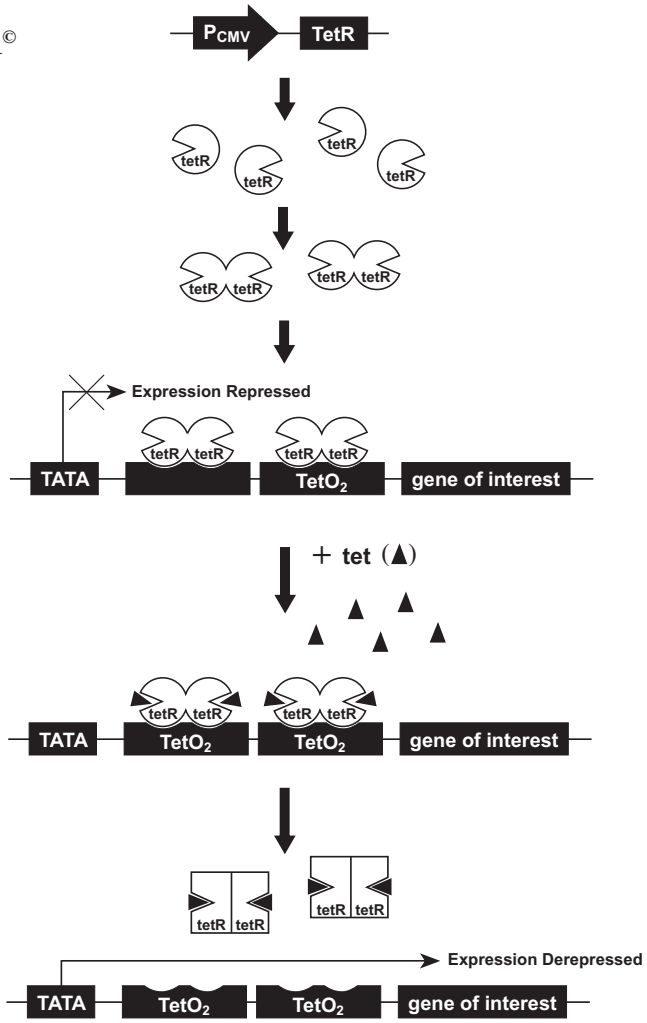
The figure illustrates the components of the T-REx™ System.

1. Tet repressor (tetR) protein is expressed from pcDNA6/TR© in cultured cells.

2. TetR homodimers bind to Tet operator 2 ($TetO_2$) sequences in the inducible expression vector, repressing transcription of the gene of interest.

3. Upon addition, 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 transcription from the gene of interest.



Methods

Designing the Single-Stranded DNA Oligos

Introduction

To use the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector with EmGFP Kit, you first need to design two single-stranded DNA oligonucleotides; one encoding the target pre-miRNA ("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 pcDNA™6.2-GW/EmGFP-miR 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 pre-miRNA will be effective in knocking down the target gene. For a given target gene, you may need to generate and screen multiple pre-miRNA sequences to identify one that is active in gene knockdown studies.

We **strongly** recommend using **Life Technologies' RNAi Designer**, an online tool to help you design and order pre-miRNA 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 pre-miRNA sequences that are compatible for use in cloning into pcDNA™6.2-GW/EmGFP-miR. Success rates exceeding 70% have been achieved with the RNAi Designer (i.e. more than 70% of designed miRNAs reduce target gene expression by at least 70%). To use the RNAi Designer, see **www.lifetechnologies.com/rnai**.

Note: Life Technologies' RNAi Designer is the only online tool for miRNA design at the moment this manual is printed. Other online RNAi designers not intended for miRNA will not necessarily design good pre-miRNA sequences.

BLOCK-iT™ miR RNAi Select

Life Technologies has pre-designed miR RNAi sequences, called BLOCK-iT™ miR RNAi Select, targeting >70% of the human, mouse and rat RefSeq genes with a guaranteed rate of success.

For additional information, visit our website at **www.lifetechnologies.com**, or contact **Technical Support** (see page 71).

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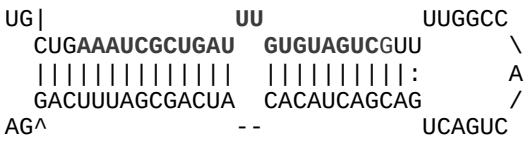
Designing the Single-Stranded DNA Oligos, Continued

Features of Pre-miRNA Insert

When designing the oligos encoding the pre-miRNA, consider that a pre-miRNA insert contains the following features (from 5′ to 3′ end):

- 5 nucleotides (TGCTG) derived from the endogenous miR-155, an endogenous murine miRNA that is the basis of the miRNA vector system developed in the laboratory of David Turner (Chung *et al.*, 2006). This also provides a four nucleotide 5′ overhang, compatible with a 4 nucleotide overhang in the provided linearized pcDNA™6.2-GW/EmGFP-miR to clone the double-stranded oligo.
- Reverse complement of the 21-nucleotide target sequence (mature miRNA sequence). When transcribed, this is the core sequence that will target your gene of interest, and therefore needs to be antisense to the targeted messenger RNA.
- 19 nucleotides derived from miR-155 to form a terminal loop with an engineered *Msc* I site to aid in sequence analysis.
- Nucleotides 1–8 and 11–21 of the sense target sequence. Note that nucleotides 9 and 10 are removed to form a short internal loop in the mature miRNA, which results in more efficient knockdown.
- 4 nucleotides derived from endogenous miR-155. This also constitutes the four nucleotide 5′ overhang, compatible with a 4 nucleotide overhang in the provided linearized pcDNA™6.2-GW/EmGFP-miR to clone the double-stranded oligo.

Upon transcription, the mature miRNA sequence and its complement form a stem of the pre-miRNA with a short internal loop, separated by a larger terminal loop. The folded pre-miRNA structure of miR-*lacZ* is shown below (*lacZ* targeting sequence in bold)



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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. These are general recommendations only; exceptions may occur.

Length: The target sequence should be 21 nucleotides in length.

Complexity:

- Make sure that the target sequence does **not** contain runs of more than three of the same nucleotide.
- Choose a sequence with low to moderate GC content (~30–50% GC content is suggested).
- Do not choose a target sequence that is a known site for RNA-protein interaction.
- Avoid the following restriction sites, which may be used for optional, advanced features later.

Restriction site	Sequence	Advanced Feature	Page
Msc I	TGGCCA	Alternate sequencing protocol	30
*Bam*H I	GGATCC	miRNA chaining	32
Bgl II	AGATCT	miRNA chaining	32
Sal I	GTCGAC	miRNA chaining	32
Xho I	CTCGAG	miRNA chaining	32
Dra I	TTTAAA	Removal of EmGFP	34

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

Orientation: Choose a target sequence encoding the **sense** sequence of the target mRNA.

Generating the Top Oligo Sequence

To generate the top oligo sequence, combine these elements (from 5′ end to 3′ end):

1. 5′ TGCTG
2. Reverse complement of the 21-nucleotide sense target sequence. This is the Mature miRNA Sequence.
3. GTTTTGGCCACTGACTGAC (terminal loop).
4. Nucleotides 1–8 (5′-3′) of sense target sequence.
5. Nucleotides 11–21 (5′-3′) of sense target sequence.

Continued on next page

Designing the Single-Stranded DNA Oligos, Continued

Generating the Bottom Oligo Sequence

To generate the bottom oligo sequence, perform the following steps:

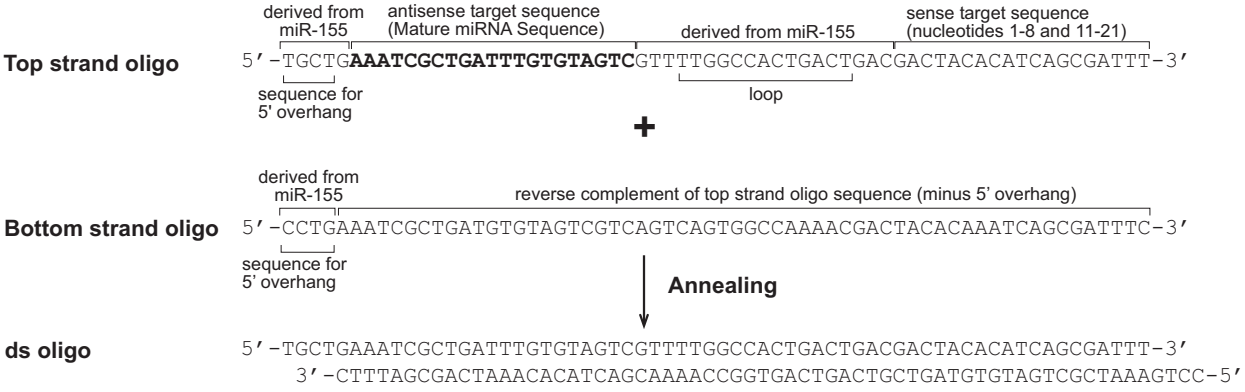
1. Remove 5′ TGCT from top oligo sequence (new sequence starts with G).
2. Take the reverse complement of the sequence from step 1.
3. Add CCTG to the 5′ end of the sequence from step 2.

Note

- We recommend using Life Technologies' RNAi Designer at **www.lifetechnologies.com/rnai**, which automatically applies the design rules, and produces a high rate of knockdown success.
- It is not necessary to add 5′ phosphates to your single stranded oligos during synthesis. The phosphate groups necessary for ligation are present in the linearized pcDNA™6.2-GW/EmGFP-miR.

Example of ss Oligo Design

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



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

Continued on next page

Designing the Single-Stranded DNA Oligos, Continued

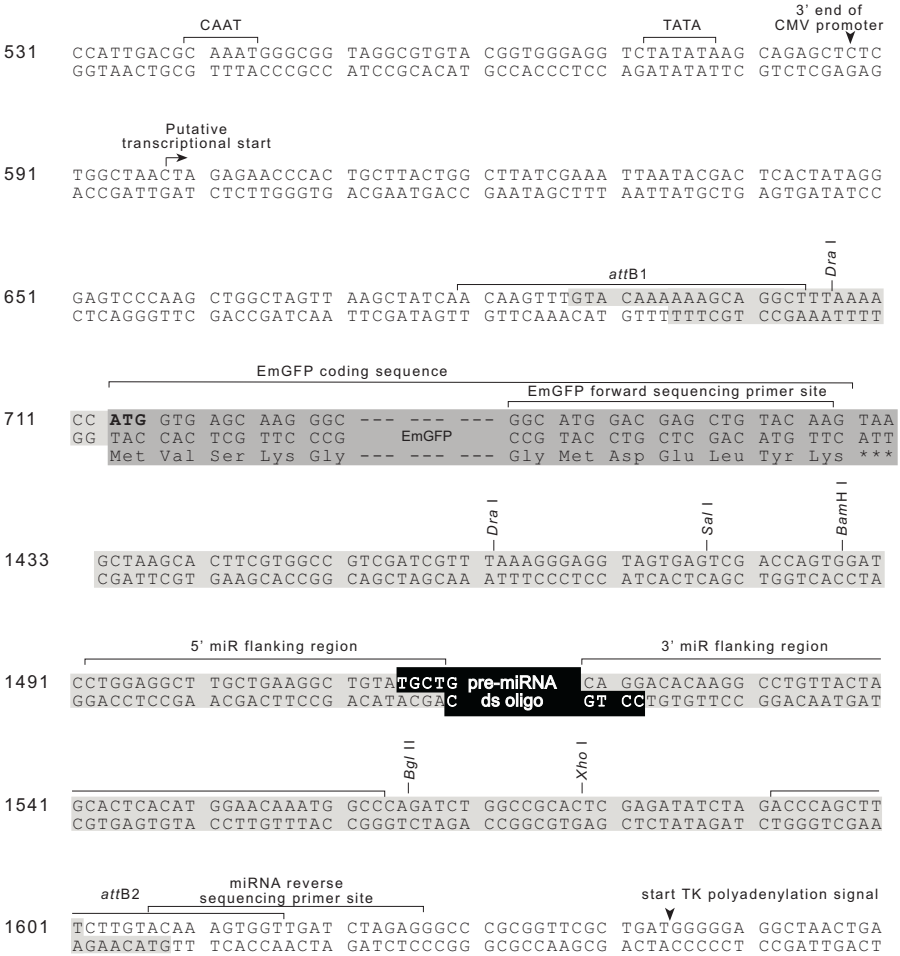
Cloning Site and Recombination Region of pcDNA™6.2-GW/EmGFP-miR

Use the diagram below to help you design suitable DNA oligonucleotides to clone into pcDNA™6.2-GW/EmGFP-miR after annealing. Note the following features in the diagram below:

- The pcDNA™6.2-GW/EmGFP-miR vector is supplied linearized between nucleotides 1518 and 1519. The linearized vector contains 4 nucleotide overhangs derived from miR-155 sequences. Note that the annealed double-stranded (ds) oligo **must** contain specific 4 nucleotide 5′ overhangs on each strand as indicated.
- The light shaded region corresponds to DNA sequences that are transferred from the initial pre-miRNA expression vector into the Gateway® destination vector (e.g., pT-REx™-DEST30 Gateway® Vector) following recombination. The dark shaded region represents the EmGFP coding sequence.

  Note: Following recombination with a Gateway® destination vector, the resulting expression clone will contain a pre-miRNA expression cassette consisting of the EmGFP coding sequence, 5′ miR flanking region, miRNA sequence, and the 3′ miR flanking region.

Note: The complete sequence of pcDNA™6.2-GW/EmGFP-miR is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71). For a map of pcDNA™6.2-GW/EmGFP-miR, see page 62.



Generating the Double-Stranded Oligo

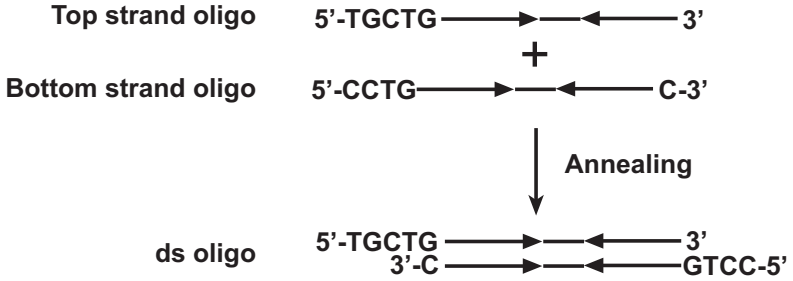
Introduction

Once you have acquired 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 pcDNA™6.2-GW/EmGFP-miR vector and for annealing. See the figure below for an illustration.

- **Top strand oligo:** Make sure that this oligo contains the sequence TGCTG at the 5′ end.
- **Bottom strand oligo:** Make sure that this oligo contains the sequence CCTG at the 5′ end, has a C at the 3′ end, and is complementary to the top strand.



Note: BLOCK-iT™ miR RNAi Select oligos have been designed to contain all these sequence elements.

Annealing BLOCK-iT™ miR RNAi Select Oligos

If you have ordered BLOCK-iT™ miR RNAi Select, you will receive up to 8 tubes containing 4 top oligos and 4 bottom DNA oligos per gene, enough to clone up to four different miR RNAi expression vectors. Each tube is marked with a unique code; an example is shown below:

Hmi123456_top_SYMB

The code consists of three parts (separated by dashes).

- A unique miR RNAi identifier, starting with Hmi for human, Mmi for mouse, and Rmi for rat RNAi oligos, followed by six digits.
- A strand indicator: "top" for the top strand, "bot" for the bottom strand
- The gene symbol (may be partly abbreviated due to space constraints)

Make sure you anneal the two matching single-stranded oligos. Anneal the two oligos marked "top" and "bot" with the **same miR RNAi identifier (Hmi, Mmi or Rmi number)** according to the instructions on the next page. The two matching BLOCK-iT™ miR RNAi Select DNA oligos may arrive in separate shipments; if only one strand is present in a shipment, please wait for the remaining strand before proceeding.

Note: BLOCK-iT™ miR RNAi Select oligos come lyophilized; store at –20°C.

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.

Continued on next page

Generating the Double-Stranded Oligo, Continued

Amount of DNA Oligo to Anneal

You will anneal equal amounts of the top and bottom strand oligos to generate the ds oligos. We perform the annealing reaction at a final single-stranded oligo concentration of 50 µM. Annealing at concentrations below 5 µM significantly reduce the efficiency. Note that the annealing step is not 100% efficient.

Re-annealing *Lac*Z2.1 Control Oligo

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

Materials Needed

- 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 miR-*lacZ* positive ds control oligo (thaw on ice)
- 10X Oligo Annealing Buffer (supplied with the kit)
- DNase/RNase-Free Water (supplied with the kit)
- 0.5-mL sterile microcentrifuge tubes
- 95°C water bath or heat block

Setting up the Annealing Reaction

Follow this procedure to set up the annealing reaction. 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	Quantity
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 miR-*lacZ* positive ds control oligo, centrifuge its tube briefly (~5 seconds), and transfer contents to a separate 0.5-mL sterile microcentrifuge tube.

Continued on next page

Generating the Double-Stranded Oligo, Continued

Annealing Procedure

Follow this procedure to anneal your single-stranded oligos to generate the ds oligo.

1. Incubate the tubes from the previous section (**Setting up the Annealing Reaction**) at 95°C for 4 minutes.
2. Remove the tube containing the annealing reaction from the water bath or the heat block and set on your laboratory bench.
3. Allow the reaction mixture to cool to room temperature for 5–10 minutes. The single-stranded oligos will anneal during this time.
4. Place the sample in a microcentrifuge and centrifuge briefly (~5 seconds). Mix gently.
5. Remove 1 µL of the annealing mixture and dilute the ds oligo as directed in **Diluting the ds Oligo**, below.
6. Store the remainder of the 50 µM ds oligo mixture at –20°C (stable for at least a year).

Diluting the ds Oligo

To clone your ds oligo or miR-*lacZ* positive ds control oligo into pcDNA™6.2-GW/EmGFP-miR, you **must** dilute the 50 µM stock to a final concentration of 10 nM (i.e., 5,000-fold dilution). We generally perform 100-fold and 50-fold serial dilutions, the first into DNase/RNase-free water and the second into the 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, above) 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) 50-fold into Oligo Annealing Buffer as follows to obtain a final concentration of 10 nM.

500 nM ds oligo	1 µL
10X Oligo Annealing Buffer	5 µL
DNase/RNase-free water	44 µL
Total volume	50 µL

3. Vortex to mix thoroughly. Store the remaining 500 nM ds oligo stock at –20°C.
4. Aliquot the 10 nM ds oligo stock and store at –20°C.

Continued on next page

Generating the Double-Stranded Oligo, Continued

Important

The undiluted ds oligos are 5,000-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**, below).
- **10 nM ds oligo (5,000-fold dilution):** Use this stock for cloning (see **Ligation Procedure**, page 26). This stock is not suitable for long-term storage.

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

Important

When using the diluted ds oligo stock solutions (i.e., 100-fold or 5,000-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 pcDNA™6.2-GW/EmGFP-miR.

If your diluted ds oligo stock solution 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 23.

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® (Life Technologies, Catalog no. G5000-04)
- **Molecular weight standard:** 10 bp DNA Ladder (Life Technologies, Catalog no. 10821-015)

Note: 4% E-Gel® resolves these fragments much better than regular 4% agarose gels.

Continued on next page

Generating the Double-Stranded Oligo, Continued

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 running around 60–70 bp.
- A faintly detectable lower molecular weight band representing unannealed oligos that form hairpins, running at around 30–35 bp.

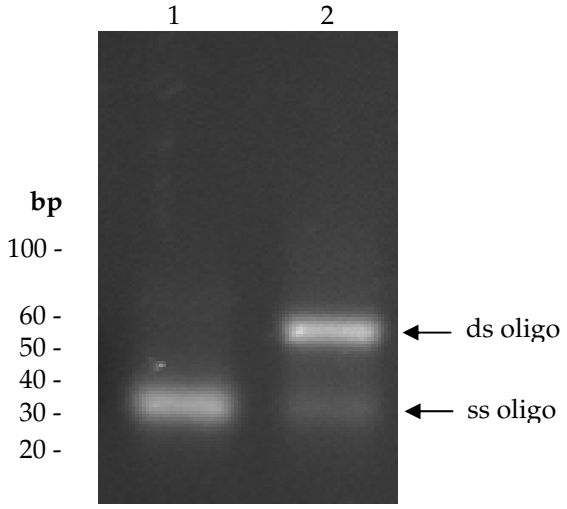
For an example of expected results obtained from agarose gel analysis, see the next page. If the band representing ds oligo is weak or if you do not see a band, see **Troubleshooting**, page 55 for tips to troubleshoot your annealing reaction.

Example of Expected Results

In this experiment, *lacZ* control oligos were annealed (50 µM final concentration) using the reagents supplied in the kit and following the procedure on page 23 to generate the *lacZ* ds control oligo. The annealing reaction was diluted 100-fold in water to a concentration of 500 nM. Aliquots of the diluted ds oligo (5 µL; 2.5 pmol) and the corresponding single-stranded oligos (mixed but not annealed; 2.5 pmol per oligo) were analyzed on a 4% E-Gel®.

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

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



Lane 1. single stranded *lacZ* oligos

Lane 2. annealed *lacZ* oligos

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 pcDNA™6.2-GW/EmGFP-miR 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 this section and **Transforming One Shot® TOP10 Competent *E. coli*** (page 28) before beginning.

Note: If you want to perform miRNA chaining, refer to page 32.

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 pcDNA™6.2-GW/EmGFP-miR 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 pcDNA™6.2-GW/EmGFP-miR 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 Pol II miR RNAi Expression Vector Kits are available separately from Life Technologies (Catalog no. 15224-017).

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

Amount of ds Oligo to Use

For optimal results, use approximately a 15:1 molar ratio of ds oligo insert: vector for ligation.

Ligation Controls

We recommend a ligation with DNase/RNase-Free Water instead of oligo as negative control for the ligation reaction.

We recommend including the miR-*lacZ* positive double-stranded (ds) control oligo supplied with the kit as a positive control in your ligation experiment. The miR-*lacZ* positive ds control oligo is supplied as a 50 µM stock in 1X Oligo Annealing Buffer, and needs to be re-annealed and diluted 5000-fold before use in a ligation reaction (see pagexx). See page xx for the sequence of each strand of the *lacZ* ds control oligo.

Note: Once you have cloned the *lacZ* ds control oligo into pcDNA™6.2-GW/EmGFP-miR, you may use the resulting expression clone as a positive control for the RNAi response in your mammalian cell line. Simply co-transfect the expression 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 10 nM ds oligo stock solution for cloning, thaw the solution on ice. **Do not thaw the ds oligo by heating or the ds oligo duplexes may melt and form intramolecular hairpin structures.** After use, return the tube to –20°C storage.

Continued on next page

Performing the Ligation Reaction, Continued

Materials Needed

- Double-stranded oligo of interest (10 nM in 1X Oligo Annealing Buffer; thaw on ice before use)
- pcDNA™6.2-GW/EmGFP-miR, linearized (5 ng/µL, supplied with the kit; thaw on ice before use)
- 5X Ligation Buffer (supplied with the kit)
- DNase/RNase-Free Water (supplied with the kit)
- T4 DNA Ligase (1 U/µL, supplied with the kit)

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	Negative control
5X Ligation Buffer	4 µL	4 µL	4 µL
pcDNA™6.2-GW/EmGFP-miR, linearized (5 ng/µL)	2 µL	2 µL	2 µL
miR-ds oligo (10 nM; i.e., 1:5,000 dilution; page 23)	4 µL	—	—
miR-*lacZ* positive ds control oligo (10 nM; i.e., 1:5,000 dilution; page 23)	—	4 µL	—
DNase/RNase-Free Water	9 µL	9 µL	13 µL
T4 DNA Ligase (1 U/µL)	1 µL	1 µL	1 µL
Total volume	20 µL	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: Extending the incubation time may result in a higher yield of colonies. Do not exceed 2 hours.

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, transform your ligation mixture into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit to facilitate transformation. Follow the guidelines and instructions provided in this section.

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

Materials Needed

- Ligation reaction (from Step 4, previous page)
- One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit, one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (supplied with the kit; warm to room temperature)
- pUC19 positive control (supplied with the kit, if desired)
- 42°C water bath
- LB plates containing 50 µg/mL spectinomycin (two for each transformation; warm at 37°C for 30 minutes before use). See **Appendix**, page 61 for recipe.
- LB plates containing 100 µg/mL ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note: Low salt LB agar plates containing 100 µg/mL Blasticidin can also be used to select for transformants. Be sure to use low salt agar plates and check pH carefully for Blasticidin to work efficiently. For more information on Blasticidin and recipes, see page 60 and 61.

One Shot® TOP10 Transformation Procedure

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

1. Add 2 µL of the ligation reaction (from Step 3, previous page) into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5–30 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 horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50–200 µL from each transformation on a pre-warmed LB agar plate containing 50 µg/mL spectinomycin and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, plate 20–100 µL of the transformation reaction on LB plates containing 100 µg/mL ampicillin.
8. An efficient ligation reaction may produce several hundred colonies.

Analyzing Transformants

Analyzing Transformants

To analyze positive clones, we recommend that you:

1. Pick 5–10 spectinomycin-resistant colonies and culture them overnight in LB or SOB medium containing 50 µg/mL spectinomycin.

   Note: Low salt LB containing 100 µg/mL Blasticidin can also be used to grow transformants. See page 61.

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, or equivalent.

Sequence each pcDNA™6.2-GW/EmGFP-miR expression 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 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: Expression clones containing mutated ds oligo inserts generally elicit a poor RNAi response in mammalian cells. Identify expression clones with the correct ds oligo sequence and use these clones for your RNAi analysis.

Sequencing

To facilitate sequencing of your pcDNA™6.2-GW/miR expression clones, use the miRNA forward sequencing primer and miRNA reverse sequencing primer supplied with the kit. For pcDNA™6.2-GW/EmGFP-miR expression clones, use EmGFP forward sequencing primer and miRNA reverse sequencing primer. See the diagram on page 20 for the location of the priming sites.

Note

If you download the sequence for pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR from our website, note that the overhang sequences will be shown already hybridized to their complementary sequences (e.g., TGCT will be shown hybridized to ACGA and CAGG will be shown hybridized to GTCC).

Continued on next page

Analyzing Transformants, Continued

In some cases, you may have difficulty sequencing the ds oligo insert in your expression 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 expression constructs, we suggest trying the following to improve your sequencing results:

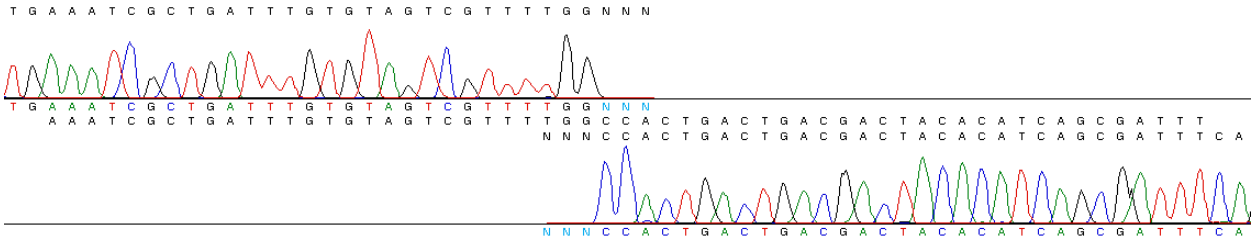
- Use high-quality, purified plasmid DNA for sequencing. We recommend preparing DNA using Life Technologies' PureLink® HQ Mini Plasmid Purification Kit.
- Add DMSO to the sequencing reaction to a final concentration of 5%.
- Increase the amount of template used in the reaction (up to twice the normal 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.

Alternative Sequencing Protocol

If sequencing problems persist, use an alternative sequencing protocol that employs *Msc* I-digestion:

1. Before sequencing, digest the vector with *Msc* I, which cuts once within the miRNA loop (and once elsewhere in the backbone).
2. Purify the digested product by cleanup on a miniprep column or extraction and precipitation (it is not necessary to gel purify).
3. Sequence the digested vector in independent runs with the forward and reverse primers. The reads will terminate in the middle of the *Msc* I site but should be very strong and clear to that point.

For an example of the sequence of the pre-miRNA insert of the positive control pcDNA™6.2-GW/ miR-*lacZ* after *Msc* I digestion see below.



BigDye® is a registered trademark of Applied Biosystems

Continued on next page

Analyzing Transformants, Continued

Long-Term Storage

Once you have identified the correct expression 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 spectinomycin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL spectinomycin.
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.

Note: Low salt LB containing 100 µg/mL Blasticidin can also be used to grow transformants.

What to Do Next

Once you have obtained your pcDNA™6.2-GW/EmGFP-miR expression clone, you have the following options:

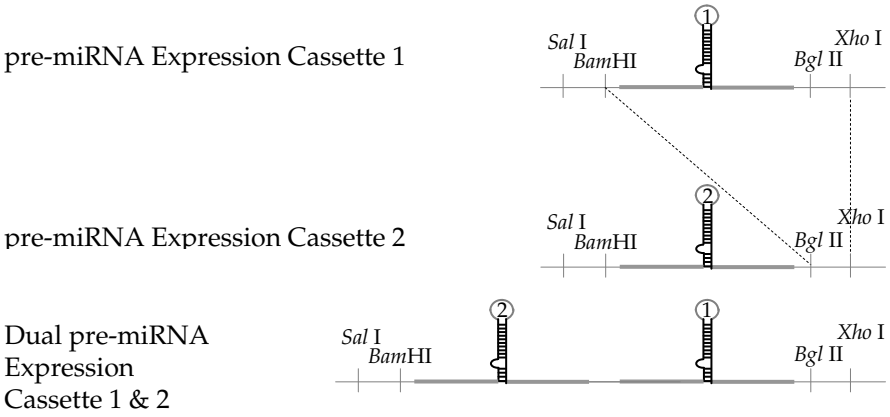
- Transfect the expression clone directly into the mammalian cell line of interest to perform transient RNAi analysis (see **Transfecting Cells**, see page 49).
- Transfect the expression clone directly into the mammalian cell line of interest and isolate **stable** transfectants that knock-down the gene of interest constitutively (see **Generating a Stable Cell Line**, page 53).
- Perform miRNA chaining to express multiple pre-miRNAs from one single construct (see **Chaining pre-miRNAs**, page 32).
- Remove the EmGFP coding sequence from your pcDNA™6.2-GW/EmGFP-miR expression clone (see **Removing EmGFP Coding Sequence**, page 34).
- Perform an LR recombination reaction with your expression construct and a suitable Gateway® destination vector to generate an expression clone in an alternative backbone (see **Performing the Rapid BP/LR Recombination Reaction**, page 38).

Chaining pre-miRNAs

Introduction

miRNAs are sometimes expressed in clusters in long primary transcripts driven by RNA Pol II (Lee *et al.*, 2004). Our vectors support chaining of miRNAs to express them in one primary transcript, thus ensuring co-cistronic expression of multiple miRNAs. In the final construct, the original pattern of restriction sites is regenerated, making the construct amenable to multiples rounds of chaining. The figure below shows the principle of chaining two miRNAs, derived from two different miRNA vectors, into one miRNA expression vector.

Note: Chaining together miRNAs targeting different genes usually results in slightly reduced knockdown of each gene. Chaining different miRNAs targeting the same gene or repeating one miRNA can enhance knock-down. Due to increased processing, EmGFP expression is attenuated by miRNA chaining.



Restriction Strategy

There are two possible strategies for restriction digestion:

1. A combination of *Bam*H I and *Xho* I to excise the pre-miRNA insert, and *Bgl* II and *Xho* I to digest the pre-miRNA expression vector used as backbone
2. A combination of *Sal* I and *Bgl* II to excise the pre-miRNA insert, and *Sal* I and *Bam*H I to digest the pre-miRNA expression vector used as backbone

Below the procedure for the first strategy is described. For the second strategy, change the restriction enzymes used in the procedure.

Before proceeding with the **Procedure for Chaining**, refer to the **Note** in **Chaining of miRNAs** (page 8)

Continued on next page

Chaining pre-miRNAs, Continued

Procedure for Chaining

Below is a protocol for chaining of miRNAs.

1. **Insert:** Digest 2 µg pcDNA™6.2-GW/EmGFP-miR-1 with 10 units *Bam*H I and 10 units *Xho* I for 2 hours at 37º C.
2. **Backbone:** Digest 1 µg pcDNA™6.2-GW/EmGFP-miR-2 with 10 units *Bgl* II and 10 units *Xho* I for 2 hours at 37º C.
3. Run fragments on 2% E-Gel® or other high percentage agarose gels.
4. Excise the backbone and insert fragments from the gel. Purify the fragments using the PureLink® Quick Gel Extraction Kit from Life Technologies or equivalent.
5. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase from Life Technologies or equivalent.
6. Transform competent cells, such as *E. coli*. One Shot® TOP10 as described on page 28.
7. Analyze resulting clones as described on pages 29.
8. Test construct for both miRNAs by transfecting cells as described on page 49.

Removing EmGFP Coding Sequence

Introduction

Depending on your experiment, you may not wish to express EmGFP from the pre-miRNA expression construct. You can remove the EmGFP coding sequence by *Dra* I digestion and self-ligation of the vector, forming a pcDNA™6.2-GW/ miR clone expressing the same pre-miRNA. This section describes a procedure for removing the EmGFP coding sequence from pcDNA™6.2-GW/EmGFP-miR.

Procedure for Removing EmGFP

Below is a protocol for removing the EmGFP Coding Sequence from pcDNA™6.2-GW/ EmGFP-miR.

1. Digest 1 µg pcDNA™6.2-GW/EmGFP-miR with 10 units *Dra* I for 2 hours at 37ºC.
2. Run fragments on 0.8% E-Gel® or other low percentage agarose gels.
3. Excise the vector fragment from the gel, purify the fragment. Purify the fragment using the PureLink® Quick Gel Extraction Kit from Life Technologies or equivalent.
4. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase from Life Technologies or equivalent.
5. Transform competent cells, such as *E. coli*. One Shot® TOP10 as described on page 28.
6. Analyze resulting clones by restriction analysis with *Dra* I; no 750 bp fragment should be visible.

Using the pT-REx™-DEST30 Vector

Introduction

Guidelines for using the pT-REx™-DEST30 vector are provided in this section.

Propagating the pT-REx™-DEST30 Vector

If you wish to propagate and maintain the pT-REx™-DEST30 vector, we recommend using Library Efficiency® DB3.1™ Competent Cells (Catalog no. 11782-018) from Life Technologies for transformation. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

Note: DO NOT use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects.

Resuspending the pT-REx™-DEST30 Vector

Before you perform the BP/LR Clonase® reaction, resuspend pT-REx™-DEST30 to 50–150 ng/µL in sterile water.

Recombination Reactions

Two recombination reactions constitute the basis of the Gateway® Technology:

BP Reaction

Facilitates recombination of an *att*B substrate (like a linearized *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone. This reaction is catalyzed by BP Clonase® II enzyme mix.

LR Reaction

Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. This reaction is catalyzed by LR Clonase® II enzyme mix.

Continued on next page

Using the pT-REx™-DEST30 Vector, Continued

Generation of new miRNA expression clones

The two recombination reactions are both needed to transfer the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR to the pT-REx™-DEST30 destination vector.

BP Reaction

pcDNA™6.2-GW/EmGFP-miR is an expression clone that contains *att*B-sites and thus needs to be recombined with a *att*P substrate (such as pDONR™221) first to form an entry clone.

LR Reaction

The destination vectors mentioned in this section all contain *att*R substrates, therefore an entry clone formed by recombination of pcDNA™6.2-GW/EmGFP-miR and a donor vector like pDONR™221 can be recombined with the destination vector to form a new miRNA expression clone.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

Transferring the Pre-miRNA Expression Cassette to Destination Vectors

Introduction

Guidelines for transferring the pre-miRNA expression cassette to you destination vector are provided in this section.

Compatible Destination Vectors

The various Gateway® vectors have widely different transcriptional and technical properties, which can be used to express the pre-miRNA. They offer custom promoter cloning, tissue-specific expression, regulated expression, and lentiviral transduction of the pre-miRNA. In addition, destination vectors providing N-terminal reporter genes can be used after removal of EmGFP. Below is a list of destination vectors that are compatible with the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits. For more information or to order the destination vectors, refer to our website (**www.lifetechnologies.com**) or contact **Technical Support** (see page 71).

Destination Vector	Quantity	Catalog no.
pLenti6.3/V5-DEST™	6 µg	V496-10
pLenti6/UbC/V5-DEST™	6 µg	V499-10
pEF-DEST51	6 µg	12285-011
pEF5/FRT/V5-DEST™ (Flp-In™)	6 µg	V6020-20
pLenti6.4/R4R2/V5-DEST™	1 kit	K4934-00
N-terminal reporter tag vectors , e.g.:		
pcDNA™6.2/nGeneBLAzer®-DEST	1 kit	12578-068
pcDNA™6.2/N-YFP-DEST	6 µg	V358-20

Note : The pLenti6.3/V5-DEST vector is also provided in the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems

Performing the Rapid BP/LR Recombination Reaction

Introduction

The Rapid BP/LR protocol is used to transfer a gene from one expression clone into another destination vector in two consecutive steps—a BP reaction using a donor vector followed by an LR recombination reaction using a destination vectors without purification of the intermediate entry clone.

Using this protocol allows you to generate expression clones more rapidly than the standard BP and LR protocols. Fewer expression clones are obtained (at least 10–20% of the total number of expression clones) using the Rapid BP/LR protocol. If you wish to maximize the number of expression clones generated, **do not** use this protocol. Use the standard BP and LR as described in the Gateway® Technology with Clonase® II manual which is available from our website (**www.lifetechnologies.com**) or by contacting **Technical Support** (see page 71).

Important

This protocol is for **experienced Gateway® users**. If you are unfamiliar with the Gateway® system, refer to the Gateway® Technology with Clonase® II manual.

Positive Control

We recommend using the pcDNA™6.2-GW/EmGFP miR-neg Control Plasmid supplied with the BLOCK-iT™ Inducible Pol II miR RNAi Expression Kits as a positive control for the Rapid BP/LR protocol. Dilute the supplied negative control plasmid 1:10 in sterile water to obtain a final concentration of 50 ng/µL.

Do not use the pEXP7-tet supplied with the BP Clonase® II Enzyme Mix or pENTR™-gus supplied with the LR Clonase® II Enzyme Mix as a positive control for the rapid protocol.

Materials Needed

- Expression clone
- pDONR™221 vector (resuspend to 150 ng/µL in sterile water).
- pT-REx™-DEST30 (150 ng/µL in TE Buffer, pH 8.0)
- pcDNA™6.2-GW/EmGFP miR-neg control, if desired (supplied with the kit)
- BP Clonase® II enzyme mix
- LR Clonase® II enzyme mix
- 2 µg/µL Proteinase K solution (supplied with Clonase® enzymes; thaw and keep on ice until use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Sterile 0.5-mL microcentrifuge tubes

Continued on next page

Performing the Rapid BP/LR Recombination Reaction, Continued

Linearizing Expression Clones

We recommend that you linearize the expression clone using *Eag* I or *Bsr*D I.

1. Linearize 1–2 µg of the expression clone with a restriction enzyme (such as *Eag* I or *Bsr*D I) that does not digest within the region of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50–150 ng/µL.

Setting Up the Rapid BP/LR Recombination Reaction

Follow this procedure to perform the Rapid BP/LR reaction between your linearized expression clone, suitable donor vector, and regular Gateway® destination vector.

1. Add the following components to sterile 0.5-mL microcentrifuge tubes at room temperature and mix.

Component	Sample	Positive Control
Linearized *att*B expression clone, (60–150 ng)	1–7 µL	—
pcDNA™6.2-GW/miR-neg control (50 ng/µL)	—	2 µL
pDONR™221 vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the BP Clonase® II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Vortex the BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. To the sample above, add 2 µL of BP Clonase® II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return BP Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate the reaction at 25°C for 1 hour.

   Important: Unlike the standard BP reaction, **do not** add Proteinase K but proceed immediately to the next step.
6. Transfer 3 µL from each of the BP reaction from Step 5 to clean, sterile 0.5-mL microcentrifuge tubes.

   Note: Save the remaining BP reaction mix at –20°C. You can transform the reaction mix into One Shot® TOP10 Chemically Competent *E. coli* as described on page 28 to check the efficiency of the BP reaction and will also allow you to isolate entry clones for future use.
7. Add the following components to the microcentrifuge tubes containing the 3 µL BP-reaction at room temperature and mix.

Component	Sample	Positive Control
pT-REx™ DEST30 Destination vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	4 µL	4 µL

Continued on next page

Performing the Rapid BP/LR Recombination Reaction, Continued

Setting Up the Rapid BP/LR Recombination Reaction Continued

Protocol continued from the previous page.

8. Remove the LR Clonase® II enzyme mix from –20°C and thaw on ice (~2 minutes).
9. Vortex the LR Clonase® II enzyme mix briefly twice (2 seconds each time).
10. To the samples above, add 2 µl of LR Clonase® II enzyme mix. Mix well by pipetting up and down.

    Reminder: Return LR Clonase® II enzyme mix to –20°C immediately after use.
11. Incubate the reaction at 25°C for 2–4 hours.

    Note: The incubation time may be extended from 4 hours to overnight, if more colonies are required.
12. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37ºC.

    Note: You may store the reaction at –20°C for up to 1 week before transformation, if desired
13. Transform an appropriate *E. coli* strain as recommended for your destination vector.

    Important: 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.
14. Isolate DNA using PureLink® HQ Mini Plasmid Purification Kit or equivlent and perform restriction analysis to find a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

What to Do Next

Once you have obtained your new expression clone, we recommend you test it by transfecting an appropriate mammalian cell line to perform transient RNAi analysis if applicable (see **Transfecting Cells**, page 49). Proceed to **General Considerations for Transfection and Regulated Expression** (next page).

General Considerations for Transfection and Regulated Expression

Introduction

Once you have generated your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector entry construct, you are ready to express your miRNA 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 49 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 miRNA of interest

Take these factors into account when designing your RNAi experiments.

miRNA Expression Options

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

Option	Procedure	Benefit
1	Obtain or generate a mammalian cell line that stably expresses the Tet repressor. Use this cell line as the host for the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct. Select for a double stable cell line, if desired.	Perform transient or stable, regulated miRNA expression experiments with multiple miRNA constructs using a cell line that consistently expresses the same amount of Tet repressor.
2	Transfer the CMV/TO RNAi cassette from BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP into a suitable Gateway® destination vector (e.g., pLenti4/TO/V5-DEST) by BP/LR recombination to generate an expression clone.	Perform other RNAi applications (e.g., regulated miRNA expression in non-dividing mammalian cells using the pLenti4/TO/V5-DEST construct).
3	Transfect the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct into any non-TetR-expressing, dividing mammalian cell line. Select for a stable cell line, if desired.	Constitutively express the miRNA of interest.

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General Considerations for Transfection and Regulated Expression, Continued

Expression of Tet Repressor (TetR)

Because tetracycline-regulated miRNA expression in the BLOCK-iT™ Inducible RNAi System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line determines the level of transcriptional repression of the Tet operator sequences in your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct. **Tet repressor levels need to be sufficiently high to suitably repress basal level transcription of the miRNA, thus suppressing target gene knockdown in uninduced cells.** In addition, the most effective repression of basal miRNA expression is achieved when Tet repressor is present in mammalian cells prior to introduction of the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector 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 BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector expression 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 miRNA expression constructs in the same mammalian cell line.
- Obtain the lowest levels of basal miRNA expression (i.e., lowest levels of target gene knockdown in the absence of tetracycline)

To generate your own stable TetR-expressing cell line, see **Generating a TetR-Expressing Host Cell Line**, page 47**.** To obtain a TetR-expressing stable cell line from Life Technologies, see the **Recommendation** below.

Several T-REx™ cell lines that stably express the Tet repressor are available from Life Technologies (see page xx 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 BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector 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 Life Technologies' 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 website (**www.lifetechnologies.com**) or contact **Technical Support** (see page 71).

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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 Life Technologies (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
  Note: If you are using sensitive cell lines, such as HeLa, we recommend using Lipofectamine®LTX (page xii).
- 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 website (**www.lifetechnologies.com**) or call **Technical Support** (see page 71).

Transient vs. Stable Expression of Your miRNA

When designing your RNAi experiment, you should consider how to assay for knockdown of the target gene. After you have transfected your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector 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 miRNA to be expressed and processed.
- Select for stably transfected cells using Geneticin®. Cells will divide once or twice in the presence of lethal doses of Geneticin® Selective Antibiotic, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 3 weeks of growth in selective medium.

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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 miRNA 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 Life Technologies in powdered form (Catalog no. Q100-19).

Using Tetracycline

To induce transcription of the miRNA 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 miRNA 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 miRNA 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 miRNA (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 Life Technologies. Contact **Technical Support** (see page 71) for more information.

Using pcDNA™6/TR

Introduction

The following section contains guidelines for maintaining and propagating the pcDNA™6/TR regulatory vector. You will use the pcDNA™6/TR vector to generate a TetR expressing host cell line.

E. coli Strain

We recommend that you propagate the pcDNA6/TR© vector in the One Shot® TOP10 Chemically Competent *E. coli*, included with the kit. However, you can use other *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). Some compatible *E.coliI* strains are provided below:

Item	Quantity	Catalog no.
One Shot® TOP10F´ (chemically competent cells)	21 × 50 µL	C3030-03
Electrocomp™ TOP10F´ (electrocompetent cells)	5 × 80 µL	C665-55
Max Efficiency® DH10B™ Chemically Competent Cells	5 × 0.2 mL	18297-010

Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of Plasmids

The pcDNA™6/TR vector contains the ampicillin and blasticidin resistance genes to allow selection of the plasmid using ampicillin or blasticidin (see pages xx for more information about pcDNA™6/TR). For more information about preparation and handling of blasticidin, refer to the **Appendix**, page 60.

To propagate and maintain the pcDNA™6/TR plasmid, we recommend using the following procedure:

1. Resuspend the vector in 20 µL sterile water to prepare a 1 µg/µL stock solution. Store the stock solution at –20°C.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F´, INV F´, or equivalent.
3. Select transformants on LB agar plates containing 50 to 100 µg/mL ampicillin or 50 µg/mL blasticidin in Low Salt LB (see the **Appendix**, page 61 for a recipe).
4. Prepare a glycerol stock of each plasmid for long-term storage (see the next page for a protocol).

Continued on next page

Selection in *E. coli*

To facilitate selection of blasticidin-resistant *E. coli*, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.0. Prepare Low Salt LB broth and plates using the recipe in the **Appendix**, page 61.

Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug unless a higher concentration of blasticidin is used.

Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50 µg/mL ampicillin or 100 µg/mL blasticidin in Low Salt LB. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin or 50 µg/mL blasticidin in Low Salt LB.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids decreasing transfection efficiency. We recommend isolating DNA using the PureLink® HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or PureLink® HiPure Plasmid Midiprep Kit (Catalog no. K2100-04).

Generating a TetR-Expressing Host Cell Line

Introduction

We recommend that you first create a stable cell line that expresses only the Tet repressor, then use that cell line to create a second cell line that expresses your miR RNAi from the inducible expression plasmid. To generate a TetR-expressing host cell line, transfect the pcDNA™6/TR plasmid into your mammalian line of interest. Use Blasticidin to select for a stable cell line.

T-REx™ cell lines that stably express the Tet repressor are available from Life Technologies (see page xiv for ordering information). If you wish to assay for tetracycline-inducible expression of your gene of interest in 293, HeLa, U2OS, and lurkat cells, you may want to use one of the T-REx™ cell lines as the host to establish your double stable cell line. For more information, visit **www.lifetechnologies.com** or contact **Technical Support** (see page 71).

Note

Reminder: When generating a stable cell line expressing the Tet repressor, you will want to select for clones that express the highest levels of Tet repressor to use as hosts for your inducible expression construct. Those clones that express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your gene of interest.

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing the Tet repressor and your miRNA, you need to determine the minimum concentration of each antibiotic (Blasticidin and Geneticin®) required to kill your untransfected host cell line. For each antibiotic, test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line. Use the protocol below to determine the minimal concentrations of Geneticin® and Blasticidin required to prevent growth of the parental cell line. Refer to the **Appendix**, page 60 for instructions on how to prepare and store blasticidin.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. For each antibiotic, prepare a set of 6–7 plates. Add the following concentrations of antibiotic to each plate in a set:
   - For blasticidin selection, test 0, 1, 3, 5, 7.5, and 10 µg/mL blasticidin
   - For Geneticin® selection, test 0, and 100–1000 µg/mL Geneticin®
2. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents growth within 1–2 weeks after addition of the antibiotic.

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Generating a TetR-Expressing Host Cell Line, Continued

Possible Sites for Linearization of pcDNA™6/TR

To obtain stable transfectants, you may choose to linearize pcDNA™6/TR before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the Tet repressor gene or other elements necessary for expression in mammalian cells. The table below lists unique sites that may be used to linearize your construct prior to transfection. Other restriction sites are possible.

Enzyme	Restriction Site (bp)	Location	Supplier
*Bst*1107 I	4470	Backbone	AGS*, Fermentas, Takara
Sap I	4733	Backbone	New England Biolabs
*Bsp*LU11 I	4849	Backbone	Boehringer-Mannheim
*Eam*1105 I	5739	Ampicillin gene	AGS*, Fermentas, Takara
Fsp I	5961	Ampicillin gene	Many

*Angewandte Gentechnologie Systeme

Selection of Stable Integrants

Once you have determined the appropriate antibiotic concentrations to use for selection, you can generate a stable cell line expressing pcDNA™6/TR and your inducible miR RNAi construct.

1. Transfect mammalian cells with pcDNA™6/TR using the desired protocol. Remember to include a plate of untransfected cells as a negative control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing blasticidin at the pre-determined concentration required for your cell line. Split the cells such that the cells are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Feed the cells with selective medium every 3–4 days until foci can be identified.
5. Pick at least 20 foci and expand them to test for tetracycline-inducible gene expression by transiently transfecting with the positive control plasmid expressing β-galactosidase. Screen for those clones that exhibit the lowest levels of basal transcription and the highest levels of β-galactosidase expression after addition of tetracycline (see below).

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

Transfecting Cells

Introduction

This section provides general guidelines to transfect your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector 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 miRNA 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 miRNA of interest, see the next section.

Factors Affecting Gene Knockdown Levels

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

- Transfection efficiency
- Transcription rate of the target gene of interest
- Stability of the target protein
- Growth characteristics of your mammalian cell line
- Efficacy of the miRNA of interest
- Activity of the promoter driving the miRNA expression cassette

Take these factors into account when designing your RNAi experiments.

Important

Reminder: For optimal results, we recommend that you transfect your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct into a mammalian cell line that stably expresses high levels of the Tet repressor (i.e., use one of Life Technologies' 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 BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector plasmid with pcDNA™6/TR into your mammalian cell line. If you wish to use this method, **we recommend using 6-fold more TetR expression plasmid DNA than BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector plasmid DNA in the co-transfection.** For example, use 600 ng of pcDNA™6/TR plasmid and 100 ng of BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector 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:BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector used for transfection.

Plasmid Preparation

Once you have obtained your expression 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® Plasmid DNA Purification Kits.

Positive and Negative Controls

If you have performed the positive control reaction and have cloned the miR-*lacZ* positive ds control oligo supplied with the kit into pcDNA™6.2-GW/EmGFP-miR, we recommend using the resulting pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression construct as a positive control to assess the RNAi response in your cell line. Simply co-transfect the pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression construct and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your mammalian cells and assay for knockdown of β-galactosidase expression 24–48 hours post-transfection using western analysis or activity assay. For optimal results, we recommend using 6-fold more expression construct DNA than reporter plasmid DNA in the co-transfection. For example, use 600 ng of pcDNA™6.2-GW/miR-*lacZ* DNA and 100 ng of pcDNA™1.2/V5-GW/*lacZ* DNA when transfecting cells plated in a 24-well format. 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.

As **negative control**, perform parallel transfections with the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid.

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

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

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

Materials Needed

- 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, maintain the cells in medium containing the appropriate concentration of Blasticidin.
- BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector expression construct (pT-REx™-DEST30)
- pcDNA™1.2/V5-GW/*lacZ* plasmid (if performing the positive control transfection; supplied with the kit)
- pT-REx/GW-30/EmGFP-miR-*lacZ* plasmid (if you have performed the positive control ligation and BP/LR reactions and are performing the positive control transfection)
- Transfection reagent of choice (e.g., Lipofectamine® 2000)
- Tetracycline (supplied with the kit, 10 mg/mL stock solution)
- Appropriate tissue culture dishes and supplies

Continued on next page

Transfecting Cells, Continued

Guidelines for Transfection and Induction

Guidelines are provided below to transfect your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector into the TetR-expressing mammalian cell line of choice and to induce expression of the miRNA 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 BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct into cells following the recommendations of the manufacturer of your transfection reagent. If you are co-transfecting the BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct and a TetR expression plasmid or the pcDNA™1.2/V5-GW/*lac*Z and pT-REx/GW-30/EmGFP-miR-*lac*Z plasmids, use the appropriate amounts of each plasmid as recommended on page 49.
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 miRNA expression. Note the following:
   - If you have transfected your cells using Lipofectamine® 2000, you may add tetracycline to induce expression of your miRNA 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 *lacZ* miRNA 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 expression clone containing the lacZ ds oligo (i.e., pT-REx/GW-30/EmGFP-miR-lacZ), you may assay for β-galactosidase expression and knockdown by western analysis or activity assay using cell-free lysates (Miller, 1972). Life Technologies 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 Life Technologies (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 website (**www.lifetechnologies.com**) or call **Technical Support** (see page 71).

Continued on next page

Detecting Fluorescence

Introduction

You can perform analysis of the EmGFP fluorescent protein from the expression clone in either transiently transfected cells or stable cell lines. Once you have transfected your expression clone into mammalian cells, you may detect EmGFP protein expression directly in cells by fluorescence microscopy or other methods that use light excitation and detection of emission. See below for recommended fluorescence microscopy filter sets.

Filters for Use with EmGFP

The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein such as the Omega XF100 filter set for fluorescence microscopy.

The spectral characteristics of EmGFP are listed in the table below:

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509

For information on obtaining these filter sets, contact Omega Optical, Inc. (www.omegafilters.com) or Chroma Technology Corporation (**www.chroma.com**).

Fluorescence Microscope

You may view the fluorescence signal of EmGFP in cells using an inverted fluorescence microscope with FITC filter or Omega XF100 filter (available from www.omegafilters.com) for viewing cells in culture or a flow cytometry system.

Color Camera

If desired, you may use a color camera that is compatible with the microscope to photograph the cells. We recommend using a digital camera or high sensitivity film, such as 400 ASA or greater.

Detecting Transfected Cells

After transfection, allow the cells to recover for 24 to 48 hours before assaying for fluorescence. Medium can be removed and replaced with PBS during viewing to avoid any fluorescence due to the medium. Be sure to replace PBS with fresh medium if you wish to continue growing the cells.

Note: Cells can be incubated further to optimize expression of EmGFP.

Note

We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable.

Generating a Stable Inducible miRNA Expressing Cell Line

Introduction

Once you have established that your miRNA can be inducibly expressed from pT-REx/GW-30/EmGFP-miR, you may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your miRNA. As with transient transfection, we recommend using a cell line that stably expresses the Tet repressor as a host for your BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector construct. Use a T-REx™ Cell Line available from Life Technologies or your own TetR-expressing cell line (see page 47 for guidelines to generate the cell line). The pT-REx™-DEST30 vector contains the neomycin resistance gene to allow selection of stable cell lines using Geneticin®. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Geneticin®. General guidelines are provided below.

To obtain stable transfectants, we recommend that you linearize your pT-REx/GW-30/EmGFP-miR construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, cut at a unique site that is neither located within a critical element nor within your gene of interest.

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern & Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® is available from Life Technologies (see page xii). Use as follows:

1. Prepare Geneticin® in a buffered solution (e.g., 100 mM HEPES, pH 7.3).
2. Use 100 to 1000 µg/mL of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin®.

Cells will divide once or twice in the presence of lethal doses of Geneticin® Selective Antibiotic, so the effects of the drug take several days to become apparent. Complete selection can take from 2–3 weeks of growth in selective medium.

Continued on next page

Generating a Stable Inducible miRNA Expressing Cell Line, Continued

Materials Needed

- 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.
- BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector (pT-REx™-DEST30)
- Transfection reagent of choice (e.g., Lipofectamine® 2000)
- Geneticin® in a buffered solution (e.g., 100 mM HEPES, pH 7.3)
- Blasticidin (to maintain the pcDNA™6/TR or pLenti6/TR construct) in the TetR-expressing cell line
- Tetracycline (supplied with the kit, 10 mg/mL stock solution)
- Appropriate tissue culture dishes and supplies

Guidelines for Transfection and Selection

Guidelines are provided below to transfect the pT-REx™-DEST30 vector into the TetR-expressing mammalian cell line of choice and to select for stable cell lines using Geneticin®.

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 pT-REx™-DEST30 vector 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 Geneticin® **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 Geneticin®
5. Replace medium with fresh medium containing Blasticidin and Geneticin® every 3–4 days until Blasticidin- and Geneticin®-resistant colonies can be identified (generally 10–14 days after selection).
6. Pick at least 10 Blasticidin- and Geneticin® -resistant colonies and expand each clone.
7. Induce expression of the miRNA of interest by adding tetracycline to a final concentration of 1 µg/mL. Assay for target gene knockdown 24–48 hours post-transfection. Compare to uninduced cells.

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 22).
	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 22.
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.
	Did not anneal or annealed incorrect BLOCK-iT™ miR RNAi Select oligos	Anneal the two oligos marked "top" and "bot" with the same miR RNAi identifier (see page 21)

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 spectinomycin-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 pcDNA™6.2-GW/EmGFP-miR: Top strand oligo: include TGCT on the 5′ end. Bottom strand oligo: include CCTG on the 5′ end.
	ds oligos were degraded	Store the 10 nM ds oligo stock in 1X Oligo Annealing Buffer. Avoid repeated freeze/thaw cycles. Aliquot the 10 nM ds oligo stock and store at –20°C.

Continued on next page

Ligation and Transformation Reactions, Continued

Problem	Reason	Solution
Few spectinomycin-resistant colonies obtained on the selective plate, continued	ds oligos stored incorrectly	Store the ds oligo stocks at –20°C.
	500 nM ds oligo stock solution diluted into water instead of 1X Oligo Annealing Buffer	To dilute the 50 µM ds oligo reaction: Dilute the 50 µM stock 100-fold into DNase/RNase-free water to generate a 500 nM stock. Dilute the 500 nM stock 50-fold into 1X Oligo Annealing Buffer to generate a 10 nM stock. Use the 10 nM stock for cloning.
	10 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 pcDNA™6.2-GW/EmGFP-miR.
	Incorrect vector: insert ratio used in ligation reaction Forgot to dilute annealed target ds oligo or miR-*lacZ* positive ds control oligo 1:5,000 before use Annealed ds oligo diluted incorrectly	Dilute the 50 µM ds oligo mixture as instructed on page 23 to generate a 10 nM stock. Use the 10 nM ds oligo stock for cloning.
	Ligation reaction not adequately mixed or incorrectly mixed prior to incubation	Mix the ligation reaction well by pipetting up and down. Note: Flicking the tube is not adequate to mix the reagents. Do not vortex the ligation reaction.
	Did not use the 5X Ligation Buffer supplied with the kit	Use the T4 DNA Ligase and 5X Ligation Buffer supplied with the kit for ligation as these reagents have been optimized to facilitate 5-minute ligation at room temperature. Important: Other T4 DNA Ligase and ligation buffers may not support 5-minute, room temperature ligation.
	Ligation reaction not incubated for long enough	Extend the incubation time of the ligation reaction up to 2 hours at room temperature.
	Ligation reaction incubated overnight at 16°C	The ligation conditions used to clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR differ from traditional ligation conditions. Incubate the ligation reaction at room temperature for 5 minutes.

Continued on next page

Troubleshooting, Continued

Ligation and Transformation Reactions, Continued

Problem	Reason	Solution
Few spectinomycin-resistant colonies obtained on the selective plate, continued	Not enough transformation mixture plated	Increase the amount of the transformation mixture plated.
	Selective plates contained too much spectinomycin	Use LB agar plates containing 50 µg/mL spectinomycin for selection.
	Did not use the competent cells supplied with the kit	Use the One Shot® TOP10 Chemically Competent E. coli supplied with the kit; transformation efficiency is >1 × 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 Life Technologies' custom primer synthesis service (see our website 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 × 10^9 cfu/µg DNA.
Poor sequencing results	Loss of sequencing signal in the hairpin region due to secondary structure formation	Use high-quality, purified plasmid DNA for sequencing. Add DMSO to the sequencing reaction to a final concentration of 5%. Increase the amount of template used for sequencing (up to twice the normal amount). Use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction. Cut your construct with Msc I and purify before sequencing (see **Alternative Sequence Protocol**, page 30)
No colonies obtained on the selective plate	Used the wrong antibiotic for selection	Select for transformants on LB agar plates containing 50 µg/mL spectinomycin.

Continued on next page

Troubleshooting, Continued

Transfection and RNAi Analysis

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

Problem	Reason	Solution
Low levels of gene knockdown observed due to low transfection efficiency	Antibiotics added to the media during transfection if using Lipofectamine® 2000 Reagent	Do not add antibiotics to the media during transfection.
	Cells too sparse at the time of transfection	Plate cells such that they will be 90–95% confluent at the time of transfection.
	Not enough plasmid DNA transfected	Increase the amount of plasmid DNA transfected.
	Not enough Lipofectamine® 2000 used	Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine® 2000 used.
Low levels of gene knockdown observed (other causes)	Didn't wait long enough after transfection before assaying for gene knockdown	Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown. Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs.
	ds oligo insert in your pcDNA™6.2-GW/EmGFP-miR construct contains mutations	When analyzing spectinomycin-resistant transformants, sequence the ds oligo insert to verify its sequence. Select constructs containing the correct ds oligo insert for use in RNAi analysis.
	miRNA sequence not optimal due to selected target region.	Select a different target region. Order BLOCK-iT™ miR RNAi Select for your target gene (see page xiii), which allows you to generate four different miR RNAi expression vectors. We guarantee that at least two out of the four BLOCK-iT™ miR RNAi Select expression vectors will result in >70% knockdown of the target gene (provided that the transfection efficiency in your experiment is at least 80%).

Continued on next page

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 or PureLink® HiPure Plamid Midiprep Kit to prepare purified plasmid DNA.
	Targeted an essential gene	Make sure that your target gene is not essential for cell viability or growth.
No gene knockdown observed	miRNA with no activity chosen	Select a different target region. Order BLOCK-iT™ miR RNAi Select for your target gene (see page xiii), which allows you to generate four different miR RNAi expression vectors. We guarantee that at least two out of the four BLOCK-iT™ miR RNAi Select expression vectors will result in >70% knockdown of the target gene (provided that the transfection efficiency in your experiment is at least 80%).
	pre-miRNA designed incorrectly	Follow the guidelines on page 16 to select the target sequence and design the single-stranded oligos.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a different target region. Order BLOCK-iT™ miR RNAi Select for your target gene (see page xiii), which are designed to limit off-target effects.
No fluorescence signal detected with expression clone containing EmGFP	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter sets for detection of fluorescence (see page 52). Be sure to use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for additional days before assaying for fluorescence. **Note:** We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable.

Appendix

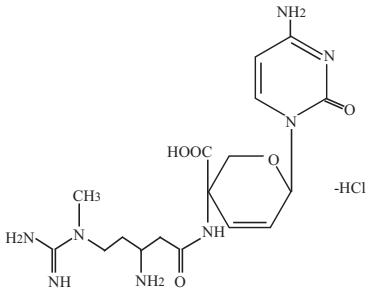
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g., a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Life Technologies (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5–10 mg/mL.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at –20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at +4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at +4°C for up to 2 weeks.

Recipes

Spectinomycin

Use this procedure to prepare a 10 mg/mL stock solution of spectinomycin.

Materials Needed

- Spectinomycin Dihydrochloride (Sigma, Catalog no. S4014)
- Sterile, deionized water

Procedure

1. Weigh out 50 mg of spectinomycin and transfer to a sterile centrifuge tube.
2. Resuspend the spectinomycin in 5 mL of sterile, deionized water to produce a 10 mg/mL stock solution.
3. Filter-sterilize.
4. Store the stock solution at +4°C for up to 2 weeks. For long-term storage, store at –20°C.

LB (Luria-Bertani) Medium and Plates

Composition:
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 at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.
5. For LB agar plates:
6. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
7. Autoclave on liquid cycle for 20 minutes at 15 psi.
8. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
9. Let harden, then invert and store at +4°C.

Low Salt LB Plates with Blasticidin

Composition:
10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.0 with 1 N NaOH and bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 g/mL final concentration.
4. Let harden, then invert and store at +4°C.

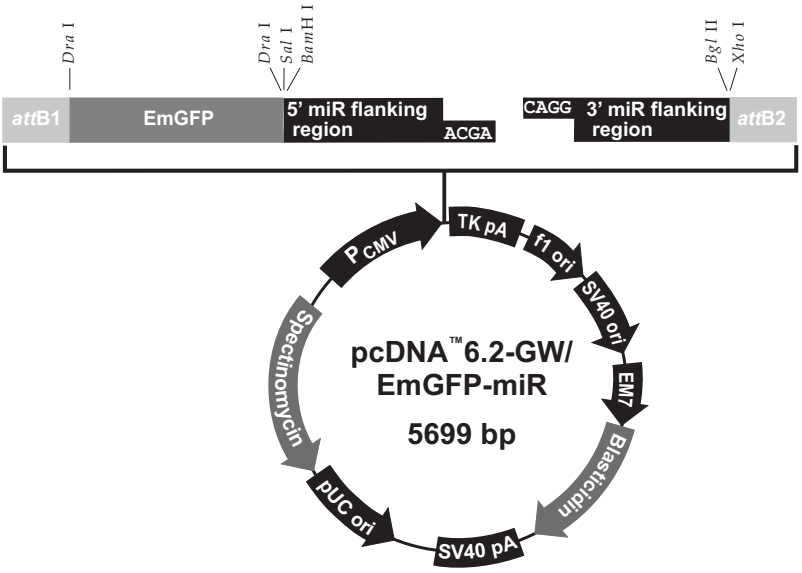
Store plates at +4°C in the dark. Plates containing Blasticidin S HCl are stable for up to 2 weeks.

Map of pcDNA™6.2-GW/ EmGFP-miR

pcDNA™6.2-GW/EmGFP-miR

The figure below shows the features of the pcDNA™6.2-GW/EmGFP-miR vector. The vector is supplied linearized between nucleotides 1518 and 1519 with 4 base pair 5′ overhangs on each strand as indicated. **The complete sequence of pcDNA™6.2-GW/ EmGFP-miR is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**

Note: For an explanation of the features see page 64.



Comments for pcDNA™ 6.2-GW/EmGFP-miR
5699 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680 - 704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
5' overhang (C): bases 1515-1518
5'overhang: bases 1519-1522
3' miR flanking region: bases 1519-1563
*att*B2 site (C): bases 1592-1616
miRNA reverse sequencing primer site (C): bases 1607-1626
TK polyadenylation signal: bases 1645-1916
f1 origin: bases 2028-2456
SV40 early promoter and origin: bases 2483-2791
EM7 promoter: bases 2846-2912
Blasticidin resistance gene: bases 2913-3311
SV40 polyadenylation signal: bases 3469-3599
pUC origin (C): bases 3737-4410
Spectinomycin resistance gene (C): bases 4480-5490
Spectinomycin promoter (C): bases 5491-5624

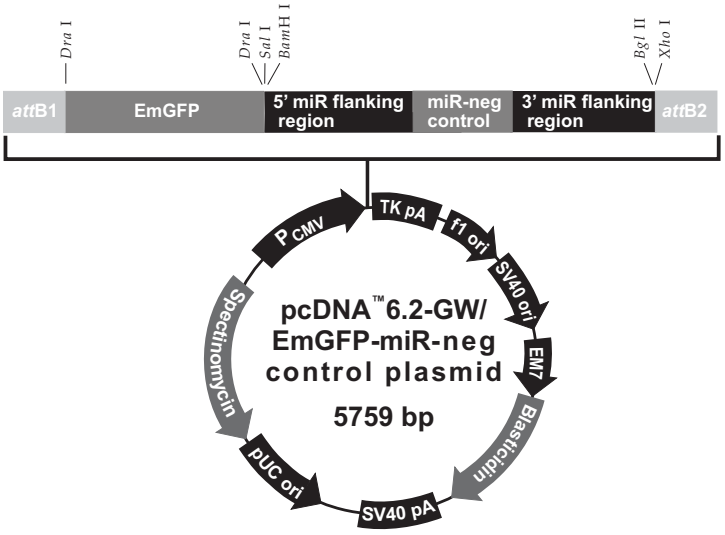
(C) = Complementary strand

Map of pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid

pcDNA™6.2-GW/EmGFP-miR-Negative Control Plasmid

The figure below shows the features of the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid. The vector contains an insert between bases 1519 and 1578 that can form a hairpin structure just as a regular pre-miRNA, but is predicted not to target any known vertebrate gene. The insert has been cloned according to the instructions in this manual (see page 16). Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with pcDNA™6.2-GW/EmGFP-miR expression vectors. **The complete sequence of pcDNA™6.2-GW/EmGFP-miR-neg control plasmid is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**

Note: For an explanation of the features see page 64.



Comments for pcDNA™ 6.2-GW/EmGFP-miR-neg control plasmid
5759 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680-704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
miR-neg control: bases 1519-1578
3' miR flanking region: bases 1579-1623
*att*B2 site (C): bases 1652-1676
miRNA reverse sequencing primer site (C): bases 1667-1686
TK polyadenylation signal: bases 1705-1976
f1 origin: bases 2088-2516
SV40 early promoter and origin: bases 2543-2851
EM7 promoter: bases 2906-2972
Blasticidin resistance gene: bases 2973-3371
SV40 polyadenylation signal: bases 3529-3659
pUC origin (C): bases 3797-4470
Spectinomycin resistance gene (C): bases 4540-5550
Spectinomycin promoter (C): bases 5551-5684

(C) = Complementary strand

Features of pcDNA™6.2-GW/EmGFP-miR

Explanation of Features

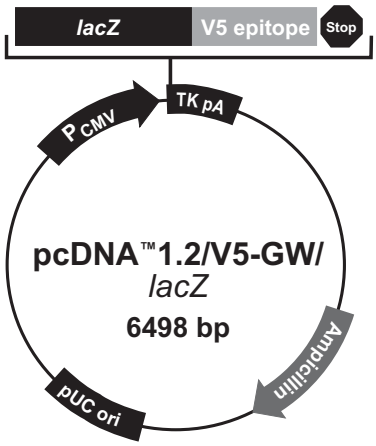
pcDNA™6.2-GW/EmGFP-miR vectors contain the following elements. All features have been functionally tested and the vectors have been fully sequenced.

Feature	Benefit
CMV promoter	Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
*att*B1 and *att*B2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the expression construct with a Gateway® destination vector (Landy, 1989).
EmGFP coding sequence	Allows visual detection of transfected mammalian cells using fluorescence microscopy
EmGFP forward sequencing primer	Allows sequencing of the insert
5′ miR flanking region	Allows formation of functional engineered pre-miRNA
5′ overhangs	Allows ligase-mediated directional cloning of the double-stranded oligonucleotide of interest.
miR-neg control	Allows formation of a pre-miRNA hairpin sequence predicted not to target any known vertebrate gene (only for pcDNA™6.2-GW/EmGFP-miR-neg control). Sequence without 5′ overhangs is shown below: `5′-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′`
3′ miR flanking region	Allows formation of functional engineered pre-miRNA
miRNA reverse sequencing primer	Allows sequencing of the insert
TK polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transfected mammalian cell lines (Kimura *et al.*, 1994).
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.
Spectinomycin resistance gene (*aad*A1)	Allows selection of the plasmid in *E. coli* (Liebert *et al.*, 1999).
Spectinomycin promoter	Allows expression of the spectinomycin resistance gene 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 Life Technologies (Catalog no. 12537-023). Briefly, a MultiSite Gateway® LR recombination reaction was performed with pDEST™R4-R3 and entry clones containing the CMV promoter, *lacZ* gene, and V5 epitope and TK polyadenylation signal (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 website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**



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

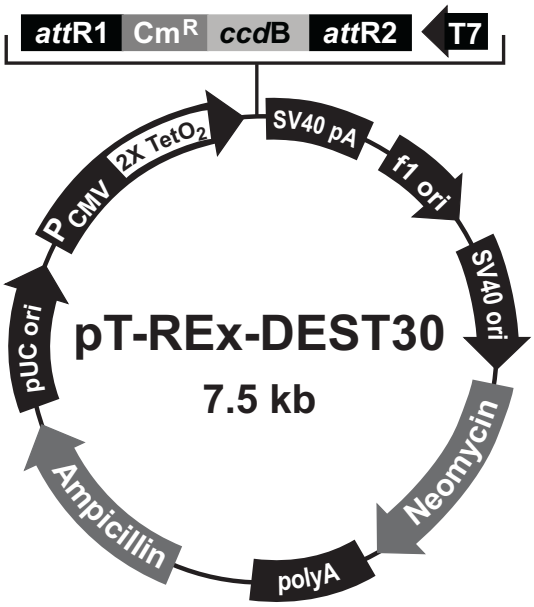
*att*B4: bases 5-25
CMV promoter: bases 137-724
*att*B1: bases 614-637
LacZ fusion protein: bases 643-3798
LacZ ORF: bases 643-3714
*att*B2: bases 3716-3739
V5 epitope: bases 3739-3780
lacZ forward 2 priming site: 840-859
lacZ reverse 2 priming site: 1820-1839 (C)
TK polyadenylation signal: bases 3807-4078
*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

Map of pT-REx™-DEST30

Map

The map below shows the elements of pT-REx™-DEST30. DNA from the entry clone replaces the region between bases 706 and 2389. **The complete sequence of pT-REx™-DEST30 is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**



Comments for pT-REx-DEST30
7544 nucleotides

CMV promoter: bases 1-588
TATA box: bases 502-508
Tetracycline operator (2X $TetO_2$) sequence: bases 518-557
*att*R1 recombination site: bases 699-823
Chloramphenicol resistance gene: bases 932-1591
*ccd*B gene: bases 1933-2238
*att*R2 recombination site: bases 2279-2403
T7 promoter: bases 2464-2483 (complementary strand)
SV40 polyadenylation region: bases 2915-3045
f1 origin: bases 3175-3603
SV40 early promoter and origin: bases 3790-4098
Neomycin resistance ORF: bases 4157-4951
Polyadenylation region: bases 5015-5063
Ampicillin (*bla*) resistance ORF: bases 5474-6334
pUC origin: bases 6479-7152

Features of the pT-REx™-DEST30 Vector

Features

pT-REx™-DEST30 (7544 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
Tetracycline operator (O_2) sequences	Two tandem 19 nucleotide repeats which serve as binding sites for Tet repressor homodimers (Hillen and Berens, 1994)
*att*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone
Chloramphenicol resistance gene	Allows counterselection of expression clones
*ccd*B gene	Allows negative selection of expression clones
T7 promoter (complementary strand)	Allows efficient *in vitro* transcription in the antisense orientation
SV40 polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin & Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
Polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
Ampicillin resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*
pUC origin	Allows high-copy number replication and growth in *E. coli*

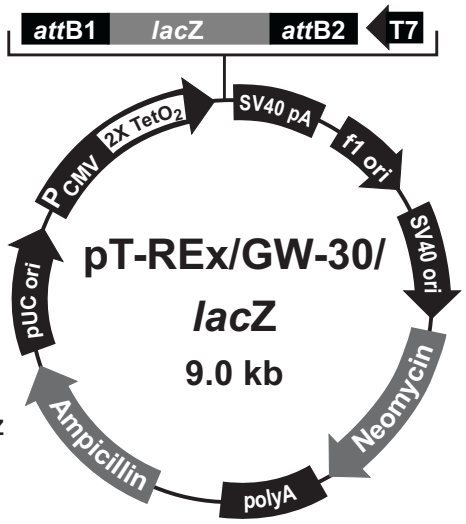
Map of pT-REx™/GW-30/lacZ

Description

pT-REx™/GW-30/*lac*Z is an 8999 bp control vector containing the gene for β-galactosidase. pT-REx™/GW-30/*lac*Z was constructed using the Gateway® LR recombination reaction between an entry clone containing the *lac*Z gene and pT-REx™-DEST30. The molecular weight of β-galactosidase is approximately 116 kDa.

Map of pT-REx/GW-30/*lac*Z

The map below shows the elements of pT-REx/GW-30/*lac*Z. **The complete sequence of pT-REx/GW-30/*lac*Z is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**



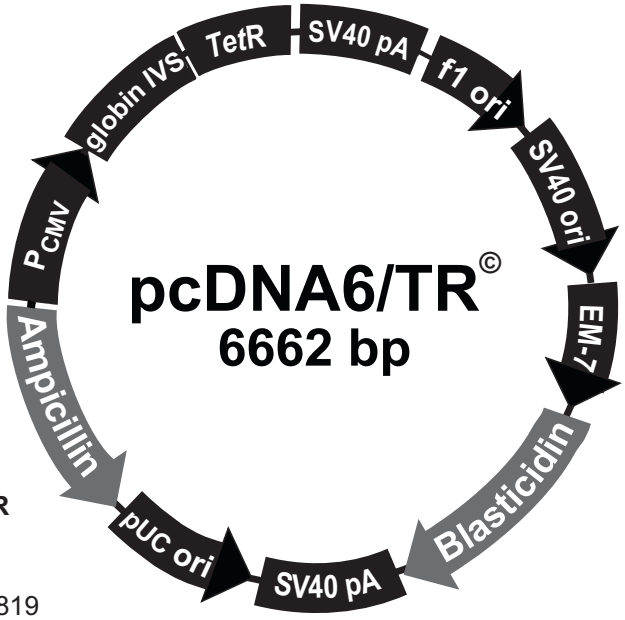
Comments for pT-REx/GW-30/*lac*Z
8999 nucleotides

CMV promoter: bases 1-588
TATA box: bases 502-508
Tetracycline operator (2X $TetO_2$) sequence: bases 518-557
*att*B1 recombination site: bases 699-723
*lac*Z ORF: bases 743-3799
*att*B2 recombination site: bases 3834-3858
T7 promoter: bases 3919-3938 (complementary strand)
SV40 polyadenylation region: bases 4370-4500
f1 origin: bases 4630-5058
SV40 early promoter and origin: bases 5245-5553
Neomycin resistance ORF: bases 5612-6406
Polyadenylation region: bases 6470-6518
Ampicillin (*bla*) resistance ORF: bases 6929-7789
pUC origin: bases 7934-8607

Map of pcDNA™6/TR Vector

Map of pcDNA™6/TR

pcDNA™6/TR is a 6662 bp vector that expresses the Tet repressor under the control of the human CMV promoter. The figure below summarizes the features of the pcDNA™6/TR vector. **The complete sequence for pcDNA™6/TR is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 71).**



Comments for pcDNA6/TR
6662 nucleotides

CMV promoter: bases 232-819
Rabbit β-globin intron II: bases 1028-1600
*Tet*R gene: bases 1684-2304
SV40 early polyadenylation sequence: bases 2346-2477
f1 origin: bases 2897-3325
SV40 promoter and origin: bases 3335-3675
EM-7 promoter: bases 3715-3781
Blasticidin resistance gene: bases 3782-4180
SV40 early polyadenylation sequence: bases 4338-4468
pUC-derived origin: bases 4851-5521
bla promoter: bases 6521-6625 (C)
Ampicillin (*bla*) resistance gene: bases 5666-6526 (C)

TetR Gene

The *Tet*R gene used in pcDNA™6/TR was originally isolated from the Tn*10* transposon which confers resistance to tetracycline in *E. coli* and other enteric bacteria (Postle *et al.*, 1984). The *Tet*R gene from Tn*10* encodes a class B Tet repressor and is often referred to as *Tet*R(B) in the literature (Hillen and Berens, 1994).

The *Tet*R gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa. For more information about the Tet repressor and its interaction with the Tet operator, please refer to the review by Hillen and Berens (1994).

Continued on next page

Features of pcDNA™6/TR Vector

Features of pcDNA™6/TR

The table below describes the relevant features of pcDNA™6/TR. The vector includes the rabbit β-globin intron II to enhance expression of the *Tet*R gene. For a more detailed description of the *Tet*R gene and the Tet repressor, please refer to the previous page. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of the *Tet*R gene (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
Rabbit β-globin intron II (IVS)	Enhances expression of the *Tet*R gene (van Ooyen *et al.*, 1979) in cultured cells
*Tet*R gene	Encodes the Tet repressor that binds to *tet* operator sequences to repress transcription of the gene of interest in the absence of tetracycline (Postle *et al.*, 1984; Yao *et al.*, 1998)
SV40 early polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen
EM-7 promoter	Synthetic prokaryotic promoter for expression of the blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura et al., 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Permits high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

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Introduction

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Life Technologies 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. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

Life Technologies 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. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

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 Life Technologies 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 Life Technologies' 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 Life Technologies' licensing department at 760-603-7200.

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Notes

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
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