USER GUIDE

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# BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP

A MultiSite Gateway®-adapted, lentiviral destination vector for high-level knockdown by miRNA-based RNAi in dividing and non-dividing mammalian cells

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MAN0000684

**For Research Use Only. Not for human or animal therapeutic or diagnostic use.**

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# Contents

# Kit Contents and Storage

## Intended Use

**For Research Use Only.** Not intended for any animal or human therapeutic or diagnostic use.

## Shipping and Storage

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

| Box | Component | Shipping | Storage |
|---|---|---|---|
| 1 | BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP | Dry ice | –20°C |
| 2 | pLenti6.4/R4R2/V5-DEST MultiSite Gateway® Vector Kit<br>• pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector<br>• pLenti6.4/CMV/V5-MSGW/*lac*Z control vector<br>• pENTR™ 5′/CMVp<br>• pENTR™ 5′/EF1αp | Dry ice | –20°C |
| 3–4 | One Shot® TOP10 Chemically Competent *E. coli* (2 kits) | Dry ice | –80°C |
| 5 | One Shot® Stbl3™ Chemically Competent *E. coli* | Dry ice | –80°C |
| 6 | pDONR™221 Vector | Room temperature | –20°C |
| 7 | ViraPower™ Lentiviral Support Kit:<br>• ViraPower™ Packaging Mix<br>• Lipofectamine® 2000 | <br>Wet ice<br>Wet ice | <br>–20°C<br>4°C **(do not freeze)** |
| 8 | Blasticidin | Room temperature | –20°C |
| 9 | Gateway® BP Clonase® II<br>• Gateway® BP Clonase® II Enzyme Mix<br>• Proteinase K solution<br>• 30% PEG8000/30 mM $MgCl_2$ Solution<br>• pEXP7-tet Positive Control | Dry ice | –20°C |
| 10 | Gateway® LR Clonase® II Plus<br>• Gateway® LR Clonase® II Plus Enzyme Mix<br>• Proteinase K solution | Dry ice | –20°C |
| 11 | 293FT Cells | Dry ice | Liquid Nitrogen |

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

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

| Reagent | Composition | Quantity |
|---|---|---|
| pcDNA™6.2-GW/EmGFP-miR, linearized | 5 ng/µL in:<br>10 mM Tris-HCl, pH 8.0<br>1 mM EDTA, pH 8.0 | 4 × 10 µL |
| 10X Oligo Annealing Buffer | 100 mM Tris-HCl, pH 8.0<br>10 mM EDTA, pH 8.0<br>1 M NaCl | 250 µL |
| DNase/RNase-Free Water | — | 3 × 1.5 mL |
| 5X Ligation Buffer | 250 mM Tris-HCl, pH 7.6<br>50 mM $MgCl_2$<br>5 mM ATP<br>5 mM DTT<br>25% (w/v) polyethylene glycol-8000 | 80 µL |
| T4 DNA Ligase | 1 (Weiss) U/µL in<br>10 mM Tris-HCl, pH 7.5<br>50 mM KCl<br>1 mM DTT<br>50% (v/v) glycerol | 20 µL |
| EmGFP forward sequencing primer | 100 ng/µL in TE Buffer, pH 8.0 | 20 µL |
| miRNA reverse sequencing primer | 100 ng/µL in TE Buffer, pH 8.0 | 20 µL |
| miR-*lacZ* positive double-stranded (ds) control oligo | 50 µM in 1X Oligo Annealing Buffer | 4 µL |
| pcDNA™1.2/V5-GW/*lacZ* control plasmid | 500 ng/µL in TE Buffer, pH 8.0 | 20 µL |
| pcDNA™6.2-GW/EmGFP-miR-neg control plasmid | 500 ng/µL in TE Buffer, pH 8.0 | 20 µL |

### Unit Definition of T4 DNA Ligase

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

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### Primer Sequences

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

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

### *lacZ* Control Oligo Sequences

The sequences of the miR-*lacZ* positive ds control oligo are listed in the following table. The miR-*lacZ* positive ds control oligo are annealed and are supplied in the kit as a 50 µM double-stranded oligo. The miR-*lacZ* positive ds control oligo needs to be reannealed and diluted 5,000-fold to 10 nM before use in the ligation reaction.

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

### pLenti6.4/R4R2/V5-DEST Multisite Gateway® Vector kit

The pLenti6.4/R4R2/V5-DEST Multisite Gateway® Vector Kit contains the following components. This kit is shipped in one box on dry ice. Upon receipt, **store components at –20°C.**

| Components | Quantity and Concentration |
|---|---|
| pLenti6.4/R4R2/V5-DEST Multisite Gateway® vector | 40 µL @ 150 ng/µL in TE Buffer, pH 8.0 |
| pLenti6.4/CMV/V5-MSGW/*lac*Z Control vector | 20 µL @ 500 ng/µL in TE Buffer, pH 8.0 |
| pENTR™5′/CMVp | 20 µL @ 500 ng/µL in TE Buffer, pH 8.0 |
| pENTR™5′/EF1αp | 20 µL @ 500 ng/µL in TE buffer, pH 8.0 |

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

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

| Reagent | Composition | Amount |
|---|---|---|
| S.O.C. Medium | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>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 8.0 | 50 µL |

### 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
**Note:** This strain is *end*A1+

### One Shot® TOP10 Chemically Competent *E. coli*

The following reagents are included in the One Shot® TOP10 Chemically Competent *E. coli* kit. Transformation efficiency is ≥ 1 × $10^9$ cfu/µg plasmid DNA. **Two boxes** of One Shot® TOP10 Chemically Competent *E. coli* are provided with each BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP. **Store reagents at −80°C.**

| Reagent | Composition | Quantity |
|---|---|---|
| S.O.C. Medium<br>(may be stored at 4°C or room temperature) | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 mL |
| TOP10 cells | — | 21 × 50 µL |
| pUC19 Control DNA | 10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0 | 50 µL |

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### Genotype of TOP10 Cells

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G

### Gateway® BP Clonase® II

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

| Reagent | Composition | Quantity |
|---|---|---|
| Gateway® BP Clonase® II Enzyme Mix | Proprietary | 40 µL |
| Proteinase K Solution | 2 µg/µL in:<br>10 mM Tris-HCl, pH 7.5<br>20 mM $CaCl_2$<br>50% Glycerol | 40 µL |
| PEG Solution | 30% PEG 8000<br>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 Plus

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

| Reagent | Composition | Quantity |
|---|---|---|
| Gateway® LR Clonase® II Plus Enzyme Mix | Proprietary | 40 µL |
| Proteinase K Solution | 2 µg/µL in:<br>10 mM Tris-HCl, pH 7.5<br>20 mM $CaCl_2$<br>50% Glycerol | 40 µL |

### pDONR™221 Gateway® Vector

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

| Vector | Composition | Quantity |
|---|---|---|
| pDONR™221 Vector | 40 µL of vector at 150 ng/µL in 10 mM Tris-HCl,<br>1 mM EDTA, pH 8.0 | 6 µg |

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## ViraPower™ Lentiviral Support Kit Contents

The ViraPower™ Lentiviral Support Kit includes the following vectors and reagents. Store the ViraPower™ Packaging Mix at −20°C. Store Lipofectamine® 2000 Reagent at 4°C.

**Important:** Do not freeze Lipofectamine® 2000, store at 4°C

| Reagent | Composition | Quantity |
|---|---|---|
| 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 | Proprietary | 0.75 mL |

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

## 293FT Cell Line

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

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

## Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* that inhibits protein synthesis in prokaryotic and eukaryotic cells. Resistance to Blasticidin is conferred by expression of either one of the 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).

For additional details on how to use Blasticidin, refer to the **Appendix**, page 68.

# Introduction

## System Summary

### Description of the System

The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP (Emerald Green Fluorescent Protein) combines the BLOCK-iT™ Pol II miR RNAi, ViraPower™ HiPerform™ Lentiviral, and MultiSite Gateway® technologies to facilitate the 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.

### pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector

The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector, included with the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP, carries the WPRE (Woodchuck Posttranscriptional Regulatory Element) from the woodchuck hepatitis virus, and cPPT (central polypurine tract) from the HIV-1 integrase gene. The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector is designed to achieve elevated EmGFP expression, higher titers and higher expression of the knockdown cassette using a choice of promoters.

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# System Summary, Continued

### Components of the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP

The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP includes the following components:

- The BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP. This vector kit is used for production of an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-micro RNA (miRNA) sequence downstream of the EmGFP site for expression in mammalian cells using an RNA Polymerase II (Pol II) promoter, the human cytomegalovirus (CMV) immediate early promoter.
- The pDONR™221 vector, which is used as an intermediate to transfer the pre-miRNA expression cassette into the lentiviral expression plasmid (see page 3) using Gateway® Technology.
- A pLenti6.4/R4R2/V5-DEST destination vector, into which the pre-miRNA cassette from the expression clone is transferred using Gateway® Technology (see page 5). The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector contains elements that allow packaging of the construct into virions, the WPRE and cPPT elements for higher titers relevant to knockdown and stronger EmGFP expression, and the Blasticidin resistance marker, which is driven by the murine PGK promoter for selection of stably transduced cell lines.
- The pENTR™5′/CMVp and pENTR™5′/EF-1αp entry vectors. Either of these vectors is introduced into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector along with the miR RNAi cassette. (As an alternative, you can also use a Pol II promoter cloned using the pENTR™5′-TOPO® entry vector, sold separately. See page 85).
- Gateway® BP Clonase® II and Gateway® LR Clonase® II Plus Enzyme Mixes are provided to facilitate the transfer of the pre-miRNA expression cassette from the expression vector along with the CMV, EF-1α, or other promoter into the pLenti6.4/R4R2/V5-DEST destination vector.
- The ViraPower™ Lentiviral Support Kit is provided for producing a replication-incompetent lentivirus that stably expresses the miRNA of interest in both dividing and non-dividing mammalian cells.

For additional information about the BLOCK-iT™ Pol II miR RNAi Technology, ViraPower™ HiPerform™ Lentiviral Technology, and Multisite Gateway® Technology, visit **www.lifetechnologies.com**, or contact **Technical Support**. (See page 87.)

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# System Summary, Continued

## Advantages of the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP

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

- The BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP provides 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.
- Multisite Gateway®-adapted vectors allow easy transfer of the miR RNAi of interest from one expression vector (pcDNA™6.2-GW/EmGFP-miR) into another (pLenti6.4/R4R2/V5-DEST) along with the promoter of choice (CMV, EF-1α, or other).
- 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).
- The pLenti6.4 vectors included with this system contain the WPRE and cPPT elements to produce higher levels of EmGFP expression and higher functional titers than vectors that do not contain these elements.
- It efficiently delivers the miR RNAi of interest to mammalian cells *in vitro* or *in vivo.*
- It provides stable, long-term expression of the miR RNAi of interest beyond that offered by traditional adenoviral-based systems.
- It produces a pseudotyped virus with a broadened host range (Yee, 1999).
- Includes multiple features designed to enhance the biosafety of the system (page 21).

## 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) and includes the use of endogenous murine miR-155 flanking sequences (see page 11 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.lifetechnologies.com/rnai** or contact **Technical Support** (see page 87).

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### ViraPower™ HiPerform™ Lentiviral Technology

The ViraPower™ HiPerform™ Lentiviral Technology facilitates highly efficient *in vitro* 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™ HiPerform™ 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™ HiPerform™ Lentiviral Expression System include:

- A pLenti-based expression vector (e.g., pLenti6.4/R4R2/V5-DEST) into which the DNA sequence of interest will be cloned. This pLenti vector contains the WPRE and cPPT elements for higher levels of gene expression, with more cells expressing EmGFP and your miR RNAi cassette. The vector also contains the elements required to allow packaging of the expression construct into virions (e.g., 5′ and 3′ LTRs, Ψ packaging signal).
- The ViraPower™ Packaging Mix which 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, refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual, which is available at **www.lifetechnologies.com**, or by contacting **Technical Support** (page 87).
- VSV Envelope Glycoprotein: Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted tropism and generally low titers. In the ViraPower™ HiPerform™ Lentiviral 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 lentiviral vector with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- 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 available at **www.lifetechnologies.com**, or by contacting **Technical Support** (page 87).

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### The MultiSite 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 transfer a single DNA sequence of interest into multiple vector systems. The MultiSite Gateway® Technology uses modifications of the Gateway® Technology to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation to create an expression construct.

In the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP, the MultiSite Gateway® Technology facilitates recombinational cloning of two DNA fragments encoding a promoter and miR RNAi of choice into the pLenti6.4/R4R2/V5-DEST lentiviral destination vector.

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

1. Clone a double-stranded oligonucleotide encoding your miR RNAi sequence of interest into the pcDNA™6.2-GW/EmGFP-miR expression vector to create an expression clone.
2. Transfect this expression clone from Step 1 directly into mammalian cells for initial screening (if desired).
3. Transfer your pre-miRNA expression cassette into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector. To transfer your pre-miRNA expression cassette:
   a. Generate an entry clone by performing a BP recombination reaction between the pcDNA™6.2-GW/EmGFP-miR expression clone and pDONR™221 donor vector, and then proceed to step 3b.
   b. Perform an LR recombination reaction between the resulting entry clone (pENTR™221/EmGFP-miR), a pENTR™5′ promoter construct, and pLenti6.4/R4R2/V5-DEST. See page 15 for more details.
4. Use your lentiviral expression clone and the reagents supplied in the kit to produce a lentiviral construct.
5. Transduce the lentiviral construct into mammalian cells to express the miR RNAi.
6. Select for stably transduced cells, if desired.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual at **www.lifetechnologies.com** or contact **Technical Support** (see page 87).

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## Purpose of this Manual

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

1. Use the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector, pENTR™5' promoter vector, and pcDNA™6.2-GW/EmGFP-miR expression clone in a Rapid BP/LR recombination reaction to generate a lentiviral expression clone containing the miR RNAi sequence of interest.
2. Co-transfect the pLenti6.4/Promoter/MSGW/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,

   *or*

   Generate a stably transduced cell line.

For details and instructions for generating a pcDNA™6.2-GW/EmGFP-miR expression clone containing the miR RNAi 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. Both of these manuals are supplied with the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP and are also available at **www.lifetechnologies.com** or by contacting **Technical Support** (see page 87).

## Note

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

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**Important**

The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmFPG is designed to help you create a lentivirus to deliver and express a miR RNAi sequence in mammalian cells for RNAi analysis. Although this system is designed to help you express your miR RNAi sequence in the simplest, most direct fashion, use of the system is geared towards users that are 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 published 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 miR RNAi 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 miR RNAi 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). RNAi 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 as small ssRNA sequences of ~22 nucleotides in length, which naturally direct gene silencing through components shared with the RNAi pathway (Bartel, 2004). Unlike shRNAs or siRNA, 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.

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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 the following section) 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).

### Using a Vector-Based System to Express Engineered miRNA

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address this 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 pcDNA™6.2-GW/EmGFP-miR expression vector (supplied in the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP) facilitates the generation of an expression clone containing a ds oligo encoding a pre-miRNA sequence (see page 11). The resulting expression construct may be introduced into dividing mammalian cells for transient expression of the miR RNAi 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 pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (or other suitable destination vector) by Gateway® recombination reactions (see page 15).

For more information about the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP, its components, and how to generate the expression construct, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.

*Continued on next page*

### The miR RNAi Vector

The BLOCK-iT™ Pol II miR RNAi Expression Vector System with EmGFP is supplied with the pcDNA™6.2-GW/EmGFP-miR vector that allows the expression of your engineered pre-miRNA. This vector 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. 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-based Vector Systems

Using miRNA-based vector systems with Pol II promoters for RNAi cassette expression offers the following advantages over traditional siRNA or shRNA expression:

- Enables co-cistronic expression of reporter genes such as GFP, allowing reliable tracking of miR RNAi expression in mammalian cells.
- Allows expression of miR RNAi from a variety of promoters, including tissue-specific and regulated promoters for *in vivo* experiments.
- Enables expression of multiple miR RNAi cassettes 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 miR RNAi 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.

*Continued on next page*

### 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.

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.

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

## Introduction

The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP facilitates highly efficient, *in vitro* delivery of an miR RNAi sequence to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus.

## Components of the System

The BLOCK-iT™ Pol II miR RNAi Expression Vector System with EmGFP contains:

- The pcDNA™6.2-GW/EmGFP-miR vector for producing an expression clone that contains elements required for expressing a double-stranded oligonucleotide encoding an miR RNAi sequence of interest in mammalian cells using a Pol II promoter. The expression vector containing the pre-miRNA expression cassette can be transfected into mammalian cells for transient RNAi analysis, or used to transfer the pre-miRNA expression cassette into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector using Gateway® Technology. For detailed information about the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit and instructions to generate an expression clone, refer to the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit manual.
- The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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 5′ and 3′ LTRs, ψ packaging signals required to allow packaging of the expression construct into virions as well as a selectable marker to allow generation of stable cell lines.
- The pENTR™ 5′ - encoding a eukaryotic promoter of interest into a MultiSite Gateway® entry vector.
- The pDONR™221 vector is used as an intermediate to transfer the pre-miRNA expression cassette into the lentiviral expression plasmid using Gateway® Technology. See page 18 for details on pDONR™221 vector.
- Gateway® BP Clonase® II and Gateway® LR Clonase® II Plus Enzyme Mixes allow the transfer of the pre-miRNA expression cassette from the expression vectors as well as a promoter of interest into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector using the Rapid BP/LR recombination reaction.
- One Shot® Stbl3™ Competent *E. coli* to obtain optimal results with lentiviral DNA after transformation.
- ViraPower™ Packaging Mix that contains an optimized mixture of, pLP1, pLP2, and pLP/VSVG. These packaging plasmids supply helper functions as well as structural and replication proteins *in trans* to produce the lentivirus.

*Continued on next page*

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

**Components of the System,** Continued

- 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™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP, this limitation is 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).
- 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 refer to the 293FT Cell Line manual.

**System Overview**

To use the BLOCK-iT™ Pol II miR RNAi Expression Vector System with EmGFP, co–transfect the ViraPower™ Packaging Mix and the pLenti6.4 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 miR RNAi 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.

*Continued on next page*

### Features of the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector

The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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)
- HIV-1 central polypurine tract (cPPT) for efficient import of the pro-virus into the nucleus of transduced cells (Park, 2001)
- Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) for high levels of expression of the viral genome during production and EmGFP after transduction and integration (Zufferey *et al.*, 1998)
- Option of using the human CMV promoter for high-level, constitutive expression of the miR RNAi, the non-viral human EF-1α promoter for lower but more ubiquitous expression in primary cells and *in vivo* with lower risk of promoter shut down, or your own promoter of interest cloned into the pENTR™5′/TOPO® vector (available separately)
- Two recombination sites, *att*R4 and *att*R2, for recombinational cloning of the miR RNAi of interest from the pcDNA™6.2-GW/EmGFP-miR expression clone using MultiSite 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*

# MultiSite Gateway® Recombination Reactions

## Introduction

The MultiSite Gateway® Technology uses modifications of the Gateway® Technology to allow simultaneous cloning of multiple DNA fragments, in a defined order and orientation, to create an expression construct. Review this section to familiarize yourself with the Multisite Gateway® recombination reactions. For details, refer to the Gateway® Technology with Clonase® II manual available at **www.lifetechnologies.com** or by contacting **Technical Support** (see page 87).

## Gateway® Vectors

Each of the vectors supplied in the BLOCK-iT™ HiPerform™ 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). To accommodate simultaneous recombinational cloning of multiple DNA fragments in the MultiSite Gateway® Technology, these *att* sites have been further modified and optimized. Modifications include alterations to both the sequence and length of the *att* sites, resulting in the creation of "new" *att* sites exhibiting enhanced specificities and the improved efficiency required to permit cloning of multiple DNA fragments in a single reaction. In the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System, the entry and destination vectors contain the following *att* sites:

- pENTR™ 5′-TOPO® containing the CMV promoter, EF-1α promoter entry clone, or your promoter of interest: *att*L4 and *att*R1
- pcDNA™6.2-GW/EmGFP-miR expression clone containing EmGFP and your miR RNAi of interest: *att*B1 and *att*B2
- pDONR™221 vector for conversion of the miR RNAi expression clone into an attL1 and attL2 entry clone
- pLenti6.4/R4R2/V5-DEST lentiviral destination vector: *att*R4 and *att*R2

**Note: To facilitate proper generation of a lentiviral expression construct, only this combination of entry clones and destination vector may be used in the MultiSite Gateway® LR recombination reaction.**

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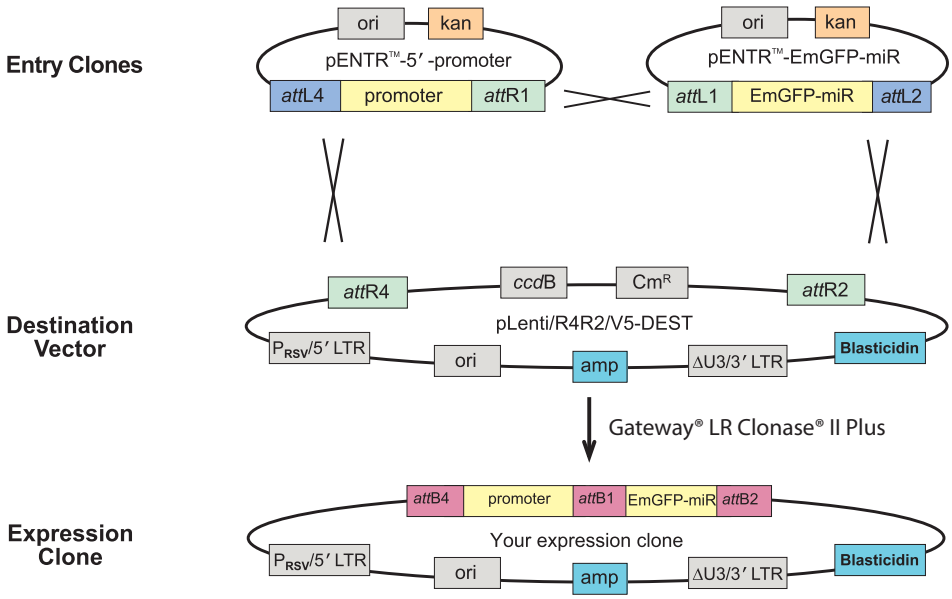
# MultiSite Gateway® Recombination Reaction, Continued

## Gateway® Vectors: Recombination Reaction




## 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 *att*L substrates (entry clones) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. This reaction is catalyzed by Gateway® LR Clonase® II Plus 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 25 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*

# MultiSite Gateway® Recombination Reaction, Continued

## Pre-miRNA Expression

Since the pcDNA™6.2-GW/EmGFP-miR expression vector contains *att*B sites, the pre-miRNA sequence **cannot** be transferred directly into the pLenti6.4/R4R2/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 pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector, 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/EmGFP-miR expression clone) and *att*P substrate (pDONR™221 vector) using the Gateway® BP Clonase® II Enzyme Mix.
2. Perform an LR recombination reaction between the resulting entry clone (*att*L substrate), a 5′ entry clone (*att*L substrate) carrying a promoter, and the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (*att*R substrate) using the Gateway® LR Clonase® II Plus Enzyme Mix.

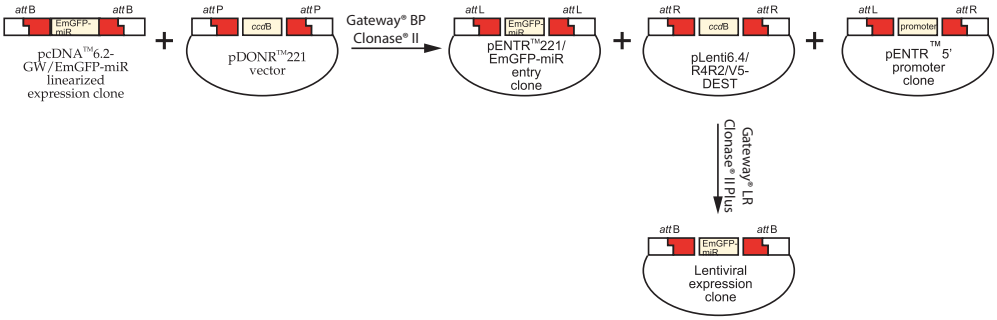
The standard BP and LR recombination reaction requires more than 2 days for completion. See below for details on expressing the miRNA from pLenti6.4/R4R2/V5-DEST destination vector using the Rapid BP/LR Recombination Reaction.

## Rapid BP/LR Recombination Reaction

To provide a faster Gateway® recombination reaction protocol to transfer the pre-miR RNAi expression cassettes into the destination vector, we have developed a Rapid BP/LR recombination reaction that allows the completion of the entire BP and LR reaction in one day. In the Rapid BP/LR Recombination Reaction, instead of isolating the entry clone after the BP reaction, the completed BP reaction is transferred directly into the LR reaction to generate expression clones within 1 day.

For **Rapid BP/LR Recombination Reactions**:

1. Perform a BP recombination reaction between the pcDNA™6.2-GW/EmGFP-miR expression clone and the pDONR™221 vector using Gateway® BP Clonase® II Enzyme Mix. This recombination reaction yields a pENTER™221/EmGFP-miR entry clone (see below).
2. Perform an LR recombination reaction between the pENTR™221/EmGFP-miR entry clone (Step 1), the pENTR™5′ promoter clone, and pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector to produce a lentiviral expression clone.

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# MultiSite 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 EmGFP gene and miR RNAi 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 77.

# 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 pLenti6.4/R4R2/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. Expression of EmGFP is significantly enhanced due to the action of the WPRE present in the pLenti6.4 vector.

## Green Fluorescent Protein (GFP)

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

## GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include amino acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescence signal, resulting in "enhanced" GFP (Zhang *et al.*, 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998).
The Emerald GFP (EmGFP) is such 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, especially after enhancement of EmGFP expression by the WPRE in pLenti6.4 clones.

*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, 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™ HiPerform™ Lentiviral RNAi Expression System with EmGFP are fourth-generation vectors based on lentiviral vectors developed by Dull *et al.*, 1998. They include a significant number of safety features designed to enhance biosafety and to minimize the relation to the wild-type, human HIV-1 virus.

## Biosafety Features of the BLOCK-iT™ HiPerform™ Lentiviral RNAi Expression System

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

- The pLenti6.4/R4R2/V5-DEST expression vector contains a deletion in the 3′ LTR (ΔU3) that does not affect the 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 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 *in trans* expression 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 pLenti6.4/R4R2/V5-DEST expression vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

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# 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 because 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.** 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 is available at:

**www.cdc.gov/biosafety/publications/index.htm**

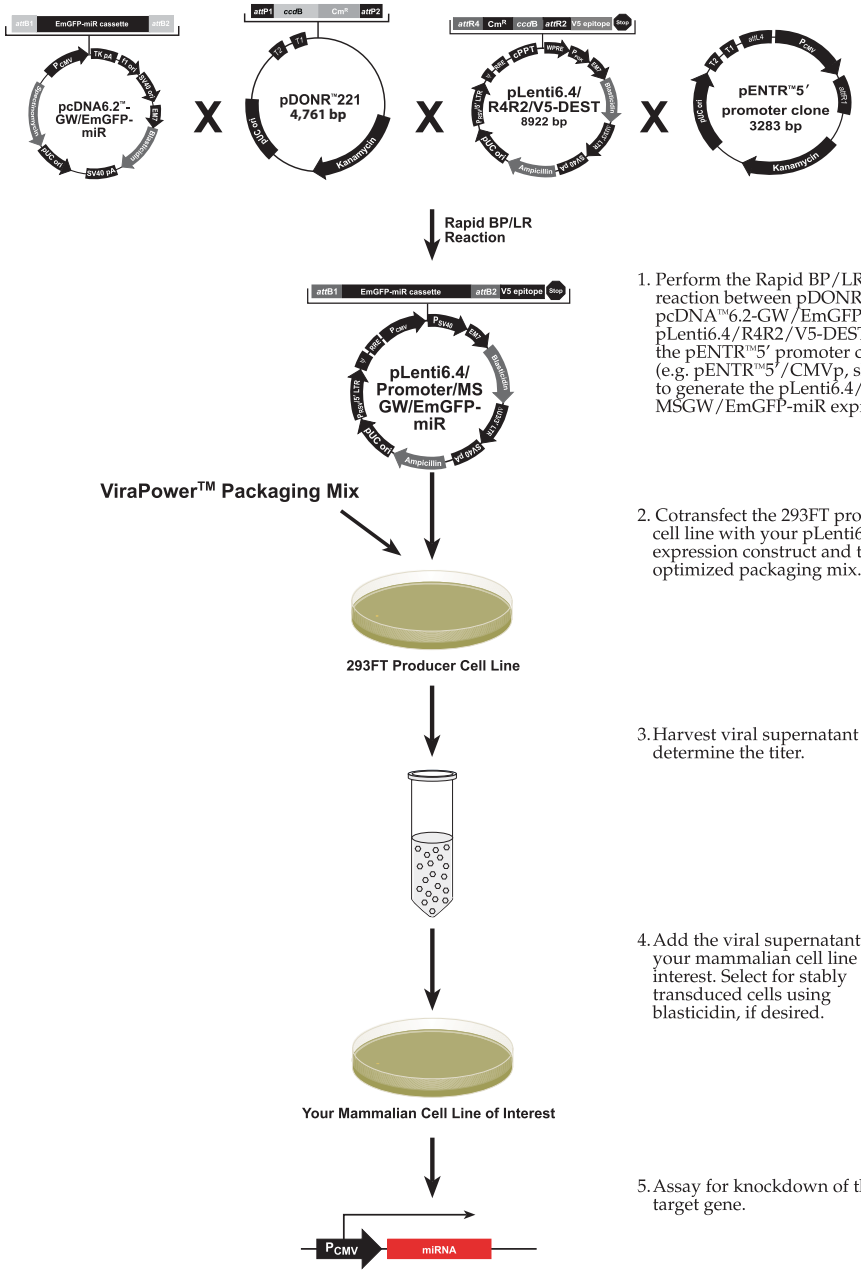
## Important

Handle all lentiviruses in compliance with established institutional guidelines. Because safety requirements for use and handling of lentiviruses may vary at individual institutions, consult the health and safety guidelines and/or officers at your institution before using the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP.

# Experimental Outline

## Flow Chart

The diagram below describes the general steps required to express your miR RNAi using the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP.




1. Perform the Rapid BP/LR recombination reaction between pDONR™221, pcDNA™6.2-GW/EmGFP-miR, pLenti6.4/R4R2/V5-DEST, and the the pENTR™5′ promoter clone (e.g. pENTR™5′/CMVp, shown above) to generate the pLenti6.4/Promoter/MSGW/EmGFP-miR expression construct.
2. Cotransfect the 293FT producer cell line with your pLenti6.4 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 miR RNAi

### Introduction

You will need to clone your miR RNAi sequence of interest contained within the engineered pre-miRNA into the 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 your miR RNAi sequence of interest from pLenti6.4/R4R2/V5-DEST.

After generating the expression clone, you will transfer the pre-miRNA expression cassette from the expression clone into the destination vector, pLenti6.4/R4R2/V5-DEST using a Rapid BP/LR recombination reaction (see page 25).

General guidelines for cloning are provided below.

### Using pcDNA™6.2-GW/EmGFP-miR

To generate an expression clone in pcDNA™6.2-GW/EmGFP-miR, you will:

- Design and synthesize two complementary oligonucleotides containing your 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/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 your 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 from **www.lifetechnologies.com** or by contacting **Technical Support** (see page 87).

# Using the pENTR™ 5' Promoter Clone

## Introduction

This section provides information on using the pENTR™ 5′ promoter clones.

## CMV Promoter

pENTR™ 5′/CMVp contains the human CMV immediate early promoter to allow high-level, constitutive expression of the gene of interest in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Although highly active in most mammalian cell lines, activity of the viral promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

## EF-1 Promoter

pENTR™ 5′/EF1αp contains the elongation factor 1α-subunit promoter (EF-1α) for high-level expression across a broad range of species and cell types (Goldman *et al.*, 1996; Mizushima & Nagata, 1990). The EF-1 promoter is expressed in a wide range of mammalian cell types, including cells where the CMV promoter expression is absent or inconsistent.

## Which Promoter to Use

pENTR™ 5′/CMVp carries the CMV promoter and is suitable for use in most cell line applications. pENTR™ 5′/EF1αp contains the EF-1α promoter and may be more appropriate for long-term gene expression in certain mouse cell lines, stem cells, primary cells, and for *in vivo* use.

## Features of the pENTR™5' Promoter Clones

Features of the pENTR™5′ promoter clones include:

- Choice of human CMV immediate early promoter or EF-1 promoter
- *att*L4 and *att*R1 sites to allow two-fragment or three-fragment recombination with appropriate entry clone(s) and a MultiSite Gateway® destination vector to generate an expression construct
- Primer binding sites within the *att*L4 and *att*R1 sites for sequencing using the GW1 and GW3 primers (see page 73 through 76 for a map and features of these sites)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

## Cloning your own Promoter

To clone your own promoter, you will need the pENTR™ 5′/TOPO® TA Cloning Kit (see page 85 for ordering information). For details on how to clone your own promoter, refer to the pENTR™ 5′/TOPO™ TA Cloning Kit manual, which is available at **www.lifetechnologies.com** or by contacting **Technical Support** (page 87).

# Creating Entry Clones for Use with pLenti6.4/R4R2/V5-DEST

## Introduction

Since the 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 pLenti6.4/R4R2/V5-DEST destination vector to perform the LR recombination reaction.

To express your miR RNAi sequence in mammalian cells using the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System, 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 the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector and pENTR™ 5′ promoter clone to generate a lentiviral expression clone.

The transfer of the miR RNAi sequence into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector can be performed using the standard BP and LR recombination reactions or Rapid BP/LR recombination reactions as described in the following table. See page 15 for an overview of the Gateway® recombination reactions.

To ensure that you obtain the best possible results, read this section, the sections entitled **Performing the Rapid BP/LR Recombination Reaction** (page 25), and **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 30) before proceeding with the procedures.

## 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 following table:

| If You Wish to…. | Then Choose….. | Described |
|---|---|---|
| To 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 61–67. |

*Continued on next page*

# Creating Entry Clones for Use with pLenti6.4/R4R2/V5-DEST,
Continued

## Rapid BP/LR Recombination Reaction

To express your miR RNAi sequence in mammalian cells using the BLOCK-iT™ HiPerform™ 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/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/EmGFP-miR), a pENTR™5′ promoter clone, and the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector using Gateway® LR Clonase® II Plus Enzyme Mix to produce a lentiviral expression clone. See page 15 for an overview on Gateway® recombination reactions.

## Experimental Outline

To generate an expression clone:

1. Perform the BP recombination reaction using the *att*B-expression clone with miR RNAi of interest and *att*P-containing pDONR™221 vector to produce a pENTR™221/EmGFP-miR entry clone.
2. Mix an aliquot of the BP reaction (containing the entry clone) with the pENTR™5′ promoter vector (*att*L4–*att*R1) and pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (*att*R4–*att*R2) perform the LR recombination reaction to produce a lentiviral expression clone.
3. Transform the reaction mixture into a suitable *E. coli* host (see page 30).
4. Select for lentiviral expression clones (see page 29 for a diagram of the recombination region of expression clones in pLenti6.4/R4R2/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 page 28 for guidelines to linearize *att*B expression clones)
- *att*P-containing donor (pDONR™221) vector

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

- Supercoiled *att*L1–*att*L2 entry vector (pENTR™221/EmGFP-miR)
- Supercoiled *att*L4–*att*R1 5′ entry vector (pENTR™5′/CMVp, pENTR™5′/EF-1αp, or other)
- Supercoiled *att*R4–*att*R2 destination vector (pLenti6.4/R4R2/V5-DEST)

*Continued on next page*

# Creating Entry Clones for Use with pLenti6.4/R4R2/V5-DEST,
Continued

## Donor Vectors

The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Kit includes the pDONR™221 vector. For a map and a description of the features of pDONR™221, see the **Appendix**, page 77. You may use other donor vectors, if desired.

## Important

The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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 pLenti6.4/R4R2/V5-DEST is **not** required to obtain optimal results for any downstream application.

## 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 and 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 pLenti6.4/R4R2/V5-DEST,
Continued

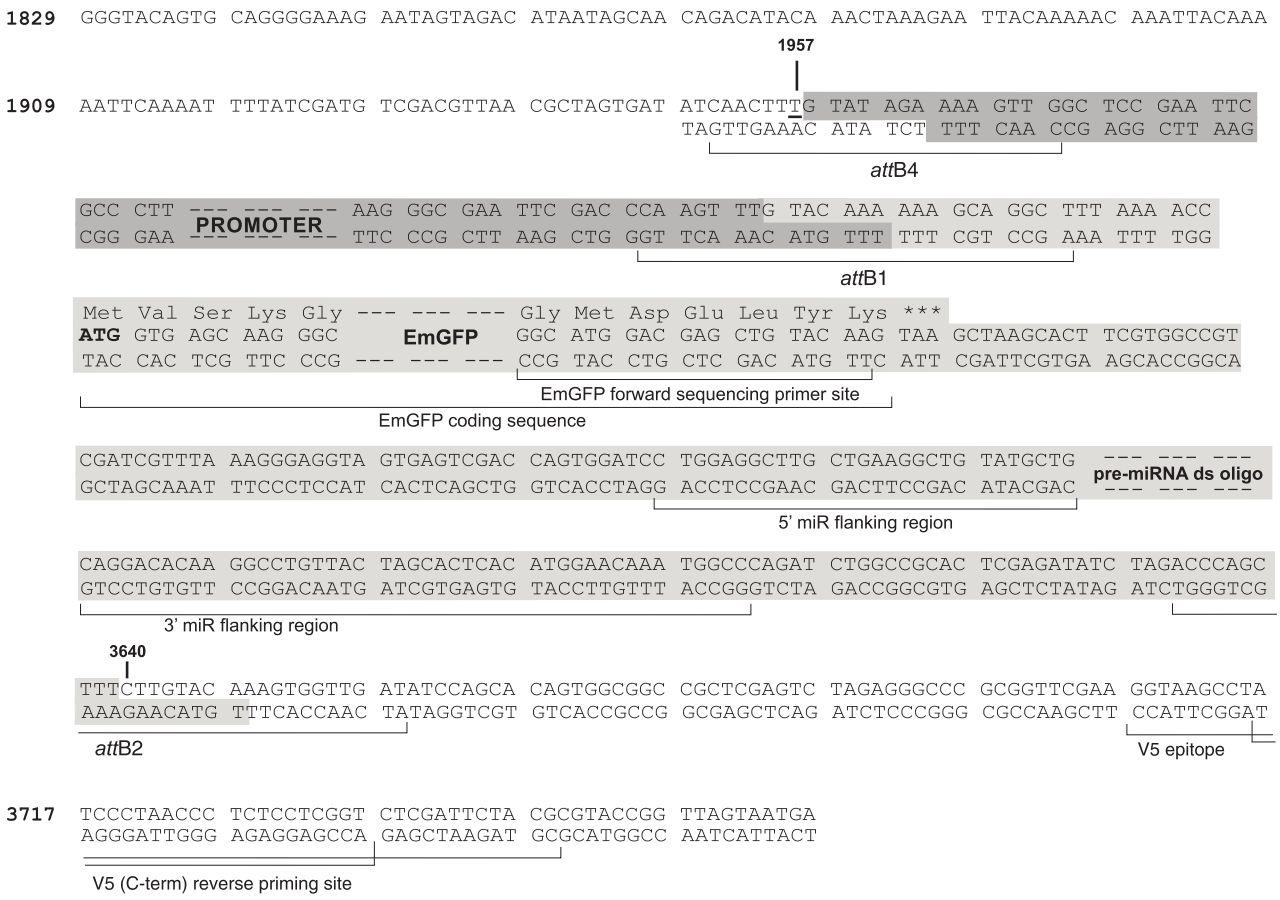
## Recombination Region of pLenti6.4/R4R2/V5-DEST

The recombination region of the lentiviral expression clone resulting from pLenti6.4/R4R2/V5-DEST × pENTR™221/EmGFP-miR entry clone plus pENTR™5′/CMVp 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 those DNA sequences transferred from the pENTR™221/EmGFP-miR and pENTR™5′/CMVp entry clones into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector by recombination. Non-shaded regions are derived from the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector.

**Note:** The DNA sequences transferred from the pENTR™221/EmGFP-miR entry clone contain the pre-miRNA expression cassette including the EmGFP coding region. The DNA sequences transferred from the pENTR™5′/CMVp entry clone contain the CMV immediate early promoter (shown as **promoter**). Since the pLenti6.4/CMV/MSGW/EmGFP-miR expression construct is expressing 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, a pENTR™5′ promoter clone, pDONR™221, and pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector.

If you wish to perform the standard BP recombination reaction followed by the standard LR recombination reaction, see page 61 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:

1. BP reaction using a donor vector.
2. LR recombination reaction using a destination vector without purification of the intermediate entry clone.

**Note:** This protocol allows you to generate expression clones more rapidly than the standard BP and LR protocols provided on page 61. 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 61.

## 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/EmGFP-miR.
2. Use a small aliquot of the BP reaction mix to perform the LR recombination reaction using a pENTR™5′ promoter clone (CMVp, EF-1αp, or other) and the pLenti6.4/R4R2/V5-DEST destination vector to generate the lentiviral expression clone, pLenti6.4/promoter/MSGW/EmGFP-miR.
3. Perform Proteinase K treatment.

## Recommended *E. coli* Host

**For optimal results, we recommend using 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 35.

## 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*

### Positive Control

We recommend using the pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid supplied with the BLOCK-iT™ Pol II miR RNAi Expression Kit 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 due to the presence of incompatible selection markers.

### Gateway® Clonase® II Enzyme Mixes

The Gateway® BP Clonase® II and Gateway® LR Clonase® II Plus enzyme mixes combine the proprietary enzyme formulation and 5X Clonase Reaction Buffer previously supplied as separate components in 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 Plus 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 procedure provided on page 33 to perform the recombination reactions using the Rapid protocol or page 61 for the standard protocol.

Gateway® BP Clonase® II and Gateway® LR Clonase® II Plus Enzyme Mixes are supplied with the kit or available separately (see page 85).

### 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:

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

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 expression clone 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:

$$(50\,\text{fmol})(2500\,\text{bp})\left(\frac{660\,\text{fg}}{\text{fmol}}\right)\left(\frac{1\,\text{ng}}{10^6\,\text{fg}}\right) = 82.5\,\text{ng of expression clone required}$$

*Continued on next page*

# 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 28)
- pENTR™5′/CMVp **or** pENTR™5′/EF-1αp **or** your own pENTR™5′ entry clone
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Sterile 0.5 mL microcentrifuge tubes

*Components supplied with the kit*

- pDONR™221 vector (150 ng/µL in sterile water)
- pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (150 ng/µL in TE Buffer, pH 8.0)
- pcDNA™6.2-GW/EmGFP-miR-neg control (if desired, Box 1)
- Gateway® BP Clonase® enzyme mix (Box 9; store at −20°C until immediately before use)
- Gateway® LR Clonase® II Plus enzyme mix (Box 10; store at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (supplied with Clonase® enzymes; thaw and keep on ice until use)

*Continued on next page*

### Setting Up the Rapid BP/LR Recombination Reaction

Follow this procedure to perform the Rapid BP/LR reaction between your linearized expression clone, pDONR™221 vector, pENTR™5′ promoter clone and the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector.

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

| Component | Sample | Positive Control |
|---|---|---|
| Linearized *att*B expression clone from page 28, (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. Instead, proceed immediately to the next step.
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/EmGFP-miR.

   **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 64 to check the efficiency of the BP reaction. This also allows you 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 |
|---|---|---|
| pENTR™5′ promoter vector (e.g., pENTR™5′/CMVp) (150 ng/µL) | 1 µL | 1 µL |
| pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (150 ng/µL) | 1 µL | 1 µL |
| TE Buffer, pH 8.0 | 3 µL | 3 µL |

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

**Setting Up the Rapid BP/LR Recombination Reaction,** Continued

*Protocol continued from the previous page.*

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

    **Reminder:** Return Gateway® LR Clonase® II Plus enzyme mix to –20°C immediately after use.
11. Incubate the reaction at room temperature (20–25°C) from 16 hours to overnight.
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 (Step 13, page 34, or Step 7, page 67)
- 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, page 34, or Step 7, page 67) 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 miR RNAi cassette from pcDNA™6.2-GW/EmGFP-miR into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector preserves the orientation of the cassette. However, if you wish to sequence your pLenti6.4 expression construct, use the following primers. Refer to the map on page 70 for the location of the primer binding sites in the expression vector.

| Primer | Sequence |
|---|---|
| CMV Forward (for CMVp) | 5′-CGCAAATGGGCGGTAGGCGTG-3′ |
| T7 Promoter (for EF-1αp) | 5′-TAATACGACTCACTATAGGA-3′ |
| V5(C-term) Reverse | 5′-ACCGAGGAGAGGGTTAGGGAT-3′ |

**Note:** For information about a convenient custom primer synthesis service go to **www.lifetechnologies.com** or call Technical Support (see page 87).

## 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 miR RNAi, first produce a lentiviral stock (containing the packaged pLenti6.4 expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti6.4/promoter/MSGW/EmGFP-miR expression construct into the 293FT Producer Cell Line. The following section provides protocols and instructions for generating a lentiviral stock.

## Experimental Outline

To produce lentivirus in 293FT Cells, you will:

1. Grow the 293FT Cells to obtain $6 \times 10^6$ 293FT cells for each sample.
2. Prepare plasmid DNA of your expression clone.
3. Cotransfect the ViraPower™ Packaging Mix and pLenti6.4/promoter/MSGW/EmGFP-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, supplied with the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Kit, facilitates 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™ HiPerform™ Lentiviral Pol II miR RNAi Kit and is also available from **www.lifetechnologies.com** or by calling Technical Support (see page 87).

**Note:** The 293FT Cell Line is available separately (page 85).


The health of your 293FT cells at the time of transfection is critical to 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 $6 \times 10^6$ 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 as an optimized mixture to facilitate viral packaging of your pLenti6.4/promoter/MSGW/EmGFP-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™ HiPerform™ Lentiviral Pol II miR RNAi Kit is sufficient to perform 20 cotransfections in 10-cm plates using the recommended protocol on page 42.

**Note:** ViraPower™ Packaging Mix is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit. (See page 85.)

### Plasmid Preparation

After generating your expression clone, you **must** isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® Plasmid Purification Kits (page 85) or CsCl gradient centrifugation.

Resuspend the purified pLenti6.4/promoter/MSGW/EmGFP-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™ HiPerform™ Lentiviral Pol II miR RNAi Kit 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 85 for ordering information).

### Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine® 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium (see page 85 for ordering information). For more information about Opti-MEM® I, go to **www.lifetechnologies.com** or call Technical Support (see page 87).

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### miR Positive Control

You may generate a 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 pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector and either the pENTR™5′/CMVp or pENTR™5′/EF-1αp vector using the Rapid BP/LR recombination reaction as described in this manual.
- Use the resulting lentiviral expression construct, pLenti6.4/promoter/MSGW/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 50) 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.

### pLenti6.4/CMV/V5-MSGW/*lacZ* Positive Control

A pLenti6.4/CMV/V5-MSGW/*lacZ* positive control vector is included with the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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 72.

To propagate and maintain the control plasmid:

1. 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 DH5**™**α**).
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 pLenti6.4/CMV/V5-MSGW/*lacZ* positive control to generate a control lentiviral stock (expressing the *LacZ* protein).
5. Use the pLenti6.4/CMV/V5-MSGW/*lacZ* lentiviral control and the pLenti6.4/promoter/MSGW/EmGFP-miR-*lacZ* lentiviral control in cotransduction experiments as a positive control for lentiviral induced RNAi analysis in your system (see page 52 for details).

*Continued on next page*

# Producing Lentivirus in 293FT Cells, Continued

## Materials Needed

- pLenti6.4/promoter/MSGW/EmGFP-miR expression construct (0.1 to 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 $6 \times 10^6$ 293FT cells for each sample.

  **Note:** D-MEM already contains 4 mM L-glutamine, which is enough to support cell growth of 293FT cells. 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 85)
- Fetal bovine serum (FBS; see page 85)
- 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 85. 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 kit*

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

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# Producing Lentivirus in 293FT Cells, Continued

## 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 $1 \times 10^5$ to $1\times 10^7$ transducing units (TU)/mL) is generally sufficient to transduce $1 \times 10^6$ to $1\times 10^8$ cells at a multiplicity of infection (MOI) of 1.

| Condition | Amount |
|---|---|
| Tissue culture plate size | 10 cm (one per lentiviral construct) |
| Number of 293FT cells to transfect | $6 \times 10^6$ cells (see **Recommendation** on previous page to prepare cells for transfection) |
| Amount of ViraPower™ Packaging Mix | 9 µg (9 µL of 1 µg/µL stock) |
| Amount of pLenti6.4/promoter/MSGW/EmGFP-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; optimization may 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 page 42).

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 43).

*Continued on next page*

### Reverse Transfection Procedure (experienced users)

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 $6 \times 10^6$ 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 pLenti6.4/promoter/MSGW/EmGFP-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, dilute 36 µL of Lipofectamine® 2000 (mix gently before use) 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 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 $1.2 \times 10^6$ 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 ($6 \times 10^6$ 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 medium containing the DNA-Lipofectamine® 2000 complexes and replace it with complete culture medium containing sodium pyruvate (see page 40).

   **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 production of the lentivirus.
6. Harvest virus-containing supernatants 48–72 hours posttransfection by removing medium to a 15-mL sterile, capped, conical tube. (The difference in viral yield is minimal whether the supernatants are collected 48 or 72 hours after transfection.)

   **Caution:** Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see pages 22 and 46 for more information).
7. Centrifuge the cells 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 and store them at −80°C.

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### Alternative (Forward) Transfection Procedure (first-time users)

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. $6 \times 10^6$ 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 it 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 Step 1, page 42.
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 on page 42.

### 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, page 42) 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 prior to concentrating your viral stock.

### Long-Term Storage

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

### Scaling Up Virus Production

You may scale up the cotransfection experiment to produce a larger volume of lentivirus. 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 proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock(s). 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 either 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 EmGFP coding sequence was retained in the pcDNA™6.2-GW/EmGFP-miR vector

## Experimental Outline

To determine the titer of a lentiviral stock:

1. Prepare 10-fold serial dilutions of your lentiviral stock.
2. Transduce the different dilutions of lentivirus into the mammalian cell line of your choice in the presence of Polybrene.
3. Based on the titering method used:
   - Select for stably transduced cells using Blasticidin. Stain and count the number of Blasticidin-resistant colonies in each dilution.
   - Determine the titer by flow cytometry 4 days post-transduction, if using EmGFP.

## Factors Affecting Viral Titer

A number of factors can influence lentiviral titers, including:

- The characteristics of the cell line used for titering (see next page for more information).
- 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 the lentiviral stock prior to use in an RNAi experiment.
- The number of freeze/thaw cycles. Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- Improper storage of your lentiviral stock. Lentiviral stocks should be aliquotted and stored at −80°C (see page 43 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 that you use for your expression studies to titer your lentiviral stocks. 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, 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, titer all of the lentiviral constructs using the same mammalian cell line.

### Blasticidin Selection

The pLenti6.4/promoter/MSGW/EmGFP-miR expression construct contains the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) of mammalian cells that have stably transduced the lentiviral construct.

Blasticidin is supplied in the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Kit. Blasticidin is also available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 85 for ordering information). For more information for preparing and handling Blasticidin, and determining Blasticidin sensitivity, refer to the **Appendix** (page 68).

### 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, 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.

*Continued on next page*

# 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 stock solutions at −20°C for long-term storage (up to 1 year). Do not freeze/thaw the stock solution more than 3 times to avoid loss of activity.

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

## Materials Needed

- Your Lenti4/promoter/MSGW/EmGFP-miR 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 20 for filters), if you are using EmGFP titering method
- Phosphate-Buffered Saline (PBS; page 85)

*Components supplied with the kit*

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

## Preparing Mammalian Cells

Initiate your mammalian cell line of choice that will be used for titering. Grow the cells in the appropriate medium. You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions). Cells should be >95% viable.


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 before disposal.
- Treat used pipettes, 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.

**Note:** If you have generated a lentiviral stock of the pLenti6.4/promoter/MSGW/EmGFP-miR-*lacZ* control construct, perform titering using the Blasticidin or EmGFP method, and if you generated a lentiviral stock of the pLenti6.4/CMV/V5-MSGW/*lacZ* control construct, use Blasticidin titering method.

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

   **Example:** When using HT1080 cells, we usually plate $2 \times 10^5$ cells per well in a 6-well plate.

2. On the day of transduction (Day 2), thaw the 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 it 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 the plate at 37°C overnight.
5. The following day (Day 3), remove the virus-containing medium and replace it with 2 mL of complete culture medium.
6. The following day (Day 4), proceed to Steps 7–8 for **EmGFP titering method** or proceed to Steps 9–14 for **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 medium 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 page 48). Determine the titer using the formula described on page 48.
9. For Blasticidin selection, remove the medium on Day 4 and replace it 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 the dish or plate 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.

*Continued on next page*

# Titering Your Lentiviral Stock, Continued

## Preparing Cells for Flow Cytometry

If you have used the EmGFP titering method, prepare the cells for flow cytometry according to protocols established at your flow cytometry facility. Refer to page 20 for spectral characteristics of EmGFP.

1. Four days after transduction, dissociate the cells from the plate by using trypsin or cell dissociation buffer.
2. Centrifuge the cells at low speed to remove residual media components, and then resuspend the cell pellet in a flow cytometry buffer such as calcium/magnesium-free PBS with 1% FBS at the required density for analysis on your flow cytometer.

   **Note:** Fixing the cells is not necessary for analysis, but may be done, if desired. 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 negative and positive samples 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). This is to avoid analyzing dilution samples containing multiple integrated lentiviral genomes, which may result in an underestimate of the viral titer, or dilution samples containing too few transduced cells, which will give inaccurate results. Titer is expressed as transducing units (TU)/mL.

Use the following formula to calculate the titer:

$$\mathbf{[F \times C/V] \times D}$$

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 page 47. 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, because 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 $2 \times 10^5$ (the number of cells in the well) divided by 1 (the volume of inoculum). Thus, the titer in this example is:
$[(0.044 \times 200{,}000)/1] \times 10^4 = 8.8 \times 10^7$ TU/mL.

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# Titering Your Lentiviral Stock, Continued

## Expected Results

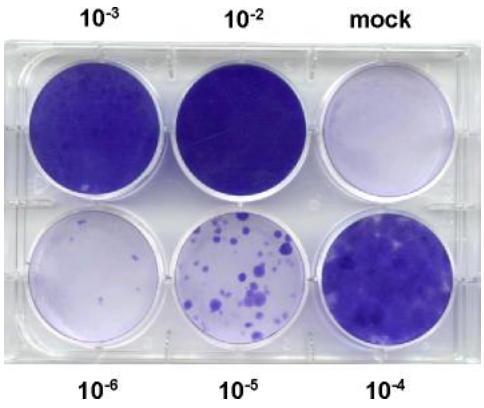
When titering pLenti6.4 lentiviral stocks using HT1080 cells, you should expect to obtain titers ranging from $5 \times 10^4$ to $5 \times 10^5$ transducing units (TU)/mL by blasticidin and $5 \times 10^5$ to $1 \times 10^7$ TU/mL using EmGFP. EmGFP titering is more sensitive and will detect more virus. However, blasticidin titering is still appropriate to use to normalize viral stocks made from different constructs at the same or at different times.

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 $5 \times 10^4$ TU/mL by blasticidin or $5 \times 10^5$ TU/mL by EmGFP, produce a new lentiviral stock. See page 37 and the **Troubleshooting** section on page 57 for more tips and guidelines for optimizing your viral yield.

## Example of Expected Results

In this experiment, a lentiviral stock was generated using the protocol on page 42. 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 in this manual. 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.




The colony counts obtained from the plate in the adjacent figure 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 $4.8 \times 10^6$ TU/mL (i.e. average of $46 \times 10^5$ and $5 \times 10^6$).

# Transduction and Analysis

## Introduction

After generating a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral construct into your mammalian cell line to express the miR RNAi of interest and perform RNAi analysis. Guidelines are provided below.

**Reminder:** Remember that your 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 miR RNAi 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 miR RNAi sequences 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 miR RNAi sequence.

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### 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 69. If you titered your lentiviral construct in the same mammalian cell line that you are using to generate a stable cell line, 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 miR RNAi, 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, miR RNAi expression levels increase as the MOI increases.

### Determining the Optimal MOI

A number of factors can influence MOI, including:

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

If you are transducing the lentiviral construct into your mammalian cell line 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 degree of target gene knockdown.

### Note

In general, non-dividing cell types transduce lentiviral constructs 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 an optimal degree of target gene knockdown.

### Preparing Mammalian Cells

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

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### Positive Controls

If you have generated two positive control lentiviral constructs (pLenti6.4/promoter/MSGW/EmGFP-miR-*lacZ* control and pLenti6.4/CMV/V5-MSGW/*lacZ* control constructs) as described on page 39, 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 pLenti6.4/promoter/MSGW/EmGFP-miR-*lacZ* to pLenti6.4/CMV/V5-MSGW/*lacZ* expression clone.

The β-galactosidase protein expressed from the pLenti6.4/CMV/V5-MSGW/*lacZ* control lentiviral construct is approximately 121 kDa in size. You may assay for β-galactosidase expression by activity assay using the 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 85 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 their transducibility. If the titer of your lentiviral stock is relatively low (less than $5 \times 10^5$ 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

- Your 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 kit*

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

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### Transduction Procedure

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

1. Plate the cells in complete medium 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 your lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of virus-containing medium as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the virus-containing medium gently by pipetting and add it to the cells.
4. Add Polybrene (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate it 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, you may incubate cells for as little as 6 hours before changing the medium.
5. The following day (Day 2), remove the virus-containing medium and replace it with fresh, complete culture medium.
6. The following day (Day 3), perform one of the following and then proceed to Step 7:

   - If you are performing transient expression experiments, harvest the cells and assay for inhibition of your target gene. If you wish to assay the cells at a later time, continue to culture the cells or replate them into larger-sized tissue culture formats as necessary.

   *or*

   - To select for stably transduced cells, remove the medium and replace it with fresh, complete medium containing the appropriate amount of Blasticidin.
7. Replace the spent medium with fresh medium containing Blasticidin every 3 to 4 days until Blasticidin-resistant colonies can be identified (generally 10 to 12 days after selection).
8. Pick at least 5 Blasticidin-resistant colonies (see **Note** on the next page) and expand each clone to assay for knockdown of the target gene.

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### 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 observe 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.

### Performing RNAi Analysis

You may use any method as appropriate to assay for knockdown of your target gene including functional analysis, immunofluorescence, western blot, or qRT-PCR with the appropriate LUX™ primers. For more information about LUX™ primers, see **www.lifetechnologies.com**.

With the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP, you can detect EmGFP fluorescence using fluorescence microscopy or flow cytometry (see page 55).

### What You Should See

When performing RNAi studies using pLenti6.4 lentiviral constructs, we generally observe 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 other factors listed on page 50. Note that 100% gene knockdown is generally not observed, but > 80% is possible with optimized conditions.

# 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 when you used pcDNA™6.2-GW/EmGFP-miR for cloning your miR RNAi 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 miR RNAi sequence.

See the following sections 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 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.

## What You Should See

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 pre-miRNA-containing vectors is reduced when compared to non-pre-miRNA containing vectors due to processing of the EmGFP transcript by Drosha.

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

# 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.

| Observation | 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 61. |
| | 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® BP and LR Clonase® II Plus enzyme mixes or Gateway® BP and LR Clonase® II Plus enzyme mixes were inactive | • Make sure to store the Gateway® BP and LR Clonase® II Plus enzyme mix at –20°C or –80°C.<br>• Do not freeze/thaw the Gateway® BP and LR Clonase® II Plus enzyme mix more than 10 times.<br>• Use the recommended amount of Gateway® BP and LR Clonase® II Plus enzyme mix (see page 33).<br>• Test another aliquot of the Gateway® BP and LR Clonase® II Plus 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. |
| | Did not include a pENTR™5′ promoter vector in the LR reaction | You must use either of the supplied pENTR™5′ promoter vectors (or a pENTR™5′ vector you have generated with your own promoter of interest) in the LR reaction. |

*Continued on next page*

### Rapid BP/LR Reaction and Transformation, continued

| Observation | 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.<br>• 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 co-transfection and titering experiments.

| Observation | Reason | Solution |
|---|---|---|
| Low viral titer | Low transfection efficiency:<br>• Used poor quality expression construct plasmid DNA (i.e. DNA from a mini-prep)<br>• Unhealthy 293FT cells; cells exhibit low viability<br>• Cells transfected in medium containing antibiotics (i.e. Geneticin®)<br>• Plasmid DNA:transfection reagent ratio incorrect<br>• 293FT cells plated too sparsely | • **Do not** use plasmid DNA from a mini-prep for transfection. Use the PureLink® HiPure Plasmid DNA Purification MidiPrep Kit to prepare plasmid DNA.<br>• Use healthy 293FT cells under passage 20; do not overgrow them.<br>• **Do not** add Geneticin® to medium during transfection as this reduces transfection efficiency and causes cell death.<br>• Use a DNA (in µg):Lipofectamine® 2000 (in µL) ratio ranging from 1:2 to 1:3.<br>• 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; see page 42). |

*Continued on next page*

### Generating the Lentiviral Stock, Continued

| Observation | 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.**<br>• Mix gently by inversion before use. **Do not vortex.** |
| | Viral supernatant harvested too early | Viral supernatants can generally be collected 48–72 hours posttransfection. 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 45. |
| | 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 antibiotic used for selection | Determine the antibiotic 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 antibiotic 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 your transduction and knockdown experiment.

| Observation | Reason | Solution |
|---|---|---|
| Low levels of gene knockdown observed | Low transduction efficiency:<br>• Polybrene not included during transduction<br>• Non-dividing cell type used | • Transduce the lentiviral construct into cells in the presence of Polybrene.<br>• 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.<br>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.<br>**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 47 before use. |
| | Viral stock stored incorrectly | • Aliquot and store stocks at −80°C.<br>• Do not freeze/thaw more than 3 times.<br>• If stored for longer than 6 months, re-titer stock before use. |
| | miR RNAi with weak activity chosen | Select a different target region. If possible, screen miR RNAi sequences first by transient transfection of the expression construct to verify its activity, then perform BP/LR recombination with the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector and proceed to generate lentivirus.<br>**Note:** Generally, transient transfection greatly overexpresses miR RNAi sequences, so moderately active expression clones may be less active when expressed from a lentiviral construct. |

*Continued on next page*

### Transduction and RNAi Analysis, Continued

| Observation | Reason | Solution |
|---|---|---|
| No gene knockdown observed | miR RNAi with no activity chosen | Select a different target region. If possible, screen miR RNAi sequences first by transient transfection of the expression construct to verify its activity, then perform BP/LR recombination with the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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.<br>• 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 experiment. 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 | Be sure to use the recommended filter sets for detection of fluorescence (see page 55). Be sure to use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for additional 1–3 days before assaying for fluorescence.<br>**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 64).

Use the standard BP reaction, if you wish 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 28 for details on 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.

*Continued on next page*

# Performing the Standard BP Recombination Reaction, Continued

## *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.

## Materials Needed

**For BP Recombination**

- Linearized *att*B expression clone, see page 28
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 85

*Components supplied with the kit*

- pDONR™221 vector (150 ng/µL)
- Gateway® BP Clonase® II Plus 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**

- 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 kit*

- One Shot® TOP10 Chemically Competent *E. coli* (or equivalent; one vial per transformation; thaw on ice before use)
- 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 28 (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 pcDNA™6.2-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 85). 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, page 65.

### 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 MultiSite LR Recombination Reaction

## Introduction

After obtaining an entry clone containing the pre-miRNA expression cassette, you will perform an LR recombination reaction between the two entry clones and a destination vector (pLenti6.4/R4R2/V5-DEST), and transform the reaction mixture into One Shot® Stbl3™ Competent *E. coli* to select for expression clones (see page 30).

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

## Experimental Outline

To generate an expression clone, you will:

1. Perform a LR recombination reaction using the entry clone containing the EmGFP-miR cassette (page 64), an entry clone containing the promoter of interest, and *att*R-containing pLenti6.4/R4R2/V5-DEST.
2. Transform the reaction mixture into a One Shot® Stbl3™ Competent *E. coli*.
3. Select for expression clones.

## Important

The pLenti6.4/R4R2/V5-DEST MultiSite Gateway® 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 pLenti6.4/R4R2/V5-DEST is not required to obtain optimal results for any downstream application.

## Recommended *E. coli* Host

**For optimal results, we recommend using 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 30).

*Continued on next page*

# Performing the Standard MultiSite 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 pENTR™5′/CMVp **or** pENTR™5′/EF-1αp **or** your own pENTR™5′ entry clone

*Components supplied with the kit*

- pENTR™5′/CMVp **or** pENTR™5′/EF-1αp (**or** your own pENTR™5′ entry clone, not supplied)
- pLenti6.4/R4R2/V5-DEST (150 ng/µL in TE, pH 8.0)
- Gateway® LR Clonase® II Plus enzyme mix (keep at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (thaw and keep on ice until use)
- One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with the kit)
- S.O.C. Medium (supplied with the competent cells, warm to room temperature)

*Continued on next page*

# Performing the Standard MultiSite LR Recombination Reaction, Continued

## 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 Plus enzyme mix (see Step 4).

| Component | Sample |
|---|---|
| miR RNAi entry clone (50–150 ng/reaction) | 1–6 µL |
| pENTR™5′/CMVp or EF-1αp or your own promoter (150 ng/reaction) | 1 µL |
| pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector (150 ng/µL) | 1 µL |
| TE Buffer, pH 8.0 | to 8 µL |

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

   **Reminder:** Return Gateway® LR Clonase® II Plus enzyme mix to −20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 µL of the Proteinase K solution to the reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 30).

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

## What You Should See

If you use *E. coli* cells with a transformation efficiency of ≥ $1 \times 10^8$ cfu/µg, the LR reaction should give > 5,000 colonies if the entire LR reaction is transformed and plated. See page 36 for confirming the expression clone.

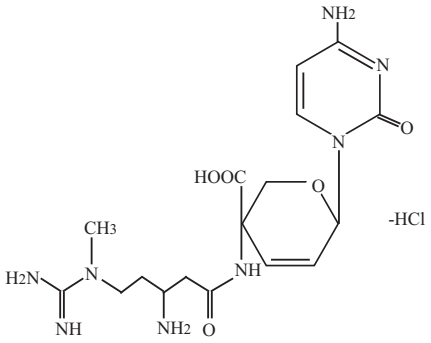
# Blasticidin

## 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.

*Continued on next page*

# Blasticidin, Continued

## Preparing and Storing Stock Solutions

Blasticidin may be obtained in 50-mg aliquots (see page 85 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 1–2 weeks 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.

## Determining Antibiotic Sensitivity

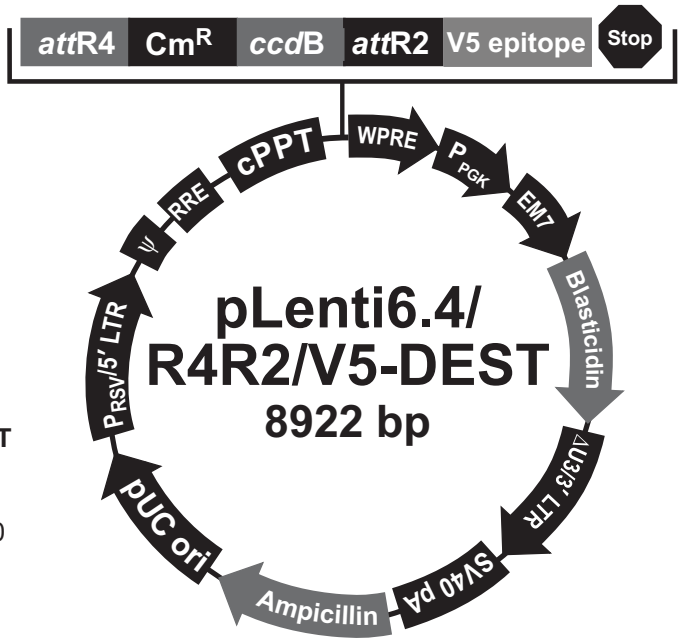
Since you will be selecting for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (i.e. perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/mL Blasticidin are 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 pLenti6.4/R4R2/V5-DEST

## Map of pLenti6.4/R4R2/V5-DEST

The map below shows the elements of pLenti6.4/R4R2/V5-DEST. DNA from the entry clone replaces the region between bases 2,447 and 4,130. **The sequence for pLenti6.4/R4R2/V5-DEST is available from www.lifetechnologies.com or by contacting Technical Support (see page 87).**




**Comments for pLenti6.4/R4R2/V5-DEST**
**8922 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
cPPT: bases 1801-1923
*att*R4 site: bases 1951-2075
Chloramphenicol resistance gene ($Cm^R$): bases 2801-3460
*ccd*B gene: bases 3184-3489
*att*R2 site: bases 3530-3654
V5 epitope: bases 3707-3748
WPRE: bases 3767-4364
PGK promoter: bases 4374-4880
EM7 promoter: bases 4891-4957
Blasticidin resistance gene: bases 4958-5356
ΔU3/3′ LTR: bases 5442-5676
ΔU3: bases 5442-5495
3′ LTR: bases 5496-5676
SV40 polyadenylation signal: bases 5748-5879
*bla* promoter: bases 6738-6836
Ampicillin (*bla*) resistance gene: bases 6837-7697
pUC origin: bases 7842-8515

*Continued on next page*

# Map and Features of pLenti6.4/R4R2/V5-DEST, Continued

## Features of the Vector

The pLenti6.4/R4R2/V5-DEST (8,922 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). |
| Polypurine Tract from HIV (cPPT) | Provides for increased viral titer (Park, 2001). |
| *att*R4 and *att*R2 sites | Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the miR RNA and promoter of interest from Gateway® entry clones (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.** |
| Woodchuck Posttranscriptional Regulatory Element (WPRE) | Provides for increased transgene expression (Zufferey et al., 1998). |
| mPGK promoter | Allows expression of the selection marker in a broad variety of mammalian cells. |
| 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*. |

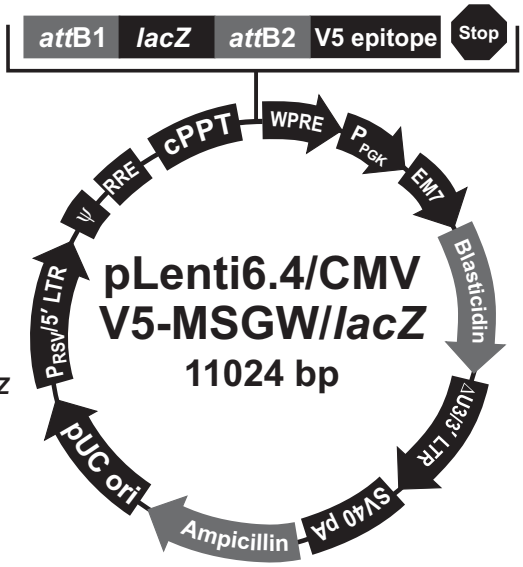
# Map of pLenti6.4/CMV/V5-MSGW/*lacZ*

## Description

pLenti6.4/CMV/V5-MSGW/*lacZ* is an 11,024 bp control vector expressing β-galactosidase, and was generated using the MultiSite Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene, an entry clone containing the CMV promoter, and pLenti6.4/R4R2/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

## Map of pLenti6.4/CMV/V5-MSGW/*lacZ*

The map below shows the elements of pLenti6.4/CMV/V5-MSGW/*lacZ*. **The sequence of the vector is available from www.lifetechnologies.com or by calling Technical Support (see page 87).**



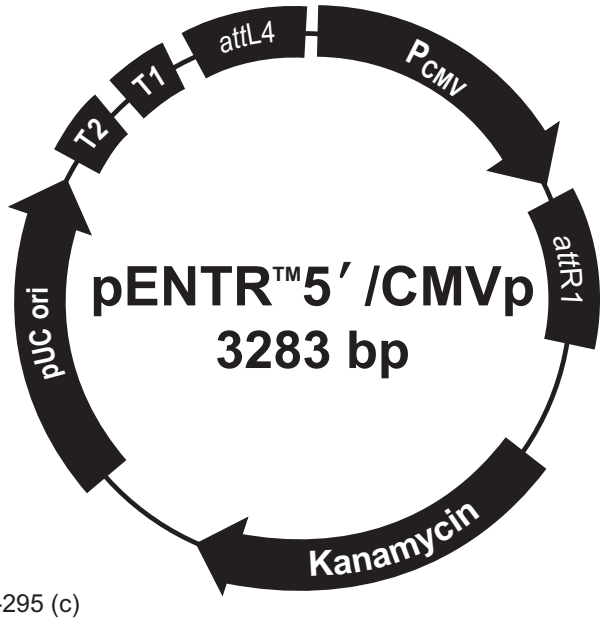

**Comments for pLenti6.4/CMV/V5-MSGW/*lacZ***
**11024 nucleotides**

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
cPPT: bases 1801-1923
CMV promoter: bases 1979-2563
*att* B1 site: bases 2611-2635
*lacZ* ORF: bases 2656-5717
*att* B2 site: bases 5732-5756
V5 epitope: bases 5809-5850
WPRE: bases 5869-6466
PGK promoter: bases 6476-6982
EM7 promoter: bases 6993-7059
Blasticidin resistance gene: bases 7060-7458
ΔU3/3′ LTR: bases 7544-7778
ΔU3: bases 7544-7597
3′ LTR: bases 7598-7778
SV40 polyadenylation signal: bases 7860-7981
*bla* promoter: bases 8840-8938
Ampicillin (*bla*) resistance gene: bases 8939-9799
pUC origin: bases 9944-10617

# Map and Features of of pENTR™5'/CMVp

## Map of pENTER™5'/CMVp

The map below shows the elements of the pENTR™ 5′/CMVp vector. **The sequence of pENTR™ 5′/CMVp is available from www.lifetechnologies.com or by contacting Technical Support (page 87).**




**Comments for pENTR™5′/CMVp**
**3283 nucleotides**

*rrn*B T2 transcription terminator: bases 268-295 (c)
*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
*att*L4: bases 592-688
GW1 priming site: bases 630-654
CMV promoter: bases 696-1280
*att*R1: bases 1329-1452
GW3 priming site: bases 1359-1388
M13 reverse priming site: bases 1548-1564
Kanamycin resistance gene: 1677-2486
pUC origin: bases 2607-3280

(c) = complementary strand

*Continued on next page*

# Map and Features of of pENTR™5'/CMVp, Continued

## Features of the Vector

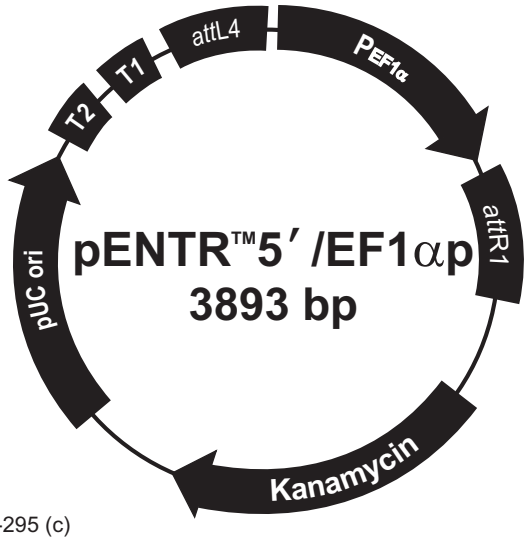
The pENTR™5'/CMVp (3,283 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

| Feature | Benefit |
|---|---|
| *rrn*B T1 and T2 transcription termination sequences | Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product. |
| M13 forward (−20) priming site | Allows sequencing of the insert. |
| GW1 priming site | Allows sequencing of the insert. |
| *att*L4 and *att*R1 sites | Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a suitable MultiSite Gateway® destination vector in conjunction with any *att*L1 and *att*L2-flanked entry clone (Landy, 1989). |
| Human cytomegalovirus (CMV) immediate-early promoter/enhancer | Permits high-level, constitutive expression of the gene of interest (Andersson *et al*., 1989; Boshart *et al*., 1985; Nelson *et al*., 1987). |
| GW3 priming site | Allows sequencing of the insert. |
| M13 reverse priming site | Allows sequencing of the insert. |
| Kanamycin resistance gene | Allows selection of the plasmid in *E. coli*. |
| pUC origin of replication (ori) | Allows high-copy replication and maintenance in *E. coli.* |

# Map and Features of pENTR™5'/EF1αp

## Map of pENTER™5'/EF1αp

The map below shows the elements of the pENTR™ 5′/EF1αp vector. **The sequence of pENTR™ 5′/EF1αp is available at www.lifetechnologies.com or by contacting Technical Support (page 87).**




**Comments for pENTR™5′/EF1αp**
**3893 nucleotides**

*rrn*B T2 transcription terminator: bases 268-295 (c)
*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
*att*L4: bases 592-688
GW1 priming site: bases 630-654
EF1α promoter: bases 704-1893
*att*R1: bases 1939-2062
GW3 priming site: bases 1969-1998
M13 reverse priming site: bases 2158-2174
Kanamycin resistance gene: 2287-3096
pUC origin: bases 3217-3890

(c) = complementary strand

*Continued on next page*

# Map and Features of pENTR™5'/EF1αp, Continued

## Features of the Vector

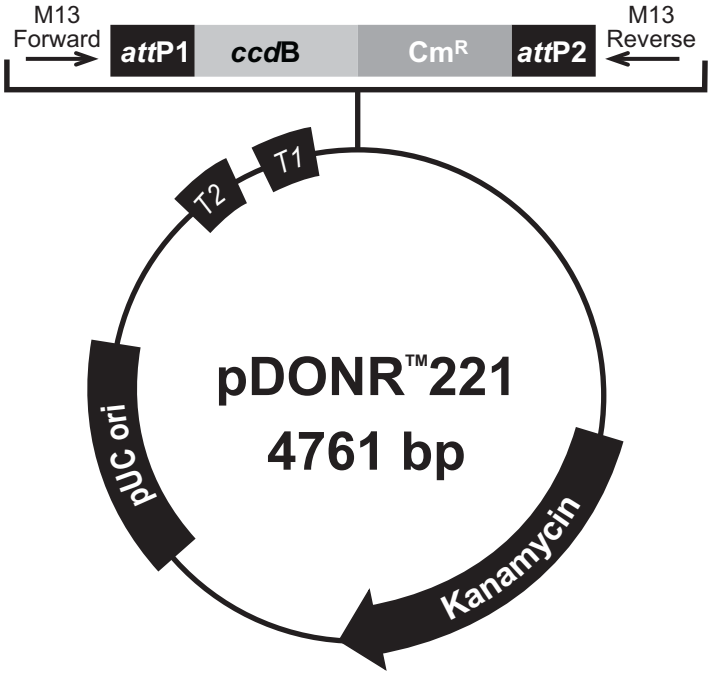
The pENTR™5'/EF1αp (3,893 bp)vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

| Feature | Benefit |
|---|---|
| *rrn*B T1 and T2 transcription termination sequences | Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product. |
| M13 forward (−20) priming site | Allows sequencing of the insert. |
| GW1 priming site | Allows sequencing of the insert. |
| *att*L4 and *att*R1 sites | Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a suitable MultiSite Gateway® destination vector in conjunction with any *att*L1 and *att*L2-flanked entry clone (Landy, 1989). |
| Elongation Factor 1 promoter (EF-1αp) | Encodes the EF-1 enzyme that catalyzes the GTP-dependent binding of aminoacyl-tRNA to ribosomes and is expressed in almost all kinds of mammalian cells. |
| GW3 priming site | Allows sequencing of the insert. |
| M13 reverse priming site | Allows sequencing of the insert. |
| Kanamycin resistance gene | Allows selection of the plasmid in *E. coli.* |
| pUC origin of replication (ori) | Allows high-copy replication and maintenance in *E. coli.* |

# 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.lifetechnologies.com or by contacting Technical Support (see page 87).**




**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

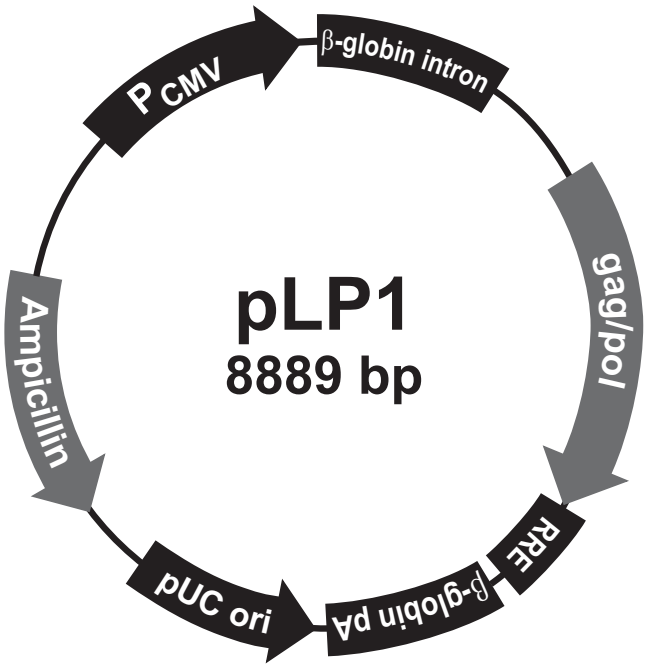
pDONR™221 (4,761 bp) contains the following elements. All features have been functionally tested and the vectors fully sequenced.

| 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.lifetechnologies.com or by contacting Technical Support (see page 87).**




**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

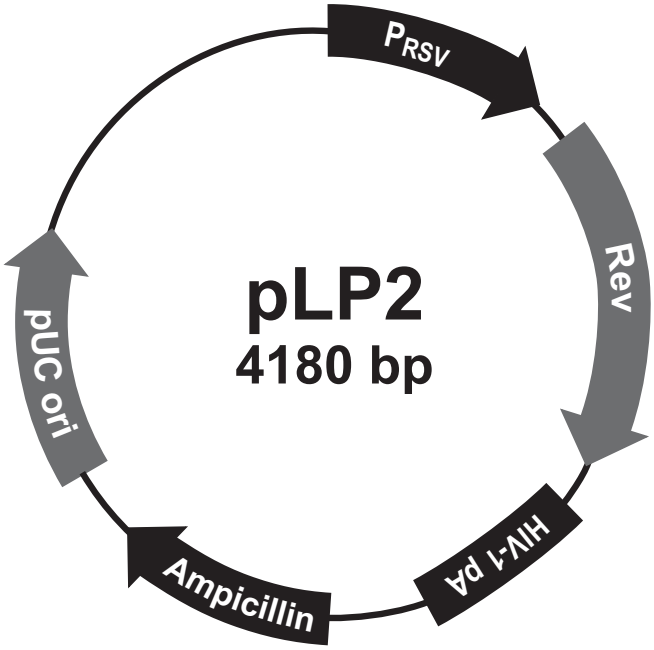
pLP1 (8,889 bp) contains the following elements. All features have been functionally tested.

| 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.lifetechnologies.com or by contacting Technical Support (see page 87).**




**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

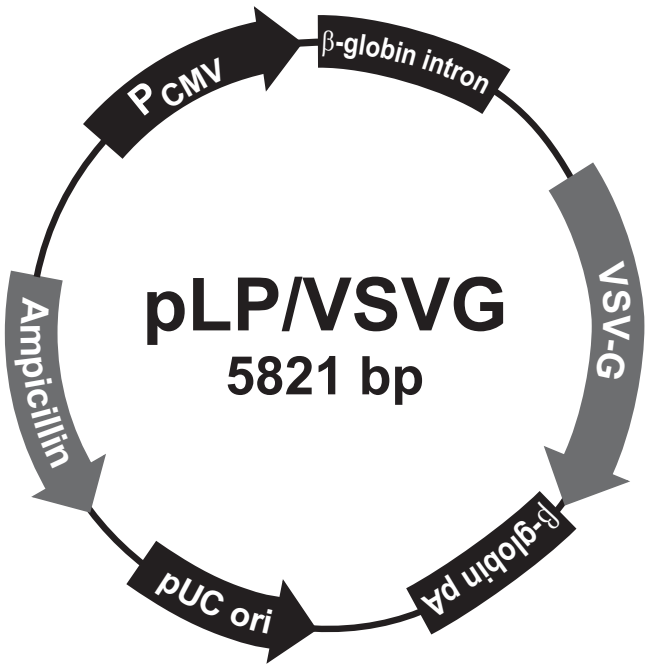
pLP2 (4,180 bp) contains the following elements. All features have been functionally tested.

| 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 pLenti6.4/R4R2/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.lifetechnologies.com or by contacting Technical Support (see page 87).**




**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*. |

# Additional Products

## Accessory Products

Many of the reagents supplied in the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Kit, as well as other products suitable for use with the kit, are available separately. Ordering information is provided below. For more information, go to **www.lifetechnologies.com** or contact Technical Support (see page 87).

| 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<br>100 reactions | 11789-020<br>11789-100 |
| Gateway® LR Clonase® II Plus Enzyme Mix | 20 reactions<br>100 reactions | 12538-120<br>12538-200 |
| One Shot® Stbl3™ Chemically Competent *E. coli* | 20 × 50 µL | C7373-03 |
| One Shot® TOP10 Chemically Competent *E. coli* | 20 x 50 µl | C4040-03 |
| ViraPower™ Bsd Lentiviral Support Kit | 20 reactions | K4970-00 |
| ViraPower™ Lentiviral Packaging Mix | 60 reactions | K4975-00 |
| Lipofectamine® 2000 Reagent | 0.75 mL<br>1.5 mL | 11668-027<br>11668-019 |
| pDONR™221 Vector | 6 µg | 12536-017 |
| **pENTR™ 5´TOPO® TA Cloning Kit with One Shot® TOP10 Chemically Competent *E. coli*** | 20 reactions | K591-20 |
| Opti-MEM® I Reduced Serum Medium | 100 mL<br>500 mL | 31985-062<br>31985-070 |
| Blasticidin | 50 mg | R210-01 |
| 293FT Cell Line | 3 × $10^6$ cells | R700-07 |
| Phosphate-Buffered Saline (PBS), pH 7.4 | 500 mL<br>1 L | 10010-023<br>10010-031 |
| Ampicillin | 200 mg | 11593-027 |
| (Millers LB Broth Base)® Luria Broth Base, powder | 500 g | 12795-027 |
| 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 |
| β-Gal Staining Kit | 1 kit | K1465-01 |
| FluoReporter® *lacZ*/Galactosidase Quantitation Kit | 1,000 assays | F2905 |

*Continued on next page*

# Additional Products, Continued

### BLOCK-iT™ RNAi Designer

The BLOCK-iT™ RNAi Designer is an online tool to help you design and order miR RNAi 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 miR RNAi 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 more details, visit the RNAi central portal or contact **Technical Support** (see page 87).

# Technical Support

## Obtaining Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

## Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

## Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

## Limited Product Warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

# Purchaser Notification

**Information for European Customers**

The 293FT Cell Line is genetically modified and carries the pUC-derived plasmid, pCMVSPORT6TAg.neo. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

**Limited Use Label License No. 358: Research Use Only**

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial services of any kind, including, without limitation, reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

# Gateway® Clone Distribution Policy

## Introduction

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

## Gateway® Entry Clones

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

## Gateway® Expression Clones

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

## Additional Terms and Conditions

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

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