invitrogen™

BLOCK-iT™ Pol II miR RNAi Expression Vector Kits

Gateway®-adapted expression vector for the expression of microRNA (miRNA) in mammalian cells under control of Pol II promoters

Catalog nos. K4935-00, K4936-00, K4937-00, K4938-00

Version F
December 29, 2010
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User Manual

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Expression Clone Generation for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits. If you are performing the annealing, cloning, or transformation procedures for the first time, follow the detailed protocols provided in the manual.

Step	Action
Design single-stranded DNA oligos	Follow the guidelines on pages 14-18 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/miR or 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.

Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Product	Catalog no.
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	K4935-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP	K4936-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System	K4937-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP	K4938-00

Kit Components

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits and BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems include the following components. For a detailed description of the contents of the BLOCK-iT™ miRNA Expression Vector Kits, see pages vii-ix. For a detailed description of the contents of the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression reagents, see the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual.

Component	Catalog no.			
	K4935-00	K4936-00	K4937-00	K4938-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	√		√	
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP		√		√
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Reagents			√	√

Shipping/Storage

The BLOCK-iT™ miRNA Expression Vector Kits are shipped as described below. Upon receipt, store each item as detailed below.

Note: For information about the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Reagents (Box 3-11) supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems, refer to the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual.

Box	Component	Shipping	Storage
1	BLOCK-iT™ Pol II miR RNAi Expression Vector Reagents **or** BLOCK-iT™ Pol II miR RNAi Expression Vector Reagents with EmGFP	Dry ice	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	-80°C

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BLOCK-iT™ Pol II miR RNAi Expression Vector Reagents

The following reagents are included with the BLOCK-iT™ Pol II miR RNAi Expression Vector Reagents or the BLOCK-iT™ Pol II miR RNAi Expression Vector Reagents with EmGFP (Box 1). **Store the reagents at -20°C.**

Reagent	Composition	Amount
pcDNA™6.2-GW/miR, linearized **or** 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 x 10 µl
10X Oligo Annealing Buffer	100 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 1 M NaCl	250 µl
DNase/RNase-Free Water	--	3 x 1.5 ml
5X Ligation Buffer	250 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP 5 mM DTT 25% (w/v) polyethylene glycol-8000	80 µl
T4 DNA Ligase	1 (Weiss) U/µl in 10 mM Tris-HCl, pH 7.5 50 mM KCl 1 mM DTT 50% (v/v) glycerol	20 µl
miRNA forward sequencing primer **or** 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/miR-neg control plasmid **or** pcDNA™6.2-GW/EmGFP-miR-neg control plasmid	500 ng/µl in TE Buffer, pH 8.0	20 µl

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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.

Primer Sequences

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

Primer	Sequence	Amount
miRNA forward sequencing primer **or** EmGFP forward sequencing primer	5′- TCCCAAGCTGGCTAGTTAAG –3′ **or** 5′- GGCATGGACGAGCTGTACAA -3′	2 µg (327 pmol) **or** 2 µg (323 pmol)
miRNA reverse sequencing primer	5′- CTCTAGATCAACCACTTTGT -3′	2 µg (332 pmol)

LacZ 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 re-annealed and diluted 5000-fold to 10 nM (see page 22) before use in the ligation reaction (page 26).

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

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One Shot® TOP10 Reagents

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

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

Genotype of TOP10 Cells

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*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

BLOCK-iT™ Lentiviral RNAi Expression Reagents

In addition to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits and the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Vector Systems also include the following components to facilitate production of a replication-incompetent lentivirus that expresses your microRNA (miRNA) of interest.

- pLenti6/V5-DEST Gateway® Vector
- Gateway® LR Clonase™ II Enzyme Mix
- Gateway® BP Clonase™ II Enzyme Mix
- One Shot® Stbl3™ Chemically Competent *E. coli*
- ViraPower™ Bsd Lentiviral Support Kit
- 293FT Cell Line
- pDONR™221

Refer to the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual supplied with Catalog nos. K4937-00 and K4938-00 for a detailed description of the lentiviral expression reagents provided with the kit and instructions to produce lentivirus. For instructions to grow and maintain the 293FT Cell Line, refer to the 293FT Cell Line manual, available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

Accessory Products

Introduction

The products listed in this section may be used with the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 65).

Accessory Products

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

Item	Amount	Catalog no.
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Vector System	20 reactions	K4937-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP	20 reactions	K4938-00
BLOCK-iT™ Pol II miR-XXXX Validated miRNA DuoPak (XXXX=gene symbol)	10 µg	V49300-01 through V49300-53
BLOCK-iT™ miR RNAi Select	50 nmol scale	See page xi
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
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plamid 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
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 x 10^6 cells, frozen	R700-07
Blasticidin	50 mg	R210-01
Purelink™ Quick Gel Extraction Kit	50 preps	K2100-12
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
LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013

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Accessory Products, Continued

Spectinomycin

For selection of pcDNA™6.2-GW/± EmGFP-miR transformants in *E. coli*, you will need to obtain spectinomycin. Spectinomycin Dihydrochloride is available from Sigma (Catalog no. S4014). For a recipe to prepare spectinomycin for use, see page 55.

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

Invitrogen 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 4 miR sequences per gene that are supplied as 8 tubes containing 4 top oligos and 4 bottom DNA oligos. Upon annealing and cloning into one of the BLOCK-iT™ Pol II miR RNAi Expression vectors, pcDNA™6.2-GW/miR or 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 lenti vectors 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%).

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

BLOCK-iT™ RNAi Products

A large variety of BLOCK-iT™ RNAi products are available from Invitrogen 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 Service (page 65).

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Gateway® Destination Vectors

A large selection of Gateway® destination vectors are available from Invitrogen to facilitate the transfer of the pre-miRNA sequence into a suitable destination vector to allow the miRNA expression in multiple systems including viral expression systems and tissue-specific expression. See below for a list of compatible destination vectors.

Destination Vector	Catalog No.
pLenti6/V5-DEST™	V496-10
pLenti6/UbC/V5-DEST™	V499-10
pEF-DEST51	12285-011
pT-REx™-DEST30	12301-016
pEF5/FRT/V5-DEST™ (Flp-In™)	V6020-20
pDEST™/R4-R3	12567-023
pLenti6/R4R2/V5-DEST™	K591-10
N-terminal reporter tag vectors , e.g.:	
pcDNA™6.2/nGeneBLAzer™-DEST	12578-068, 12578-050
pcDNA™6.2/N-YFP-DEST	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.

Introduction

Overview

Introduction

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits facilitate the expression of microRNA (miRNA) for use in RNA interference (RNAi) analysis of a target gene in mammalian cells. The kits provide a Gateway®-adapted expression vector designed to allow efficient transient or stable expression of miRNA. If more specialized expression is required, the vector allows easy recombination with other suitable destination vectors allowing tissue-specific, regulated, or lentiviral expression of the miRNA in mammalian cells.

Note: The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems include the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits as well as the pLenti6/V5-DEST destination vector and other reagents required to generate a lentiviral RNAi construct. For more information about the pLenti6/V5-DEST vector and how to generate lentivirus, refer to the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual. This manual is supplied with the BLOCK-iT™ Lentiviral RNAi Expression System, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

System Components

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits include:

- pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR (collectively referred to as pcDNA™6.2-GW/± EmGFP-miR)
- Reagents for production of an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA (oligos have to be ordered separately).
- Positive and negative controls for the generation and use of the expression clone.

Note: The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems additionally include components for Gateway® recombination and lentiviral production. Refer to the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual, supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System, available for downloading from our Web site (www.invitrogen.com), or available from Technical Service (see page 65).

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Overview, continued

Advantages of the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits

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

- Offers a rapid and efficient way to clone ds oligo duplexes encoding a desired miRNA target sequence into a vector containing a Pol II promoter for use in RNAi analysis.
- Allows **transient** or **stable** expression of miRNA into mammalian cells.
- Enables targeting multiple genes or increasing knockdown of a single target gene with one construct.
- Permits visual or automated selection of cells expressing the pre-miRNA through co-cistronic expression of EmGFP(in the BLOCK-iT™ Pol II miR RNAi Expression Vector with 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

The BLOCK-iT™ 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 flexible expression of knockdown cassettes driven by RNA Polymerase II (Pol II) promoters in mammalian cells. See page 5 for more details.

The BLOCK-iT™ 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 (see page 7 for details).

A variety of BLOCK-iT™ RNAi products are available from Invitrogen 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.invitrogen.com/rnai or contact Technical Service (see page 65).

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Alternative Expression Systems

The pcDNA™6.2-GW/± EmGFP-miR vectors express 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 Gateway® Technology.

Gateway® Technology

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 (see page 8) into the destination vector, first 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 (such as pDONR™221), then perform an LR recombination reaction between the resulting entry clone and a destination vector of choice. See page 40 for more details. For more in depth information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available from our web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

Purpose of this Manual

This manual provides the following information:

- An overview of the pathway by which miRNA facilitates gene knockdown in mammalian cells.
- Rules 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*.
- Guidelines to transfect your pcDNA™6.2-GW/± EmGFP-miR construct into mammalian cells for transient analysis or to generate stable cell lines.
- 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 from pcDNA™6.2-GW/ EmGFP-miR
- Information to perform a Rapid BP/LR recombination reaction with a suitable Gateway® destination vector to generate an expression clone.

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Important

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits are 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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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™ Pol II miR RNAi Expression Vector Kits (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 vectors facilitate the generation of an expression clone containing a ds oligo encoding a pre-miRNA sequence (see page 8). The resulting expression construct may be introduced into mammalian cells for transient expression of the miRNA sequence, or stable transfectants can be generated. If desired, the pre-miRNA sequence may be easily and efficiently transferred into the pLenti6/V5-DEST vector or other suitable destination vector by Gateway® recombination reactions (see page 40).

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Types of miRNA Vectors

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits are supplied with one of the following expression vectors that allow the expression of your engineered pre-miRNA:

- pcDNA™6.2-GW/miR

  Allows expression of the 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

- pcDNA™6.2-GW/EmGFP-miR

  This vector is similar to pcDNA6.2™-GW/miR, except 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™ Pol II miR RNAi Expression Vectors 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).

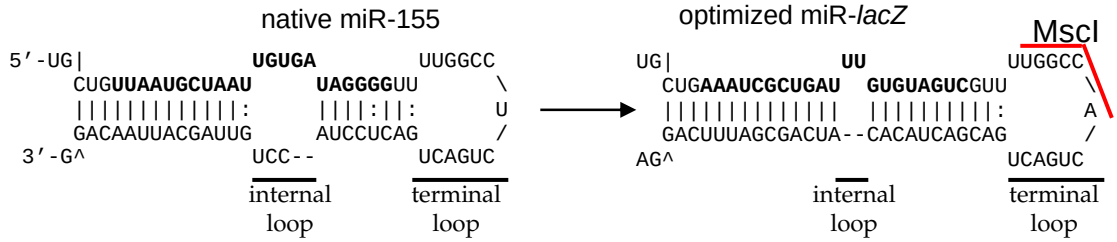
We have chosen the human CMV promoter to control vector-based expression of miRNA molecules in mammalian cells for the following reasons:

- 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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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 (Δ2) 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 page 14. We recommend using Invitrogen's RNAi Designer at www.invitrogen.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™ Pol II miR RNAi Expression Vectors that is flanked on either side with sequences from murine miR-155 to allow proper processing of the engineered pre-miRNA sequence (see page 18-19 for the flanking region sequences).

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 (see figure on page 18-19).

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. See page 38 for details.

BLOCK-iT™ Pol II miR RNAi Expression Vector Kits

Description of the System

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits facilitate the generation of an expression construct that permits high-level expression of a pre-miRNA in mammalian cells for RNAi analysis of a target gene. The kit contains the following major components:

- The pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR linearized plasmids into which a ds oligo encoding the pre-miRNA will be cloned to generate an expression clone that contains the elements required for expression of the miRNA in mammalian cells. 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. The resulting expression clone containing the pre-miRNA expression cassette (see page 8) may be transfected into mammalian cells for transient or stable RNAi analysis, or used to transfer the pre-miRNA expression cassette into a suitable destination vector using Gateway® Technology.
- T4 DNA Ligase and an optimized ligation buffer to allow 5-minute room temperature ligation of the ds oligo insert into the pcDNA™6.2-GW/± EmGFP-miR vector.
- One Shot® TOP10 Chemically Competent *E. coli* for high efficiency transformation of the ligation reaction.

Controls

The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits also includes a negative control plasmid and a ds positive control oligo.

- 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 61-62):

```
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.

Continued on next page

Generating an miRNA Expression Vector Using the Kit

Using the reagents supplied in the BLOCK-iT™ 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 xi).
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, isolate stable cell lines expressing the miRNA, or perform a Gateway® recombination reaction with a suitable Gateway® destination vector to generate a different expression clone.

Features of the pcDNA™6.2-GW/± EmGFP-miR Vectors

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

Additionally, the vector pcDNA™6.2-GW/EmGFP-miR also contains an EmGFP coding sequence for co-cistronic expression with the pre-miRNA.

Green Fluorescent Protein

Description

The BLOCK-iT™ 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.

After transferring the pre-miRNA expression cassette into pLenti6/V5-DEST, you may produce lentiviruses that simultaneously express the EmGFP protein and miRNA, allowing you to visually track the cells in which knockdown is occurring or sort the cells using a flow cytometer.

Green Fluorescent Protein (GFP)

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.

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.

Continued on next page

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.

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

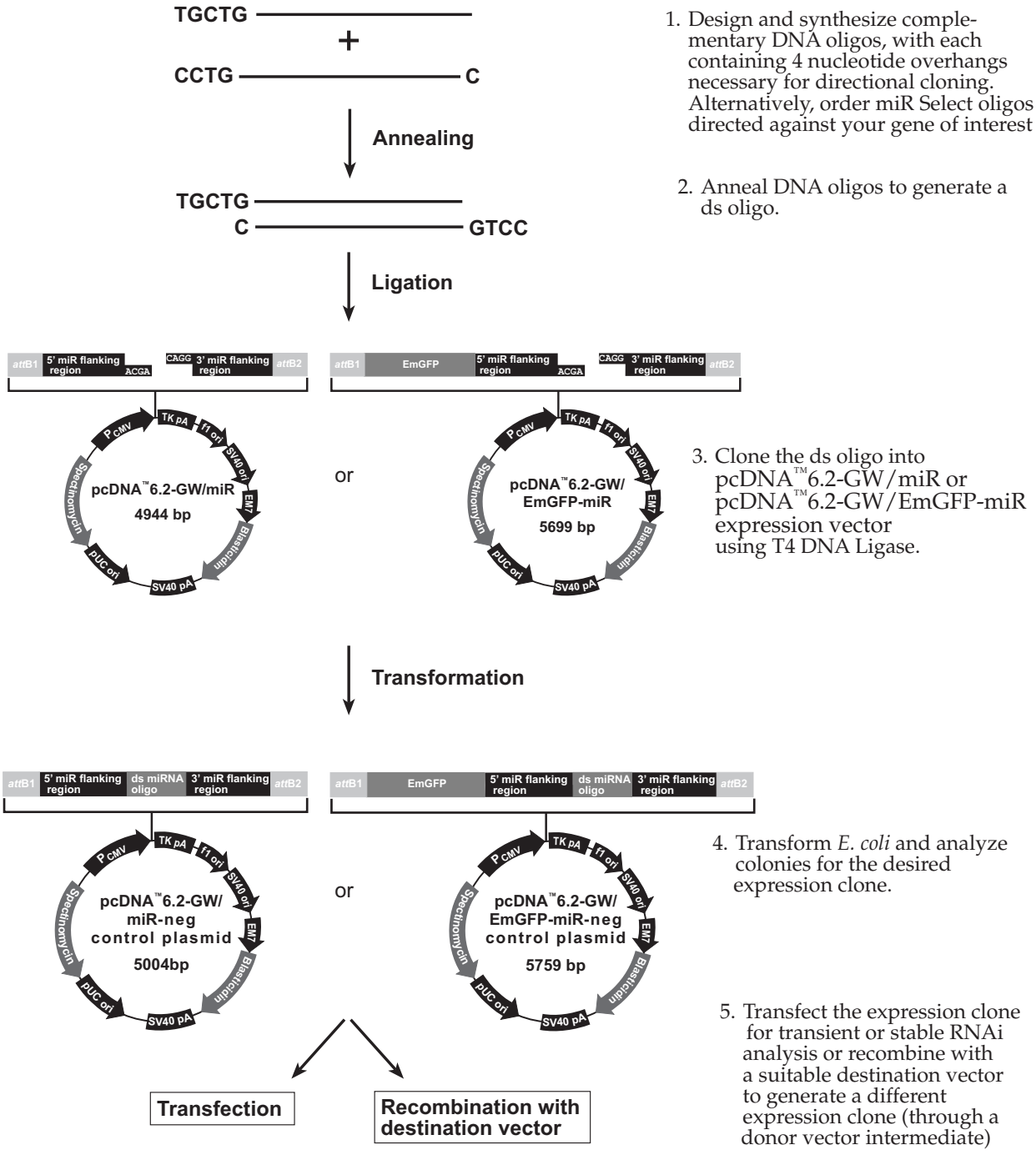
The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein. The filter set for fluorescence microscopy and the manufacturer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

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

Experimental Outline

Flow Chart

The figure below illustrates the major steps necessary to produce a pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression clone using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits.



1. Design and synthesize complementary DNA oligos, with each containing 4 nucleotide overhangs necessary for directional cloning. Alternatively, order miR Select oligos directed against your gene of interest

2. Anneal DNA oligos to generate a ds oligo.

3. Clone the ds oligo into pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression vector using T4 DNA Ligase.

4. Transform *E. coli* and analyze colonies for the desired expression clone.

5. Transfect the expression clone for transient or stable RNAi analysis or recombine with a suitable destination vector to generate a different expression clone (through a donor vector intermediate)

Methods

Designing the Single-Stranded DNA Oligos

Introduction

To use the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits, you will 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 **Invitrogen's 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.invitrogen.com/rnai.

Note: Invitrogen's 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

Invitrogen 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.

See page xi for more details.

Continued on next page

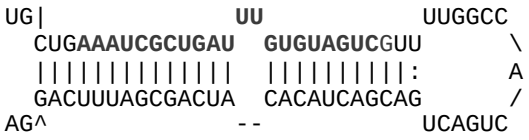
Designing the Single-Stranded DNA Oligos, continued

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	38
Bgl II	AGATCT	miRNA chaining	38
Sal I	GTCGAC	miRNA chaining	38
Xho I	CTCGAG	miRNA chaining	38
Dra I	TTTAAA	Removal EmGFP	40

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:

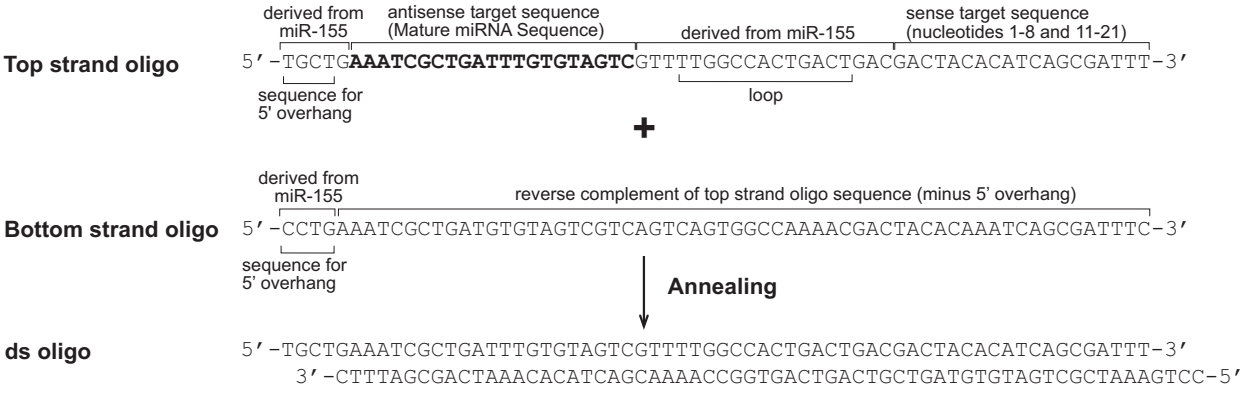
1. Remove 5′ TGCT from top oligo sequence (new sequence starts with G).
2. Take the reverse complement of the sequence from step 1.
3. Add CCTG to the 5′ end of the sequence from step 2.

Note

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

Continued on next page

Designing the Single-Stranded DNA Oligos, continued

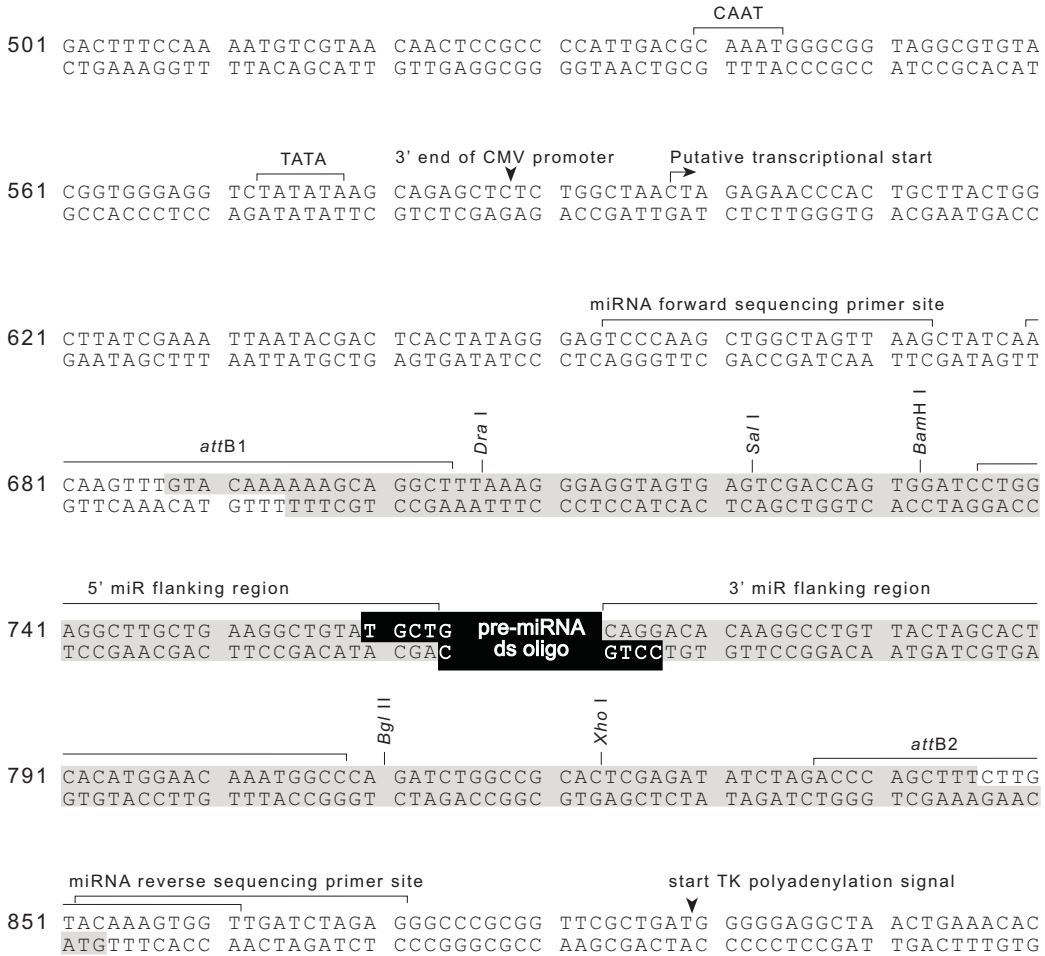
Cloning Site and Recombination Region of pcDNA™6.2-GW/miR

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

- The pcDNA™6.2-GW/miR vector is supplied linearized between nucleotides 763 and 764. 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 those DNA sequences that will be transferred from the initial pre-miRNA expression vector into the Gateway® destination vector (*e.g.* pLenti6/V5-DEST Gateway® Vector) following recombination.

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

The complete sequence of pcDNA™6.2-GW/miR is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65). For a map of pcDNA™6.2-GW/miR, see the **Appendix**, page 56.



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:

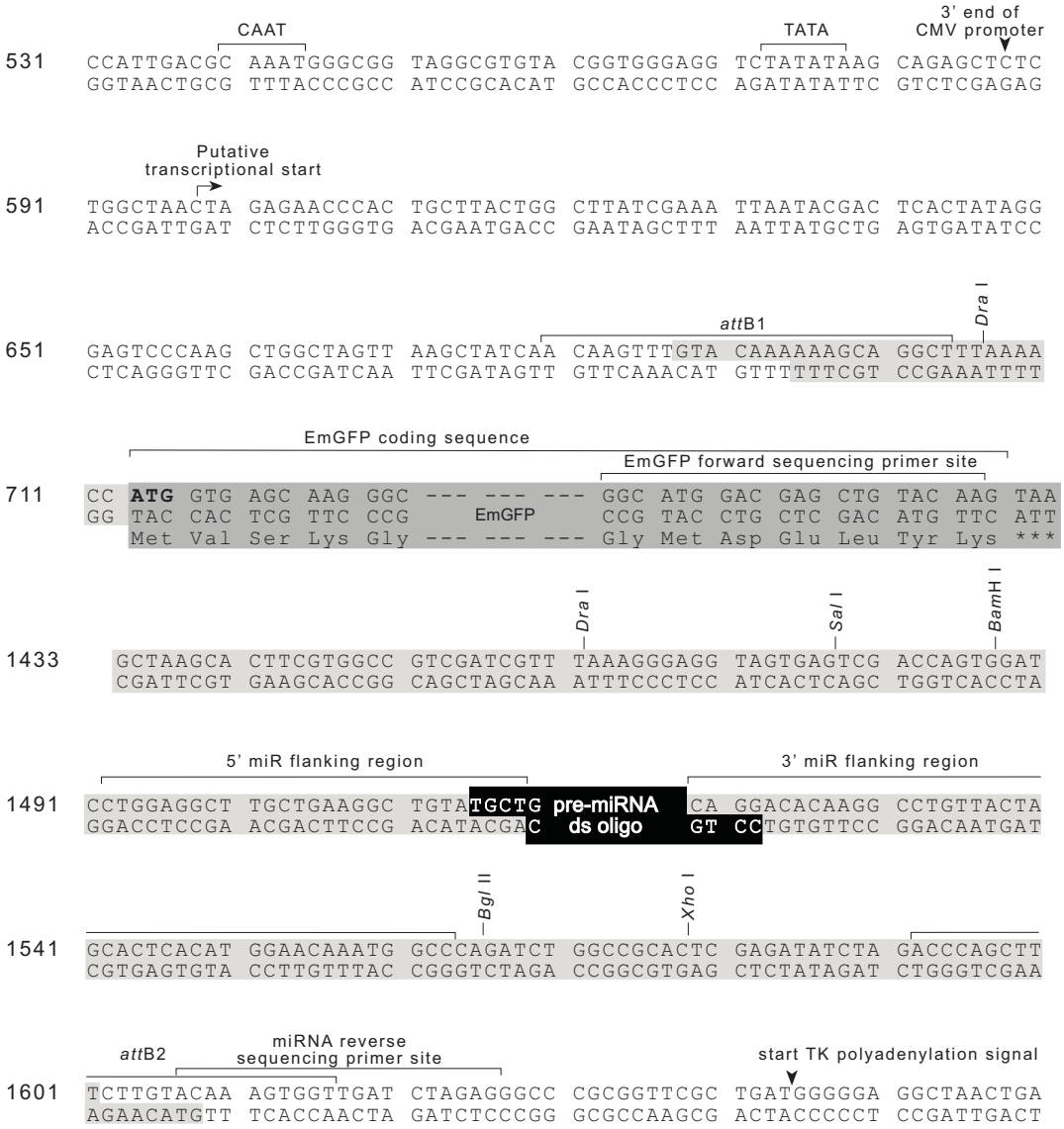
- The pcDNA™6.2-GW/EmGFP-miR vector is supplied linearized between nucleotides 1518 and 1519. The linearized vector contains 4 nucleotide overhangs derived from miR-155 sequences. Note that the annealed double-stranded (ds) oligo **must** contain specific 4 nucleotide 5′ overhangs on each strand as indicated.
- The light shaded region corresponds to those DNA sequences that will be transferred from the initial pre-miRNA expression vector into the Gateway® destination vector (*e.g.* pLenti6/V5-DEST 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.

The complete sequence of pcDNA™6.2-GW/EmGFP-miR is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65). For a map of pcDNA™6.2-GW/EmGFP-miR, see the **Appendix**, page 60.

Continued on next page

Designing the Single-Stranded DNA Oligos, continued



Generating the Double-Stranded Oligo

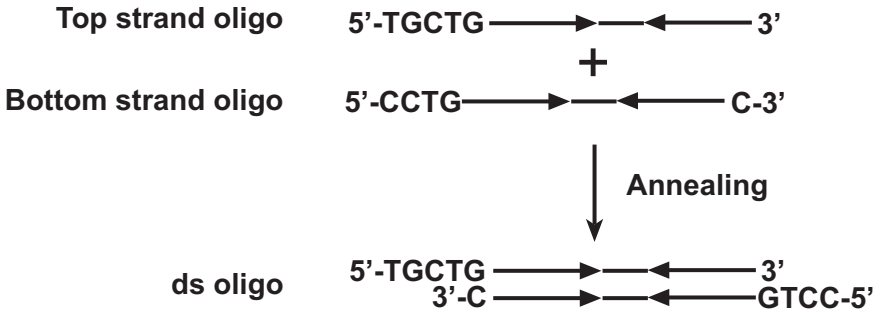
Introduction

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 LacZ2.1 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 1 x Oligo Annealing Buffer, re-anneal the miR-*lacZ* positive ds control oligo without further dilution.

Materials Needed

Have the following materials on hand before beginning:

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

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

2. If re-annealing the 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

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**, next page.
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, previous page) 100-fold into DNase/RNase-free water to obtain a final concentration of 500 nM. Vortex to mix thoroughly.

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

2. Dilute the 500 nM ds oligo mixture (from Step 1) 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

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**, next page).
- **10 nM ds oligo (5,000-fold dilution):** Use this stock for cloning (see **Ligation Procedure**, page 27). This stock is not suitable for long-term storage.

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

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 22.

Checking the Integrity of the ds Oligo

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

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

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.

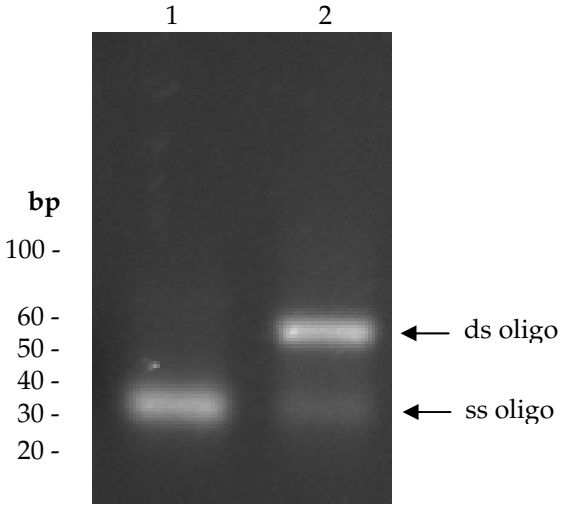
For an example of expected results obtained from agarose gel analysis, see the next page. If the band representing ds oligo is weak or if you do not see a band, see **Troubleshooting**, page 49 for tips to troubleshoot your annealing reaction.

Example of Expected Results

In this experiment, *lacZ* control oligos (see page viii for the sequence of each DNA oligo) were annealed (50 µM final concentration) using the reagents supplied in the kit and following the procedure on page 22 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 the sections entitled **Performing the Ligation Reaction** (pages 26-27) and **Transforming One Shot® TOP10 Competent *E. coli*** (page 28) before beginning.

Note: If you want to perform miRNA chaining, refer to page 38.

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™ Pol II miR RNAi Expression Vector Kits are available separately from Invitrogen (Catalog no. 15224-017).

- Traditional ligation reactions are performed at 16°C overnight. **This is not recommended for this application.** Follow the ligation procedure on page 27.

Amount of ds Oligo to Use

For optimal results, use 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 page 22). See page viii 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

Have the following reagents on hand before beginning:

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

Ligation Procedure

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

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

Reagent	Sample	Positive control	Negative control
5X Ligation Buffer	4 µl	4 µl	4 µl
pcDNA™6.2-GW/miR, linearized (5 ng/µl) **or** 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, you will transform your ligation mixture into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation. Follow the guidelines and instructions provided in this section.

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

Materials to Have on Hand

You will need the following materials on hand before beginning:

- Ligation reaction (from Step 3, previous page)
- One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit, Box 2; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (supplied with the kit, Box 2; warm to room temperature)
- pUC19 positive control (supplied with the kit, Box 2; if desired)
- 42°C water bath
- LB plates containing 50 µg/ml spectinomycin (two for each transformation; warm at 37°C for 30 minutes before use). See page 55 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 54 and 55.

One Shot® TOP10 Transformation Procedure

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

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

   Note: Longer incubations seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake 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 54 and 55.

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.

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 (Box 1). For pcDNA™6.2-GW/EmGFP-miR expression clones, use EmGFP forward sequencing primer and miRNA reverse sequencing primer. See the diagram on page 18-19 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 Web site, 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

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:

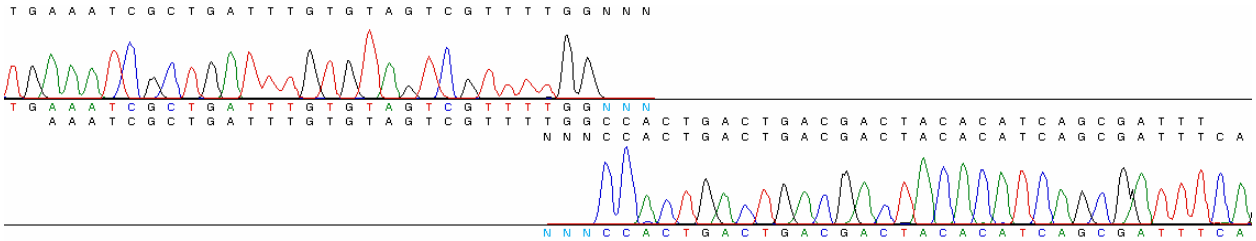
- Use high-quality, purified plasmid DNA for sequencing. We recommend preparing DNA using Invitrogen's PureLink HQ Mini Plasmid Purification Kit.
- Add DMSO to the sequencing reaction to a final concentration of 5%.
- Increase the amount of template used in the reaction (up to twice the normal concentration).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



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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**, next page).
- 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 36).
- Perform miRNA chaining to express multiple pre-miRNAs from one single construct (see **Chaining multiple pre-miRNAs**, page 38).
- Remove the EmGFP coding sequence from your pcDNA™6.2-GW/EmGFP-miR expression clone (see **Removing EmGFP Coding Sequence**, page 36).
- 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 40).

Transfecting Cells

Introduction

This section provides general guidelines to transfect your pcDNA™6.2-GW/± EmGFP-miR expression construct into the mammalian cell line of interest to perform transient 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
- Test the effect of gene knock-down on your particular transient assay

Once you have tested various miRNA target sequences using transient transfection, you may use the most efficient miRNA expression clone for further transient assays, generate stable transfectants, or transfer the optimal miRNA expression cassettes into suitable destination vectors for use in other RNAi applications (e.g. use of alternative promoters and/or viral transduction).

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.

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™ HQ Mini Plasmid Purification Kit, PureLink™ HiPure Plamid Midiprep Kit, or CsCl gradient centrifugation.

Continued on next page

Transfecting Cells, continued

Methods of Transfection

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

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

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

- Provides the highest transfection efficiency in many mammalian cell types.
- DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes, medium change, or medium addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity.

For more information on Lipofectamine™ 2000 Reagent, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 65)

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 blot analysis or activity assay. For more information about the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid, recommendations for transfection, and methods to assay for β-galactosidase activity, see the next page.

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

Continued on next page

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

The pcDNA™1.2/V5-GW/*lacZ* reporter plasmid is supplied with the kit for use as a positive control to assay for the RNAi response in your mammalian cell line. In this vector, β-galactosidase is expressed as a C-terminally tagged fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). See page 64 for more information.

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

Transfecting the *LacZ*-Containing Reagents

To perform RNAi analysis using the *lacZ* control reagents, you will co-transfect the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and the pcDNA™6.2-GW/± EmGFP-miR-*lacZ* expression construct that you have generated into your mammalian cell line. 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.

Assaying for β-galactosidase Expression

If you perform RNAi analysis using the control expression clone containing the *lacZ* ds oligo (*i.e.* pcDNA™6.2-GW/miR-*lacZ* or pcDNA™6.2-GW/EmGFP-miR-*lacZ*), you may assay for β-galactosidase expression by western blot analysis using β-gal Antiserum (Catalog no. R901-25), by activity assay using cell-free lysates (Miller, 1972) and FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Catalog no. F-2905), or by staining the cells for activity using the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression. For an example of results obtained from a β-galactosidase knockdown experiment, see page 46.

Note

The β-galactosidase protein expressed from the pcDNA™1.2/V5-GW/*lacZ* control plasmid is fused to a V5 epitope and is approximately 119 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (*e.g.* Anti-V5-HRP Antibody; Catalog no. R961-25 or Anti-V5-AP Antibody, Catalog no. R962-25) for detection. For more information, refer to our Web site (www.invitrogen.com) or call Technical Support (see page 65).

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.

What You Should See

See the Expected Results Section, page 47

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 Cell Line

Introduction

Once you have determined that the miRNA in your pcDNA™6.2-GW/± EmGFP-miR expression clone is functional, you may wish to establish stable cell lines that constitutively express your miRNA. As negative control, establish cell lines expressing pcDNA™6.2-GW/± EmGFP-miR-neg control plasmid.

Blasticidin Selection

The pcDNA™6.2-GW/± EmGFP-miR expression construct contains the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) of mammalian cells that are stably transfected with the pcDNA™6.2-GW/± EmGFP-miR construct.

Blasticidin is available separately from Invitrogen (see page x for ordering information). For more information about how to prepare and handle Blasticidin, and determine the Blasticidin sensitivity, refer to the **Appendix**, page 54.

Determining Antibiotic Sensitivity

Since you will be selecting for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransfected mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2-10 µg/ml Blasticidin are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin (*e.g.* 0, 2, 4, 6, 8, 10 µg/ml Blasticidin).
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10-14 days after addition of antibiotic.

Continued on next page

Generating a Stable Cell Line, continued

Materials Needed

Have the following materials on hand before beginning:

- Mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)
- pcDNA™6.2-GW/± EmGFP-miR expression clone
- pcDNA™6.2-GW/± EmGFP-miR-neg control plasmid
- Transfection reagent of choice (*e.g.* Lipofectamine™ 2000)
- Blasticidin (5 to 10 mg/ml)
- Appropriate tissue culture dishes and supplies

Guidelines for Transfection and Selection

Guidelines are provided below to transfect your pcDNA™6.2-GW/± EmGFP-miR expression clone and pcDNA™6.2-GW/± EmGFP-miR-neg control plasmid into the mammalian cell line of choice and to select for stable cell lines using Blasticidin.

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 pcDNA™6.2-GW/± EmGFP-miR expression construct and pcDNA™6.2-GW/± EmGFP-miR-neg control plasmid into cells following the recommendations of the manufacturer of your transfection reagent. Use separate wells for separate constructs.
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.

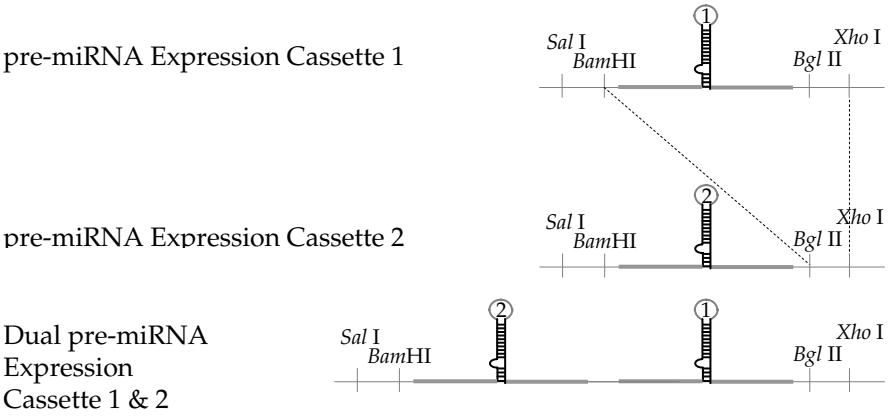
   Example: If transfecting cells in a 6-well format, trypsinize and replate cells into 10 cm tissue culture plates in medium containing Blasticidin.
5. Replace medium with fresh medium containing Blasticidin every 3-4 days until Blasticidin- resistant colonies can be identified (generally 10-14 days after selection).
6. Pick at least 10 Blasticidin-resistant colonies per construct and expand each clone.
7. Assay for target gene knockdown, compare to uninduced cells and cells stably transfected with pcDNA™6.2-GW/± EmGFP-miR-neg control plasmid

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

Two strategies of restriction digestions are possible:

- 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
- 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.

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 Invitrogen or equivalent.
5. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase from Invitrogen 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 29-31
8. Test construct for both miRNAs by transfecting cells as described on page 32 and page 46

For an example of results obtained from knockdown by a chained miRNA vector experiment, see page 48.

Removing EmGFP Coding Sequence

Introduction

Depending on your experiment, it may not wish to express EmGFP from the pre-miRNA expression construct. If you have previously established a pcDNA™6.2-GW/EmGFP-miR clone that works well, 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.

Note

The EmGFP coding sequence is not present on the pcDNA™6.2-GW/miR vector and therefore does not have to be removed.

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 Invitrogen or equivalent.
4. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase from Invitrogen 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.

Transferring the Pre-miRNA Expression Cassette to Destination Vectors

Introduction

pcDNA™6.2-GW/± EmGFP-miR expression vectors are Gateway® compatible. The pre-miRNA is transcribed by RNA Pol II; the pre-miRNA expression cassette can be transferred to other Gateway® adapted destination vectors utilizing Pol II promoters, which allows expression of the pre-miRNA.

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 Web site (www.invitrogen.com) or contact Technical Service (see page 65).

Destination Vector	Catalog No.
pLenti6/V5-DEST™	V496-10
pLenti6/UbC/V5-DEST™	V499-10
pEF-DEST51	12285-011
pT-REx™-DEST30	12301-016
pEF5/FRT/V5-DEST™ (Flp-In™)	V6020-20
pDEST™/R4-R3	12567-023
pLenti6/R4R2/V5-DEST™	K591-10
N-terminal reporter tag vectors , e.g.:	
pcDNA™6.2/nGeneBLAzer™-DEST	12578-068, 12578-050
pcDNA™6.2/N-YFP-DEST	V358-20

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

Important

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 because these vectors do not carry a Pol II promoter upstream of the *att*R1 site. Transfer to pLenti6/V5-DEST as described in the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual, available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

Continued on next page

Transferring the Pre-miRNA Expression Cassette to Destination Vectors, continued

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.

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 a new 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 these destination vectors to form a new miRNA expression clone.

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 web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

Note: For **Multisite Gateway® destination vectors**, follow the alternate protocol at page 56.

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™ 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

You will need the following materials:

- Expression clone (see page 29)
- pDONR™221 vector, or other suitable donor vector (resuspend to 150 ng/µl in sterile water).
- Appropriate destination vector (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
Donor 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
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 32). After that, refer to the manual provided with the destination vector to take advantage of the features of the new expression clone.

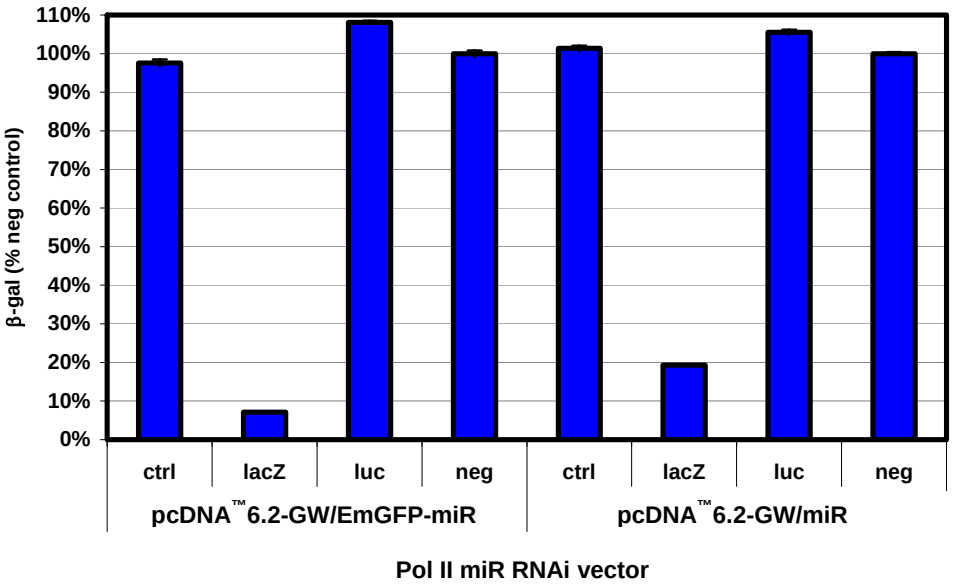
Expected Results

Knockdown of Reporter Gene

In this experiment, pcDNA™6.2-GW/ EmGFP-miR or pcDNA™6.2-GW/ miR expression vectors containing ds oligo encoding miRNA targeting the *lacZ*, luciferase reporter genes or a negative control (neg) were generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits. Note that the miR-*lacZ* positive double-stranded (ds) control oligo and negative control vectors used in this experiment are supplied with the kit.

GripTite™ 293 MSR cells (Invitrogen, Catalog no. R795-07) were grown to 90% confluence. Individual wells in a 24-well plate were transfected using Lipofectamine™ 2000 Reagent with 100 ng of the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and co-transfected with 300 ng of the *lacZ*, luc or neg pre-miRNA expression vectors as indicated. Non-specific plasmid DNA was added to a total of 500 ng DNA. Cell lysates were prepared 48 hours after transfection and assayed for β-galactosidase activity using the FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Catalog no. F-2905),.

Results: Potent and specific inhibition of β-galactosidase activity is evident from the *lacZ*-derived miRNA and not from the luciferase-derived or negative control miRNA for both the pcDNA™6.2-GW/EmGFP-miR and pcDNA™6.2-GW/miR expression vectors.



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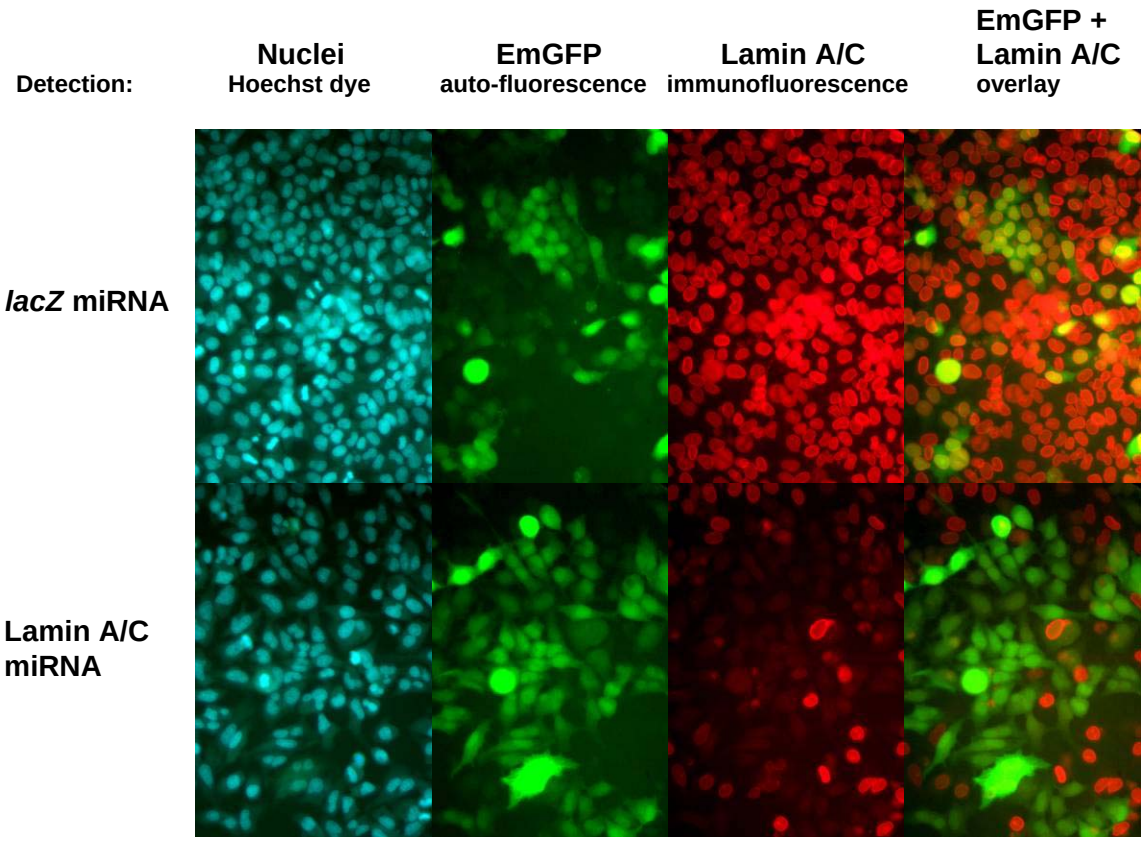
Knockdown of Endogenous Lamin A/C

HeLa cells transfected with pcDNA™6.2-GW/EmGFP-miR vectors containing *lacZ*- (top panel) or lamin A/C-directed (bottom panel) miRNA inserts were fixed and stained four days after transfection. A single field of cells is shown in each row to reveal nuclei (Hoechst dye), EmGFP (auto-fluorescence), lamin A/C (immunofluorescence using mouse monoclonal sc-7292, Santa Cruz Biotechnology), and the overlay of EmGFP and lamin A/C signal.

Results: The lamin A/C-miRNA transfected cells in which EmGFP fluorescence is detectable show markedly reduced lamin A/C staining, indicating tight correlation between EmGFP expression and lamin knockdown. In the control *lacZ*-miRNA transfected wells, lamin signals in non-EmGFP and EmGFP expressing cells are similar, indicating no effect of the *lacZ*-miRNA on lamin A/C expression.



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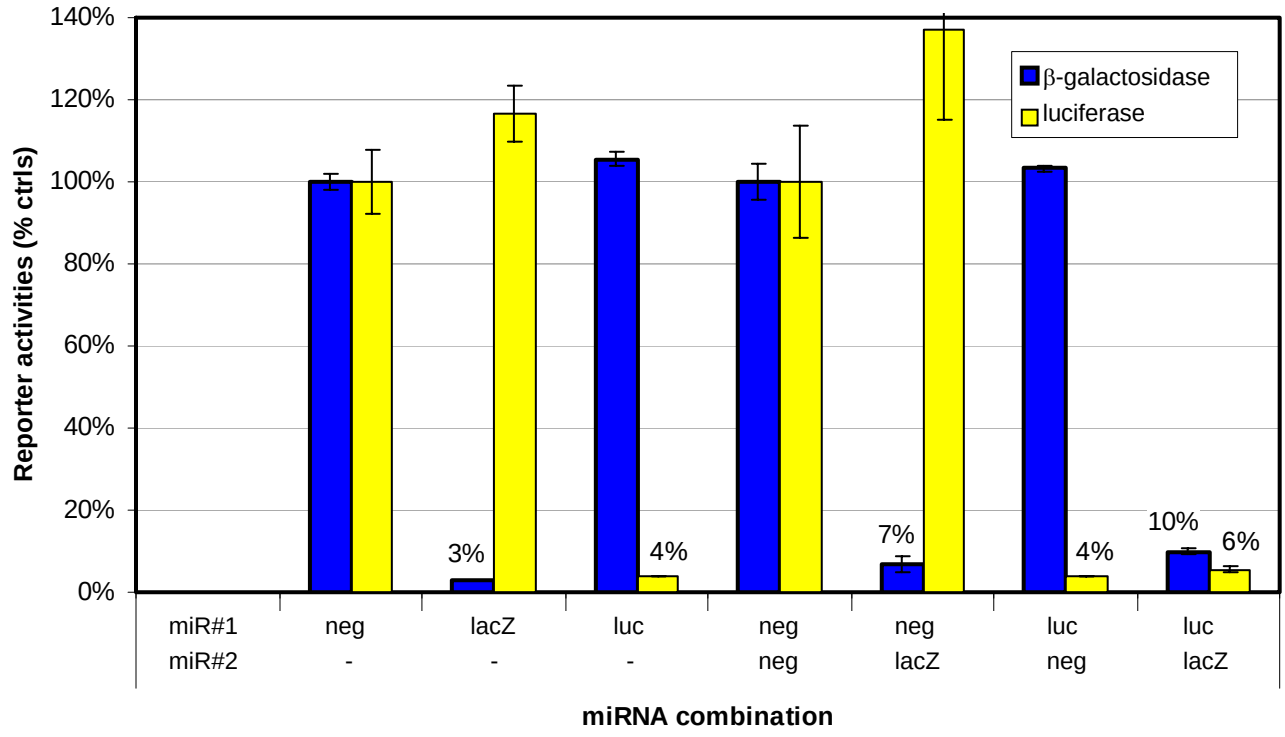
Knockdown by Chained miRNA Vector

Results of experiment co-transfecting luciferase and *lacZ* reporter plasmids with single- or dual-miRNA vectors with the indicated inserts.

GripTite™ 293 MSR cells (Invitrogen, Catalog no. R795-07) were grown to 90% confluence. Individual wells in a 24-well plate were transfected using Lipofectamine™ 2000 Reagent with 100 ng each of the pcDNA™1.2/V5-GW/*lacZ* and pcDNA™5/FRT/luc reporter plasmid and co-transfected with 300 ng of the indicated pre-miRNA expression vectors as indicated. Cell lysates were prepared 48 hours after transfection and assayed for β-galactosidase and luciferase activity. Luciferase and β-galactosidase activities are normalized to the single (neg) or dual (neg/neg) miRNA negative control inserts.

Results: Both for single and dual miRNA expressing vectors, vectors expressing miRNA-*lacZ* inhibit β-galactosidase activity, while vectors expressing miRNA-luc inhibit luciferase activity.



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 x 109 cfu/µg DNA.
	Not enough of the ligation reaction transformed	Increase the amount of ligation reaction transformed.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the bacterial culture for 1 hour at 37°C with shaking before plating.
Many clones contain inserts with sequence mutations	Poor quality single-stranded oligos used Oligo preparation contains mutated sequences Oligo preparation contains contaminants	Use mass spectrometry to check for peaks of the wrong mass. Order HPLC or polyacrylamide gel (PAGE)-purified oligos. Order oligos from Invitrogen's custom primer synthesis service (see our Web site for more information).
	Did not use the competent cells supplied with the kit	Use the One Shot® TOP10 Chemically Competent E. coli supplied with the kit; transformation efficiency is > 1 x 109 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 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

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 xi), 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 xi), 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 pages 14-18 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 xi), 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 35). 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

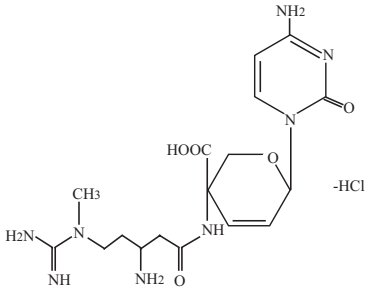
Blasticidin

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 Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 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.

Performing the Rapid BP/LR Recombination Reaction for Multisite Gateway® Vectors

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 for **Multisite Gateway® destination vectors**. 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 web site (www.invitrogen.com) or by contacting Technical Service (see page 65).

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™ 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

You will need the following materials:

- Expression clone (see page 29)
- pDONR™221 vector, or other suitable donor vector (resuspend to 150 ng/µl in sterile water).
- Appropriate Multisite Gateway® vectors (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™ Plus 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 for Multisite Gateway® Vectors 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: Multisite Gateway®

Follow this procedure to perform the Rapid BP/LR reaction between your linearized expression clone, pDONR™221 vector, and Multisite 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 6 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.

Continued on next page

Performing the Rapid BP/LR Recombination Reaction for Multisite Gateway® vectors, Continued

Setting Up the Rapid BP/LR Recombination Reaction: Multisite Gateway® continued

Protocol continued from the previous page.

7. Add the following components to the microcentrifuge tubes containing the 6 µl

Component	Sample	Positive Control
Multisite Gateway® Destination vector (60ng/µl)	1 µl	1 µl
5′ pENTR vector (60ng/µl)	1 µl	1 µl
3′ pENTR vector (60ng/µl)	1 µl	1 µl
5X LR Clonase Plus Buffer	3 µl	3 µl
TE Buffer, pH 8.0	4 µl	4 µl

   BP-reaction at room temperature and mix.

8. Remove the LR Clonase™ Plus enzyme mix from -20°C and thaw on ice (~ 2 minutes).
9. Vortex the LR Clonase™ Plus enzyme mix briefly twice (2 seconds each time).
10. To the samples above, add 4 µl of LR Clonase™ Plus enzyme mix. Mix well by pipetting up and down.

    Reminder: Return LR Clonase™ Plus enzyme mix to -20°C immediately after use.

11. Incubate the reaction at 25°C **overnight.**
12. Add 1 µl of the 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. 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).

    Note: You may store the reaction at -20°C for up to 1 week before transformation, if desired.

What to Do Next

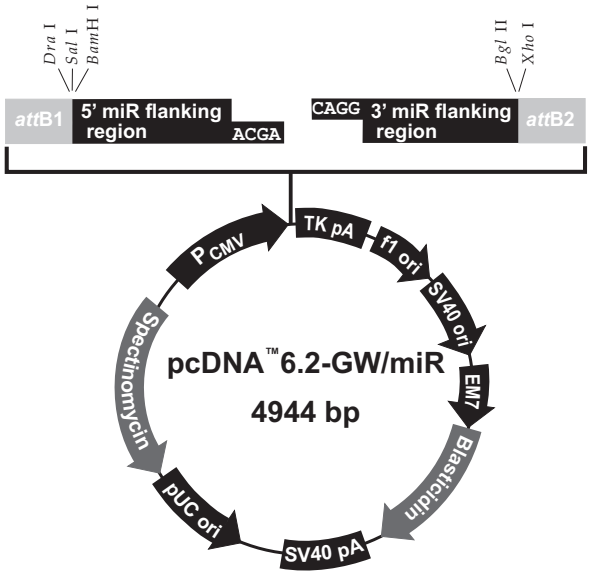
Once you have obtained your new expression clone, we recommend you test it by transfecting an appropriate mammalian cell line to perform transient RNAi analysis if applicable (see **Transfecting Cells**, page 32). After that, refer to the manual provided with the destination vector to take advantage of the features of the new expression clone.

Map and Features of pcDNA™6.2-GW/miR

pcDNA™6.2-GW/miR

The figure below shows the features of the pcDNA™6.2-GW/miR vector. The vector is supplied linearized between nucleotides 763 and 764 with 4 base pair 5′ overhangs on each strand as indicated. **The complete sequence of pcDNA™6.2-GW/miR is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).**

Note: For an explanation of the features see page 63.



Comments for pcDNA™ 6.2-GW/miR
4944 nucleotides

CMV promoter: bases 1-588
miRNA forward sequencing primer site: bases 654-673
*att*B1 site: bases 680-704
5' miR flanking region: bases 737-763
5' overhang (C): bases 760-763
5'overhang: bases 764-767
3' miR flanking region: bases 764-808
*att*B2 site (C): bases 837-861
miRNA reverse sequencing primer site (C): bases 852-871
TK polyadenylation signal: bases 890-1161
f1 origin: bases 1273-1701
SV40 early promoter and origin: bases 1728-2036
EM7 promoter: bases 2091-2157
Blasticidin resistance gene: bases 2158-2556
SV40 polyadenylation signal: bases 2714-2844
pUC origin (C): bases 2982-3655
Spectinomycin resistance gene (C): bases 3725-4735
Spectinomycin promoter (C): bases 4736-4869

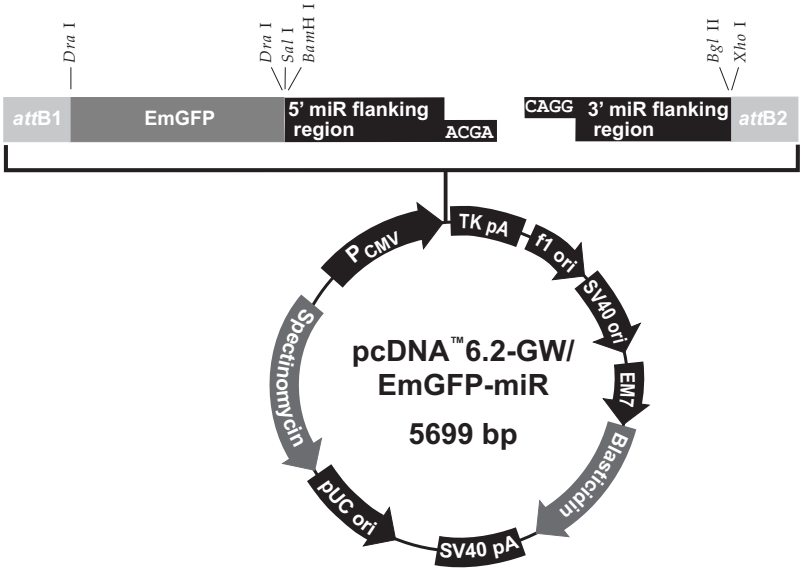
(C) = Complementary strand

Map and Features 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 Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).**

Note: For an explanation of the features see page 63.



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

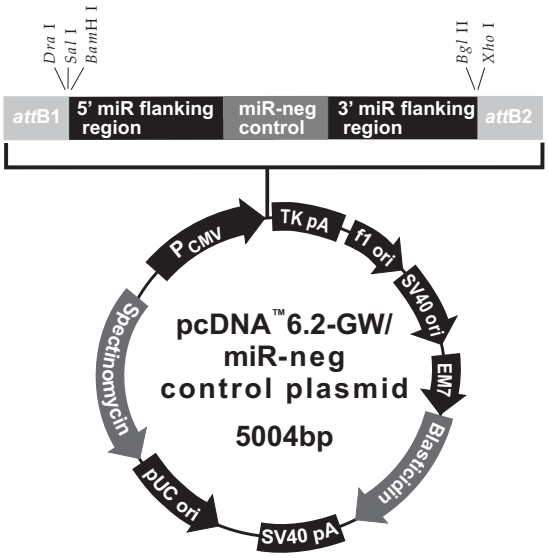
(C) = Complementary strand

Map and Features of pcDNA™6.2-GW/miR-neg control plasmid

pcDNA™6.2-GW/miR-neg control plasmid

The figure below shows the features of the pcDNA™6.2-GW/miR-neg control plasmid. The vector contains an insert between bases 764 and 823 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 14). Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with pcDNA™6.2-GW/miR expression vectors. **The complete sequence of pcDNA™6.2-GW/miR-neg control plasmid is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).**

Note: For an explanation of the features see page 63.



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

CMV promoter: bases 1-588
miRNA forward sequencing primer site: bases 654-673
*att*B1 site: bases 680-704
5' miR flanking region: bases 737-763
miR-neg control: bases 764-823
3' miR flanking region: bases 824-868
*att*B2 site (C): bases 897-921
miRNA reverse sequencing primer site (C): bases 912-931
TK polyadenylation signal: bases 950-1221
f1 origin: bases 1333-1761
SV40 early promoter and origin: bases 1788-2096
EM7 promoter: bases 2151-2217
Blasticidin resistance gene: bases 2218-2616
SV40 polyadenylation signal: bases 2774-2904
pUC origin (C): bases 3042-3715
Spectinomycin resistance gene (C): bases 3785-4795
Spectinomycin promoter (C): bases 4796-4929

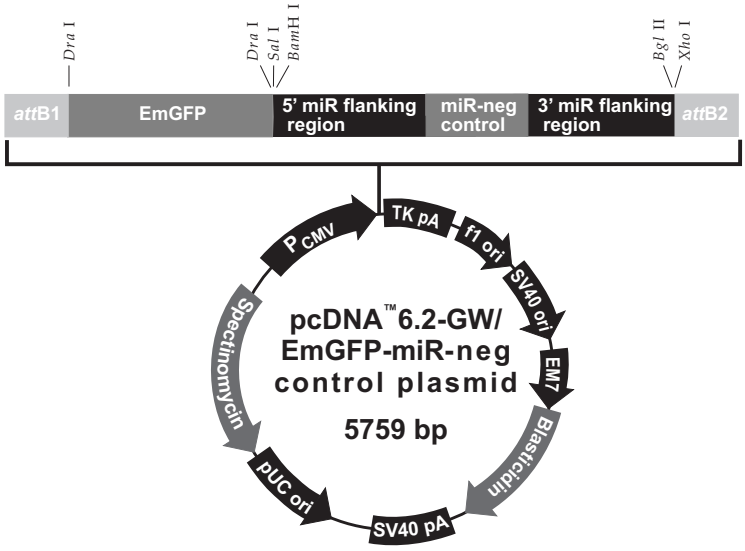
(C) = Complementary strand

Map and Features of pcDNA™6.2-GW/EmGFP-miR-neg control plasmid

pcDNA™6.2-GW/EmGFP-miR-neg 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 14). 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 Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).**

Note: For an explanation of the features see page 63.



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

Explanation of Features of pcDNA™6.2-GW/± EmGFP-miR

Explanation of Features

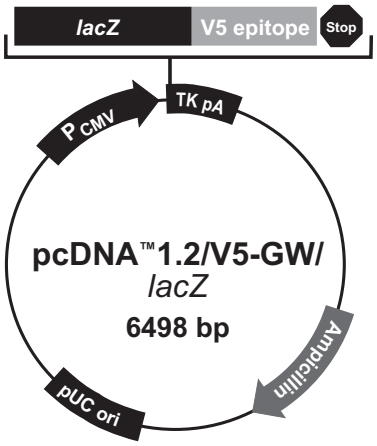
pcDNA™6.2-GW/± EmGFP-miR vectors contain the following elements. All features have been functionally tested and the vectors have been fully sequenced.

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).
miRNA forward sequencing primer	Allows sequencing of the insert (for pcDNA™6.2-GW/miR vectors)
*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 (for pcDNA™6.2-GW/EmGFP-miR vector)
EmGFP forward sequencing primer	Allows sequencing of the insert (for pcDNA™6.2-GW/EmGFP-miR vector)
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 Invitrogen (Catalog no. 12537-023). Briefly, a MultiSite Gateway® LR recombination reaction was performed with pDEST™R4-R3 and entry clones containing the CMV promoter, *lacZ* gene, and V5 epitope and TK polyadenylation signal (Cole & Stacy, 1985) to generate the pcDNA™1.2/V5-GW/*lacZ* vector. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 119 kDa. **The complete sequence of pcDNA™1.2/V5-GW/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 65).**



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

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

(C) = complementary strand

Technical Service

Web Resources

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

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
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Contact Us

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Certificate of Analysis

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

Material Data Safety Sheets (MSDSs)

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 69.

Continued on next page

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Continued on next page

Limited Use Label License No. 271: miRNA Constructs

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

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