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BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System

A Gateway®-adapted, lentiviral destination vector for high-level expression of microRNA in dividing and non-dividing mammalian cells

Cat. nos. K4937-00 and K4938-00

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User Manual

Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Cat. no.
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System	K4937-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP	K4938-00

Intended Use

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

Kit Components

The BLOCK-iT™ Lentiviral Pol II miR RNAi Kits include the following components. For a detailed description of the contents of each component, see the following pages. For a detailed description of the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit contents and how to use the reagents supplied, see the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual. For detailed instructions to grow and maintain the 293FT Cell Line, see the 293FT Cell Line manual.

Components	Cat. no.	
	K4937-00	K4938-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	✓	
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP		✓
pLenti6/V5-DEST Gateway® Vector Kit	✓	✓
Gateway® BP Clonase® II Enzyme Mix	✓	✓
Gateway® LR Clonase® II Enzyme Mix	✓	✓
One Shot® Stbl3™ Chemically Competent *E. coli*	✓	✓
One Shot® TOP10 Chemically Competent *E. coli*	✓	✓
ViraPower™ Bsd Lentiviral Support Kit	✓	✓
293FT Cell Line	✓	✓
pDONR™221	✓	✓

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Kit Contents and Storage, Continued

Shipping and Storage

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

Box	Component	Shipping	Storage
1	BLOCK-iT™ Pol II miR RNAi Expression Vector Kit **or** BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP	Dry ice	–20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	–80°C
3	pLenti6/V5-DEST Gateway® Vector Kit	Blue ice	–20°C
4	pDONR™221 Vector	Room temperature	–20°C
5–7	ViraPower™ Bsd Lentiviral Support Kit: ViraPower™ Packaging Mix Lipofectamine® 2000 Blasticidin	 Blue ice Blue ice Room temperature	 –20°C 4°C **(do not freeze)** –20°C
8	One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
9–10	Gateway® BP Clonase® II Enzyme Mix Gateway® LR Clonase® II Enzyme Mix	Dry ice Dry ice	–20°C –20°C
11	293FT Cell Line	Dry ice	Liquid nitrogen

BLOCK-iT™ Pol II miR RNAi Expression Vector Kit

The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kits include an expression vector for expression of your microRNA (miRNA) sequence. The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits contain:

- BLOCK-iT™ Pol II miR RNAi Expression Vector **or** BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP Reagents (Box 1)
- One Shot® TOP10 Chemically Competent *E. coli* (Box 2)

Refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual for a detailed description of the reagents provided with the kit and instructions to produce the Gateway® expression vector construct.

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Kit Contents and Storage, Continued

Vectors

The following vectors are included with the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kits (Boxes 3 and 4). **Store the vectors at –20°C.**

Vector	Composition	Amount
pLenti6/V5-DEST Gateway® Vector	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pLenti6/V5-GW/*lacZ* control	20 µL of vector at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg
pDONR™221 Vector	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg

ViraPower™ Bsd Lentiviral Support Kit Contents

The following reagents are included with the ViraPower™ Bsd Lentiviral Support Kit (Boxes 5–7). Store the ViraPower™ Packaging Mix and Blasticidin at –20°C. Store Lipofectamine® 2000 Reagent at 4°C.

Important: Do not freeze Lipofectamine® 2000 Reagent.

Reagent	Composition	Amount
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, 1 µg/µL in TE, pH 8.0	195 µg
Lipofectamine® 2000 Reagent	Proprietary	0.75 mL
Blasticidin	Powder	50 mg

One Shot® Stbl3™ Chemically Competent *E. coli*

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit (Box 8). Transformation efficiency is ≥ 1×10^8 cfu/µg plasmid DNA. **Store Box 8 at –80°C.**

Reagent	Composition	Amount
S.O.C. Medium	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
Stbl3™ Cells	--	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

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Genotype of Stbl3™ Cells

F⁻ *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ⁻ *leu mtl*-1

Gateway® LR Clonase® II Enzyme Mixes

The following reagents are included with the Gateway® Clonase® II Enzyme Mixes (Boxes 9–10).

Store Box 9 and 10 at –20°C for up to 6 months. For long-term storage, store at –80°C.

Gateway® BP Clonase® II

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

Gateway® LR Clonase® II

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

Note: The pENTR™-gus control included with the Gateway® LR Clonase® II Enzyme Mix may be used as a positive control for the LR recombination reaction **only** (see page 61).

293FT Cell Line

The 293FT Cell Line (Box 11) is used for the production of lentiviral stocks. The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 mL of Freezing Medium. **Upon receipt, store in liquid nitrogen.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see the 293FT Cell Line manual.

Introduction

System Summary

Description of the System

The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System combines BLOCK-iT™ Pol II miR RNAi and ViraPower™ Lentiviral technologies to facilitate creation of a replication-incompetent lentivirus that delivers a microRNA (miRNA) sequence of interest to dividing or non-dividing mammalian cells for RNA interference (RNAi) analysis.

System Components

The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System includes:

- A BLOCK-iT™ Pol II miR RNAi Expression Vector Kit for producing an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA sequence (see page 8) for expression in mammalian cells using an RNA Polymerase II (Pol II) promoter, the human cytomegalovirus (CMV) immediate early promoter. The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits are supplied with a choice of pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression vectors (collectively referred to as pcDNA™6.2-GW/± EmGFP-miR).
- The pDONR™221 vector is used as an intermediate to transfer the pre-miRNA expression cassette (see page 8 for details) into the lentiviral expression plasmid (see below) using Gateway® Technology.
- A pLenti6/V5-DEST destination vector into which the pre-miRNA cassette from the expression clone is transferred using Gateway® Technology (see below). This expression plasmid contains elements that allow packaging of the construct into virions and the Blasticidin resistance marker for selection of stably transduced cell lines.
- Gateway® BP and LR Clonase® II Enzyme Mixes that facilitate the transfer of the pre-miRNA expression cassette from the expression vectors into the pLenti6/V5-DEST destination vector.
- Components of the ViraPower™ Lentiviral System for production of a replication-incompetent lentivirus that stably expresses the miRNA of interest in both dividing and non-dividing mammalian cells.

For more information about the BLOCK-iT™ Pol II miR RNAi Technology, ViraPower™ Lentiviral Technology, and Gateway® Technology, see pages 2–3.

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System Summary, Continued

Advantages of the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System

Using BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kits to facilitate lentiviral-based delivery of miRNA to mammalian cells provides the following advantages:

- The BLOCK-iT™ Pol II miR RNAi Expression Vectors provide 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.
- Gateway®-adapted vectors for easy transfer of the miRNA of interest from one expression vector (pcDNA™6.2-GW/±EmGFPmiR) into another (pLenti6/V5-DEST vector).
- Generates a replication-incompetent lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential RNAi applications beyond those of other traditional retroviral systems (Naldini, 1998).
- Efficiently delivers the miRNA of interest to mammalian cells in culture or *in vivo*.
- Provides stable, long-term expression of the miRNA of interest beyond that offered by traditional adenoviral-based systems.
- Produces a pseudotyped virus with a broadened host range (Yee, 1999).
- Includes multiple features designed to enhance the biosafety of the system.

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 miRNA-based knockdown cassettes driven by RNA Polymerase II (Pol II) promoters in mammalian cells.

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 8 for details).

A variety of BLOCK-iT™ RNAi products are available 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 Support (see page 79).

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System Summary, Continued

ViraPower™ Lentiviral Technology

ViraPower™ Lentiviral Technology facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene or RNA to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ Lentiviral Technology possesses features which enhance its biosafety while allowing high-level expression in a wider range of cell types than traditional retroviral systems. The main components of the ViraPower™ Lentiviral Expression System include:

- A pLenti-based expression vector (*e.g.*, pLenti6/V5-DEST) into which the DNA sequence of interest will be cloned. This vector contains elements required to allow packaging of the expression construct into virions and an antibiotic resistance marker to allow selection of stably transduced cell lines. For more information, see page 9.
- The ViraPower™ Packaging Mix, an optimized mixture of the three packaging plasmids required for production of the lentivirus.
- An optimized 293FT producer cell line to facilitate optimal production of virus.

For more information about these components, see page 9. For more information about the biosafety features of the system, see page 16.

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 into multiple vector systems.

To express your miRNA of interest in mammalian cells using the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System and Gateway® Technology, simply:

1. Clone a double-stranded oligonucleotide encoding your miRNA sequence of interest into the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression vector to create an expression clone. Transfect this expression clone directly into mammalian cells for initial screening, if desired.
2. To transfer your pre-miRNA expression cassette into pLenti6/V5-DEST vector, generate an entry clone by performing a BP recombination reaction between the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression clone and pDONR™221 donor vector, then perform a LR recombination reaction between the resulting entry clone (pENTR™221/miR) and pLenti6/V5-DEST. See page 25 for more details.
3. Use your lentiviral expression clone and the reagents supplied in the kit to produce a lentiviral construct.
4. Transduce the lentiviral construct into mammalian cells to express the miRNA. Select for stably transduced cells, if desired.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual which is available at www.invitrogen.com or by contacting Technical Support (see 79).

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Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kits and provides instructions and guidelines to:

1. Use the pLenti6/V5-DEST vector and expression clone (pcDNA™6.2-GW/±EmGFP-miR) in a Rapid BP/LR recombination reaction to generate a lentiviral expression clone containing the pre-miRNA sequence of interest.
2. Co-transfect the pLenti6/V5-GW/±EmGFP-miR expression construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
3. Titer the lentiviral stock.
4. Transduce the lentiviral construct into mammalian cells and perform "transient" RNAi analysis
5. Generate a stably transduced cell line, if desired.

For detailed instructions to generate a pcDNA6.2™-GW/±EmGFP-miR expression clone containing the pre-miRNA expression cassette, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual. These manuals are supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kits, but are also available at www.invitrogen.com or by contacting Technical Support (see page 79).

Note

The One Shot® Stbl3™ Chemically Competent *E. coli*, Gateway® LR Clonase® II Enzyme Mix, and Lipofectamine® 2000 Reagent included in the BLOCK-iT™ Lentiviral RNAi Expression System are available separately and are supplied with individual documentation detailing general use of the product. **For instructions to use these products specifically with the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits, follow the recommended protocols in this manual.**

Important

The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Kit is designed to help you create a lentivirus to deliver and express an miRNA sequence in mammalian cells for RNAi analysis. Although the system is designed to help you express your miRNA sequence in the simplest, most direct fashion, use of the system is intended for users familiar with the principles of retrovirus biology and gene silencing. We highly recommend that users possess a working knowledge of viral and tissue culture techniques, lipid-mediated transfection, Gateway® Technology, and the RNAi pathway. For more information about the following topics, refer to these references:

- Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal, 2000 and Luciw, 1996.
- Retroviral and lentiviral vectors: see Naldini, 1999, Naldini, 1998, and Yee, 1999.
- RNAi pathway and expression of miRNA in mammalian cells: see published references (Brummelkamp *et al.*, 2002; Cullen, 2004; Kim, 2005; McManus & Sharp, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Zeng *et al.*, 2002)

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 (Lund *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

A major limitation of using siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is the transient nature of siRNA. To address this limitation, 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 the 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 pcDNA6.2™-GW/±EmGFP-miR expression vectors (supplied in the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits) 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 dividing mammalian cells for transient expression of the miRNA sequence and initial RNAi screening, if desired. Once initial screening is complete, the pre-miRNA sequence may then be easily and efficiently transferred into the pLenti6/V5-DEST vector (or other suitable destination vector) by Gateway® recombination reactions (see page 11).

For more information about the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits, included components, and generating the expression construct, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.

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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.

Advantages of Using Pol II miRNA Vector-Based Systems

miRNA vector-based systems that use Pol II promoters for RNAi cassette expression offer the following advantages over traditional siRNA or shRNA expression:

- Enables co-cistronic expression of reporter genes such as GFP (see above) allowing reliable tracking of miRNA expression in mammalian cells
- Allows expression of miRNA from a variety of promoters, including tissue-specific and regulated promoters for *in vivo* experiments
- Enables expression of multiple miRNAs from a single transcript allowing the knockdown of more than one gene simultaneously (see the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual for details)
- Permits design of predictable RNAi constructs with a high rate of success

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.

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Structure of the Engineered pre-miRNA

The BLOCK-iT™ Pol II miR RNAi Expression 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. For details on miR-155 and stem-loop optimization, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector manual.

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

- Two 4 nucleotides, 5′ overhangs 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

The structural features are depicted in the figure below.

For more details on the structure and guidelines to design the oligonucleotides, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.

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 23 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. During the Gateway® recombination reactions, the pre-miRNA expression cassette is transferred between vectors.

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 23).

The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System

Components of the System

The system includes the following major components

1. The BLOCK-iT™ Pol II miR RNAi Expression Vector Kits containing the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR vector for producing an expression clone that contains elements required for expression of a double-stranded oligonucleotide encoding an miRNA sequence of interest in mammalian cells using a Pol II promoter. The expression vector containing the pre-miRNA expression cassette may be transfected into mammalian cells for transient RNAi analysis or used to transfer the pre-miRNA expression cassette into the pLenti6/V5-DEST vector (see below) using Gateway® Technology. For detailed information about the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits and instructions for generating an expression clone, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual supplied with the kit.
2. The pLenti6/V5-DEST vector allows easy transfer of the pre-miRNA expression cassette from the expression clone into a lentiviral destination vector for use with the Lentiviral system components. The destination vector contains the elements required to allow packaging of the expression construct into virions (*e.g.*, 5′ and 3′ LTRs, ψ packaging signal) and a selectable marker to allow generation of stable cell lines. For more information about the pLenti6/V5-DEST vector, see page 65.
3. The pDONR™221 vector is used as an intermediate to transfer the pre-miRNA expression cassette into the lentiviral expression plasmid (see above) using Gateway® Technology. See page 13 for details on pDONR™221 vector.
4. Gateway® BP and LR Clonase® II Enzyme Mixes that allow the transfer of the pre-miRNA expression cassette from the expression vectors into the pLenti6/V5-DEST vector using the Rapid BP/LR recombination reaction.
5. One Shot® Stbl3™ Competent *E. coli* to obtain optimal results with lentiviral DNA after transformation.
6. The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see the **Appendix**, pages 70–75.
7. An optimized 293FT producer cell line that stably expresses the SV40 large T-antigen under the control of the human CMV promoter and facilitates optimal production of virus. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual.

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The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System,
Continued

System Overview

You will co-transfect the ViraPower™ Packaging Mix and the pLenti6/V5-DEST expression construct containing the pre-miRNA expression cassette into 293FT cells to produce a replication-incompetent lentivirus, which can then be transduced into the mammalian cell line of interest. Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). Once the lentiviral construct has integrated into the genome, the miRNA is constitutively expressed, allowing you to perform transient RNAi analysis or use Blasticidin selection to generate a stable cell line for long-term knockdown studies.

VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as delivery vehicles by their restricted tropism and generally low titers. In the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System, this limitation has been overcome by use of the G glycoprotein gene from Vesicular Stomatitis Virus (VSV-G) as a pseudotyping envelope, thus allowing production of a high titer lentivirus with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).

Features of the pLenti6/V5-DEST Vector

The pLenti6/V5-DEST vector contains the following elements:

- Rous Sarcoma Virus (RSV) enhancer/promoter for Tat-independent production of viral mRNA in the producer cell line (Dull *et al.*, 1998)
- Modified HIV-1 5′ and 3′ Long Terminal Repeats (LTR) for viral packaging and reverse transcription of the viral mRNA (Dull *et al.*, 1998; Luciw, 1996)

  Note: The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- Human CMV promoter for high-level, constitutive expression of the miRNA from an RNA Polymerase II-dependent promoter
- Two recombination sites, *att*R1 and *att*R2, for recombinational cloning of the miRNA of interest from the pcDNA™6.2-GW/±EmGFP-miR expression clone using Gateway® Technology
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- The *ccd*B gene located between the *att*R sites for negative selection
- Blasticidin resistance gene (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) for selection in *E. coli* and mammalian cells
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

Gateway® Recombination Reactions

Introduction

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move a DNA sequence of interest into multiple vector systems.

Review the information in this section to briefly familiarize yourself with the Gateway® recombination reactions. For details, refer to the Gateway® Technology with Clonase® II manual available from at www.invitrogen.com or by contacting Technical Support (see page 79).

Gateway® Vectors

Each of the vectors supplied in the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System is Gateway®-adapted, *i.e.* contains the appropriate *att* sites that allow site specific recombination to facilitate the transfer of heterologous DNA sequences between vectors.

Recombination Reactions

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

BP Reaction

Facilitates recombination of an *att*B substrate (*att*B-PCR product or 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 Gateway® 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 Gateway® LR Clonase® II enzyme mix.

Note

If you are an experienced user of Gateway® Technology and wish to perform the Rapid BP/LR recombination reaction, we recommend that you review the information on page 20 before proceeding with the experiment.

Do not use the standard recombination reaction conditions to perform the Rapid BP/LR recombination reaction.

Continued on next page

Gateway® Recombination Reaction, Continued

Pre-miRNA Expression

Since the pcDNA™6.2-GW/miR and pcDNA™6.2-GW/EmGFP-miR expression vectors contain *att*B sites, the expressions vectors containing the pre-miRNA sequence **cannot** be transferred directly into the pLenti6/V5-DEST destination vector using a single recombination reaction.

To transfer your pre-miRNA expression cassette from pcDNA™6.2-GW/±EmGFP-miR expression clone into the pLenti6/V5-DEST vector, you need to perform the two Gateway® recombination reactions as follows:

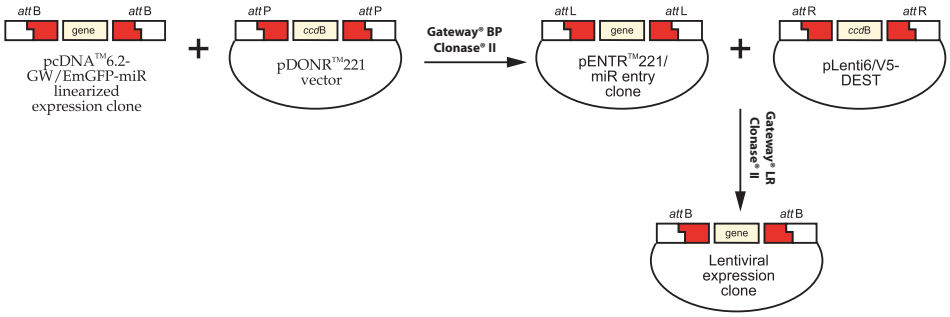
1. Generate an entry clone by performing a BP recombination reaction between the *att*B substrate (pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression clone) and *att*P substrate (pDONR™221 vector) using Gateway® BP Clonase® II Enzyme Mix.
2. Perform an LR recombination reaction between the resulting entry clone (*att*L substrate) and pLenti6/V5-DEST vector (*att*R substrate) using Gateway® LR Clonase® II Enzyme Mix.

The standard BP and LR recombination reaction requires more than 2 days for completion. See below for details on expressing the miRNA from pLenti6/V5-DEST destination vector using the Rapid BP/LR Recombination Reaction.

Rapid BP/LR Recombination Reaction

We have developed a Rapid BP/LR recombination reaction to transfer the pre-miRNA expression cassettes into the destination vector that allows the completion of the entire BP and LR reaction in a day. In the Rapid BP/LR Recombination Reaction, instead of isolating the entry clone after BP reaction, the completed BP reaction is transferred directly into the LR reaction to generate expression clones.

For **Rapid BP/LR Recombination Reactions**, perform a BP recombination reaction between the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression clone and pDONR™221 donor vector using Gateway® BP Clonase® II Enzyme Mix, then perform an LR recombination reaction between the resulting entry clone (pENTR™221/miR) and pLenti6/V5-DEST vector using Gateway® LR Clonase® II Enzyme Mix (see below) to produce a lentiviral expression clone.



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Gateway® Recombination Reaction, Continued

Features of pDONR™221

The pDONR™221 vector contains the following elements:

- *rrn*B T1 and T2 transcription terminators for protection of the cloned gene or miRNA from expression by vector-encoded promoters
- Two recombination sites, *att*P1 and *att*P2, for recombinational cloning of the gene of interest from a Gateway® expression clone or *att*B PCR product
- *ccd*B gene located between the two *att*P sites for negative selection
- Chloramphenicol resistance gene located between the two *att*P sites for counterselection
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for replication and maintenance of the plasmid in *E. coli*

For a map of pDONR™221, see page 68.

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 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

Green Fluorescent Protein, Continued

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)

Biosafety Features of the System

Introduction

The lentiviral and packaging vectors supplied in the BLOCK-iT™ Lentiviral RNAi Expression System are third-generation vectors based on lentiviral vectors developed by Dull *et al.*, 1998. This third-generation lentiviral system includes a significant number of safety features designed to enhance biosafety and to minimize relation to the wild-type, human HIV-1 virus. These safety features are discussed below.

Biosafety Features of the BLOCK-iT™ Inducible H1 Lentiviral RNAi Expression System

The BLOCK-iT™ Lentiviral Pol II miR RNAi System includes the following key safety features:

- The pLenti6/V5-DEST expression vector contains a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.
- The number of genes from HIV-1 that are used in the system has been reduced to three (*i.e. gag*, *pol*, and *rev*).
- The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- Genes encoding the structural and other components required for packaging the viral genome are separated onto four plasmids. All four plasmids have been engineered not to contain any regions of homology with each other to prevent undesirable recombination events that could lead to the generation of a replication-competent virus (Dull *et al.*, 1998).
- Although the three packaging plasmids allow expression *in trans* of proteins required to produce viral progeny (*e.g.*, gal, pol, rev, env) in the 293FT producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus can be produced.
- The lentiviral particles produced in this system are replication-incompetent and only carry the gene of interest. No other viral species are produced.
- Expression of the *gag* and *pol* genes from pLP1 has been rendered Rev-dependent by virtue of the HIV-1 RRE in the gag/pol mRNA transcript. Addition of the RRE prevents gag and pol expression in the absence of Rev (Dull *et al.*, 1998).
- A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti6/V5-DEST expression vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

Continued on next page

Biosafety Features of the System, Continued

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this system can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus that express shRNA targeting human genes involved in controlling cell division (*e.g.*, tumor suppressor genes).

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

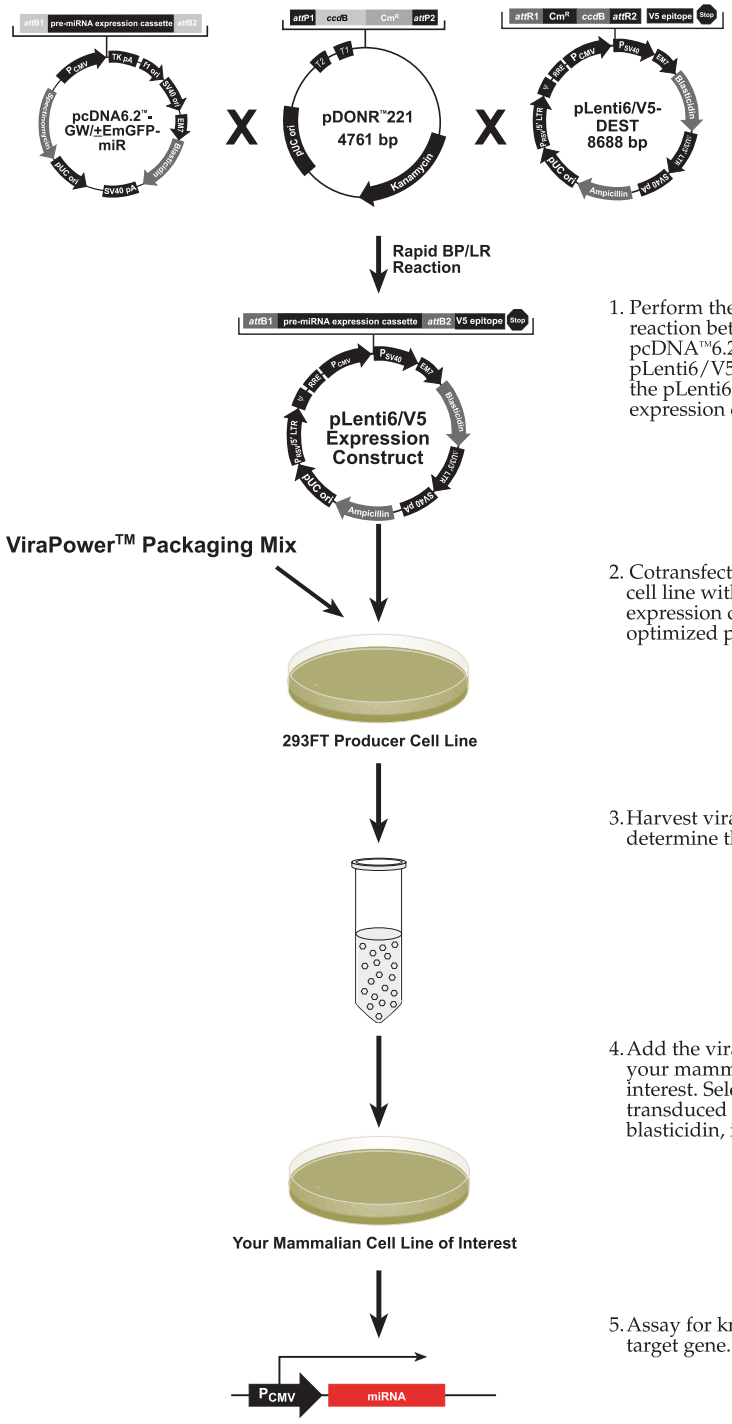
Important

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System.

Experimental Outline

Flow Chart

The diagram below describes the general steps required to express an shRNA of interest using the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System.



1. Perform the Rapid BP/LR recombination reaction between pDONR™221, pcDNA™6.2-GW/±EmGFP-miR, and pLenti6/V5-DEST to generate the pLenti6/V5-GW/±EmGFP-miR expression construct.
2. Cotransfect the 293FT producer cell line with your pLenti6/V5 expression construct and the optimized packaging mix.
3. Harvest viral supernatant and determine the titer.
4. Add the viral supernatant to your mammalian cell line of interest. Select for stably transduced cells using blasticidin, if desired.
5. Assay for knockdown of the target gene.

Methods

Cloning miRNA

Introduction

You will need to clone your miRNA sequence of interest contained within the engineered pre-miRNA into the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression vector using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits to generate an expression clone prior to expressing the miRNA sequence of interest from pLenti6/V5-DEST.

After generating the expression clone, you will transfer the pre-miRNA expression cassette from the expression clone into the destination vector, pLenti6/V5-DEST using a Rapid BP/LR recombination reaction (see next page).

General guidelines for cloning are provided below.

Using pcDNA6.2-GW/±EmGFP-miR

To generate an expression clone in pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR:

- Design and synthesize two complementary oligonucleotides containing the miRNA target sequence according to specified guidelines
- Anneal the oligonucleotides to create a double-stranded oligonucleotide
- Clone the double-stranded oligonucleotide into pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR using an optimized 5-minute ligation procedure
- Transform competent *E. coli* and select for expression clones

For detailed instructions and guidelines for generating an expression clone, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual. This manual is supplied with the kits and is also available at www.invitrogen.com or by contacting Technical Support (see page 79).

Creating Entry Clones for Use with pLenti6/V5-DEST

Introduction

Since the resulting pcDNA™6.2-GW/miR and pcDNA™6.2-GW/EmGFP-miR expression vectors contain *att*B sites, the expression vectors containing the pre-miRNA expression cassette **cannot** be used directly with the pLenti6/V5-DEST destination vector to perform the LR recombination reaction.

To express your miRNA sequence in mammalian cells using the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System, you need to first generate an entry clone containing *att*L sites by performing a BP recombination reaction, then use the entry clone in an LR recombination reaction with pLenti6/V5-DEST vector to generate a lentiviral expression clone.

The transfer of the miRNA sequence into the pLenti6/V5-DEST vector can be performed using the standard BP and LR recombination reactions or Rapid BP/LR recombination reactions as described below. See page 11 for an overview of the Gateway® recombination reactions.

To ensure that you obtain the best possible results, we recommend that you read this section, the sections entitled **Performing the Rapid BP/LR Recombination Reaction** (page 25), and **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 29) before beginning.

Choosing a Suitable Protocol

Based on your experimental needs, you may choose between the standard or Rapid BP/LR recombination reactions as described in the table below:

If You Wish to….	Then Choose…..	Described
Generate the expression clones using a fast protocol but obtain at least 10% fewer expression clones than the standard protocol	Rapid BP/LR Recombination Protocol	In this section.
Maximize the number of expression clones generated and isolate entry clones for future use	Standard BP and LR Protocols	On pages 57–62.

Rapid BP/LR Recombination Reaction

To express your miRNA sequence in mammalian cells using the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System, perform the Rapid BP/LR recombination reactions as follows:

Perform a BP recombination reaction between the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR expression clone and pDONR™221 donor vector using Gateway® BP Clonase® II Enzyme Mix. Then perform a LR recombination reaction between the resulting entry clone (pENTR™221/miR) and pLenti6/V5-DEST vector using Gateway® LR Clonase® II Enzyme Mix to produce a lentiviral expression clone. See page 11 for an overview on Gateway® recombination reactions.

Continued on next page

Creating Entry Clones for Use with pLenti6/V5-DEST, Continued

Experimental Outline

To generate an expression clone, you will:

1. Perform the BP recombination reaction using the *att*B-expression clone with miR of interest and *att*P-containing pDONR™221 vector to produce a pENTR™221/miR entry clone.
2. Mix an aliquot of the BP reaction (containing the entry clone) with the *att*R-containing pLenti6/V5-DEST vector and perform the LR recombination reaction to produce a lentiviral expression clone.
3. Transform the reaction mixture into a suitable *E. coli* host (see page 29).
4. Select for lentiviral expression clones (see page 23 for a diagram of the recombination region of expression clones in pLenti6/V5-DEST).

Substrates for the Recombination Reactions

To perform a BP recombination reaction, you need the following substrates:

- Linearized *att*B-containing expression clones (see the next page for guidelines to linearize *att*B expression clones)
- *att*P-containing donor (pDONR™221) vector (see below)

To perform an LR recombination reaction, you need the following substrates:

- Supercoiled *att*L entry vector (pENTR™221/miR)
- Supercoiled *att*R destination vector (pLenti6/V5-DEST)

Donor Vectors

The BLOCK-iT™ Lentiviral Pol II miR RNAi Kits includes the pDONR™221 vector. For a map and a description of the features of pDONR™221, see the **Appendix**, page 69. You may use other donor vectors, if desired.

Important

The pLenti6/V5-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual previously recommended using a linearized destination vector for more efficient LR recombination, further testing has found that linearization of pLenti6/V5-DEST is **not** required to obtain optimal results for any downstream application.

Continued on next page

Creating Entry Clones for Use with pLenti6/V5-DEST, Continued

Propagating the Donor and Destination Vectors

To propagate and maintain the pDONR™221 and pLenti6/V5-DEST vectors, use One Shot® *ccd*B 2 T1^R Chemically Competent *E. coli* (see page 77) for transformation. The One Shot® *ccd*B Survival 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

Donor Vector

To maintain the integrity of pDONR™221, select for transformants in media containing 50 µg/mL kanamycin and 15 µg/mL chloramphenicol.

Destination Vector

To maintain integrity of pLenti6/V5-DEST, select for transformants in media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.

Note: Do not use general *E. coli* cloning strains including Stbl3™, TOP10, or DH5α for propagation and maintenance, as these strains are sensitive to CcdB effects.

Linearizing Expression Clones

For best results, linearize the expression clone using *Eag* I or *Bsr*D I (see the guidelines below).

1. Linearize 1–2 µg of the expression clone with a restriction enzyme (*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.

Continued on next page

Creating Entry Clones for Use with pLenti6/V5-DEST, Continued

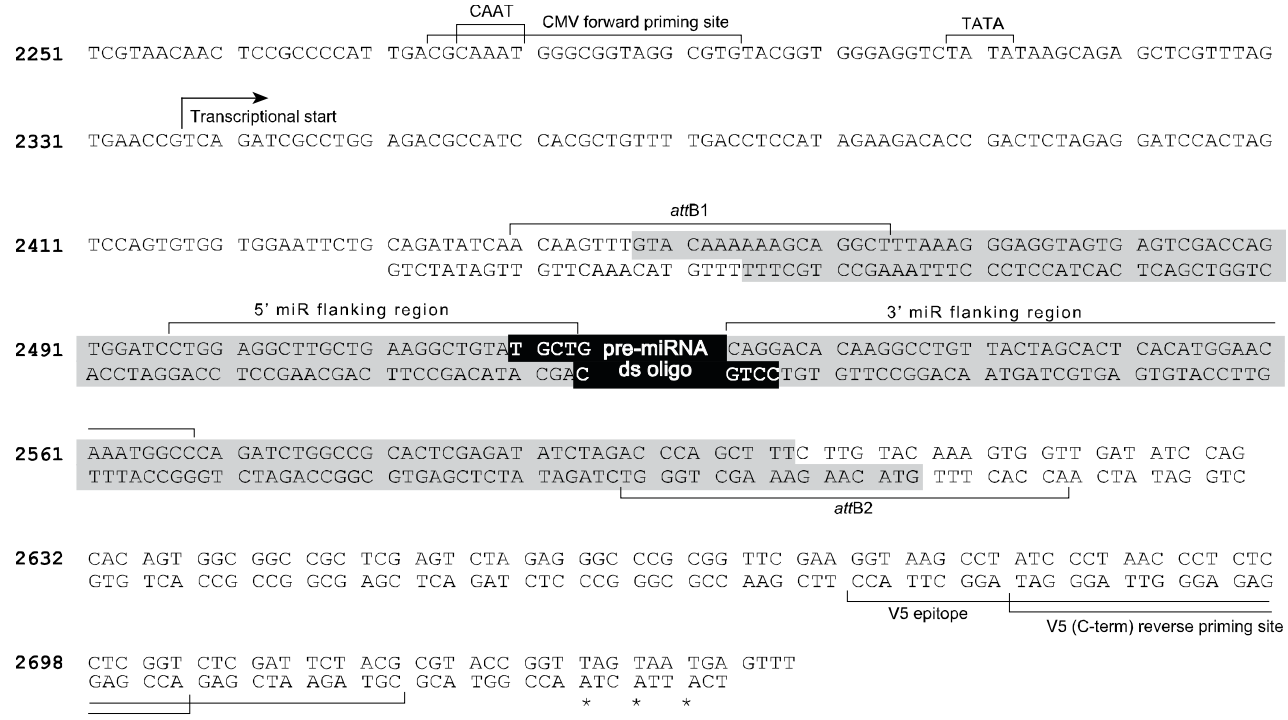
Recombination Region of pLenti6/V5-DEST

The recombination region of the lentiviral expression clone resulting from pLenti6/V5-DEST × pENTR™221/miR is shown below. The pENTR™221/miR entry clone is obtained by transferring the pre-miRNA expression cassette from pcDNA™6.2-GW/miR into the pDONR™221 vector.

Features of the Recombination Region:

Shaded regions correspond to DNA sequences transferred from the pENTR™221/miR entry clone into the pLenti6/V5-DEST vector by recombination. Non-shaded regions are derived from the pLenti6/V5-DEST vector.

Note: The DNA sequences transferred from the pENTR™221/miR entry clone contain the pre-miRNA expression cassette.



Note

Since the pLenti6-V5-GW/miR expression construct expresses a pre-miRNA sequence that is processed to form a mature miRNA and not a protein, the V5 epitope is not expressed.

Continued on next page

Creating Entry Clones for Use with pLenti6/V5-DEST, Continued

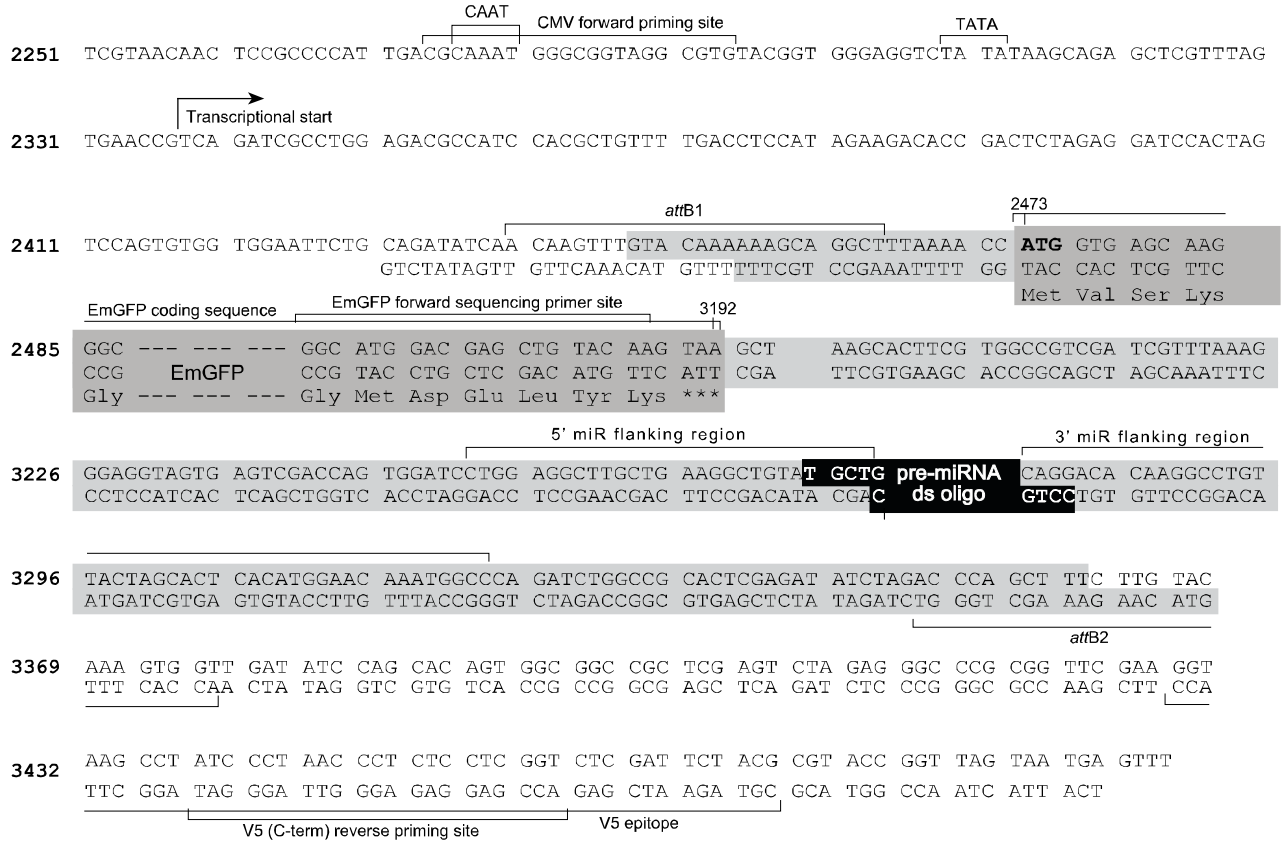
Recombination Region of pLenti6/V5-DEST, continued

The recombination region of the lentiviral expression clone resulting from pLenti6/V5-DEST × pENTR™221/EmGFP-miR is shown below. The pENTR™221/EmGFP-miR entry clone is obtained by transferring the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR into the pDONR™221 vector.

Features of the Recombination Region:

Shaded regions correspond to DNA sequences transferred from the pENTR™221/EmGFP-miR entry clone into the pLenti6/V5-DEST vector by recombination. Non-shaded regions are derived from the pLenti6/V5-DEST vector. Bases 2,473 and 3,192 indicate the coding sequence of EmGFP.

Note: The DNA sequences transferred from the pENTR™221/miR entry clone contain the pre-miRNA expression cassette including EmGFP coding sequence.



Note

Since the pLenti6-V5-GW/EmGFP-miR expression construct expresses a pre-miRNA sequence that is processed to form a mature miRNA and not a protein, the V5 epitope is not expressed.

Performing the Rapid BP/LR Recombination Reaction

Introduction

Follow the guidelines and instructions in this section to perform the Rapid BP/LR recombination reaction using the expression clone containing your pre-miRNA expression cassette, pDONR™221, and pLenti6/V5-DEST vector.

If you wish to perform the standard BP recombination reaction followed by the standard LR recombination reaction, see page 57 for details.

Rapid BP/LR Protocol

The Rapid BP/LR protocol is used to transfer a gene from one expression clone into another destination vector in 2 consecutive steps: a BP reaction using a donor vector followed by an LR recombination reaction using a destination vector without purification of the intermediate entry clone.

Note: The Rapid BP/LR protocol allows you to generate expression clones more rapidly than the standard BP and LR protocols provided on page 57. Fewer expression clones are obtained (~10% 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 recombination protocols on page 57.

Experimental Outline

To perform the Rapid BP/LR protocol, you will:

1. Perform a BP recombination reaction using the linearized expression clone containing your pre-miRNA sequence and pDONR™221 to generate the entry clone, pENTR™221/miR.
2. Use a small aliquot of the BP reaction mix to perform the LR recombination reaction using the pLenti6/V5-DEST destination vector to generate the lentiviral expression clone, pLenti6/V5-GW/miR.
3. Perform Proteinase K treatment.

Recommended *E. coli* Host

For optimal results, use Stbl3™ *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA, which contains direct repeats. One Shot® Stbl3™ Chemically Competent *E. coli* are included in the kit for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 29.

Important

Do not transform the BP or 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.

Continued on next page

Performing Performing the Rapid BP/LR Recombination Reaction, Continued

Positive Control

We recommend using the pcDNA™6.2-GW/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 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 Gateway® BP Clonase® II Enzyme Mix or pENTR™-gus supplied with the Gateway® LR Clonase® II Enzyme Mix as positive controls for the Rapid protocol due to the presence of incompatible selection markers.

Gateway® Clonase® II Enzyme Mixes

The Gateway® BP and Gateway® LR Clonase® II enzyme mixes combine the proprietary enzyme formulation and 5X Clonase Reaction Buffer previously supplied as separate components in Gateway® Clonase® enzyme mixes into an optimized single-tube format for easier set-up of the BP or LR recombination reaction. The Gateway® LR Clonase® II Enzyme catalyzes the *att*L x *att*R Gateway® recombination reaction while the Gateway® BP Clonase® II Enzyme catalyzes the *att*B x *att*P Gateway® recombination reaction. Use the protocol provided on page 27 to perform the recombination reactions using the Rapid protocol or page 57 using the standard protocol.

Gateway® BP and LR Clonase® II Enzyme Mixes are supplied with the kit and are also available separately (see page 77).

Note: You may perform the BP or LR recombination reaction with the Gateway® BP or LR Clonase® enzyme mix, if desired. To use the Gateway® BP or LR Clonase® enzyme mix, follow the protocol provided with the product. Do not use the protocol for Gateway® LR Clonase® II enzyme mix provided in this manual.

Converting Femtomoles (fmol) to Nanograms (ng)

Use the following formula to convert femtomoles (fmol) of DNA to nanograms (ng) of DNA required for BP reaction:

$$\text{ng} = (\text{fmol})(\text{N})(\frac{660\ \text{fg}}{\text{fmol}})(\frac{1\ \text{ng}}{10^6\ \text{fg}})$$

where N is the size of the DNA in bp. For an example, see below.

In this example, you need to use 50 fmol of an *att*B-PCR product in the BP reaction. The *att*B-PCR product is 2.5 kb in size. Calculate the amount of *att*B-PCR product required for the reaction (in ng) by using the above equation:

$$(50\ \text{fmol})(2500\ \text{bp})(\frac{660\ \text{fg}}{\text{fmol}})(\frac{1\ \text{ng}}{10^6\ \text{fg}}) = 82.5\ \text{ng of PCR product required}$$

Continued on next page

Performing Performing the Rapid BP/LR Recombination Reaction, Continued

Materials Needed

- Linearized expression clone (50–150 ng/µL in TE Buffer, pH 8.0, see page 22)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 77
- Sterile 0.5 mL microcentrifuge tubes

Components supplied with the kits

- pDONR™221 vector
- pLenti6/V5-DEST vector
- pcDNA™6.2-GW/±EmGFP-miR-neg control (if desired, supplied with BLOCK-iT™ Pol II miR RNAi Expression Vector Kits, Box 1)
- Gateway® BP Clonase® II enzyme mix (Box 9; store at −20°C until immediately before use)
- Gateway® LR Clonase® II enzyme mix (Box 10; store at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (supplied with Gateway® BP and LR Clonase® enzymes; thaw and keep on ice until use)

Setting Up the Rapid BP/LR Recombination Reaction

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 from page 22, (20–50 fmol)	1–7 µL	–
pcDNA™6.2-GW/±EmGFPmiR-neg control (diluted to 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 Gateway® BP Clonase® II enzyme mix from −20°C and thaw on ice (~ 2 minutes).
3. Vortex the Gateway® BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. To the samples above, add 2 µL of Gateway® BP Clonase® II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return Gateway® 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 to the samples.

Continued on next page

Performing Performing the Rapid BP/LR Recombination Reaction, Continued

Setting Up the Rapid BP/LR Recombination Reaction, Continued

6. Transfer 3 µL from each BP reaction from Step 5 to clean, sterile 0.5 mL microcentrifuge tubes. This reaction mix contains the resulting **entry clone**, pENTR™221/miR with or without EmGFP.

   Note: Save the remaining BP reaction mix at –20ºC for up to 1 week. You can treat the samples with Proteinase K and transform the reaction mix into One Shot® TOP10 Chemically Competent *E. coli* as described on page 60 to check the efficiency of the BP reaction and to isolate entry clones for future use. For transformation of the BP reaction only, you can use any *E. coli* including TOP10.

7. Add the following components to the tubes containing 3 µL BP reaction from Step 6 at room temperature and mix.

Component	Sample	Positive Control
pLenti6/V5-DEST vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	4 µL	4 µL

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

    Reminder: Return Gateway® 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.
13. Proceed to **Transforming One Shot® Stbl3™ Competent *E. coli***, next page.

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

Transforming One Shot® Stbl3™ Competent *E. coli*

Introduction

Follow the instructions in this section to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli* (Box 8) included with the kit. The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is $\geq 1 \times 10^8$ cfu/µg plasmid DNA.

Materials Needed

- LR recombination reaction (from Step 13, previous page or Step 7, page 62)
- LB Medium (if performing the pUC19 control transformation)
- 42°C water bath
- LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 37°C shaking and non-shaking incubator

Components supplied with the kit

- One Shot® Stbl3™ Chemically Competent *E. coli* (Box 8; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (Box 8; warm to room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency; Box 8)

One Shot® Stbl3™ Transformation Procedure

Use this procedure to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.

1. Thaw, on ice, one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2 to 3 µL of the LR recombination reaction (from Step 13, previous page or Step 7, page 62) into a vial of One Shot® Stbl3™ cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place them on ice for 2 minutes.
6. Add 250 µL of pre-warmed S.O.C. Medium to each vial.
7. Cap the vial(s) tightly and shake horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 25–100 µL of the transformation mix on a pre-warmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (*e.g.*, add 100 µL of the transformation mix to 900 µL of LB Medium) and plate 25–100 µL.
9. Store the remaining transformation mix at 4°C. Plate out additional cells the next day, if desired.

Continued on next page

Expected Results

When using One Shot® Stbl3™ Chemically Competent cells for transformation, the LR recombination reaction should result in greater than 4,000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

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

Sequencing

Sequencing the expression construct is not required as transfer of the miRNA cassette from pcDNA™6.2-GW/±EmGFP-miR into the pLenti6/V5-DEST vector preserves the orientation of the cassette. However, if you wish to sequence the pLenti6/V5 expression construct, we recommend using the following primers. Refer to the diagram on page 23 for the location of the primer binding sites in the expression vector.

Primer	Sequence
CMV Forward	5′-CGCAAATGGGCGGTAGGCGTG-3′
V5(C-term) Reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Note: For information about a convenient custom primer synthesis service go to www.invitrogen.com or call Technical Support (see page 79).

Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the expression clone in LB medium containing 100 µg/mL ampicillin.

Producing Lentivirus in 293FT Cells

Introduction

Before creating a stably transduced cell line expressing your shRNA of interest, first produce a lentiviral stock (containing the packaged pLenti6/V5 expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti6/V5-GW/miR expression construct into the 293FT Producer Cell Line.

Experimental Outline

To produce lentivirus in 293FT Cells, you will:

1. Grow the 293FT Cells to obtain 6×10^6 293FT cells for each sample.
2. Prepare plasmid DNA of your expression clone.
3. Cotransfect the ViraPower™ Packaging Mix and pLenti6/V5-GW/miR expression plasmid DNA into 293FT Cells using Lipofectamine® 2000.
4. Harvest virus-containing supernatants 48–72 hours post-transfection.

293FT Cell Line

The human 293FT Cell Line is supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T-antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin®. For more information about pCMVSPORT6TAg.neo and how to culture and maintain 293FT cells, refer to the 293FT Cell Line manual. This manual is supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits and is also available at www.invitrogen.com or by contacting Technical Support (see page 79).

Note: The 293FT Cell Line is also available separately (see page 77).

The health of your 293FT cells at the time of transfection has a critical effect on the success of lentivirus production. Use of "unhealthy" cells can negatively affect the transfection efficiency, resulting in production of a low titer lentiviral stock. For optimal lentivirus production (*i.e.* producing lentiviral stocks with the expected titers), follow the guidelines below to culture 293FT cells before use in transfection:

- Make sure that cells are greater than 90% viable.
- Subculture and maintain cells as recommended in the 293FT Cell Line manual. Do not allow cells to overgrow before passaging. You will need 6×10^6 293FT cells for each sample.
- Use cells that have been subcultured for less than 20 passages.

Continued on next page

ViraPower™ Packaging Mix

The pLP1, pLP2, pLP/VSVG plasmids are provided in an optimized mixture to facilitate viral packaging of the pLenti6/V5-GW/miR expression vector following cotransfection into 293FT producer cells.

The amount of the packaging mix (195 µg) and Lipofectamine® 2000 Reagent (0.75 mL) supplied in the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits is sufficient to perform 20 cotransfections in 10 cm plates using the recommended protocol on page 36.

Note: The ViraPower™ Packaging Mix is available separately (page 77) or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 77).

Plasmid Preparation

Once you have generated the 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 and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. Isolate plasmid DNA using the PureLink™ Plasmid Purification Kits (page 77) or CsCl gradient centrifugation.

Resuspend the purified pLenti6/V5-GW/miR expression plasmid in sterile water or TE Buffer, pH 8.0, to a final concentration ranging from 0.1–3.0 µg/µL. You will need 3 µg of the expression plasmid for each transfection.

Important: Do not use mini-prep plasmid DNA for transfection.

Lipofectamine® 2000

The Lipofectamine® 2000 reagent supplied with the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells (Ciccarone *et al.*, 1999). Using Lipofectamine® 2000 to transfect 293FT cells offers the following advantages:

- Provides the highest transfection efficiency in 293FT cells
- DNA-Lipofectamine® 2000 complexes can be added directly to cells in culture medium in the presence of serum
- Removal of complexes or medium change or addition following transfection is not required, although complexes can be removed after 4–6 hours without loss of activity

Note: Lipofectamine® 2000 is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 77).

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine® 2000 complexes, use Opti-MEM® I Reduced Serum Medium (see page 77). For more information about Opti-MEM® I, see www.invitrogen.com or contact Technical Support (see page 79).

Continued on next page

miR Positive Control

Generate the miR Positive Control using the reagents included in the kit as follows:

- Generate the pcDNA™6.2-GW/±EmGFP-miR-*lacZ* expression control using the *lacZ* double-stranded oligo and pcDNA™6.2-GW/±EmGFP-miR expression vector included with the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit and as described in the expression vector manual.
- Use the pcDNA™6.2-GW/±EmGFP-miR-*lacZ* expression control to generate the lentiviral construct with pLenti6-V5-DEST vector using the Rapid BP/LR recombination reaction as described in this manual.
- Use the resulting lentiviral expression construct, pLenti6/V5-GW/±EmGFP-miR-*lacZ*, to generate a miR control lentiviral stock (*lacZ* targeting miRNA).

Once generated, the miR control lentivirus may be transduced into mammalian cell lines (see page 47) to express an miRNA targeted to the human *lacZ* gene, and may be used as a control for the RNAi response in these cell lines.

pLenti6/V5-GW/*lacZ* Positive Control

A pLenti6/V5-GW/*lacZ* positive control vector is included with the pLenti6/V5-DEST vector for use as an expression control in the ViraPower™ Lentiviral Expression System. The β-galactosidase is expressed as a C-terminally tagged fusion protein that may be easily detected by western blot or functional assay. For details on the vector, see page 67.

To propagate and maintain the control plasmid:

1. Use the vector stock solution to transform a *rec*A, *end*A *E. coli* strain like Stbl3™, TOP10, DH5α™-T1^R, or equivalent. Use 10 ng of plasmid for transformation.
2. Select transformants on LB agar plates containing 100 µg/mL ampicillin (for **Stbl3**™ cells) or LB agar plates containing 100 µg/mL ampicillin and 50 µg/mL Blasticidin (for **TOP10 or DH5α**).
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage. Propagate the plasmid in LB containing 100 µg/mL ampicillin.
4. Use the pLenti6/V5-GW/*lacZ* positive control to generate a control lentiviral stock (expressing the LacZ protein).
5. Use the pLenti6/V5-GW/*lacZ* lentiviral control and the pLenti6/V5-GW/±EmGFP-miR-*lacZ* lentiviral control in cotransduction experiments as a positive control for lentiviral induced RNAi analysis in your system (see page 46 for details).

Continued on next page

Producing Lentivirus in 293FT Cells, Continued

Materials Needed

- pLenti6/V5-GW/miR expression construct (0.1–3.0 µg/µL in sterile water or TE Buffer, pH 8.0)
- Positive controls (see previous page to generate positive controls; resuspend in sterile water to a concentration of 1 µg/µL)
- 293FT cells cultured in the appropriate medium (*i.e.* D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin/streptomycin). You will need 6×10^6 293FT cells for each sample.

  Note: D-MEM already contains 4 mM L-glutamine, which is enough to support cell growth of the 293FT Cell Line. However, since L-glutamine slowly decays over time, supplement the medium with 2 mM L-glutamine to ensure that the concentration of L-glutamine will not get too low over time due to its slow degradation. 293FT cells grow well in 6 mM L-glutamine, but higher concentrations of L-glutamine may reduce growth.
- Opti-MEM® I Reduced Serum Medium (pre-warmed; see page 32)
- Fetal bovine serum (FBS; see page 77)
- Complete growth medium containing sodium pyruvate (*i.e.* D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available separately, see page 77. See note above for L-glutamine concentration.
- Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)
- Sterile, tissue culture supplies
- 5 and 15 mL sterile, capped, conical tubes
- Cryovials

Components supplied with the kits

- ViraPower™ Packaging Mix
- Lipofectamine® 2000 transfection reagent (store at 4°C and mix gently before use)

Continued on next page

Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the **optimized** transfection conditions shown below. The amount of lentivirus produced using these recommended conditions (at a titer of 1×10^5 to 1×10^7 transducing units (TU)/mL) is generally sufficient to transduce 1×10^6 to 1×10^8 cells at a multiplicity of infection (MOI) = 1.

Condition	Amount
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	6×10^6 cells (see **Recommendation** on page 31 to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg (9 µL of 1 µg/µL stock)
Amount of pLenti6/V5-GW/miR expression plasmid	3 µg
Amount of Lipofectamine® 2000 Reagent to use	36 µL

Note: You may produce lentiviral stocks using other tissue culture formats, but keep in mind that optimization will be necessary to obtain the expected titers.

The recommended procedure to co-transfect 293FT cells differs from the traditional Lipofectamine® 2000 transfection procedure in that you will:

- First prepare DNA:Lipofectamine® 2000 complexes and add them to plates containing growth media, then
- Add the 293FT cells to the media containing DNA:Lipofectamine® 2000 complexes and allow the cells to attach and transfect overnight (see details on the next page).

Using this procedure, we consistently obtain lentiviral stocks with titers that are **3 to 4-fold higher** than lentiviral stocks generated using the traditional Lipofectamine® 2000 transfection procedure (*i.e.* plating cells first followed by transfection with DNA:Lipofectamine® 2000 complexes). You may use the traditional Lipofectamine® 2000 transfection procedure, if desired, but keep in mind that the viral titer obtained may be lower (see **Alternative Transfection Procedure**, page 37).

Continued on next page

Transfection Procedure

Follow the procedure below to cotransfect 293FT cells. Include a negative control (no DNA, no Lipofectamine® 2000) in your experiment to help evaluate results. You will need 6×10^6 293FT cells for each sample.

1. **For each transfection sample,** prepare DNA-Lipofectamine® 2000 complexes as follows:
   a. In a sterile 5 mL tube, combine 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti6/V5-GW/miR expression plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 5 mL tube, mix Lipofectamine® 2000 gently before use, then dilute 36 µL in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine® 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine® 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
2. While DNA-lipid complexes are forming, trypsinize and count the 293FT cells. Resuspend the cells at a density of 1.2×10^6 cells/mL in growth medium containing serum (or Opti-MEM® I Medium containing serum).
3. Add the DNA-Lipofectamine® 2000 complexes to a 10 cm tissue culture plate containing 5 mL of growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not add antibiotics to the medium.**
4. Add 5 mL of the 293FT cell suspension (6×10^6 total cells) to the plate containing media and DNA-Lipofectamine® 2000 complexes and mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.
5. The next day, remove the media containing the DNA-Lipofectamine® 2000 complexes and replace with complete culture medium containing sodium pyruvate (see page 34).

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of multinucleated syncitia. This morphological change is normal and does not affect lentivirus production.
6. Harvest virus-containing supernatants 48–72 hours posttransfection by removing medium to a 15 mL sterile, capped, conical tube.

   Note: Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours posttransfection.

   Caution: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see pages 17 and 40 for more information).
7. Centrifuge at 3000 rpm for 5 minutes at 4°C to pellet cell debris. Perform filtration step, if desired (see **Note** on the next page).
8. Pipet viral supernatants into cryovials in 1 mL aliquots. Store viral stocks at −80°C. Proceed to **Titering Your Lentiviral Stock**, page 38.

Continued on next page

Alternative Transfection Procedure

An alternative transfection procedure is provided below to cotransfect 293FT cells. Note that use of this procedure generally results in production of lentiviral stocks with a slightly lower titer that those produced when using the recommended **Transfection Procedure**, previous page.

1. The day before transfection, plate 293FT cells in a 10 cm tissue culture plate such that they will be 90–95% confluent on the day of transfection (*i.e.* 6×10^6 cells in 10 mL of growth medium containing serum).
2. On the day of transfection, remove the culture medium from the 293FT cells and replace with 5 mL of growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not include antibiotics in the medium.**
3. Prepare DNA-Lipofectamine® 2000 complexes as instructed in the recommended **Transfection Procedure**, Step 1, previous page.
4. Add the DNA-Lipofectamine® 2000 complexes dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.
5. Follow Steps 5–8 as instructed in the recommended **Transfection Procedure**, previous page.

Note

If you plan to use your lentiviral construct for *in vivo* applications, filter your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (see Step 7, previous page) to remove any remaining cellular debris. We recommend using Millex®-HV 0.45 µm PVDF filters (Millipore, Cat. no. SLHV033RB) for filtration.

If you wish to concentrate your viral stock to obtain a higher titer, perform the filtration step first before concentrating your viral stock.

Long-Term Storage

Place lentiviral stocks at −80°C for long-term storage. Repeated freezing and thawing is not recommended as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, re-titer your viral stocks before transducing the mammalian cell line of interest.

Scaling Up Virus Production

It is possible to scale up the cotransfection experiment to produce a larger volume of lentivirus, if desired. For example, we have scaled up the cotransfection experiment from a 10 cm plate to a T-175 cm^2 flask and harvested up to 30 mL of viral supernatant. To scale up your cotransfection, increase the number of cells plated and the amounts of DNA, Lipofectamine® 2000, and medium used in proportion to the difference in surface area of the culture vessel.

Titering Your Lentiviral Stock

Introduction

Before transducing a mammalian cell line of interest and expressing the miRNA for RNAi analysis, we highly recommend determining the titer of your lentiviral stock. While this procedure is not required for some applications, it is necessary if:

- You wish to control the number of integrated copies of the lentivirus
- You wish to generate reproducible gene knockdown results

Titering Methods

You can determine the titer of your lentiviral stock using any of the following methods:

- Blasticidin selection (usually takes 2 weeks to determine the titer)
- EmGFP detection (usually takes 4 days post-transduction to determine the titer), if the miRNA sequence was cloned into pcDNA™6.2-GW/EmGFP-miR vector

Experimental Outline

To determine the titer of a lentiviral stock, you will:

1. Prepare 10-fold serial dilutions of your lentiviral stock
2. Transduce the different dilutions of lentivirus into a mammalian cell line of choice in the presence of Polybrene®
3. Select for stably transduced cells using the appropriate selection agent
4. Stain and count the number of antibiotic-resistant colonies in each dilution

Factors Affecting Viral Titer

A number of factors can influence lentiviral titers including:

- The characteristics of the cell line used for titering (see the next page).
- The age of your lentiviral stock. Viral titers may decrease with long-term storage at −80°C. If your lentiviral stock has been stored for longer than 6 months, titer or re-titer your lentiviral stock prior to use in an RNAi experiment.
- Number of freeze/thaw cycles. Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- Improper storage of the lentiviral stock. Lentiviral stocks should be aliquotted and stored at −80°C (see page 37 for recommended storage conditions).

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Titering Your Lentiviral Stock, Continued

Selecting a Cell Line

You may titer your lentiviral stock using any mammalian cell line of choice. Generally, we recommend using the same mammalian cell line to titer your lentiviral stock as you will use to perform your expression studies. However, in some instances, you may wish to use a different cell line to titer your lentivirus (*e.g.*, if you are performing RNAi studies in a non-dividing cell line or a primary cell line). In these cases, we recommend that you choose a cell line with the following characteristics to titer your lentivirus:

- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18–25 hours
- Non-migratory

We generally use the HT1080 human fibrosarcoma cell line (ATCC, Cat. no. CCL-121) for titering purposes.

Important: You may use other cell lines, including HeLa and NIH/3T3, to titer your lentivirus. However, note that the titer obtained when using HeLa cells or NIH/3T3 cells is approximately **10-fold lower** than the titer obtained when using HT1080 cells.

Note

The titer of a lentiviral construct may vary depending on which cell line is chosen (see **Selecting a Cell Line**, above). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line.

Blasticidin Selection

The pLenti6/V5-GW/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 have stably transduced the lentiviral construct.

If you are using the BLOCK-iT™ Lentiviral Pol II miR RNAi Kits, Blasticidin is supplied with the kit. Blasticidin is also available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 77 for ordering information). For more information about how to prepare and handle Blasticidin, and determine the Blasticidin sensitivity, refer to the **Appendix**, page 63.

Using Polybrene® During Transduction

Transduction of lentivirus into mammalian cells may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene®). For best results, we recommend performing transduction in the presence of Polybrene®. Note however, that some cells are sensitive to Polybrene® (*e.g.*, primary neurons). Before performing any transduction experiments, you may want to test your cell line for sensitivity to Polybrene®. If your cells are sensitive to Polybrene® (*e.g.*, exhibit toxicity or phenotypic changes), do not add Polybrene® during transduction. In this case, cells should still be successfully transduced.

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Titering Your Lentiviral Stock, Continued

Preparing and Storing Polybrene®

Follow the instructions below to prepare Polybrene® (Sigma-Aldrich, Cat. no. H9268):

1. Prepare a 6 mg/mL stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 mL aliquots into sterile microcentrifuge tubes.
3. Store at –20°C for long-term storage. Stock solutions may be stored at –20°C for up to 1 year. Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

   Note: The working stock may be stored at 4°C for up to 2 weeks.

Materials Needed

- Your Lenti6/V5-DEST lentiviral stock (store at –80°C until use)
- Adherent mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- 6-well tissue culture plates
- Crystal violet (Sigma-Aldrich®, Cat. no. C3886; prepare a 1% crystal violet solution in 10% ethanol), if you are using Blasticidin selection for titering
- Inverted fluorescence microscope and appropriate filters for EmGFP visualization (see page 15 for filters), if you are using EmGFP titering method
- Phosphate-Buffered Saline (PBS; page 77)

Components supplied with the kits

- Blasticidin (10 mg/mL stock), if you are using Blasticidin selection for titering

Remember that you will be working with media containing infectious virus. Follow the recommended Federal and institutional guidelines for working with BL-2 organisms.

- Perform all manipulations within a certified biosafety cabinet.
- Treat media containing virus with bleach.
- Treat used pipets, pipette tips, and other tissue culture supplies with bleach and dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

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Titering Your Lentiviral Stock, Continued

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of your choice.

Note: You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions).

- If you have generated a lentiviral stock of the pLenti6-V5-GW/miR-*lacZ* control construct with or without EmGFP, perform titering using the Blasticidin or EmGFP method.
- If you generated a lentiviral stock of the pLenti6-V5-GW/*lacZ* control construct, use the Blasticidin titering method.

1. The day before transduction (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C overnight.

   Example: When using HT1080 cells, we plate 2×10^5 cells per well in a 6-well plate.

2. On the day of transduction (Day 2), thaw your lentiviral stock and prepare 10-fold serial dilutions ranging from 10^{-2} to 10^{-6}. For each dilution, dilute the lentiviral construct into complete culture medium to a final volume of 1 mL. **DO NOT** vortex.

   Note: You may prepare a wider range of serial dilutions (10^{-2} to 10^{-8}), if desired.

3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 mL).
4. Add Polybrene® (if desired) to each well to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. The following day (Day 3), remove the media containing virus and replace with 2 mL of complete culture medium.
6. The following day (Day 4), proceed to Steps 7–8 for the **EmGFP titering method** or proceed to Steps 9–14 for the **Blasticidin titering method**.
7. Determine the titer by flow cytometry on Day 4 for titering EmGFP. For each viral dilution well of the 6 well plate, trypsinize and resuspend the cells in complete media at a concentration of 10–500 cells/µL.
8. Using a flow cytometry system, determine the percentage of GFP-positive cells for each dilution (see next page). Determine the titer using the formula described on the next page.
9. For Blasticidin selection, remove the medium on Day 4 and replace with complete culture medium containing the appropriate amount of Blasticidin to select for stably transduced cells.
10. Replace medium with fresh medium containing Blasticidin every 3–4 days.
11. After 10–12 days of selection (day 14–16), you should see no live cells in the mock well and discrete Blasticidin-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
12. Add crystal violet solution (1 mL for 6-well dish; 5 mL for 10 cm plate) and incubate for 10 minutes at room temperature.
13. Remove the crystal violet stain and wash the cells with PBS. Repeat wash.
14. Count the blue-stained colonies and determine your lentiviral stock titer.

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Titering Your Lentiviral Stock, Continued

Preparing Cells for Flow Cytometry

If you have used the EmGFP titering method, prepare cells for flow cytometry according to the established protocols in use at your flow cytometry facility. Refer to page 15 for spectral characteristics of EmGFP. The steps below provide general guidelines, but other methods may be suitable.

1. At day 4 post-transduction, dissociate the cells from the plate by using trypsin or cell dissociation buffer.
2. Spin the cells at low speed to remove residual media components and resuspend the cell pellet in flow cytometry buffer such as calcium/magnesium-free PBS with 1% FBS at the required density for analysis on your flow cytometer. Fixing the cells is not necessary for analysis, but may be done, if desired.

   Note: To fix your cells before flow cytometry, use 2% formaldehyde or paraformaldehyde in calcium/magnesium-free PBS. However, these fixatives may increase autofluorescence of cells; thus, it is critical to include fixed, mock-transduced cells as a negative control for flow cytometry.
3. Use the mock-transduced cells and the lowest dilution of virus (*i.e.* 10^{-2}) as the negative and positive samples, respectively, to set up the parameters of your flow cytometer.

Calculating Lentiviral Titer

Calculate the EmGFP lentivirus titers from the dilutions at which the percentage of EmGFP-positive cells fall within the range of 1–30% (Sastry *et al.*, 2002; White *et al.*, 1999) to avoid analyzing dilution samples containing multiple integrated lentiviral genomes, which may result in underestimating the viral titer, or dilution samples containing too few transduced cells, which will give inaccurate results. The titer is expressed as transducing units (TU)/mL.

Use the following formula to calculate the titer:

[F × C/V] × D

F = frequency of GFP-positive cells (percentage obtained divided by 100)

C = total number of cells in the well at the time of transduction

V = volume of inoculum in mL

D = lentivirus dilution

An example for calculating the lentiviral titer is provided below. An EmGFP lentiviral stock was generated using the protocol on the previous page. The following data were generated after performing flow cytometry analysis:

Lentivirus Dilution	% EmGFP Positive Cells
10^{-2}	91.5%
10^{-3}	34.6%
10^{-4}	4.4%

In the above example, the 10^{-4} dilution is used to calculate the titer since the percentage of EmGFP-positive cells falls into the desired range of 1–30%. The frequency of EmGFP-positive cells is 4.4/100 = 0.044, multiplied by 2×10^5 (the number of cells in the well) divided by 1 (the volume of inoculum). Thus the calculation is as follows:

$$[(0.044 \times 200{,}000)/1] \times 10^4$$

The lentiviral titer for this example is 8.8×10^7 TU/mL.

Continued on next page

Titering Your Lentiviral Stock, Continued

Expected Results

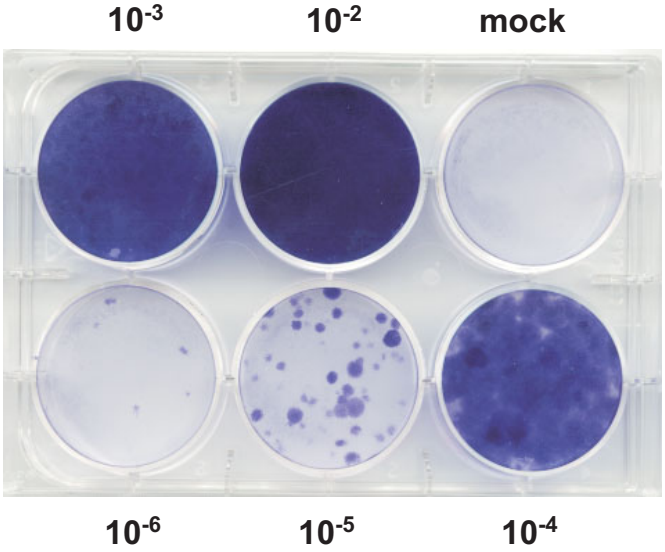
When titering pLenti/V5 lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 5×10^5 to 2×10^7 transducing units (TU)/mL.

For an example of expected results obtained from a typical titering experiment using Blasticidin, see below.

Note: If the titer of your lentiviral stock is less than 1×10^5 TU/mL, we recommend producing a new lentiviral stock. See page 31 and the **Troubleshooting** section, page 52 for more tips and guidelines to optimize the viral yield.

Example of Expected Results

In this experiment, a pLenti6 lentiviral stock was generated using the protocol on page 36. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock) following the protocol on page 41. Forty-eight hours post-transduction, the cells were placed under Blasticidin selection (10 µg/mL). After 10 days of selection, the cells were stained with crystal violet (see plate below), and colonies were counted.



In the plate above, the colony counts were:

- Mock: no colonies
- 10^{-2} dilution: confluent; undeterminable
- 10^{-3} dilution: confluent; undeterminable
- 10^{-4} dilution: confluent; undeterminable
- 10^{-5} dilution: 46
- 10^{-6} dilution: 5

Thus, the titer of this lentiviral stock is 4.8×10^6 TU/mL (*i.e.* average of 46×10^5 and 5×10^6).

Transduction and Analysis

Introduction

Once you have generated lentiviral stocks with suitable titers, you are ready to transduce the lentiviral constructs into a mammalian cell line of choice to inducibly express the miRNA of interest and perform RNAi analysis.

Reminder: Remember that each lentiviral construct contains a deletion in the 3′ LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once integrated into the genome, the lentivirus can no longer produce packageable virus.

Experimental Outline

To perform transduction, you will:

1. Determine the Multiplicity of Infection (MOI) and antibiotic sensitivity for your cell line.
2. Grow the mammalian cell line of choice.
3. Transduce the mammalian cell line of choice with your lentiviral construct in the presence of Polybrene®.
4. Harvest cells after 48–96 hours to perform transient knockdown experiments or select for stably transduced cells using Blasticidin.
5. Expand at least 5 Blasticidin-resistant colonies and analyze each clone to assay for knockdown of the target gene.

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:

- Transduction efficiency
- MOI used to transduce cells
- Transcription rate of the target gene of interest
- Stability of the target protein
- Growth characteristics of your mammalian cell line
- Activity of your miRNA in transient transfections

Take these factors into account when designing your transduction and RNAi experiments.

Transient vs. Stable Expression

After transducing your lentiviral construct into the mammalian cell line of choice, you may assay for target gene knockdown in the following ways:

- Pool a heterogeneous population of cells and test for gene knockdown directly after transduction (*i.e.* "transient" RNAi analysis). Note that you must wait for a minimum of 48–72 hours after transduction before harvesting your cells to allow expressed miRNA molecules to accumulate in transduced cells.
- Select for stably transduced cells using Blasticidin. This requires a minimum of 10–12 days after transduction, but allows generation of clonal cell lines that stably express the miRNA sequence.

Continued on next page

Determining Antibiotic Sensitivity for Your Cell Line

Before selecting for stably transduced cells, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). For guidelines to perform a kill curve experiment, see page 64. If you titered your lentiviral construct in the same mammalian cell line that you are using to generate a stable cell line, then you may use the same concentration of Blasticidin for selection that you used for titering.

Multiplicity of Infection (MOI)

To obtain optimal expression of your miRNA and therefore, the highest degree of target gene knockdown, you will need to transduce the lentiviral construct into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with the number of integration events and as a result, expression. Typically, miRNA expression levels increase as the MOI increases.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including:

- The nature of your mammalian cell line (*e.g.*, non-dividing vs. dividing cell type; see **Note**, below)
- The transduction efficiency of your mammalian cell line
- The nature of your target gene of interest

If you are transducing the lentiviral construct into a mammalian cell line of choice for the first time, use a range of MOIs (*e.g.*, 0, 1, 5, 10, 50) to determine the MOI required to obtain the optimal level of target gene knockdown.

Note

In general, lentiviral constructs transduce non-dividing cell types less efficiently than actively dividing cell lines. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI to achieve optimal target gene knockdown.

Preparing Mammalian Cells

Initiate your mammalian cell line of choice that will be used for titering. Grow the cells in the appropriate medium. Cells should be >95% viable.

Continued on next page

Positive Control

If you have generated two positive control lentiviral constructs (pLenti6/V5-GW/±EmGFP-miR-*lacZ* control and pLenti6/V5-GW/*lacZ* control constructs) as described on page 33, you may use the controls in cotransduction experiments to verify the lentiviral induced RNAi response in mammalian cells.

For cotransductions, use a 3:1 MOI ratio of pLenti6/V5-GW/miR-*lacZ* to pLenti6/V5-GW/*lacZ* expression clone. For expected results, see page 50.

The β-galactosidase protein expressed from the pLenti6/V5-GW/*lacZ* control lentiviral construct is approximately 121 kDa in size. You may assay for β-galactosidase expression by activity assay FluoReporter® *lacZ*/Galactosidase Quantitation Kit or by staining the cells for activity using the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression, see page 77 for ordering information.

Important

Remember that viral supernatants are generated by harvesting spent media containing virus from the 293FT producer cells. Spent media lacks nutrients and may contain some toxic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (*e.g.*, 1 mL of viral supernatant per well in a 6-well plate), note that growth characteristics or morphology of the cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting the transducibility of the lentivirus. If the titer of your lentiviral stock is relatively low (less than 5×10^5 TU/mL) and your experiment requires that you use a large volume of viral supernatant (*e.g.*, a relatively high MOI), you may wish to concentrate your virus before proceeding to transduction. For details and guidelines to concentrate your virus, refer to published reference sources (Yee, 1999).

Materials Needed

- Titered lentiviral stock (store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- Appropriately sized tissue culture plates for your application

Components supplied with the kits

- 10 mg/mL Blasticidin stock (if selecting for stably transduced cells)

Continued on next page

Transduction Procedure

Follow the procedure below to transduce your mammalian cell line of choice with your lentiviral construct.

1. Plate cells in complete media as appropriate for your application.
   When determining the density at which to plate cells, remember to take into account the length of time cells will be cultured prior to performing RNAi analysis (*e.g.*, 48 hours vs. 120 hours).
2. On the day of transduction (Day 1), thaw the lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

   Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 6 hours prior to changing medium.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.
6. The following day (Day 3), perform one of the following:
   - Harvest the cells and assay for inhibition of your target gene if you are performing transient expression experiments. If you wish to assay the cells at a later time, you may continue to culture the cells or replate them into larger-sized tissue culture formats as necessary.
   - Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin to select for stably transduced cells. Proceed to Step 7.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days until Blasticidin-resistant colonies can be identified (generally 10–12 days after selection).
8. Pick at least 5 Blasticidin-resistant colonies (see **Note**, below) and expand each clone to assay for knockdown of the target gene.

Note

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of target gene knockdown from different Blasticidin-resistant clones. Test at least 5 Blasticidin-resistant clones and select the clone that provides the optimal degree of gene knockdown for further studies.

Continued on next page

Performing RNAi Analysis

Use any method as appropriate to assay for knockdown of your target gene including functional analysis, immunofluorescence, western blot, or real-time quantitative RT-PCR (qRT-PCR) with the appropriate fluorogenic LUX™ primers. For more information about LUX™ primers or to design LUX™ primers, see www.invitrogen.com/lux.

If you have used the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP, you can detect EmGFP fluorescence using fluorescence microscopy or flow cytometry (see the next page).

Expected Results

When performing RNAi studies using pLenti6/V5 lentiviral constructs, we generally observe significant inhibition of gene expression within 48 to 120 hours after transduction. The degree of gene knockdown depends on the time of assay, stability of the protein of interest, and on the other factors listed on page 44. Note that 100% gene knockdown is generally not observed, but > 80% is possible with optimized conditions

For an example of results obtained from RNAi experiments using the pLenti6-V5-DEST, see page 50.

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, if you used pcDNA™6.2-GW/EmGFP-miR for cloning your miRNA sequence. 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. The EmGFP expression is essentially 100% correlated with the expression of your miRNA.

See below for recommended fluorescence microscopy filter sets.

Filters for Use with EmGFP

EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein, such as the Omega XF100-2 or XF100-3 filter set for fluorescence microscopy.

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

Excitation (nm)	Emission (nm)
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 an 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.

Detecting Transfected Cells

After transduction, 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.

Expected Results

Cells expressing EmGFP will appear brightly labeled and will emit a green fluorescence signal that should be easy to detect above the background fluorescence.

Note: The fluorescence signal of EmGFP from miRNA-containing vectors is reduced when compared to non-miRNA containing vectors.

Cells with bright fluorescence will demonstrate highest knockdown with a functional miRNA. However, cells with reduced fluorescence may still express the miRNA and demonstrate knockdown since the expression levels required to observe gene knockdown are generally lower than that required to detect EmGFP expression.

Examples of Expected Results

Example of Expected Results

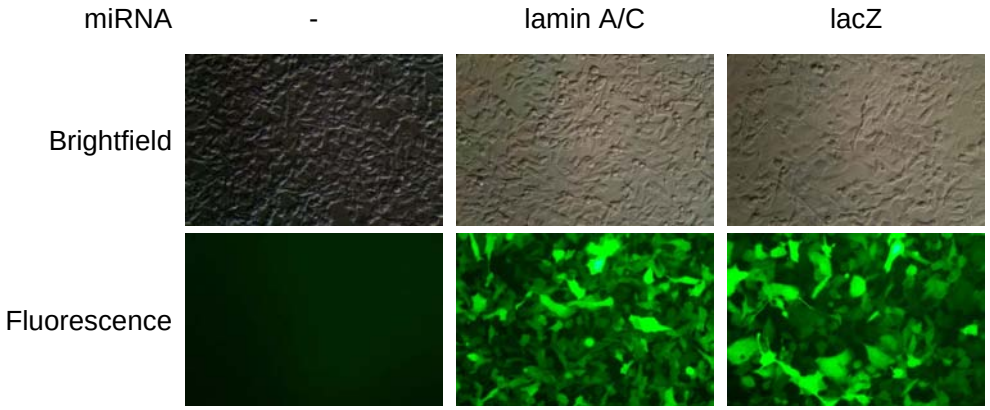
In this experiment, a double-stranded miR-*lac*Z control oligo (directed towards exogenous LacZ) and a double-stranded miR-lamin oligo (directed towards endogenous lamin) were cloned into pcDNA6.2™-GW/EmGFP-miR expression vector using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit. The miR-*lac*Z and miR-lamin cassettes were transferred into the pLenti6/V5-DEST vector using the Rapid BP/LR recombination reaction to generate the pLenti6-GW/EmGFP-miR-*lacZ* and pLenti6-GW/EmGFP-miR-lamin expression constructs, respectively. Lentiviral stocks were generated and titered in HT1080 cells following the protocols in this manual.

HT1080 cells plated in a 12-well plate were co-transduced with the following lentiviral particles:

- Expressing the *lacZ*-directed miRNA with EmGFP at an MOI of 30 and pLenti6/V5-GW/*lacZ* at an MOI of 10
- Expressing the lamin A/C-directed miRNA with EmGFP at an MOI of 30 and pLenti6/V5-GW/*lacZ* at an MOI of 10.

Cells were harvested 48 hours post-transduction and subjected to fluorescence microscopy using the appropriate filters as described in this manual. Fluorescence microscopy results are shown in figure A below and indicate the expression of EmGFP in virtually all cells transduced with the miRNA lentiviruses.

Figure A



Cell lysates were then prepared from duplicate wells 48 hours (*i.e.* 2 days) after transduction. β-galactosidase activity was determined from equivalent amounts of cell lysate using the FluoReporter® *lacZ*/Galactosidase Quantitation Kit (see page 77). Results are shown in figure B, next page.

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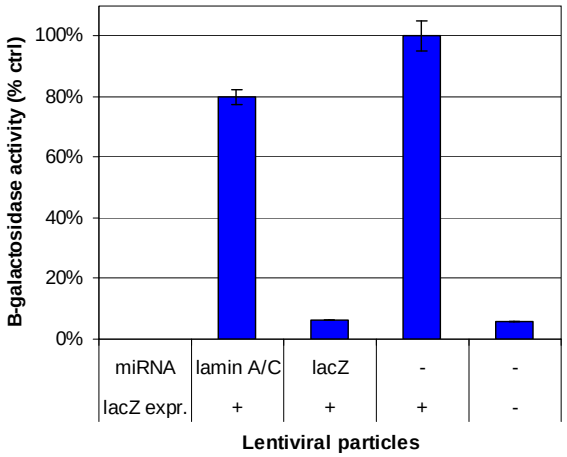
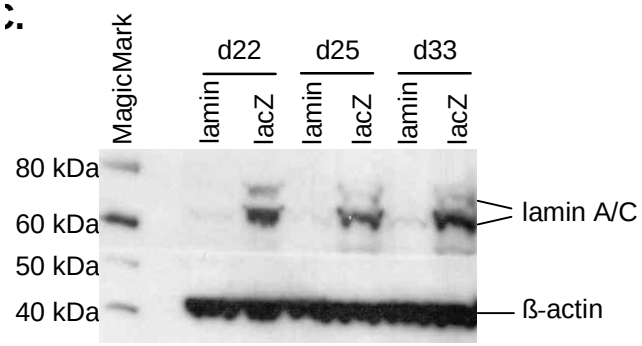
Example of Expected Results, Continued

Figure B depicts a dramatic decrease in β-galactosidase activity only in cells co-transduced with the *lacZ*-directed miRNA and not lamin-directed miRNA.



HeLa cells were transduced with the *lacZ* and lamin miRNA lentiviruses and stable cell lines were generated using Blasticidin selection as described in this manual. Cell lysates were prepared from stably transduced cells after 22–33 days and subjected to western blot analysis. The western blot was cut in half and one half was probed with Anti-Lamin A/C Antibody (1:1,000 dilution, BD Biosciences, Cat. no. 612162) and the other half was probed with Anti-β-Actin Antibody (1:5,000 dilution, Abcam, Cat. no. ab6276). The blot was developed using WesternBreeze® Chemiluminescent Kit, see page 77. Western blot results are shown in figure C, below. The results show substantial decrease in the lamin A/C protein isoforms (molecular weight of lamin A is 70 kDa and lamin C is 65 kDa) even 33 days post-transduction indicating the reliable and stable transduction of lamin-directed miRNA in lentiviral system.

Figure C



Troubleshooting

Introduction

Review the information in this section to troubleshoot your lentiviral expression experiments. To troubleshoot oligo design and cloning experiments, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.

Rapid BP/LR Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the Rapid BP/LR recombination and transformation procedures.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 100 µg/mL ampicillin.
	Rapid BP/LR reaction may not work for your insert	Use the standard BP and LR recombination reactions as described on page 57.
	BP recombination reaction is treated with Proteinase K	**Do not** treat reaction the BP reaction with Proteinase K before the LR reaction.
	Did not use the suggested Gateway® Clonase® II enzyme mixes or Gateway® Clonase® II enzyme mixes were inactive	• Make sure to store the Gateway® BP and LR Clonase® II enzyme mix at –20°C or –80°C. • Do not freeze/thaw the Gateway® BP and LR Clonase® II enzyme mix more than 10 times. • Use the recommended amount of Gateway® BP and LR Clonase® II enzyme mix (see page 27). • Test another aliquot of the Gateway® Clonase® II enzyme mix.
	Not enough LR reaction transformed	Transform 2–3 µL of the LR reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	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 transformation mixture for 1 hour at 37°C with shaking before plating.
	Too much BP reaction used in the LR reaction	Use 3 µL BP reaction for the LR reaction.

Continued on next page

Rapid BP/LR Reaction and Transformation, Continued

Problem	Reason	Solution
Different sized colonies (*i.e.* large and small) appear when using TOP10 *E. coli* for transformation	Some transformants contain plasmids in which unwanted recombination has occurred between 5′ and 3′ LTRs	Always use the One Shot® Stbl3™ Chemically Competent *E. coli* supplied with the kit for transformation. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally give rise to fewer unwanted recombinants.
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	• Store the One Shot® Stbl3™ Chemically Competent *E. coli* at –80°C. • Thaw a vial of One Shot® cells on ice immediately before use.
	After addition of DNA, competent cells mixed by pipetting up and down	After adding DNA, mix competent cells gently. **Do not mix by pipetting up and down.**

Generating the Lentiviral Stock

The table below lists some potential problems and possible solutions that may help you troubleshoot your co-transfection and titering experiments.

Problem	Reason	Solution
Low viral titer	Low transfection efficiency: • Used poor quality expression construct plasmid DNA (*i.e.* DNA from a mini-prep) • Unhealthy 293FT cells; cells exhibit low viability • Cells transfected in media containing antibiotics (*i.e.* Geneticin®) • Plasmid DNA:transfection reagent ratio incorrect • 293FT cells plated too sparsely	• **Do not** use plasmid DNA from a mini-prep for transfection. Use PureLink™ Plasmid Purification Kits or CsCl gradient centrifugation to prepare plasmid DNA. • Use healthy 293FT cells under passage 20; do not overgrow. • **Do not** add Geneticin® in the media during transfection as this reduces transfection efficiency and causes cell death. • Use a DNA (in µg):Lipofectamine® 2000 (in µL) ratio ranging from 1:2 to 1:3. • Plate cells such that they are 90–95% confluent at the time of transfection OR use the recommended transfection protocol (*i.e.* add cells to media containing DNA:lipid complexes; page 36).

Continued on next page

Generating the Lentiviral Stock, Continued

Problem	Reason	Solution
Low viral titer	Transfected cells not cultured in media containing sodium pyruvate	One day after transfection, remove media containing DNA:lipid complexes and replace with complete media containing sodium pyruvate. Sodium pyruvate provides an extra energy source for the cells.
	Lipofectamine® 2000 Reagent handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion before use. **Do not vortex.**
	Viral supernatant harvested too early	Viral supernatants can generally be collected 48–72 hours post-transfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant.
	Viral supernatant too dilute	Concentrate virus using any method of choice (Yee, 1999).
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 3 times.
	Poor choice of titering cell line	Use HT1080 cells or another adherent cell line with the characteristics discussed on page 39.
	Target gene is essential for cell viability	Make sure that your target gene is not essential for cell viability or growth by performing a transient transfection with the entry construct containing the miRNA of interest.
	Polybrene® not included during titering procedure	Transduce the lentiviral construct into cells in the presence of Polybrene®.
No colonies obtained upon titering	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve experiment. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
	Viral stocks stored incorrectly	Aliquot and store stocks at −80°C. Do not freeze/thaw more than 3 times.
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
Titer indeterminable; cells confluent	Too little Blasticidin used for selection	Increase amount of Blasticidin used for selection.
	Viral supernatant not diluted sufficiently	Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.*, 10^{-2} to 10^{-8}).

Continued on next page

Transduction and RNAi Analysis

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

Problem	Reason	Solution
Low levels of gene knockdown observed	Low transduction efficiency: • Polybrene® not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene®. • Transduce your lentiviral construct into cells using a higher MOI.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
	Cells harvested and assayed too soon after transduction	Do not harvest cells until at least 48–72 hours after transduction to allow expressed miRNA to accumulate in transduced cells. If low levels of knockdown are observed at 48 hours, culture cells for a longer period of time before assaying for gene knockdown or place cells under Blasticidin selection. **Note:** Placing cells under Blasticidin selection can improve gene knockdown results by killing untransduced cells.
	Target gene is important for cell viability	Make sure that your target gene is not essential for cell viability or growth.
	Viral stocks not titered	Titer the lentiviral stock using the procedure on page 41 before use.
	Viral stock stored incorrectly	• Aliquot and store stocks at –80°C. • Do not freeze/thaw more than 3 times. • If stored for longer than 6 months, re-titer stock before use.
	miRNA with weak activity chosen	Select a different target region. If possible, screen miRNA first by transient transfection of the expression construct to verify its activity, then perform BP/LR recombination with the pLenti6/V5-DEST vector and proceed to generate lentivirus. **Note:** Generally, transient transfection greatly overexpresses miRNA, so moderately active expression clones may be less active when expressed from a lentiviral construct.

Continued on next page

Transduction and RNAi Analysis, Continued

Problem	Reason	Solution
No gene knockdown observed	miRNA with no activity chosen	Select a different target region. If possible, screen miRNA first by transient transfection of the expression construct to verify activity, then perform BP/LR recombination with the pLenti6/V5-DEST vector and proceed to generate lentivirus.
	Viral stocks stored incorrectly	Aliquot and store stocks at −80°C. Do not freeze/thaw more than 3 times.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
Cytotoxic effects observed after transduction	Target gene is essential for cell viability	Make sure that your target gene is not essential for cell viability or growth.
	Large volume of viral supernatant used for transduction	• Remove the "spent" media containing virus and replace with fresh, complete media. • Concentrate the virus (Yee, 1999).
	Polybrene® used during transduction	Verify the sensitivity of your cells to Polybrene®. If cells are sensitive, omit the Polybrene® during transduction.
	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a different target region.
No fluorescence signal detected with expression clone containing EmGFP	Incorrect filters used to detect fluorescence	Use the recommended filter sets for detection of fluorescence (see page 49). Use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for an additional 1–3 days before assaying for fluorescence. **Note:** The expression levels required to observe gene knockdown are generally lower than that required to detect EmGFP expression. Knockdown may still occur in non-EmGFP positive cells.

Appendix

Performing the Standard BP Recombination Reaction

Introduction

General guidelines and instructions are provided below and in the next section to perform a standard BP recombination reaction using the linearized expression clone containing your pre-miRNA expression cassette (*att*B substrate) and a donor vector, and to transform the reaction mixture into One Shot® TOP10 Chemically Competent *E. coli* host to select for entry clones (page 60).

Use the standard BP reaction to obtain the maximum number of colonies or generate an entry clone for future use.

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction using the linearized *att*B-containing expression clone and *att*P-containing pDONR™221 vector.
2. Transform the reaction mixture into competent *E. coli* host.
3. Select for entry clones.

Donor Vector and Expression Clone

See page 22 for details on the donor vector, propagating the donor vector, and for linearizing the expression clone.

Positive Control

pEXP7-tet is provided as a positive control for the BP reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. The efficiency of the BP recombination reaction can easily be determined by streaking entry clones onto LB plates containing 20 µg/mL tetracycline.

E. coli Host Strain

You may use any *rec*A, *end*A *E. coli* strain including TOP10, OmniMAX™ 2-T1^R or equivalent for transformation. **Do not** use *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.

Continued on next page

Performing the Standard BP Recombination Reaction, Continued

Materials Needed

For BP Recombination

- Linearized *att*B expression clone, see page 22
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 77

Components supplied with the kits

- pDONR™221 vector (150 ng/µL)
- Gateway® BP Clonase® II enzyme mix (keep at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (keep on ice until use)
- pEXP7-tet positive control (50 ng/µL)

For transformation

- One Shot® TOP10 Chemically Competent *E. coli* (or equivalent; one vial per transformation; thaw on ice before use), see page 77
- LB Medium
- LB plates containing 50 µg/mL kanamycin (two for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Components supplied with the kits

- S.O.C. Medium (warm to room temperature)
- Positive control (*e.g.*, pUC19, use as a control for transformation if desired)

Continued on next page

Performing the Standard BP Recombination Reaction, Continued

Setting Up the BP Recombination Reaction

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

   Note: To include a negative control, set up a second sample reaction and omit the Gateway® BP Clonase® II enzyme mix (see Step 4).

Components	Sample	Positive Control
Linearized *att*B expression clone from Step 4, page 22 (20–50 fmol)	1–7 µL	–
pDONR™221 vector (150 ng/µL)	1 µL	1 µL
pEXP7-tet positive control (50 ng/µL)	–	2 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the Gateway® BP Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the Gateway® BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of Gateway® BP Clonase® II enzyme mix to the sample and positive control vials. Do not add Gateway® BP Clonase® II to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).

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

   Note: For most applications, a 1 hour incubation will yield a sufficient number of entry clones. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. An overnight incubation typically yields 5–10 times more colonies than a 1 hour incubation.
6. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transformation Protocol**, next page.

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

Continued on next page

One Shot® TOP10 Transformation Protocol

Use this procedure to transform the BP recombination reaction into One Shot® TOP10 Chemically Competent *E. coli*. If you are using any other competent cell, follow the manufacturer's protocol.

1. Thaw, on ice, one vial of One Shot® TOP10 chemically competent cells for each transformation.
2. Add 1 µL of the BP recombination reaction (from, Step 6, previous page) into a vial of One Shot® TOP10 cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C bath and place them on ice for 2 minutes.
6. Add 250 µL of room temperature S.O.C. medium to each vial.
7. Cap the vial(s) tightly and shake horizontally (225 rpm) at 37°C for 1 hour.
8. Before plating, dilute the transformation mix 1:10 into LB Medium (*e.g.*, remove 20 µL of the transformation mix and add to 180 µL of LB Medium)
9. Spread 20 µL and 100 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C.

An efficient BP recombination reaction may produce hundreds of colonies (> 1,500 colonies if the entire BP reaction is transformed and plated).

Verifying Entry Clones

You may verify the entry clones by performing restriction digestion analysis. Sequencing of the entry clone is not required as transfer of the pre-miRNA expression cassette from pcDNA6.2™-GW/±EmGFP-miR into the pDONR™221 vector preserves the orientation of the cassette.

After verifying the entry clone, isolate plasmid DNA using your method of choice. We recommend using PureLink™ Plasmid Purification Kits (see page 77). For the LR recombination reaction, you will need purified plasmid DNA at a concentration of 50–150 ng/µL in TE, pH 8.0. Proceed to the LR recombination reaction, next page.

Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your BP recombination reaction, you may transform One Shot® TOP10 competent cells using the protocol on the previous page. The efficiency of the BP reaction may then be assessed by streaking entry clones onto LB agar plates containing 20 µg/mL tetracycline. True entry clones should be tetracycline-resistant.

Performing the Standard LR Recombination Reaction

Introduction

After obtaining an entry clone containing the pre-miRNA expression cassette, perform an LR recombination reaction between the entry clone (previous page) and a destination vector (pLenti6/V5-DEST), and transform the reaction mixture into One Shot® Stbl3™ Competent *E. coli* to select for expression clones (see page 29).

Use the standard LR reaction, if the Rapid BP/LR protocol produces fewer (~20–30) colonies.

Experimental Outline

To generate an expression clone, you will:

1. Perform a LR recombination reaction using the *att*L-containing entry clone (previous page) and *att*R-containing pLenti6/V5-DEST.
2. Transform the reaction mixture into a One Shot® Stbl3™ Competent *E. coli*.
3. Select for expression clones.

Destination Vector

See page 22 for details on the destination vector and instructions for propagating the destination vector.

Important

The pLenti6/V5-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual previously recommended using a linearized destination vector for more efficient LR recombination, further testing has found that linearization of pLenti6/V5-DEST is not required to obtain optimal results for any downstream application.

Recommended *E. coli* Host

For optimal results, use Stbl3™ *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats. One Shot® Stbl3™ Chemically Competent *E. coli* are included in the kit for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 29.

Positive Control

The pENTR™-gus plasmid is provided with the Gateway® LR Clonase® II Enzyme Mix for use as a positive control for recombination. Using the pENTR™-gus entry clone in an LR recombination reaction with a destination vector will allow you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991).

Recombination Region of pLenti6/V5-DEST

The recombination region of the expression clone resulting from pLenti6/V5-DEST × entry clone is shown on page 23.

Continued on next page

Performing the LR Recombination Reaction, Continued

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE, pH 8.0)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- LB agar plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37ºC for 30 minutes before use) to select for expression clones

Components supplied with the kits

- pLenti6/V5-DEST (supplied with the kit, 150 ng/µL in TE, pH 8.0)
- Gateway® LR Clonase® II enzyme mix (keep at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µL)
- One Shot® Stbl3™ Chemically Competent *E. coli*
- S.O.C. Medium (warm to room temperature)

Setting Up the LR Recombination Reaction

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

   Note: To include a negative control, set up a second sample reaction and omit the Gateway® LR Clonase® II enzyme mix (see Step 4).

Component	Sample	Positive Control
Entry clone (50–150 ng/reaction)	1–7 µL	--
pLenti6/V5-DEST vector (150 ng/µL)	1 µL	1 µL
pENTR™-gus (50 ng/µL)	--	2 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the Gateway® LR Clonase® II enzyme mix from −20°C and thaw on ice (~ 2 minutes).
3. Vortex the Gateway® LR Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of Gateway® LR Clonase® II enzyme mix to the sample and positive control vials. Do not add Gateway® LR Clonase® II enzyme mix to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return Gateway® LR Clonase® II enzyme mix to −20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: For most applications, 1 hour will yield a sufficient number of colonies for analysis. For large plasmids (≥ 10 kb), longer incubation times (*i.e.* 18 hour incubation) will yield more colonies and are recommended.
6. Add 1 µL of the Proteinase K solution. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 29).

Expected Results

If you use *E. coli* cells with a transformation efficiency of ≥ 1×10^8 cfu/µg, the LR reaction should give > 5,000 colonies if the entire LR reaction is transformed and plated. See page 30 for confirming the expression clone.

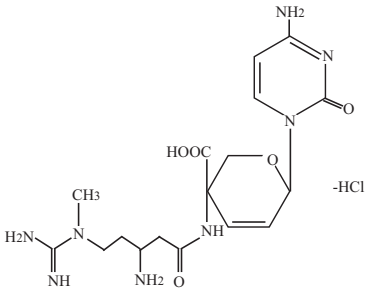
Blasticidin

Description

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 in 50 mg aliquots (see page 77 for ordering information).

- Blasticidin is soluble in water and acetic acid.
- Prepare a stock solution of 5 to 10 mg/mL Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use 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 week at 4°C and 6–8 weeks at −20°C.
- pH of the aqueous solution should not exceed 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 discard the unused portion.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Continued on next page

Determining Antibiotic Sensitivity

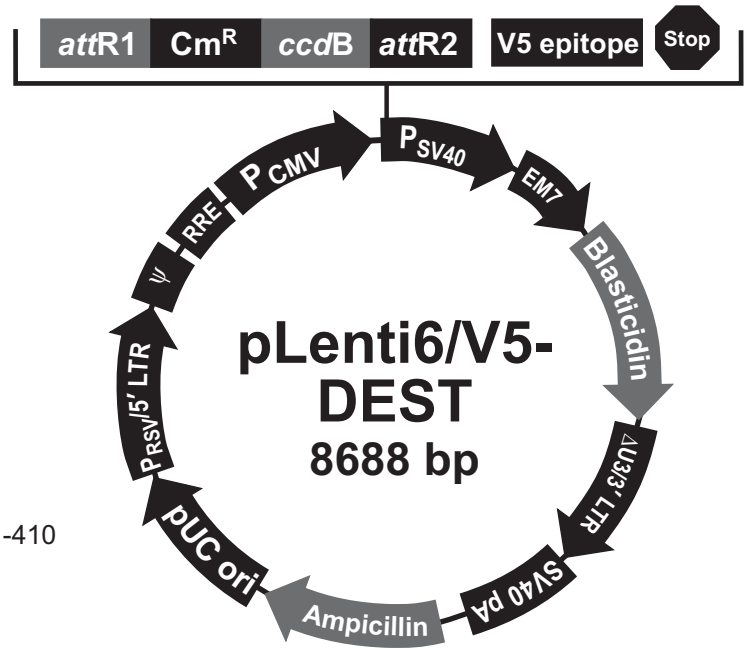
Since you will be selecting for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/mL Blasticidin are sufficient to kill most untransduced mammalian cell lines. 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.

Map and Features of pLenti6/V5-DEST

Map of pLenti6/V5-DEST

The map below shows the elements of pLenti6/V5-DEST. DNA from the entry clone replaces the region between bases 2,447 and 4,130. **The sequence for pLenti6/V5-DEST is available at www.invitrogen.com or by contacting Technical Support (see page 79).**



Comments for pLenti6/V5-DEST
8688 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1809-2392
*att*R1 site: bases 2440-2564
Chloramphenicol resistance gene (Cm^R): bases 2673-3332
*ccd*B gene: bases 3674-3979
*att*R2 site: bases 4020-4144
V5 epitope: bases 4197-4238
SV40 early promoter and origin: bases 4293-4602
EM7 promoter: bases 4657-4723
Blasticidin resistance gene: bases 4724-5122
ΔU3/3′ LTR: bases 5208-5442
    ΔU3: bases 5208-5261
    3′ LTR: bases 5262-5442
SV40 polyadenylation signal: bases 5514-5645
bla promoter: bases 6504-6602
Ampicillin (*bla*) resistance gene: bases 6603-7463
pUC origin: bases 7608-8281

Continued on next page

Map and Features of pLenti6/V5-DEST, Continued

Features of the Vector

The pLenti6/V5-DEST (8,688 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
CMV promoter	Permits high-level, constitutive expression of the gene or miRNA of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991). **This feature is not used when expressing miRNAs.**
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 transduced mammalian cell lines (Kimura *et al.*, 1994).
ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

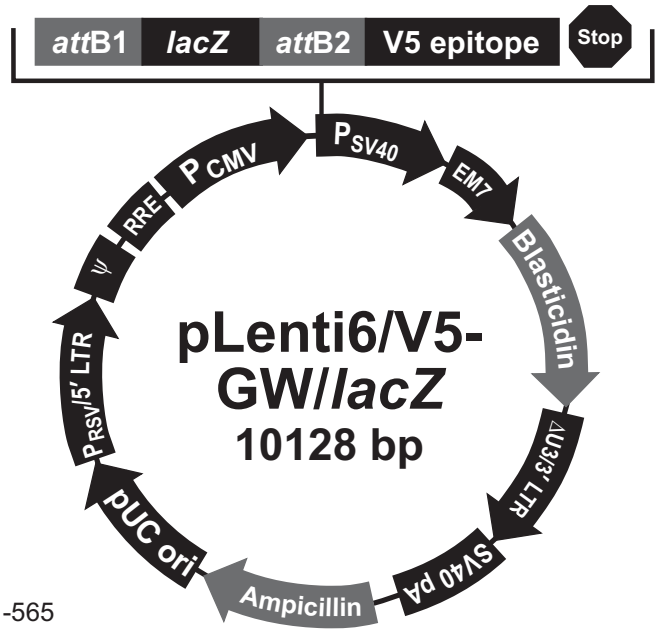
Map of pLenti6/V5-GW/*lacZ*

Description

pLenti6/V5-GW/*lacZ* is a 10,128 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pLenti6/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

Map of pLenti6/V5-GW/*lacZ*

The map below shows the elements of pLenti6/V5-GW/*lacZ*. **The sequence of the vector is available at www.invitrogen.com or by contacting Technical Support (see page 79).**



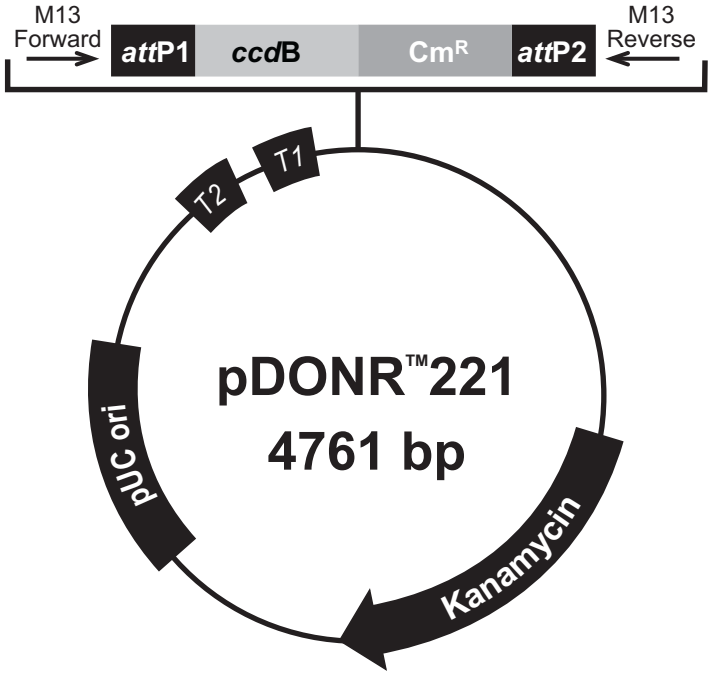
Comments for pLenti6/V5-GW/*lacZ*
10128 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1809-2392
*att*B1 site: bases 2440-2464
lacZ ORF: bases 2484-5540
*att*B2 site: bases 5560-5584
V5 epitope: bases 5637-5678
SV40 early promoter and origin: bases 5733-6042
EM7 promoter: bases 6097-6163
Blasticidin resistance gene: bases 6164-6562
ΔU3/HIV-1 3′ LTR: bases 6648-6882
ΔU3: bases 6648-6701
Truncated HIV-1 3′ LTR: bases 6702-6882
SV40 polyadenylation signal: bases 6954-7085
bla promoter: bases 7944-8042
Ampicillin (*bla*) resistance gene: bases 8043-8903
pUC origin: bases 9048-9721

Map and Features of pDONR™221

Map of pDONR™221

The map below shows the elements of pDONR™221 vector. **The sequence of pDONR™221 is available at www.invitrogen.com or by contacting Technical Support (see page 79).**



Comments for pDONR™221
4761 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295 (C)
*rrn*B T1 transcription termination sequence: bases 427-470 (C)
M13 Forward (-20) priming site: bases 537-552
*att*P1:bases 570-801
*ccd*B gene: bases 1197-1502 (C)
Chloramphenicol resistance gene: bases 1825-2505 (C)
*att*P2: bases 2753-2984 (C)
M13 Reverse priming site: bases 3026-3042
Kanamycin resistance gene: bases 3155-3964
pUC origin: bases 4085-4758

(C) = complementary strand

Continued on next page

Map and Features of pDONR™221, Continued

Features of pDONR™221

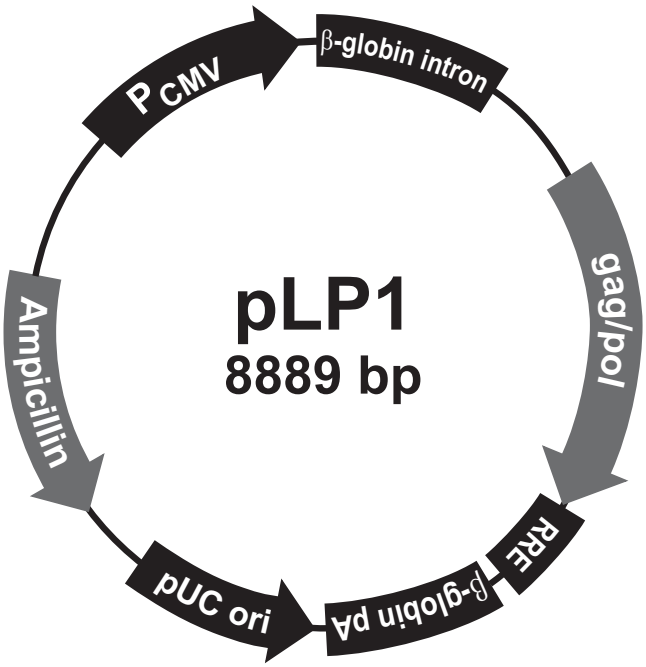
pDONR™221 (4,761 bp) contains the following elements. All features have been functionally tested and the vectors fully sequenced.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Protects the cloned gene or miRNA from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz *et al.*, 1991).
M13 Forward (–20) priming site	Allows sequencing in the sense orientation.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived DNA recombination sequences that allow recombinational cloning of the gene of interest from a Gateway® expression clone or *att*B PCR product (Landy, 1989).
*ccd*B gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli.*
pUC origin	Allows high-copy replication and maintenance in *E. coli.*

Map and Features of pLP1

pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is self-cleaved by the viral protease into individual Gag and Pol polyproteins. The sequence of pLP1 is available at **www.invitrogen.com** or by contacting Technical Support (see page 79).



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
    TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
    gag coding sequence: bases 1355-2857
    gag/pol frameshift: base 2650
    pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

Continued on next page

Map and Features of pLP1, Continued

Features of pLP1

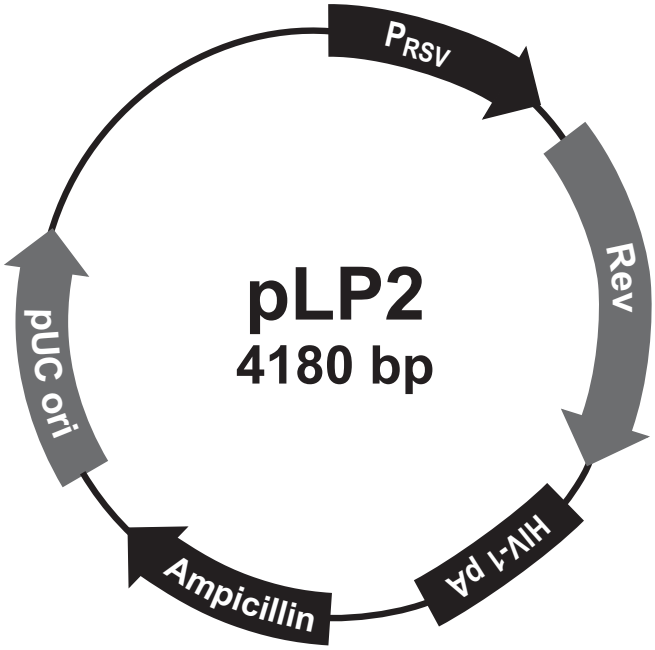
pLP1 (8,889 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) promoter	Permits high-level expression of the HIV-1 *gag* and *pol* genes in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the *gag* and *pol* genes in mammalian cells.
HIV-1 *gag* coding sequence	Encodes the viral core proteins required for forming the structure of the lentivirus (Luciw, 1996).
HIV-1 *pol* coding sequence	Encodes the viral replication enzymes required for replication and integration of the lentivirus (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent expression of the *gag* and *pol* genes.
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli.*
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli.*

Map and Features of pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. The sequence of pLP2 is available at **www.invitrogen.com** or by contacting Technical Support (see page 79).



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

Continued on next page

Map and Features of pLP2, Continued

Features of pLP2

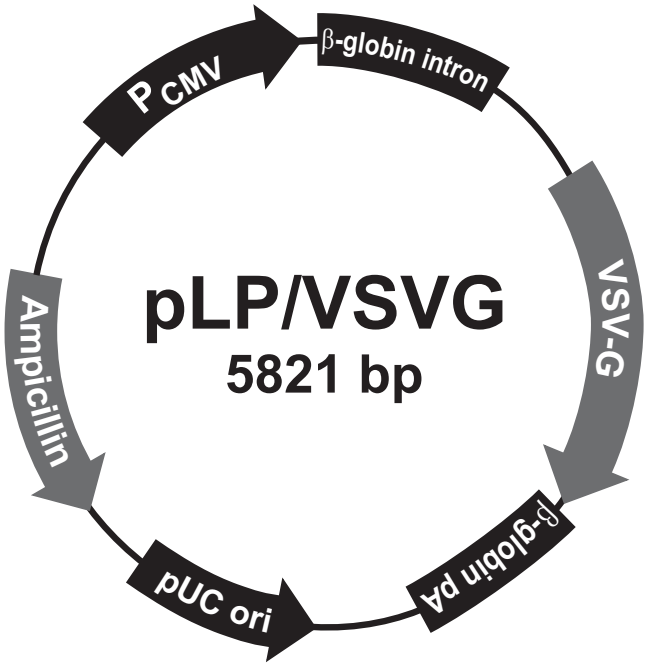
pLP2 (4,180 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
RSV enhancer/promoter	Permits high-level expression of the *rev* gene (Gorman *et al.*, 1982).
HIV-1 Rev ORF	Encodes the Rev protein which interacts with the RRE on pLP1 to induce Gag and Pol expression, and on the pLenti6/V5-DEST expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles.
HIV-1 LTR polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli.*
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli.*

Map and Features of pLP/VSVG

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. The sequence of pLP/VSVG is available at **www.invitrogen.com** or by contacting Technical Support (see page 79).



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

Continued on next page

Map and Features of pLP/VSVG, Continued

Features of pLP/VSVG

pLP/VSVG (5,821 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human CMV promoter	Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the VSV-G gene in mammalian cells.
VSV G glycoprotein (VSV-G)	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

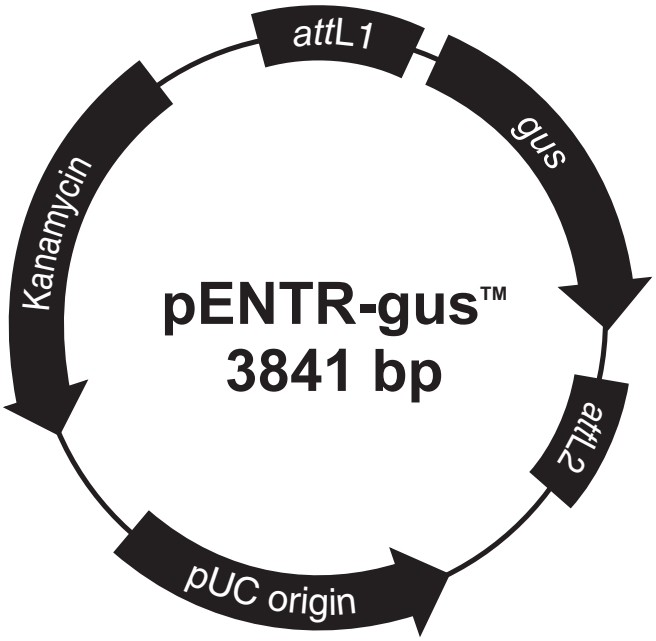
Map of pENTR™-gus

Description

pENTR™-gus is a 3,841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR201™ to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase® II manual which is available at www.invitrogen.com or by contacting Technical Support (see page 79).

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The sequence for pENTR™-gus is available** at www.invitrogen.com or by contacting Technical Support (see page 79).



Comments for pENTR-gus™ 3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Additional Products

Accessory Products

Many of the reagents supplied in the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System as well as other products suitable for use with the kits are available separately. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Support (see page 79).

Product	Amount	Cat. no.
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	20 constructions	K4935-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP	20 constructions	K4936-00
Gateway® BP Clonase™ II Enzyme Mix	20 reactions 100 reactions	11789-020 11789-100
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µL	C7373-03
One Shot® TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
OmniMAX™ 2-T1^R	20 × 50 µL	C854003
Blasticidin	50 mg	R21001
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 reactions	A10460
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
Lipofectamine® 2000 Reagent	0.75 mL 1.5 mL	11668-027 11668-019
pDONR™221 Vector	6 µg	12536-017
Opti-MEM® I Reduced Serum Medium	100 mL 500 mL	31985-062 31985-070
293FT Cell Line	3 × 10^6 cells	R700-07
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL 1 L	10010-023 10010-031
FluoReporter® *lacZ*/Galactosidase Quantitation Kit	1,000 assays	F-2905
β-Gal Staining Kit	1 kit	K1465-01
Ampicillin	200 mg	11593027
TE, pH 8.0	500 mL	AM9849
PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit	25 reactions	K2100-04
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
MEM Sodium Pyruvate Solution (100X)	100 mL	11360-070
WesternBreeze® Chemiluminescent Kit Anti-Mouse	1 kit	WB7104

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Additional Products, Continued

BLOCK-iT™ RNAi Designer

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™ RNAi Products

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

For details, visit the RNAi central portal or contact Technical Support (see page 79).

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
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Introduction

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