invitrogen™

# BioModule™ BLOCK-iT™ Units with Pol II miR RNAi Expression System

## Gateway®-adapted expression vector and lentiviral destination vector for high-level expression of microRNA in mammalian cells

**Catalog nos. WFGE07, WFGE08**

**Version B**
28 December 2010
*25-0883*

User Manual

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# Experienced Users Procedure to Generate Expression Clone

## Introduction

A quick procedure to generate an expression clone is provided for experienced users of the BioModule™ BLOCK-iT™ RNAi Kits. If you are performing this procedure for the first time or for additional protocols, refer to this manual.

| Step | Action |
|---|---|
| Design single-stranded DNA oligos | Follow the guidelines on page 37 to design single-stranded DNA oligos encoding the pre-miRNA of interest. |
| Anneal the single-stranded oligos to generate a ds oligo | 1. Set up the following annealing reaction.<br>200 µM top strand oligo 5 µl<br>200 µM bottom strand oligo 5 µl<br>10X Oligo Annealing Buffer 2 µl<br>DNase/RNase-free water 8 µl<br>Total volume 20 µl<br>2. Heat the reaction mixture at 95°C for 4 minutes.<br>3. Remove the sample and set on the laboratory bench. Allow the reaction to cool to room temperature for 5-10 minutes.<br>4. Spin down the sample in a microcentrifuge for 5 seconds. Mix gently.<br>5. Dilute the ds oligo mixture 5,000-fold by performing serial 100-fold and 50-fold dilutions: the first into DNase/RNase-free water and the second into 1X Oligo Annealing Buffer. Final concentration is 10 nM. |
| Clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR Vector | 1. Set up the following ligation reaction.<br>5X Ligation Buffer 4 µl<br>pcDNA™6.2-GW/EmGFP-miR (5 ng/µl), linearized 2 µl<br>ds oligo (10 nM; 1:5,000 dilution) 4 µl<br>DNase/RNase-Free water 9 µl<br>T4 DNA Ligase (1 U/µl) 1 µl<br>Total volume 20 µl<br>2. Mix reaction well and incubate for 5 minutes at room temperature.<br>3. Place reaction on ice and proceed to transform *E. coli*, below. |
| Transform One Shot® TOP10 Chemically Competent *E. coli* | 1. Add 2 µl of the ligation reaction into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently.<br>2. Incubate on ice for 5-30 minutes.<br>3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.<br>4. Add 250 µl of room temperature S.O.C. Medium.<br>5. Incubate at 37°C for 1 hour with shaking.<br>6. Spread 20-100 µl of bacterial culture on a pre-warmed LB agar plate containing 50 µg/ml spectinomycin and incubate overnight at 37°C. |
| To transfect the miRNA construct into mammalian cells, transfer the miRNA construct into destination vectors, and lentiviral expression, see detailed protocols in this manual. | |

# Kit Contents and Storage

## Types of Kits

This manual is supplied with the following products.

| Product | Catalog no. |
|---|---|
| BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector | WFGE07 |
| BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System | WFGE08 |

## Kit Components

The BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector and BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System (referred to as BioModule™ BLOCK-iT™ RNAi Units) include the following components. For detailed contents, see the following pages.

The BioModule™ BLOCK-iT™ RNAi Units are shipped as described below. Upon receipt, store each item as detailed below.

| Box | Component | Catalog no. | | Shipping | Storage |
|---|---|---|---|---|---|
| | | WFGE07 | WFGE08 | | |
| 1 | BLOCK-iT™ Pol II miR RNAi Expression Vector Kit | √ | √ | Dry ice | -20ºC |
| 2 | One Shot® TOP10 Chemically Competent *E. coli* | √ | √ | Dry ice | -80ºC |
| 3 | PureLink™ HQ Mini Plasmid DNA Purification Kit | √ | √ | Room temperature | Room temperature |
| 4 | Lipofectamine™ 2000 Reagent | √ | | Blue ice | 4°C **(do not freeze)** |
| 5 | OptiMEM® I Reduced Serum Medium | √ | √ | Room temperature | 2 to 8ºC (in the dark) |
| 6 | Spectinomycin | √ | √ | Dry ice | -20ºC |
| 7 | Blasticidin | √ | | -20ºC | -20ºC |
| 8 | Kanamycin | √ | √ | Dry ice | -20ºC |
| 9 | Ampicillin | | √ | Room temperature | 4ºC |
| 10 | LB Broth (1X), liquid | √ | √ | Room temperature | Room temperature |
| 11 | 4% E-Gel® Starter Pak | √ | √ | Room temperature | Room temperature |
| 12 | 1.2% E-Gel® Agarose Gels | | √ | Room temperature | Room temperature |
| 13 | TrackIt™ 10 bp DNA Ladder | √ | √ | Room temperature | Room temperature |

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### Kit Components, continued

| Box | Component | Catalog no. | | Shipping | Storage |
|---|---|---|---|---|---|
| | | WFGE07 | WFGE08 | | |
| 14 | TrackIt™ 10 bp DNA 1 Kb Plus DNA Ladder | | √ | Room temperature | Room temperature |
| 15 | pLenti6/V5-DEST Gateway® Vector Kit | | √ | Blue ice | -20ºC |
| 16 | pDONR™221 | | √ | Room temperature | -20ºC |
| 17 | Gateway® BP Clonase™ II Enzyme Mix | | √ | Dry ice | -20ºC |
| 18 | Gateway® LR Clonase™ II Enzyme Mix | | √ | Dry ice | -20ºC |
| 19 | One Shot® Stbl3™ Chemically Competent *E. coli* | | √ | Dry ice | -80ºC |
| 20-21 | ViraPower™ Bsd Lentiviral Support Kit: | | √ | | |
| | ViraPower™ Packaging Mix | | | Blue ice | -20ºC |
| | Lipofectamine™ 2000 Reagent | | | Blue ice | +4°C **(do not freeze)** |
| | Blasticidin | | | Room temperature | -20ºC |
| 22 | 293FT Cell Line | | √ | Dry ice | Liquid nitrogen |
| 23 | S.N.A.P.™ MidiPrep Kit | | √ | Room temperature | Room temperature |

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

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

| Reagent | Composition | Amount |
|---|---|---|
| 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 x 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 x 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 | 10 nM in 1X Oligo Annealing Buffer | 50 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 |

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# Kit Contents and Storage, Continued

## Unit Definition of T4 DNA Ligase

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

## Primer Sequences

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

| Primer | Sequence | Amount |
|---|---|---|
| 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 below. The miR-*lacZ* top and bottom strand oligos are annealed and are supplied in the kit as a 10 nM double-stranded oligo that is ready-to-use in the ligation reaction (page 51).

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

## One Shot® TOP10 Reagents

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

| Reagent | Composition | Amount |
|---|---|---|
| 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 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 50 µl |

## Genotype of TOP10 Cells

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

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# Kit Contents and Storage, Continued

## PureLink™ HQ Mini Plasmid Purification Kit

The components included in the PureLink™ HQ Mini Plasmid Purification Kit (Box 3) are listed below.

Upon receipt, store all components at room temperature, except store the Resuspension Buffer after addition of RNase at +4°C.

| Component | Amount |
|---|---|
| Resuspension Buffer | 120 ml |
| Lysis Buffer | 120 ml |
| Neutralization/Binding Buffer | 2 × 85 ml |
| Wash Buffer | 16 ml |
| Elution Buffer (10 mM Tris-HCl, pH 8.5) | 15 ml |
| RNase A | 12 mg |
| Spin Columns | 100 |
| Collection Tubes (2 ml) | 100 |
| Elution Tubes (1.7 ml) | 100 |

## Lipofectamine™ 2000 Reagent and Opti-MEM® I Reduced Serum Medium

**Lipofectamine™ 2000 Reagent** (Box 4) is a transfection reagent with proprietary formulation. **Store at 4°C. Do not freeze.**

**Note:** 0.75 ml Lipofectamine™ 2000 Reagent is included in the ViraPower™ Bsd Lentiviral Support Kit for Cat. no. WFGE08.

**Opti-MEM® I Reduced Serum Medium** (Box 5) is used for dilution of the lipid: DNA complexes during transfection. 100 ml Opti-MEM® I Reduced Serum Medium is included with each BioModule™ BLOCK-iT™ RNAi Unit. The formulation of the medium is described below.

Opti-MEM® I Reduced Serum Medium is a modification of Eagle's Minimal Essential Medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine or GLUTAMAX, trace elements and growth factors. The protein level is minimal (15 µg/ml); insulin and transferrin are the only protein supplements. Phenol red is included at a reduced concentration as a pH indicator.

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### LB Broth and Antibiotics

The following antibiotics and medium are included with the BioModule™ BLOCK-iT™ RNAi Units (Boxes 6-10).

| Antibiotic | Composition | Amount |
|---|---|---|
| Spectinomycin | 100 mg/ml in deionized water | 1 ml |
| Kanamycin (only with WFGE08) | 25 mg/ml in deionized water | 1 ml |
| Ampicillin (only with WFGE08) | Powder | 200 mg |
| Blasticidin | Powder | 50 mg |
| LB Broth (1X), liquid | **Formulation per one liter:**<br>10 g SELECT Peptone 140<br>5 g SELECT Yeast Extract<br>5 g sodium chloride | 500 ml |

### E-Gel® Agarose Gels and DNA Ladders

The following E-Gel® agarose gels and DNA Ladders are included with the BioModule™ BLOCK-iT™ RNAi Units (Boxes 11-14).
**Store at room temperature.**

| Item | Composition | Amount |
|---|---|---|
| E-Gel® 4% Starter Pak | See page 15 for details | 6 gels and one E-Gel® PowerBase™ |
| E-Gel® 1.2% 18 Pak (only with WFGE08) | See page 15 for details | 18 gels |
| TrackIt™ 10 bp DNA Ladder | 0.5 µg/µl in:<br>10 mM Tris-HCl, pH 7.5<br>10 mM EDTA, pH 8.0<br>0.06% XCFF<br>0.4% Orange G<br>5% glycerol | 20 applications |
| TrackIt™ 1 Kb Plus DNA Ladder (only with WFGE08) | 0.1 µg/µl in:<br>10 mM Tris-HCl, pH 7.5<br>10 mM EDTA, pH 8.0<br>0.06% XCFF<br>0.6% tartrazine<br>5% glycerol<br>5 mM NaCl | 100 applications |

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## Vectors

The following vectors are included with the BioModule™ BLOCK-iT™ RNAi Units (Boxes 15 and 16). For details on pcDNA™6.2-GW/EmGFP-miR vector, see page viii. **Store the vectors at -20°C.**

| Reagent | Composition | Amount |
|---|---|---|
| pLenti6/V5-DEST Gateway® Vector | Lyophilized in TE Buffer, pH 8.0 | 6 µg |
| pLenti6/V5-GW/*lacZ* control | Lyophilized in TE Buffer, pH 8.0 | 10 µg |
| pDONR™221 Vector | Lyophilized in TE Buffer, pH 8.0 | 6 µg |

## Gateway® Clonase™ II Enzyme Mixes

The following reagents are included with the Gateway® Clonase™ II Enzyme Mixes (Boxes 17-18).

**Store Box 17 and 18 at -20°C for up to 6 months.** For long-term storage, store at -80°C.

**BP Clonase™ II**

| Reagent | Composition | Amount |
|---|---|---|
| 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 |

**LR Clonase™ II**

| Reagent | Composition | Amount |
|---|---|---|
| Gateway® LR 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 |
| pENTR™-gus Positive Control | 50 ng/µl in TE Buffer, pH 8.0 | 20 µl |

**Note:** The pENTR™-gus control included with the LR Clonase™ II Enzyme Mix may be used as a positive control for the LR recombination reaction **only** (page 139).

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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 (Box 19). Transformation efficiency is ≥ 1 x $10^8$ cfu/µg plasmid DNA. **Store Box 19 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 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 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 $\lambda^-$ *leu mtl*-1

### ViraPower™ Bsd Lentiviral Support Kit

The following reagents are included with the ViraPower™ Bsd Lentiviral Support Kit (Boxes 20-21). Store the ViraPower™ Packaging Mix and Blasticidin at -20°C. Store Lipofectamine™ 2000 Reagent at +4°C.

**Important:** Do not freeze Lipofectamine™ 2000 Reagent.

| Reagent | Composition | Amount |
|---|---|---|
| ViraPower™ Packaging Mix | Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, lyophilized in TE Buffer, pH 8.0 | 195 µg |
| Lipofectamine™ 2000 Reagent | Proprietary | 0.75 ml |
| Blasticidin | Powder | 50 mg |

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### 293FT Cell Line

The 293FT Cell Line (Box 22) is used for the production of lentiviral stocks. The 293FT Cell Line is supplied as one vial containing 3 x $10^6$ frozen cells in 1 ml of Freezing Medium. **Upon receipt, store in liquid nitrogen until use.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see page 96.

### S.N.A.P.™ MidiPrep Kit

The following items are included in the S.N.A.P.™ MidiPrep Kit (Box 23).

Store the kit at room temperature, except store the following components at +4°C after opening the kit:

- Resuspension Buffer
- Precipitation Salt
- RNase A

| Item | Composition | Quantity |
|---|---|---|
| S.N.A.P.™ MidiPrep | -- | |
| Column A (Filtering) | | 20 |
| Column B (Binding) | | 20 |
| Resuspension Buffer | 50 mM Tris-HCl, pH 8.0, 10 mM EDTA | 80 ml |
| RNase A, lyophilized | -- | 6 mg |
| Lysis Buffer | 0.2 M NaOH, 1% SDS | 80 ml |
| Precipitation Salt | 3 M Potassium acetate, pH 5.2 | 80 ml |
| Binding Buffer | 7.5 M Guanidine-HCl | 2 x 120 ml |
| Wash Buffer | 5 M Guanidine-HCl, 50 mM MOPS, pH 7.0 | 100 ml |
| 4X Final Wash | 400 mM NaCl | 80 ml |

**Note**

- Some reagents in the units may be provided in excess of the amount needed.
- Individual documentations detailing general use are included with some of the products supplied in the BioModule™ BLOCK-iT™ RNAi Units. **To use the products specifically with the BioModule™ BLOCK-iT™ RNAi Units, follow the recommended protocols in this manual.**

# Introduction

## Overview

### Introduction

The BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector (Cat. no. WFGE07) facilitates the expression of microRNA (miRNA) for use in RNA interference (RNAi) analysis of a target gene in mammalian cells. The kit includes a Gateway®-adapted expression vector designed for transient or stable expression of your miRNA and allows for efficient recombination with suitable destination vectors allowing tissue-specific, regulated, or lentiviral expression of the miRNA in mammalian cells using Gateway® Technology.

The BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System (Cat. no WFGE08) combines Invitrogen's BLOCK-iT™ Pol II miR RNAi and ViraPower™ Lentiviral technologies to facilitate creation of a replication-incompetent lentivirus that delivers a miRNA sequence of interest to dividing or non-dividing mammalian cells for RNAi analysis.

The BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector and BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System (referred to as BioModule™ BLOCK-iT™ RNAi Units) provide qualified reagents and validated protocols to express your miRNA in mammalian cells for RNAi analysis.

For details on the BLOCK-iT™ Pol II miR RNAi, Gateway®, and ViraPower™ Lentiviral technologies, see page 5.

### BioModule™ Units for Gene Expression Profiling

The BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology is one of the several BioModule™ Units available from Invitrogen (page 160) for gene expression profiling. Each of the BioModule™ Units for gene expression profiling includes high-quality reagents and validated protocols with relevant controls for each step of the workflow (see next page). Each unit is designed to provide an integrated workflow that allows you to perform various steps seamlessly during expression analysis.

Gene expression profiling comprises multiple steps employing various technologies such as microarray analysis or quantitative PCR (qPCR) for analysis at the nucleic acid level; western immunodetection and immunohistochemistry for analysis at the protein level; and RNAi for functional analysis.

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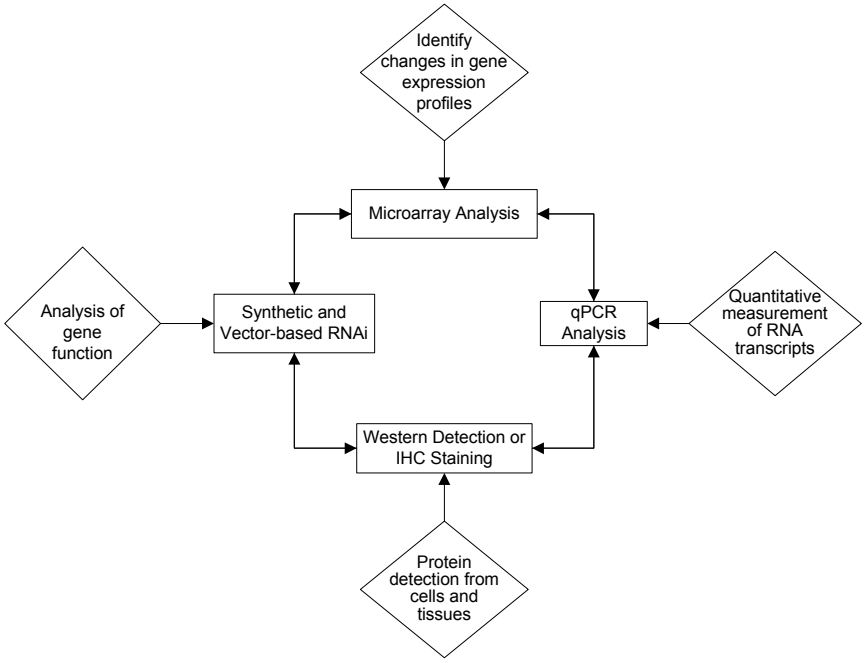
## Gene Expression Profiling Workflow

The gene expression profiling is shown below.

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### System Components

The BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector includes:

- pcDNA™6.2-GW/EmGFP-miR expression vector for production of an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA sequence for expression in mammalian cells using an RNA Polymerase II (Pol II) promoter, the human cytomegalovirus (CMV) immediate early promoter.
- Reagents for production of an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA (need to order oligos separately).
- Positive and negative controls for the generation and use of the expression clone.
- Reagents for plasmid DNA purification and Lipofectamine™ 2000 Reagent for efficient transfection into mammalian cells.

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

- A BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector for production of an expression clone containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA sequence (see above).
- The pDONR™221 vector is used as an intermediate to transfer the pre-miRNA expression cassette (see page 11 for details) into the lentiviral expression plasmid (see below) using Gateway® Technology.
- A pLenti6/V5-DEST destination vector into which the pre-miRNA cassette from the expression clone is transferred using Gateway® Technology (see below). This expression plasmid contains elements that allow packaging of the construct into virions and the Blasticidin resistance marker for selection of stably transduced cell lines.
- Gateway® BP and LR Clonase™ II Enzyme Mixes that facilitate the transfer of the pre-miRNA expression cassette from the expression vector into the pLenti6/V5-DEST destination vector.
- Components of the ViraPower™ Lentiviral System for production of a replication-incompetent lentivirus that stably expresses the miRNA of interest in both dividing and non-dividing mammalian cells.
- Reagents for lentiviral plasmid DNA purification and Lipofectamine™ 2000 Reagent for efficient transfection into mammalian cells.

For more information about the BLOCK-iT™ Pol II miR RNAi Technology, ViraPower™ Lentiviral Technology, and Gateway® Technology, see page 5.

For details on system components, see page 12.

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## Advantages of the BioModule™ BLOCK-iT™ RNAi Units

Use of the BioModule™ BLOCK-iT™ RNAi Units for vector-based expression of miRNA provides the following advantages:

- The BLOCK-iT™ Pol II miR RNAi Expression Vector provides a rapid and efficient way to clone a ds oligo duplex encoding a desired miRNA target sequence into a vector containing a Pol II promoter for use in RNAi analysis.
- Gateway®-adapted vector for easy transfer of the miRNA of interest from one expression vector (pcDNA™6.2-GW/EmGFP-miR) into other destination vectors.
- Enables co-cistronic expression of the pre-miRNA of interest with EmGFP to allow visualization of cells expressing the miRNA.
- Efficiently delivers the miRNA of interest to mammalian cells in culture or *in vivo*.

**Lentiviral System**

- Generates a replication-incompetent lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential of RNAi applications beyond those of other traditional retroviral systems (Naldini, 1998).
- Provides stable, long-term expression of the miRNA of interest beyond that offered by traditional adenoviral-based systems.
- Produces a pseudotyped virus with a broadened host range (Yee, 1999).
- Includes multiple features designed to enhance the biosafety of the system.

## The BLOCK-iT™ Pol II miR RNAi Technology

The BLOCK-iT™ Pol II miR RNAi Technology is a next generation RNAi technology employing miRNA expression vectors that allow flexible expression of miRNA-based knockdown cassettes driven by RNA Polymerase II (Pol II) promoters in mammalian cells.

The BLOCK-iT™ Pol II miR RNAi Expression Vector is specially designed to allow expression of miRNA sequences and contain specific miR flanking sequences that allow proper processing of the miRNA. The expression vector design is based on the miRNA vector system developed in the laboratory of David Turner (U.S. Patent Publication No. 2004/0053876) and includes the use of endogenous murine miR-155 flanking sequences (see page 10 for details).

A variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis in mammalian and invertebrate systems. For more information about any of the BLOCK-iT™ RNAi products, see the RNAi Central application portal at www.invitrogen.com/rnai or contact Technical Service (page 163).

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### ViraPower™ Lentiviral Technology

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

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

For more information about these components, see page 12. For more information about the biosafety features of the system, see page 28.

### Gateway® Technology

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

To express your miRNA of interest in mammalian cells using the BioModule™ BLOCK-iT™ RNAi Units and Gateway® Technology, simply:

1. Clone a double-stranded oligonucleotide encoding your miRNA sequence of interest into the pcDNA™6.2-GW/EmGFP-miR expression vector to create an expression clone. Transfect this expression clone directly into mammalian cells for initial screening, if desired.
2. To transfer your pre-miRNA expression cassette into a destination vector including pLenti6/V5-DEST vector, 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.
3. Then perform an LR recombination reaction between the resulting entry clone (pENTR™221/miR) and any destination vector including pLenti6/V5-DEST. See page 80 for more details.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available from our web site (www.invitrogen.com) or by contacting Technical Service (page 163).

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### Purpose of this Manual

This manual provides an overview of the pathway by which miRNA facilitates gene knockdown in mammalian cells and includes instructions and guidelines to:

- Design the appropriate single-stranded oligonucleotides representing the target gene.
- Clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR expression vector.
- Purify high-quality plasmid DNA for mammalian transfection.
- Transfect your pcDNA™6.2-GW/EmGFP-miR construct into mammalian cells for transient RNAi analysis or to generate stable cell lines.
- Perform a Rapid BP/LR recombination reaction with a destination vector of choice to generate an expression clone containing the pre-miRNA sequence of interest.

Lentiviral expression

- Co-transfect the pLenti6/V5-GW/EmGFP-miR expression construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
- Titer the lentiviral stock.
- Transduce the lentiviral construct into mammalian cells and perform "transient" RNAi analysis
- Generate a stably transduced cell line, if desired.

Sections on troubleshooting and examples of expected results are also included.

# Using miRNA for RNAi Analysis

## Introduction

RNA interference (RNAi) describes the phenomenon by which short, homologous RNA duplexes induce potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA). RNAi functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et al.*, 1994; Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990) and quelling in fungi (Cogoni & Macino, 1997; Cogoni & Macino, 1999; Romano & Macino, 1992).

In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li & Ding, 2001; Voinnet *et al.*, 1999). In experimental settings, RNAi is widely used to silence genes through transfection of RNA duplexes or introduction of vector-expressed short hairpin RNA (shRNA).

## The RNAi Pathway

In eukaryotic organisms, dsRNA produced *in vivo*, introduced by pathogens, or through research, is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001).

Each siRNA then incorporates into an RNA-induced silencing complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation, or translational repression (Hammond *et al.*, 2000; Nykanen *et al.*, 2001). MicroRNAs (miRNAs) are endogenous RNAs that trigger gene silencing (Ambros, 2001; Carrington & Ambros, 2003).

## miRNA Pathway

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

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

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### Translational Repression versus Target Cleavage

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

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

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

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

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

To overcome the limitations with siRNA and shRNA, we have developed Gateway®-adapted expression vectors that enable the expression of engineered miRNA sequences from Pol II promoters. The pcDNA6.2™-GW/EmGFP-miR expression vector (supplied in the BioModule™ Unit with BLOCK-iT™ Pol II miR RNAi Expression Vector) facilitates the generation of an expression clone containing a ds oligo encoding a pre-miRNA sequence (page 10). The resulting expression construct may be introduced into dividing mammalian cells for transient expression of the miRNA sequence and initial RNAi screening, if desired. Once initial screening is complete, the pre-miRNA sequence may then be easily and efficiently transferred into the pLenti6/V5-DEST vector (or other suitable destination vector) by Gateway® recombination reactions (page 23).

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# Using miRNA for RNAi Analysis, Continued

## miRNA Vector

The pcDNA™6.2-GW/EmGFP-miR Expression Vector included with the unit 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 Vector-Based Systems

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

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

## Human CMV Promoter

The pcDNA™6.2-GW/EmGFP-miR expression vector contains the human cytomegalovirus (CMV) immediate early promoter to allow high-level, constitutive miRNA expression in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).

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

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

**Note:** Although highly active in most mammalian cell lines, activity of the viral CMV promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

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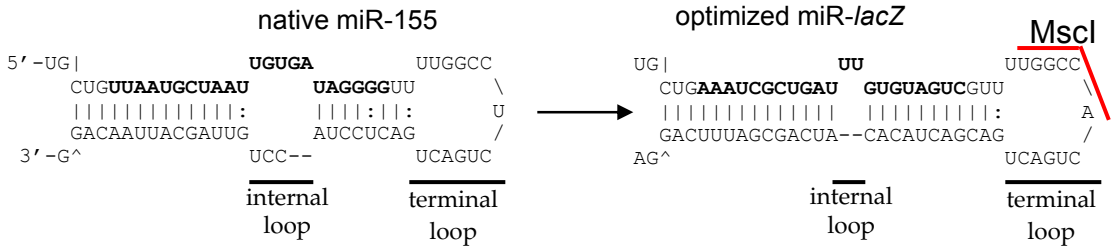
## Design of the Engineered Pre-miRNA

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




## Structure of the Engineered pre-miRNA

The pcDNA™6.2-GW/EmGFP-miR expression vector is 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, see above.

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

- Two 4 nucleotide, 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 page 37.

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### Pre-miRNA Expression Cassette

The engineered pre-miRNA sequence is cloned into the cloning site of the BLOCK-iT™ Pol II miR RNAi Expression Vector that is flanked on either side with sequences from murine miR-155 to allow proper processing of the engineered pre-miRNA sequence (see page 38 for the flanking region sequences).

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


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

**Note:** The 21 nucleotides are derived from the target sequence while the 3′ most nucleotide is derived from the native miR-155 sequence (see figure on page 41).

### Chaining of miRNAs

miRNAs are sometimes expressed in clusters in long primary transcripts driven by RNA Pol II (Lee *et al.*, 2004). Our vectors support chaining of miRNAs to express multiple miRNAs in one primary transcript, thus ensuring co-cistronic expression of multiple miRNAs. See page 57 for details.

# BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector

## Description of the System

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

- The pcDNA™6.2-GW/EmGFP-miR linearized plasmid into which a ds oligo encoding the pre-miRNA is cloned to generate an expression clone that contains the elements required for expression of the miRNA in mammalian cells. The pcDNA™6.2-GW/EmGFP-miR vector is supplied linearized with 4-nucleotide 5′ overhangs on each strand to facilitate directional cloning of the ds oligo insert. The resulting expression clone containing the pre-miRNA expression cassette may be transfected into mammalian cells for transient or stable RNAi analysis, or used to transfer the pre-miRNA expression cassette into a suitable destination vector using Gateway® Technology.
- T4 DNA Ligase and an optimized ligation buffer to allow 5-minute room temperature ligation of the ds oligo insert into the pcDNA™6.2-GW/EmGFP-miR vector.
- One Shot® TOP10 Chemically Competent *E. coli* for high efficiency transformation of the ligation reaction.
- PureLink™ HQ Mini Plasmid Purification Kit for high-quality plasmid DNA purification suitable for transfection into mammalian cells.
- Lipofectamine™ 2000 Reagent and Opti-MEM® Reduced Serum Medium for efficient transfection of your construct into mammalian cells.
- E-Gel® agarose gels and DNA ladders for checking integrity of the ds oligo by gel electrophoresis.

## Controls

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

- The pcDNA™6.2-GW/EmGFP-miR-neg control plasmid contains an insert that can form a hairpin structure that is processed into mature miRNA, but is predicted not to target any known vertebrate gene. Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with pcDNA™6.2-GW/EmGFP-miR expression vector. The negative control sequence without 5′ overhangs is shown below (page 144 for a map):

```
5′-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′
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- The miR-*lacZ* positive double-stranded (ds) control oligo serves as a positive control during the miRNA expression vector generation. Use this oligo to generate a pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression clone.
- Co-transfecting the resulting pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression clone targeting the *lacZ* gene and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into mammalian cells provide a means to assess the RNAi response in your cell line by assaying for knockdown of β-galactosidase.

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### Generating an miRNA Expression Vector Using the Kit

Using the reagents supplied in the unit, you will perform the following steps to generate an expression clone in pcDNA™6.2-GW/EmGFP-miR:

1. Design and synthesize two complementary single-stranded DNA oligonucleotides, with one encoding the miRNA of interest.
2. Anneal the single-stranded oligonucleotides to generate a double-stranded oligo (ds oligo).
3. Clone the ds oligo into the linearized pcDNA™6.2-GW/EmGFP-miR vector.
4. Transform the ligation reaction into One Shot® TOP10 Chemically Competent *E. coli* and select for spectinomycin-resistant transformants.
5. Use the pcDNA™6.2-GW/EmGFP-miR expression construct for transient RNAi analysis in mammalian cells, isolate stable cell lines expressing the miRNA, or perform a Gateway® recombination reaction with a suitable Gateway® destination vector to generate a different expression clone.

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

The pcDNA™6.2-GW/EmGFP-miR Vector contains the following features:

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

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### Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 is a proprietary formulation for the delivery of plasmid DNA and RNAi oligomers to mammalian cells for RNAi analysis (Gitlin *et al.*, 2002; Yu *et al.*, 2002). Using Lipofectamine™ 2000 to transfect eukaryotic cells offers the following advantages:

- Highest transfection efficiency in many cell types and formats (*e.g.* 96-well). Refer to the Cell Lines database at www.invitrogen.com for a list of cell types successfully transfected.
- Nucleic acid-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium, in the presence or absence of serum.
- It is not necessary to remove complexes or change/add medium after transfection, but complexes may be removed after 4-6 hours.

### Opti-MEM® I Reduced Serum Medium

Opti-MEM® I Reduced Serum Medium is a versatile, chemically-defined medium used for diluting the lipid and nucleic acid during transfection.

The Opti-MEM® I Reduced Serum Medium is a multi-purpose medium proven to be useful in reducing serum requirements for a wide variety of cell lines and applications and has been effective in the growth and maintenance of adherent and non-adherent cell lines. When supplemented with 2-4% fetal bovine serum or alternative sera, Opti-MEM® I Reduced Serum Medium supports proliferative rates and maximal cell densities comparable to, and in some cases superior to, conventional media supplemented with 10% fetal bovine serum. Relatively non-fastidious cell lines may be maintained in long-term culture with even more substantial serum reduction. If using adherent cell lines and less than 2% serum supplementation or in an agitated system such as in roller bottles, the medium should be further supplemented with 100 mg/l $CaCl_2$.

The versatility of Opti-MEM® I Reduced Serum Medium in the propagation of various cell types makes this medium the optimal choice for many cell culture requirements. For details on using the medium for cell culture, download the Opti-MEM® I Reduced Serum Medium manual from www.invitrogen.com.

### PureLink™ HQ Mini Plasmid Purification Kit

The PureLink™ HQ Mini Plasmid Purification Kit is designed for the isolation of high-quality plasmid DNA that is suitable for restriction enzyme digestion, PCR, sequencing, bacterial cell transformation, and mammalian cell transfection. Use the kit to isolate high-quality plasmid DNA for mammalian transfection.

Using the kit, plasmid DNA can be isolated from varying amounts of bacterial cells. The high quality of the isolated plasmid DNA is demonstrated by its low genomic DNA contamination, high supercoiled to nicked forms ratio, and reliable performance in demanding downstream applications such as mammalian cell transfection.

Bacterial cells are lysed, the lysate is then neutralized and conditions are adjusted for subsequent binding. After clarification by centrifugation, the lysate is processed through the PureLink™ spin column. The DNA binds to the silica-based membrane in the column, and impurities are removed by a single wash step. The DNA is then eluted in Elution Buffer or water.

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## E-Gel® System

The E-Gel® agarose gel electrophoresis system is a complete bufferless system for agarose gel electrophoresis of DNA samples. Use the E-Gel® agarose system to verify the integrity of your ds oligos after annealing and prior to cloning the ds oligo into the pcDNA™6.2-GW/EmGFP-miR Expression Vector.

The major components of the E-Gel® system are:

- E-Gel® pre-cast agarose gels
- Electrophoresis base

E-Gel® pre-cast agarose gels are self-contained gels that include electrodes packaged inside a dry, disposable, UV-transparent cassette. Each gel contains an ion generating system (TAE buffer system), a pH balancing system, and ethidium bromide for DNA staining. The E-Gel® agarose gels run in a specially designed electrophoresis base, E-Gel® PowerBase™ v.4.

The E-Gel® PowerBase™ v.4 is a base and a power supply in one device. The E-Gel® PowerBase™ connects directly to an electrical outlet using the adaptor supplied with the base.

## E-Gel® System Specifications

**E-Gel®**

| | |
|---|---|
| Cassette Dimensions: | 8 cm × 10 cm x 0.6 cm thick |
| Gel Thickness: | 3 mm |
| Gel Volume: | 20 ml |
| No. of Wells: | 12 |
| Run length: | 5.8 cm |
| Sample volume: | 20 µl |
| Separation Range: | 100 bp-5 kb (1.2% gel) |
| | 20-500 bp (4% gel) |

Each well is 4.1 mm wide and the space between wells is 1 mm.

**E-Gel® PowerBase™ v.4**

| | |
|---|---|
| Dimensions: | 12.5 cm x 13 cm x 13.5 cm |
| Weight: | 1.19 lbs (540 g) with adaptor |
| Safety: | UL listed and CE certified |
| Temperature: | Ambient 15°C to 40°C |
| Built-in Features: | Alarm, light LED |

The E-Gel® PowerBase™ v.4 is designed for use with an adaptor included with the PowerBase™. Use only UL Listed Class 2 Direct Plug-in Adaptor included with the PowerBase™. Input and Output supplied by the adaptor are shown in the table below.

| Country | Input | Output |
|---|---|---|
| U.S. and Canada | 110-120 V AC, 60 Hz | 12 V DC, 880 mA |
| Europe | 220-240 V AC, 50 Hz | 12 V DC, 880 mA |

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### TrackIt™ DNA Ladders

The TrackIt™ 10 bp and 1 Kb Plus DNA Ladders are suitable for sizing double-stranded DNA fragments on agarose gels. The TrackIt™ DNA Ladders are formulated with unique tracking dyes, Xylene Cyanol FF (XCFF), tartrazine, or Orange G, that allow you to visually track DNA migration during electrophoresis and also indicate when maximum resolution is achieved.

The important features of the ladder are listed below:

- **10 bp** Ladder consists of 33 fragments ranging in the size of 10-200 bp and an additional fragment at 1668 bp
- **1 Kb Plus** Ladder consists of 12 bands in 1000 bp increments ranging in size from 1-12 kb, a 1650 bp fragment, and vector DNA fragments from 100-850 bp
- **10 bp Ladder** includes a 100 bp reference band that is ~2-fold brighter for easy band size determination
- **1 Kb Plus Ladder** includes an orientation doublet at 1650 bp and 2000 bp for easy reference
- Formulated with unique tracking dyes
- Designed for use with E-Gel® agarose gels and TBE or TAE agarose gels
- Supplied in a ready-to-load format
- Visualized with ethidium bromide or SYBR® Green staining

### Spectinomycin

Spectinomycin is an antibiotic isolated from *Streptomyces spectabilis* which inhibits protein synthesis (elongation) by interfering with peptidyl tRNA translocation in prokaryotic cells. Resistance is conferred by expression of the adenylyltransferase *aad*A gene that prevents binding of Spectinomycin.

The formula for Spectinomycin (dihydrochloride pentahydrate) is $C_{14}H_{24}N_2O_7.2HCl.5H_2O$ and the molecular weight is 495.35. Recommended concentration for selection in bacteria is 50 µg/ml.

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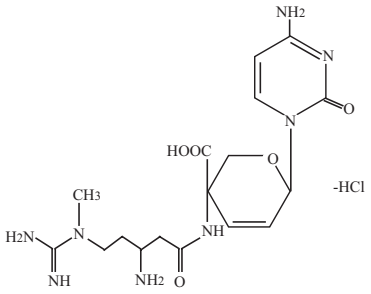
## Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991). See page 134 for handling and preparing Blasticidin.

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. Recommended concentration is 100 µg/ml for bacterial cells and 2-10 µg/ml for mammalian cells. The structure of Blasticidin is shown below.




## LB Broth (1X)

The LB Broth is supplied ready-to-use as a liquid at 1X concentration. The formulation per one liter at 1X: 10 g SELECT Peptone 140, 5 g SELECT Yeast Extract, 5 g sodium chloride. Add the desired antibiotic at the required concentration to the LB Broth and use for growth of bacterial cultures.

**Note:** The resulting LB Broth is low salt LB medium suitable for use with Blasticidin as well as other antibiotics.

# BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System

## Introduction

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

## Components of the System

The system includes the following major components

- The BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector containing the pcDNA™6.2-GW/EmGFP-miR expression vector for production of an expression clone that allows expression of your miRNA sequence of interest in mammalian cells using a Pol II promoter.
- The pLenti6/V5-DEST vector allows easy transfer of the pre-miRNA expression cassette from the expression clone into a lentiviral destination vector for use with the lentiviral system components. The destination vector contains the elements required to allow packaging of the expression construct into virions (*e.g.* 5′ and 3′ LTRs, ψ packaging signal) and a selectable marker to allow generation of stable cell lines. For more information about the pLenti6/V5-DEST vector, see page 147.
- The pDONR™221 vector is used as an intermediate to transfer the pre-miRNA expression cassette into the lentiviral expression plasmid (see above) using Gateway® Technology. See page 25 for details on pDONR™221 vector.
- Gateway® BP and LR Clonase™ II Enzyme Mixes that allow the transfer of the pre-miRNA expression cassette from the expression vectors into the pLenti6/V5-DEST vector using the Rapid BP/LR recombination reaction.
- One Shot® Stbl3™ Competent *E. coli* to obtain optimal results with lentiviral DNA after transformation.
- The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see pages 152-156.
- 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, see page 22.
- PureLink™ HQ Mini Plasmid Purification Kit for high-quality plasmid DNA purification suitable for transfection into mammalian cells and S.N.A.P.™ MidiPrep Kit for plasmid DNA purification from lentiviral vectors.
- Lipofectamine™ 2000 Reagent and Opti-MEM® Reduced Serum Medium for efficient transfection of your construct into mammalian cells.
- E-Gel® agarose gels and DNA ladders for checking integrity of the ds oligo by gel electrophoresis and for analysis of restriction digests to validate lentiviral expression clones.

*Continued on next page*

## System Overview

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

## VSV Envelope Glycoprotein

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

## Features of the pLenti6/V5-DEST Vector

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

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

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

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# BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System, Continued

### Gateway® Clonase™ II Enzyme Mixes

The BP and LR Clonase™ II 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 LR Clonase™ II Enzyme catalyzes the *att*L x *att*R Gateway® recombination reaction while the BP Clonase™ II Enzyme catalyzes the *att*B x *att*P Gateway® recombination reaction. Use the protocol provided on page 80 to perform the recombination reactions using the Rapid protocol or page 133 using the standard protocol.

BP and LR Clonase™ II Enzyme Mixes are supplied with the kit or available separately from Invitrogen.

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

The Stbl3™ *E. coli* strain is derived from the HB101 *E. coli* strain and is recommended for use when cloning unstable inserts such as lentiviral DNA containing direct repeats. The transformation efficiency of One Shot® Stbl3™ Chemically Competent cells is greater than 1 x $10^8$ cfu/µg DNA.

**Note:** One Shot® Stbl3™ cells require IPTG to induce expression from the *lac* promoter. If blue/white screening is required to select for transformants, make sure that selective plates contain 50 µg/ml X-gal and 1 mM IPTG.

### ViraPower™ Packaging Mix

The ViraPower™ Packaging Mix contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see pages 152-156.

### S.N.A.P.™ MidiPrep Kit

The S.N.A.P.™ MidiPrep Kit allows isolation of highly pure plasmid DNA that is suitable for transfection, manual or automated sequencing, PCR, restriction mapping, ligation, and transformation. The S.N.A.P.™ resin is supplied in a column and preferentially binds supercoiled plasmid DNA. Use the S.N.A.P.™ MidiPrep Kit to prepare plasmid DNA from lentiviral vectors.

*Continued on next page*

## Antibiotics

**Kanamycin Sulfate**

Kanamycin is effective as a bacteriocidal agent by inhibiting ribosomal translocation and eliciting miscoding. Resistance is conferred by the KanR-*Tn*5 gene product (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents interaction with ribosomes.

The formula for Kanamycin Sulfate is $C_{18}H_{36}N_4O_{11}.H_2SO_4$ and the molecular weight is 583. Recommended concentration for use is 50 µg/ml.

**Ampicillin**

Ampicillin is a semi-synthetic penicillin derived from the penicillin nucleus, 6-amino-penicillanic acid and causes cell death by inhibiting cell wall biosynthesis. Resistance is conferred by β-lactamase cleavage of the β-lactam ring (*bla* gene).

The formula for Ampicillin is $C_{16}H_{18}N_3O_4S_2Na$ and molecular weight is 371.4. Recommended concentration for use is 100 µg/ml.

For details on Spectinomycin and Blasticidin, see page 16.

**Note**

For details on PureLink™ HQ Mini Plasmid Purification Kit, Lipofectamine™ 2000 Reagent, Opti-MEM® Reduced Serum Medium, LB Broth, E-Gel® agarose gels, and DNA ladders, see page 12.

# 293FT Cell Line

## Introduction

The 293FT Cell Line is derived from the 293F Cell Line (see below) and stably expresses the SV40 large T antigen from the pCMVSPORT6TAg.neo plasmid. Expression of the SV40 large T antigen is controlled by the human cytomegalovirus (CMV) promoter and is high-level and constitutive. For more information about pCMVSPORT6TAg.neo, see page 158.

## Use of the Cell Line

Studies have demonstrated maximal virus production in human 293 cells expressing SV40 large T antigen (Naldini *et al.*, 1996), making the 293FT Cell Line a particularly suitable host for generating lentiviral constructs using the ViraPower™ Lentiviral Expression System including BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System.

## Parental Cell Lines

The 293 Cell Line is a permanent line established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA (Graham *et al.*, 1977; Harrison *et al.*, 1977). The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein.

The 293-F Cell Line available from Invitrogen (Catalog no. 11625) is a fast-growing variant of the 293 cell line, and was originally obtained from Robert Horlick at Pharmacopeia.

## Antibiotic Resistance

293FT cells stably express the neomycin resistance gene from pCMVSPORT6TAg.neo and should be maintained in medium containing 500 µg/ml Geneticin®. Expression of the neomycin resistance gene in 293FT cells is controlled by the SV40 enhancer/promoter. Geneticin® is available separately from Invitrogen (page 160).

# Gateway® Recombination Reactions

## Introduction

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

Review the information in this section to briefly familiarize yourself with the Gateway® recombination reactions. For details, refer to the Gateway® Technology with Clonase™ II manual available from our web site at www.invitrogen.com or by contacting Technical Service (page 163).

## Gateway® Vectors

Each of the vectors supplied in the BioModule™ BLOCK-iT™ RNAi Units is Gateway®-adapted *i.e.* contains the appropriate *att* sites that allow site specific recombination to facilitate the transfer of heterologous DNA sequences between vectors.

## Recombination Reactions

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

**BP Reaction**

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

**LR Reaction**

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

**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 this page before proceeding with the experiment.

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

*Continued on next page*

# Gateway® Recombination Reaction, Continued

## Pre-miRNA Expression

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

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

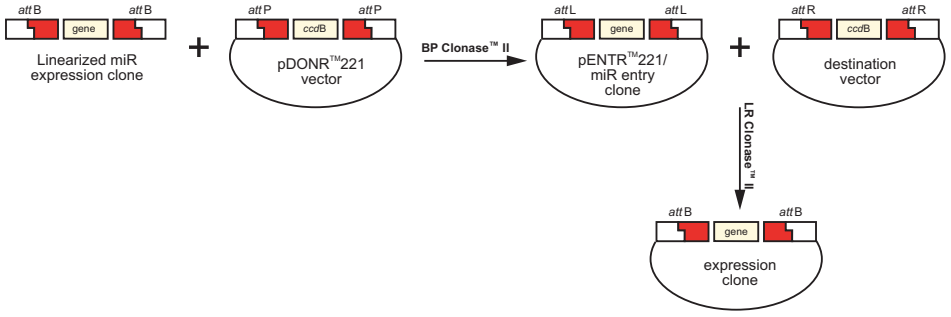
1. Generate an entry clone by performing a BP recombination reaction between the *att*B substrate (pcDNA™6.2-GW/EmGFP-miR expression clone) and *att*P substrate (pDONR™221 vector) using BP Clonase™ II Enzyme Mix.
2. Perform an LR recombination reaction between the resulting entry clone (*att*L substrate)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

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

## Rapid BP/LR Recombination Reaction

To develop a faster Gateway® recombination reaction protocol that transfers the pre-miRNA expression cassette 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 a day. In the Rapid BP/LR Recombination Reaction, instead of isolating the entry clone after BP reaction, the completed BP reaction is transferred directly into the LR reaction to generate expression clones within a day.

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




*Continued on next page*

### Features of pDONR™221

The pDONR™221 vector (included with Cat. no. WFGE08) contains the following elements:

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

For a map of pDONR™221, see page 150.

# Green Fluorescent Protein

## Description

The pcDNA™6.2-GW/EmGFP-miR expression vector 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 a destination vector, you may produce lentiviruses that simultaneously express the EmGFP protein and miRNA, allowing you to visually track the cells in which knockdown is occurring or sort the cells using a flow cytometer.

## Green Fluorescent Protein (GFP)

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

## GFP and Spectral Variants

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

**Note**

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

*Continued on next page*

# Green Fluorescent Protein, Continued

## EmGFP

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

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

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

## EmGFP Fluorescence

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

| Excitation (nm) | Emission (nm) |
|---|---|
| 487 | 509 |

## Filter Sets for Detecting EmGFP Fluorescence

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

| Filter Set | Manufacturer |
|---|---|
| Omega XF100 | Omega<br>(www.omegafilters.com) |

# Biosafety Features of the Lentiviral System

## Introduction

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

## Biosafety Features of the BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System

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

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

*Continued on next page*

# Biosafety Features of the System, Continued

### Biosafety Level 2


Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this System can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus that express miRNA 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", 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded from the Web at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

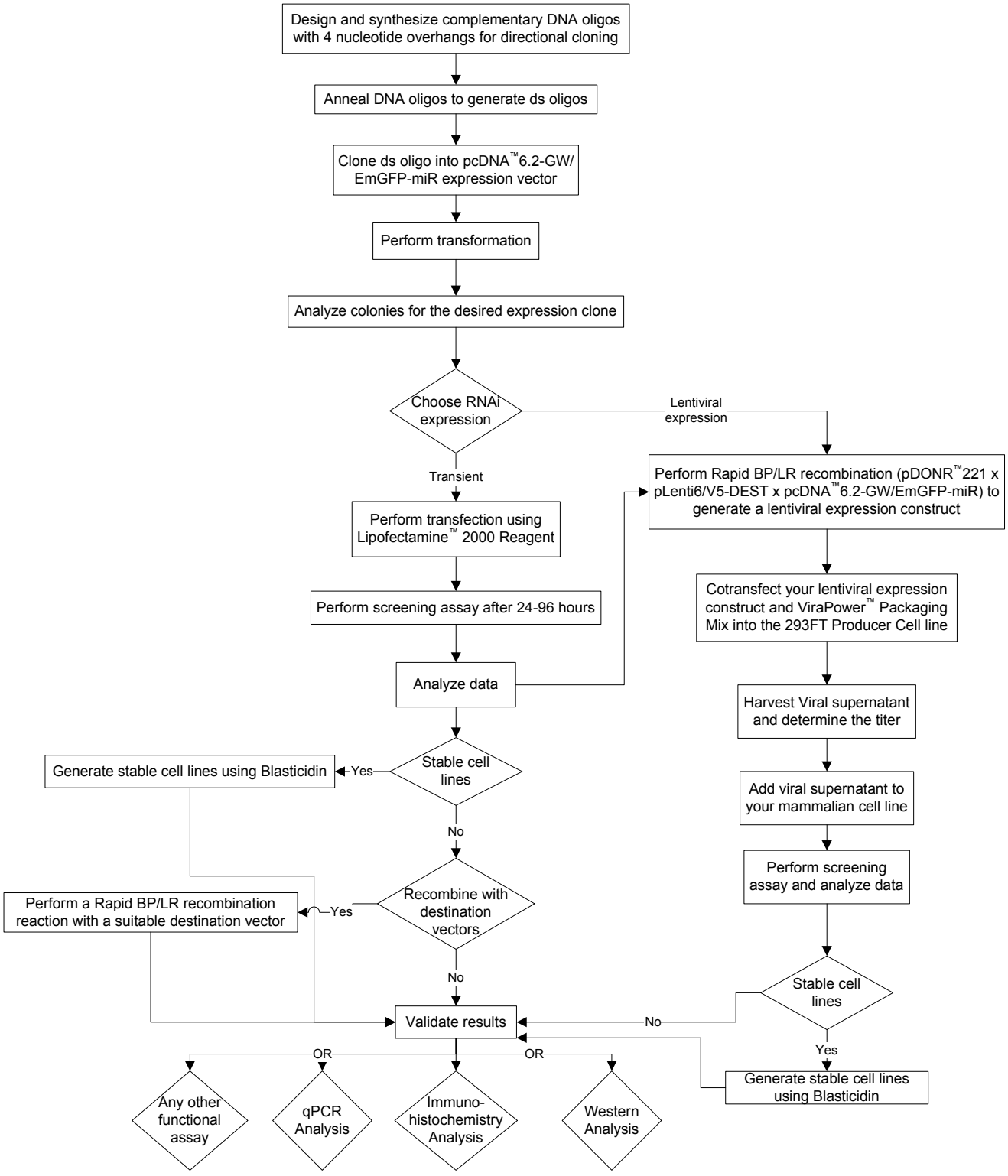
**Important**

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

# Experimental Outline

## Workflow

The experimental workflow for using BioModule™ BLOCK-iT™ RNAi Units is shown below.

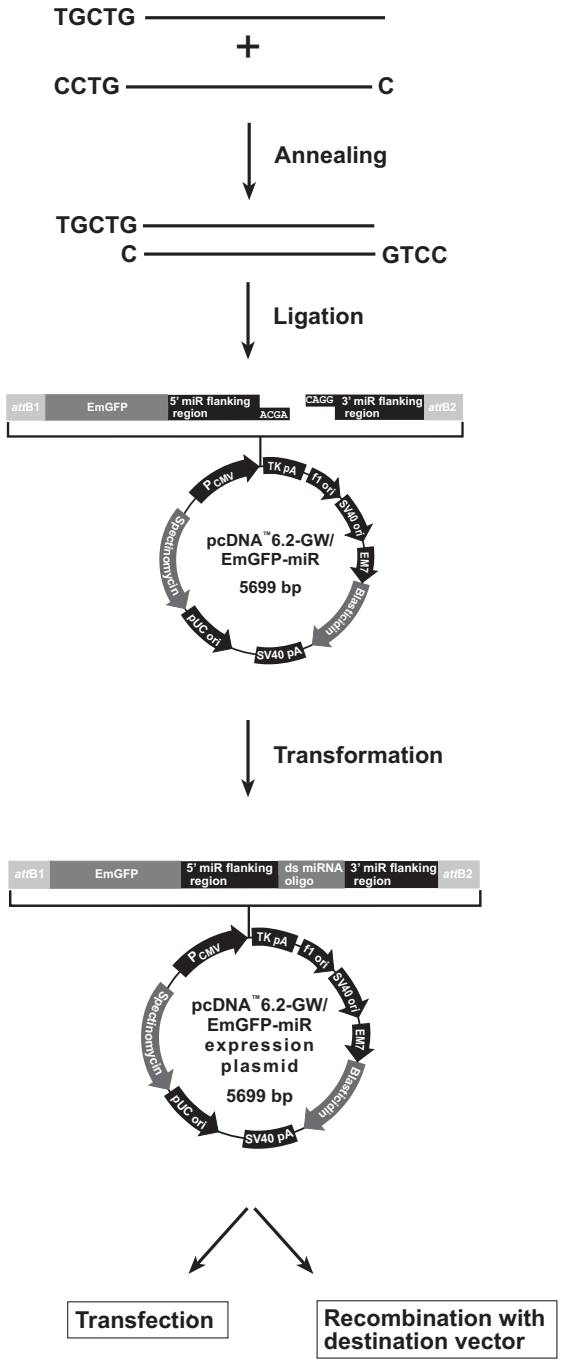



*Continued on next page*

# Experimental Outline, Continued

## Flow Chart for Cloning and Expression

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



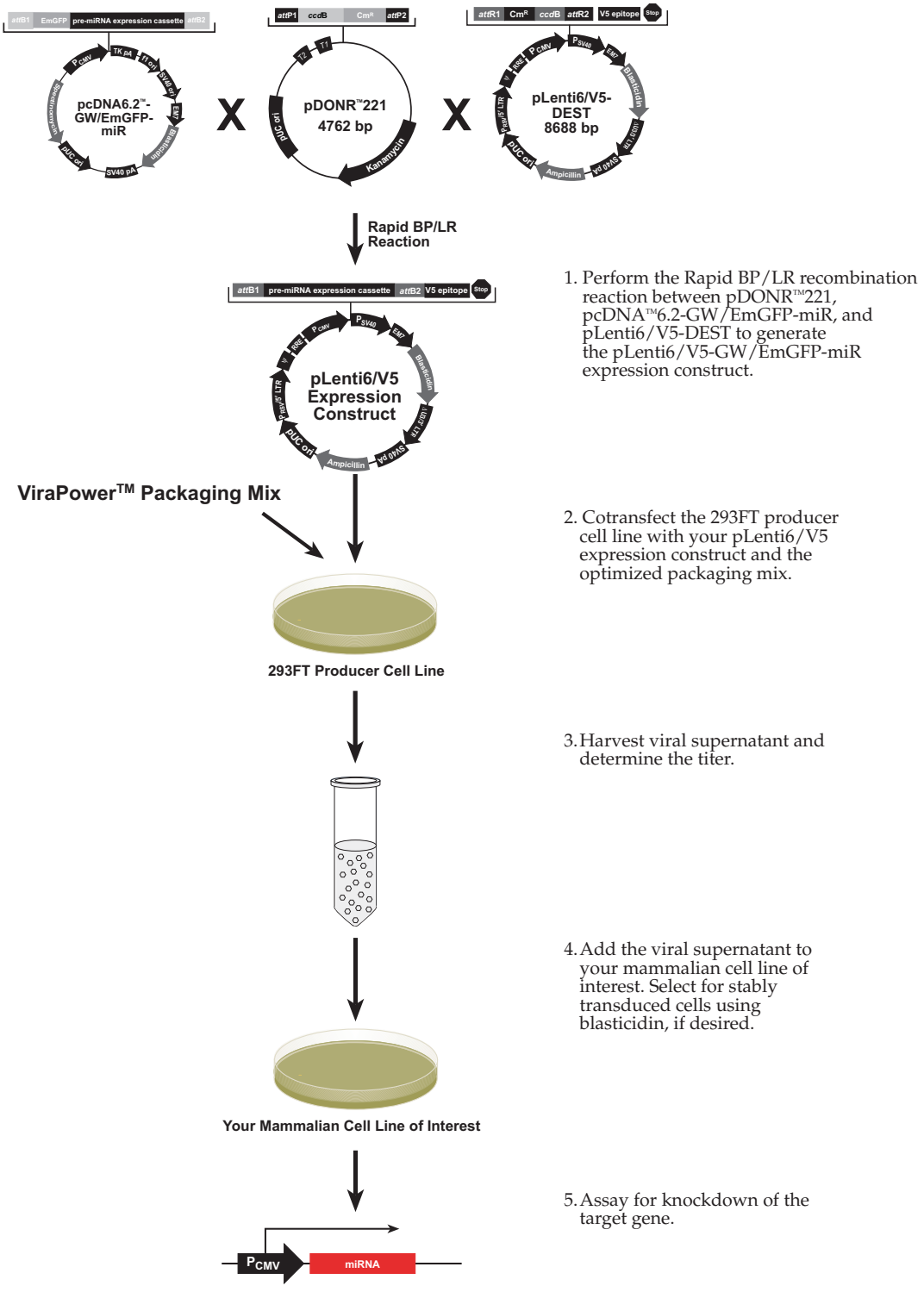

1. Design and synthesize complementary DNA oligos, with each containing 4 nucleotide overhangs necessary for directional cloning.
2. Anneal DNA oligos to generate a ds oligo.
3. Clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR expression vector using T4 DNA Ligase.
4. Transform *E. coli* and analyze colonies for the desired expression clone.
5. Transfect the expression clone for transient or stable RNAi analysis or recombine with a suitable destination vector to generate a different expression clone (through a donor vector intermediate).

*Continued on next page*

# Experimental Outline, Continued

## Flow Chart for Lentiviral Expression

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




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

*Continued on next page*

# Experimental Outline, Continued

## Materials Needed

Materials supplied with the BioModule™ BLOCK-iT™ RNAi Units and User Supplied materials are listed below. Ordering information is on page 160.

| Step | Supplied in the kit | User Supplied |
|---|---|---|
| Generating ds Oligo | • 10X Oligo Annealing Buffer<br>• DNase/RNase-Free Water | • Synthesize your "top strand" and "bottom strand" single-stranded oligo (200 µM in water or TE Buffer)<br>• 95°C water bath or heat block |
| Checking the Integrity of ds Oligo | • 4% E-Gel®<br>• E-Gel® PowerBase™<br>• TrackIt™ 10 bp DNA Ladder | *Optional:* Sample Buffer |
| Ligation Reaction | • pcDNA™6.2-GW/EmGFP-miR, linearized<br>• 5X Ligation Buffer<br>• DNase/RNase-Free Water<br>• T4 DNA Ligase (1 U/µl) | Double-stranded oligo of interest |
| Transformation | • One Shot® TOP10 Chemically Competent *E. coli*<br>• S.O.C. Medium and LB Broth<br>• pUC19 positive control<br>• Spectinomycin<br>• Ampicillin | • 42°C water bath<br>• 37°C shaking and non-shaking incubator |
| Plasmid DNA Purification | PureLink™ HQ Mini Plasmid Purification Kit | 64 ml 100% ethanol |
| Transfection | • Lipofectamine™ 2000 Reagent<br>• Opti-MEM® I Reduced Serum Medium | • Mammalian cell line of interest<br>• Appropriate tissue culture plates and supplies |
| Rapid BP/LR Recombination Reaction | • pDONR™221 vector<br>• pLenti6/V5-DEST<br>• BP Clonase™ II enzyme mix<br>• LR Clonase™ II enzyme mix<br>• Proteinase K solution<br>• One Shot® Stbl3™ Competent *E. coli* | • TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)<br>If you are not performing lentiviral expression, then you will need:<br>• Destination vector of choice<br>• Appropriate competent cells |

*Continued on next page*

# Experimental Outline, Continued

| Step | Supplied in the kit | User Supplied |
|---|---|---|
| Lentiviral Plasmid DNA Purification | S.N.A.P.™ MidiPrep Kit | • 240 ml 95% ethanol<br>• Sterile water or TE |
| Lentivirus Production | • ViraPower™ Packaging Mix<br>• 293FT cells<br>• Lipofectamine™ 2000 Reagent<br>• Opti-MEM® I Reduced Serum Medium | • Fetal bovine serum (FBS)<br>• Complete growth medium for 239FT Cell<br>• Appropriate tissue culture plates and supplies |
| Transduction | • Blasticidin | • Mammalian cell line of choice<br>• Complete culture medium for your cell line<br>• 6 mg/ml Polybrene®, if desired<br>• Appropriate tissue culture plates and supplies |

# Methods

## General Information

### Using Appropriate Protocols

This manual supports protocols to express your miRNA sequence in pcDNA™6.2-GW/EmGFP-miR expression vector for RNAi analysis in mammalian cells **and** express your miRNA sequence in mammalian cells with lentivirus-based delivery using the two BioModule™ BLOCK-iT™ RNAi Units.

Be sure to use protocols that are appropriate for the experiment that you wish to perform as shown in the table below:

| Experiment | Choose Protocols on |
|---|---|
| To express your miRNA sequence in pcDNA™6.2-GW/EmGFP-miR | page 36 (cloning miRNA) |
| Perform transient or stable RNAi analysis in mammalian cells | page 60 (transfection and RNAi analysis) |
| Transfer the pre-miRNA expression cassette into a destination vector including pLenti6/V5-DEST | page 74 (transfer pre-miRNA expression cassette to destination vectors) |
| To express your miRNA sequence in dividing or non-dividing mammalian cells using lentivirus-based delivery | page 92 (lentivirus production, transduction, and RNAi analysis) |

**Important**

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

The BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression is designed to help you create a lentivirus to deliver and express an miRNA sequence in mammalian cells for RNAi analysis. 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 on the following topics, refer to published references:

- RNAi pathway and expression of miRNA in mammalian cells: see published references (Brummelkamp *et al.*, 2002; Cullen, 2004; Kim, 2005; McManus & Sharp, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Zeng *et al.*, 2002)
- 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.
- General Molecular Biology handbooks, such as *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

# Cloning miRNA

## Experimental Outline

The experimental steps necessary to clone the miRNA of interest into pcDNA™6.2-GW/EmGFP-miR expression vector are outlined below. We recommend performing the steps as described below to achieve the best results.

| Step | Action | Page no. |
|---|---|---|
| 1 | Designing and synthesizing the single-stranded oligos. | 37 |
| 2 | Anneal the single-stranded oligo to generate the double-stranded oligo. Dilute the oligo to the required concentration. | 43 |
| 3 | Check the integrity of the double-stranded oligo by gel electrophoresis. | 47 |
| 4 | Clone the double-stranded oligo into pcDNA™6.2-GW/EmGFP-miR expression vector. | 51 |
| 5 | Transform the ligation mixture into One Shot® TOP10 Chemically Competent *E. coli.* | 53 |
| 6 | Analyze transformants by sequencing. | 54 |
| 7 | **Optional:** | |
| | Perform chaining of miRNA. | 57 |
| | Remove EmGFP Coding Sequence. | 59 |

# Designing the Single-Stranded DNA Oligos

## Introduction

To use the BioModule™ BLOCK-iT™ RNAi Units, you need to design two single-stranded DNA oligonucleotides; one encoding the target pre-miRNA ("top strand" oligo) and the other its complement ("bottom strand" oligo). You will then anneal the top and bottom strand oligos to generate a double-stranded oligonucleotide (ds oligo) suitable for cloning into the pcDNA™6.2-GW/EmGFP-miR vector.

The design of the single-stranded oligonucleotides (ss oligos) is critical to the success of both the cloning procedure and ultimately, the RNAi analysis. General guidelines are provided in this section to help you choose the target sequence and to design the ss oligos. Note, that simply following these guidelines does not guarantee that the pre-miRNA will be effective in knocking down the target gene. For a given target gene, you may need to generate and screen multiple pre-miRNA sequences to identify one that is active in gene knockdown studies.


We **strongly** recommend using **Invitrogen's RNAi Designer**, an online tool to help you design and order pre-miRNA sequences for any target gene of interest. The RNAi Designer incorporates the guidelines provided in this manual as well as other design rules into a proprietary algorithm to design pre-miRNA sequences that are compatible for use in cloning into pcDNA™6.2-GW/EmGFP-miR. Success rates exceeding 70% have been achieved with the RNAi Designer (i.e. more than 70% of designed miRNAs reduce target gene expression by at least 70%). To use the RNAi Designer, go to www.invitrogen.com/rnaidesigner.

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

*Continued on next page*

# Designing the Single-Stranded DNA Oligos, continued

### Features of Pre-miRNA Insert

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

- 5 nucleotides (TGCTG) derived from the endogenous miR-155, an endogenous murine miRNA that is the basis of the miRNA vector system developed in the laboratory of David Turner (U.S. Patent Publication No. 2004/0053876). This also provides a four nucleotide 5′ overhang, compatible with a 4 nucleotide overhang in the provided linearized pcDNA™6.2-GW/EmGFP-miR to clone the double-stranded oligo.
- Reverse complement of the 21-nucleotide target sequence (mature miRNA sequence). When transcribed, this is the core sequence that will target your gene of interest, and needs to be antisense to the targeted messenger RNA.
- 19 nucleotides derived from miR-155 to form a terminal loop with an engineered *Msc* I site to aid in sequence analysis.
- Nucleotides 1-8 and 11-21 of the sense target sequence. Note that nucleotides 9 and 10 are removed to form a short internal loop in the mature miRNA, which results in more efficient knockdown.
- 4 nucleotides derived from endogenous miR-155. This also constitutes the four nucleotide 5′ overhang, compatible with a 4 nucleotide overhang in the provided linearized pcDNA™6.2-GW/EmGFP-miR to clone the double-stranded oligo.

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

```
UG|              UU            UUGGCC
  CUGAAAUCGCUGAU  GUGUAGUCGUU       \
  ||||||||||||||  ||||||||||:       A
  GACUUUAGCGACUA  CACAUCAGCAG       /
AG^               --            UCAGUC
```

*Continued on next page*

# Designing the Single-Stranded DNA Oligos, continued

## Choosing the Target Sequence

When performing RNAi analysis on a particular gene, your choice of target sequence can significantly affect the degree of gene knockdown observed. We recommend following the guidelines below when choosing your target sequence. These are general recommendations only; exceptions may occur.

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

**Complexity:**

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

| Restriction site | Sequence | Advanced Feature | Page |
|---|---|---|---|
| *Msc* I | TGGCCA | Alternate sequencing protocol | 55 |
| *Bam*H I | GGATCC | miRNA chaining | 57 |
| *Bgl* II | AGATCT | miRNA chaining | 57 |
| *Sal* I | GTCGAC | miRNA chaining | 57 |
| *Xho* I | CTCGAG | miRNA chaining | 57 |
| *Dra* I | TTTAAA | Removal of EmGFP | 59 |

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

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

## Generating the Top Oligo Sequence

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

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

*Continued on next page*

# Designing the Single-Stranded DNA Oligos, continued

## Generating the Bottom Oligo Sequence

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

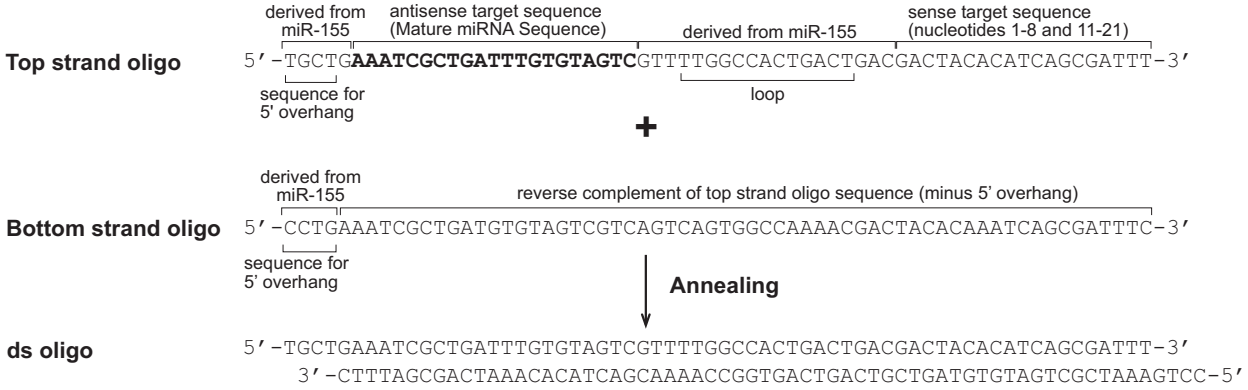
1. Remove 5′ TGCT from top oligo sequence (new sequence starts with G).
2. Take the reverse complement of the sequence from step 1.
3. Add CCTG to the 5′ end of the sequence from step 2.

**Note**

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

## Example of ss Oligo Design

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




**Recommendation**

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

*Continued on next page*

# Designing the Single-Stranded DNA Oligos, continued

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

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

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

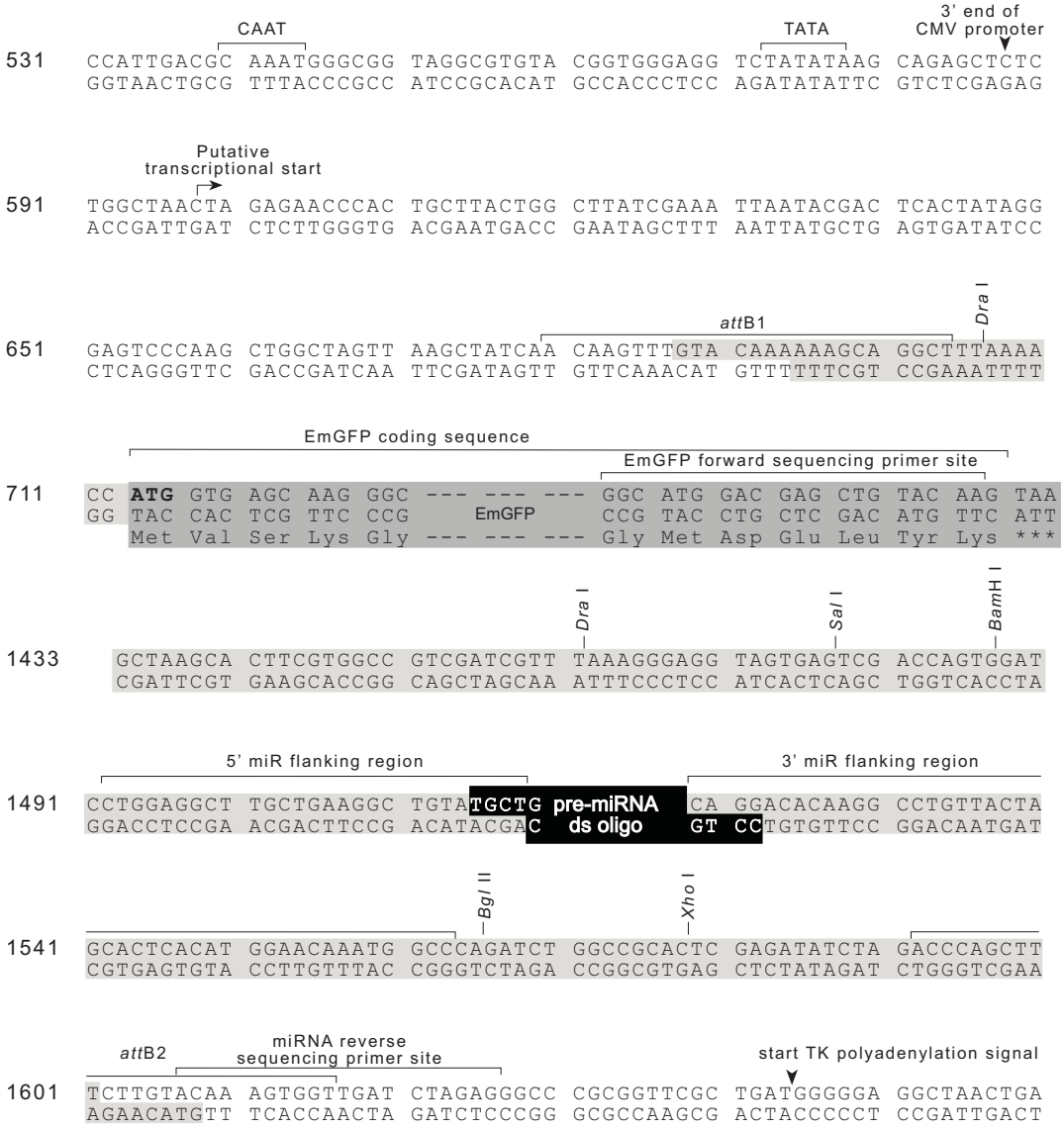
  **Note:** Following recombination with a Gateway® destination vector, the resulting expression clone will contain a pre-miRNA expression cassette consisting of the EmGFP coding sequence, 5′ miR flanking region, miRNA sequence, and the 3′ miR flanking region.

**The complete sequence of pcDNA™6.2-GW/EmGFP-miR is available for downloading from www.invitrogen.com or by contacting Technical Service (page 163).** For a map of pcDNA™6.2-GW/EmGFP-miR, see page 143.

*Continued on next page*

# Designing the Single-Stranded DNA Oligos, continued

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



# Generating the Double-Stranded Oligo

## Introduction

Once you have synthesized the appropriate complementary single-stranded DNA oligos, you will anneal equal amounts of each single-stranded oligo to generate a double-stranded oligo (ds oligo). Guidelines and instructions are provided in this section.

## Experimental Outline

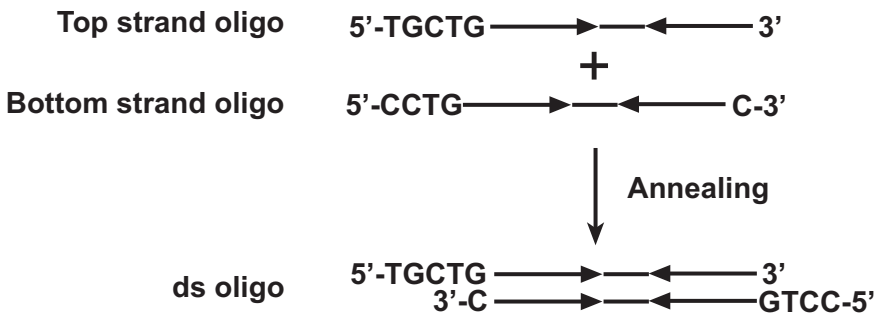
To generate the double-stranded oligo, you will:

1. Calculate the amount of single-stranded oligos needed.
2. Anneal the oligonucleotides to create a double-stranded oligonucleotide.
3. Dilute the double-stranded oligo to a final concentration of 10 nM.

## Single-Stranded Oligos

Before beginning, make sure that you have synthesized the single-stranded oligos with the appropriate sequences required for cloning into the pcDNA™6.2-GW/EmGFP-miR vector and for annealing. See the figure below for an illustration.

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




## Amount of DNA Oligo to Anneal

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

## Resuspending the Oligos

If your single-stranded oligos are supplied lyophilized, resuspend them in water or TE Buffer to a final concentration of 200 µM before use.

*Continued on next page*

# Generating the Double-Stranded Oligo, continued

## Materials Needed

You will need the following materials:

- Your "top strand" single-stranded oligo (200 µM in water or TE Buffer)
- Your "bottom strand" single-stranded oligo (200 µM in water or TE Buffer)
- 10X Oligo Annealing Buffer (supplied with the kit, Box 1)
- DNase/RNase-Free Water (supplied with the kit, Box 1)
- 0.5 ml sterile microcentrifuge tubes
- 95°C water bath or heat block

## Annealing Procedure

Follow this procedure to anneal your single-stranded oligos to generate the ds oligo. Note that the final concentration of the oligo mixture is 50 µM.

1. In a 0.5 ml sterile microcentrifuge tube, set up the following annealing reaction at room temperature.

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

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

*Continued on next page*

# Generating the Double-Stranded Oligo, continued

### Diluting the ds Oligo

To clone your ds oligo into pcDNA™6.2-GW/EmGFP-miR, you **must** dilute the 50 µM stock to a final concentration of **10 nM** (*i.e.* 5,000-fold dilution). We generally perform 100-fold and 50-fold serial dilutions, the first into DNase/RNase-free water and the second into the Oligo Annealing Buffer supplied with the kit. Follow the procedure below to dilute the ds oligo.

1. Dilute the 50 µM ds oligo mixture (from **Annealing Procedure**, Step 5, previous page) 100-fold into DNase/RNase-free water (*i.e.* 1 µl of 50 µM ds oligo into 99 µl of DNase/RNase-free water) to obtain a final concentration of 500 nM. Vortex to mix thoroughly.
2. Dilute the 500 nM ds oligo mixture (from Step 1) 50-fold into Oligo Annealing Buffer as follows to obtain a final concentration of 10 nM.

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

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

**Important**

The undiluted ds oligos are 5,000-fold more concentrated than the working concentration. **When performing the dilutions, be careful not to cross-contaminate the different ds oligo stocks.** Remember to wear gloves and change pipette tips after every manipulation.

### Storing the ds Oligo

Once you have diluted your ds oligo, you should have three stocks of annealed ds oligo. Use each stock as follows:

- **50 µM ds oligo (undiluted):** Use this stock to prepare new diluted ds oligo stocks if existing stocks become denatured or cross-contaminated.
- **500 nM ds oligo (100-fold dilution):** Use this stock for gel analysis (see **Checking the Integrity of the ds Oligo**, page 47).
- **10 nM ds oligo (5,000-fold dilution):** Use this stock for cloning (see **Ligation Procedure**, page 52).

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

*Continued on next page*

**Important**

When using the diluted ds oligo stock solutions (*i.e.* 100-fold or 5,000-fold diluted stocks), thaw the solutions on ice. **Do not** heat or allow the ds oligo solutions to reach greater than room temperature as this causes the ds oligos to melt. The concentration of the oligos in the diluted solutions is not high enough to permit re-annealing and instead favors the formation of intramolecular hairpin structures. These intramolecular hairpin structures **will not clone** into pcDNA™6.2-GW/EmGFP-miR.

If your diluted ds oligo stock solution is heated, discard the ds oligo solution and prepare new diluted stocks using the procedure on the previous page.

**Note:** If the 50 μM ds oligo solution (undiluted stock) becomes heated, the oligos are sufficiently concentrated and may be re-annealed following the annealing procedure on page 44.

# Checking the Integrity of the ds Oligo

## Introduction

You may verify the integrity of your annealed ds oligo using 4% E-Gel® Starter Pak included in the kit (Box 11) for agarose gel electrophoresis. We suggest running an aliquot of the annealed ds oligo and comparing it to an aliquot of each starting single-stranded oligo. **Note:** 4% E-Gel® resolves these fragments much better than regular 4% agarose gels.

## Experimental Outline

To perform agarose gel electrophoresis, you will:

1. Prepare your samples for electrophoresis.
2. Analyze the samples on 4% E-Gel® agarose gels.
3. Visualize the results.

## Materials Needed

You will need the following materials:

- 4% E-Gel® (supplied with the kit)
- E-Gel® PowerBase™ (supplied with the kit)
- TrackIt™ 10 bp DNA Ladder (supplied with the kit)
- Sample Buffer (page 160)


Follow these recommendations to obtain the best results with E-Gel® agarose gels:

- All wells in the gel must contain sample or water. Avoid introducing bubbles while loading, as bubbles will cause bands to distort.
- The One-Step Loading method described on the next page is routinely used to load E-Gel® gels. Use the Two-Step Loading method, if the One-Step Loading method produces fuzzy or indistinct bands, or the gel was removed from its plastic pouch for more than 30 minutes. Refer to the E-Gel® Technical Guide (available from www.invitrogen.com) for details on the Two-Step Loading method.

## Preparing Samples

You may prepare DNA samples for E-Gel® agarose gels in deionized water or loading buffer (recommended final loading buffer concentration is 10 mM Tris-HCl; 1 mM EDTA, pH 7.5; 0.005% bromophenol blue; and 0.005% xylene cyanol FF). If you wish to use 10X BlueJuice™ Gel Loading Buffer or TrackIt™ Loading Buffer (page 160), dilute this buffer 50- to 200-fold to obtain the optimal dye concentration.

Prepare each sample for analysis in total sample volume of 20 µl as follows:

- Annealed ds oligo: To 5 µl of the 500 nM stock, add 15 µl deionized water or loading buffer
- Each starting single-stranded oligo: Dilute the 200 µM stock 400-fold to 500 nM. To 5 µl of the 500 nM stock, add 15 µl deionized water or loading buffer
- Mix 2 µl TrackIt™ 10 bp DNA Ladder with 18 µl deionized water. Loading undiluted TrackIt™ DNA Ladder on an E-Gel® will result in loss of resolution.

*Continued on next page*

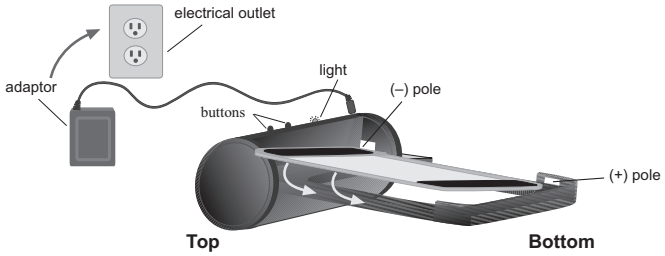
# Checking the Integrity of the ds Oligo, Continued

## Running E-Gel®

Pre-run the E-Gel® agarose gel for 2 minutes **with the comb in place** before loading your samples to ensure proper resolution of your DNA fragments.

Each E-Gel® cassette is supplied individually wrapped and ready for use. To set up and use an E-Gel®, follow the instructions below:

1. Plug the E-Gel® PowerBase™ v.4 into an electrical outlet using the adaptor plug.




2. Open the package containing the gel and insert the gel **(with the comb in place)** into the apparatus right edge first. Press firmly at the top and bottom to seat the gel in the base. You should hear a snap when it is in place. The Invitrogen logo should be located at the bottom of the base, close to the positive pole. See the above diagram. A **steady, red light** illuminates when the E-Gel® is correctly inserted (Ready Mode).
3. **Press and hold** either button until the **red** light turns to a **flashing green light**. This indicates the start of the 2-minute pre-run.
4. At the end of the pre-run, current automatically shuts off. The **flashing green** light changes to a **flashing red** light and the PowerBase™ **beeps rapidly**.
5. **Press and release** either button to stop the beeping. The light changes from a **flashing red** light to a **steady red** light.
6. Remove the comb from the E-Gel® using both hands to lift the comb gently by rolling the comb slowly towards you. Remove any excess fluid using a pipette.
7. Load 20 µl of sample into each well (see previous page for sample preparation).
8. Load 20 µl of the diluted TrackIt™ 10 bp DNA Ladder (see previous page) on the well. Load 20 µl of water into any remaining empty wells.
9. Proceed immediately to electrophoresis, next page.

*Continued on next page*

# Checking the Integrity of the ds Oligo, Continued

### Electrophoresis of E-Gels®

1. Choose the 30-minute run for single-comb gels on the E-Gel® PowerBase™ v.4. For the 30-minute run, **press and release** the 30-min button to start the 30-minute electrophoresis run. The light changes to a **steady green** light.

   **Note:** The actual running time of the E-Gel® gel may vary between 30-33 minutes for single-comb gels.

2. Current through the E-Gel® gel automatically shuts off at the end of each run. The E-Gel® PowerBase™ v.4 signals the end of the run with a **flashing red** light and **rapid beeping**.
3. **Press and release** either button to stop the beeping. The light turns to a **steady red** light.
4. At the end of the run, remove the gel cassette from the power unit and analyze your results using a UV transilluminator.

**Note**

E-Gel® agarose gels can only be used once. Do not re-use them.

### What You Should See

When analyzing an aliquot of the annealed ds oligo reaction by agarose gel electrophoresis, we generally see the following:

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

For an example of expected results obtained from agarose gel analysis, see the next page. If the band representing ds oligo is weak or if you do not see a band, see **Troubleshooting**, page 121 for tips to troubleshoot your annealing reaction.

*Continued on next page*

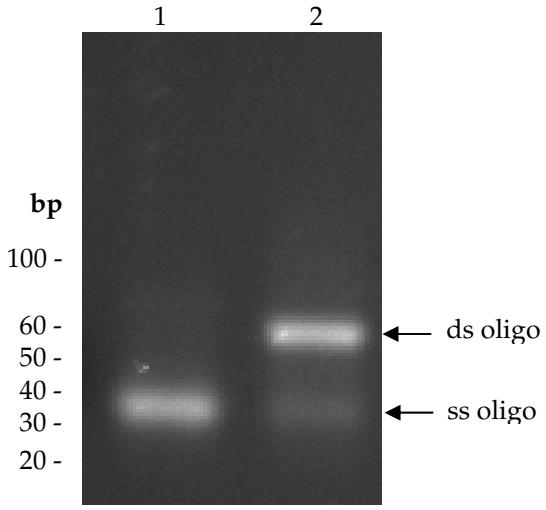
# Checking the Integrity of the ds Oligo, continued

## Example of Expected Results

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

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

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




**Lane 1.** single stranded *lacZ* oligos

**Lane 2.** annealed *lacZ* oligos

# Performing the Ligation Reaction

## Introduction

After generating your ds oligo and diluting to the appropriate concentration, clone the ds oligo into the pcDNA™6.2-GW/EmGFP-miR vector and transform your ligation reaction into competent TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best results. We suggest that you read the sections entitled **Performing the Ligation Reaction** (pages 51-52) and **Transforming One Shot® TOP10 Competent *E. coli*** (page 53) before beginning.

**Note:** If you want to perform miRNA chaining, refer to page 57.

## Important

You will use T4 DNA Ligase and a 5X Ligation Buffer supplied with the kit to facilitate ligation of your ds oligo with the linearized pcDNA™6.2-GW/EmGFP-miR vector. When performing the ligation reaction, note the following:

- The T4 DNA Ligase and the 5X Ligation Buffer supplied with the kit have been optimized to permit ligation of the ds oligo into the pcDNA™6.2-GW/EmGFP-miR vector in **5 minutes at room temperature**. T4 DNA Ligase preparations and reaction buffers available from other manufacturers may not be appropriate for use in this application.
- Traditional ligation reactions are performed at 16°C overnight. **This is not recommended for this application.** Follow the ligation procedure on the next page.

## Amount of ds Oligo to Use

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

## Ligation Controls

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

We recommend including the miR-*lacZ* positive double-stranded (ds) control oligo supplied with the kit as a positive control in your ligation experiment. The miR-*lacZ* positive ds control oligo is supplied ready-to-use as a 10 nM stock in 1X Oligo Annealing Buffer. See page ix for the sequence of each strand of the *lacZ* ds control oligo.

**Note:** Once you have cloned the *lacZ* ds control oligo into pcDNA™6.2-GW/EmGFP-miR, you may use the resulting expression clone as a positive control for the RNAi response in your mammalian cell line. Simply co-transfect the expression clone and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your mammalian cell line and assay for knockdown of β-galactosidase expression.

## Important

**Reminder:** When using the 10 nM ds oligo stock solution for cloning, thaw the solution on ice. **Do not thaw the ds oligo by heating or the ds oligo duplexes may melt and form intramolecular hairpin structures.** After use, return the tube to -20°C storage.

*Continued on next page*

# Performing the Ligation Reaction, continued

## Materials Needed

You will need the following materials:

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

## Ligation Procedure

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

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

| Reagent | Sample | Positive control | Negative control |
|---|---|---|---|
| 5X Ligation Buffer | 4 µl | 4 µl | 4 µl |
| pcDNA™6.2-GW/EmGFP-miR, linearized (5 ng/µl) | 2 µl | 2 µl | 2 µl |
| miR-ds oligo (10 nM; *i.e.* 1:5,000 dilution; page 45) | 4 µl | -- | -- |
| miR-*lacZ* positive ds control oligo (10 nM) | -- | 4 µl | -- |
| DNase/RNase-Free Water | 9 µl | 9 µl | 13 µl |
| T4 DNA Ligase (1 U/µl) | 1 µl | 1 µl | 1 µl |
| Total volume | 20 µl | 20 µl | 20 µl |

2. Mix reaction well by pipetting up and down.

   **Note:** The presence of PEG and glycerol (supplied by the Ligation Buffer and the T4 DNA Ligase) will make the reaction mixture viscous. Be sure to mix the reaction thoroughly by pipetting up and down. **Do not vortex.**

3. Incubate for 5 minutes at room temperature.

   **Note:** Extending the incubation time may result in a higher yield of colonies. Do not exceed 2 hours.

4. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent *E. coli***, next page.

   **Note:** You may store the ligation reaction at -20°C overnight.

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

## Introduction

Once you have performed the ligation reaction, transform your ligation mixture into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation. Follow the guidelines and instructions provided in this section.

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

## Materials Needed

You will need the following materials:

- Ligation reaction (from Step 3, previous page)
- One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit, Box 2; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (supplied with the kit, Box 2; warm to room temperature)
- pUC19 positive control (supplied with the kit, Box 2; if desired)
- 42°C water bath
- LB plates containing 50 µg/ml spectinomycin (two for each transformation; warm at 37°C for 30 minutes before use). See page 133 for recipe.
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

**Note:** Low salt LB agar plates containing 100 µg/ml Blasticidin can also be used to select for transformants. Be sure to use low salt agar plates and check pH carefully for Blasticidin to work efficiently. For more information on Blasticidin and recipes, see page 134.

## One Shot® TOP10 Transformation Procedure

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

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

   **Note:** Longer incubations seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a pre-warmed LB agar plate containing 50 µg/ml spectinomycin and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, plate 20-100 µl of the transformation reaction on LB plates containing 100 µg/ml ampicillin.
8. An efficient ligation reaction may produce several hundred colonies.
9. Proceed to analyzing transformants, next page.

# Analyzing Transformants

## Analyzing Transformants

To analyze positive clones, we recommend that you:

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

   **Note:** Low salt LB containing 100 µg/ml Blasticidin can also be used to grow transformants. See page 134.

2. Isolate plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit included with the BioModule™ units. See page 61 for protocol details.

3. Sequence each pcDNA™6.2-GW/EmGFP-miR expression construct (see below) to confirm the following:

   a. The presence and correct orientation of the ds oligo insert.

   b. The sequence of the ds oligo insert.

   **Note:** Because of the small size of the ds oligo insert, we do not recommend using restriction enzyme analysis to screen transformants.

**Important**

**We highly recommend sequencing positive transformants to confirm the sequence of the ds oligo insert.** When screening transformants, we find that up to 20% of the clones contain mutated inserts (generally 1 or 2 bp deletions within the ds oligo). The reason for this is not known, but may be due to triggering of repair mechanisms within *E. coli* as a result of the inverted repeat sequence within the ds oligo insert.

**Note:** Expression clones containing mutated ds oligo inserts generally elicit a poor RNAi response in mammalian cells. Identify expression clones with the correct ds oligo sequence and use these clones for your RNAi analysis.

## Sequencing

To facilitate sequencing of your pcDNA™6.2-GW/EmGFP-miR expression clones, use the EmGFP forward sequencing primer and miRNA reverse sequencing primer supplied with the kit (Box 1). See the diagram on page 41 for the location of the priming sites.

**Note**

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

*Continued on next page*

In some cases, you may have difficulty sequencing the ds oligo insert in your expression construct. This is because the hairpin sequence is an inverted repeat that can form secondary structure during sequencing, resulting in a drop in the sequencing signal when entering the hairpin. If you have difficulty sequencing your expression constructs, we suggest trying the following to improve your sequencing results:

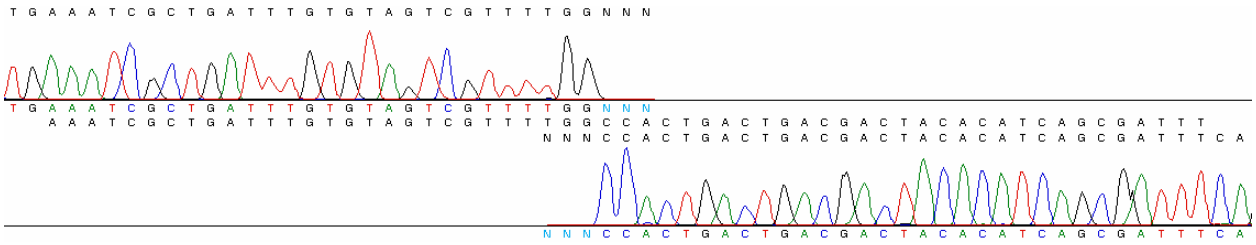
- Use the PureLink HQ Mini Plasmid Purification Kit to isolate high-quality, plasmid DNA for sequencing.
- Add DMSO to the sequencing reaction to a final concentration of 5%.
- Increase the amount of template used in the reaction (up to twice the normal concentration).
- Standard sequencing kits typically use dITP in place of dGTP to reduce G:C compression. Other kits containing dGTP are available for sequencing G-rich and GT-rich templates. If you are using a standard commercial sequencing kit containing dITP, obtain a sequencing kit containing dGTP (*e.g.* dGTP BigDye® Terminator v3.0 Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Catalog no. 4390229) and use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction.

### Alternative Sequencing Protocol

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

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

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




*Continued on next page*

### Long-Term Storage

Once you have identified the correct expression clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for a single colony on an LB plate containing 50 µg/ml spectinomycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml spectinomycin.
3. Grow until the culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store the glycerol stock at -80°C.

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

### What to Do Next

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

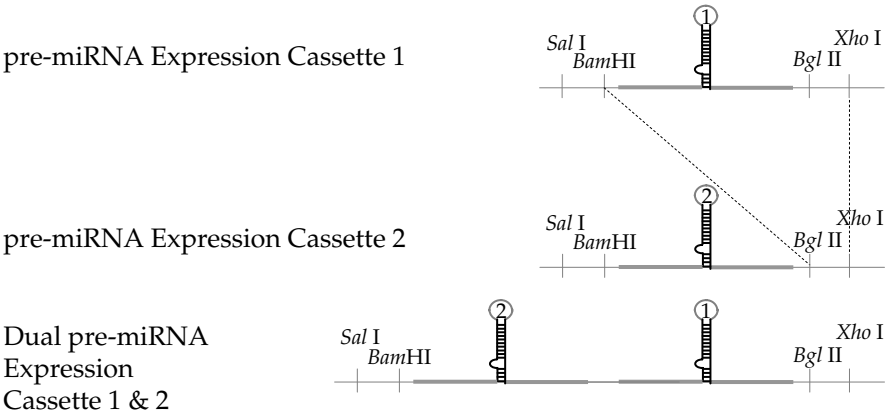
- Transfect the expression clone directly into the mammalian cell line of interest to perform transient RNAi analysis (see **Transfecting Cells**, page 64).
- Transfect the expression clone directly into the mammalian cell line of interest and isolate **stable** transfectants that knock down the gene of interest constitutively (see **Generating a Stable Cell Line**, page 70).
- Perform miRNA chaining to express multiple pre-miRNAs from one single construct (see **Chaining multiple pre-miRNAs**, next page).
- Remove the EmGFP coding sequence from your pcDNA™6.2-GW/EmGFP-miR expression clone (see **Removing EmGFP Coding Sequence**, page 59).
- Perform an LR recombination reaction with your expression construct and a suitable Gateway® destination vector to generate an expression clone in an alternative backbone (see **Performing the Rapid BP/LR Recombination Reaction**, page 80).
- Perform an LR recombination reaction with your expression construct and a pLenti5/V5-DEST destination vector to generate an expression clone to perform lentiviral expression (see **Performing the Rapid BP/LR Recombination Reaction**, page 80).

# Chaining pre-miRNAs

## Introduction

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

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




## Restriction Strategy

Two strategies of restriction digestions are possible:

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

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

The procedure for the first strategy is described on the next page. For the second strategy, change the restriction enzymes used in the procedure.

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# Chaining pre-miRNAs, continued

## Materials Needed

You will need the following materials:

- Appropriate restriction enzymes
- 2% E-Gel® agarose gel
- PureLink™ Quick Gel Extraction Kit (page 160)
- One Shot® TOP10 Competent Cells (page 160)

## Procedure for Chaining

Below is a protocol for chaining miRNAs.

1. **Insert:** Digest 2 µg pcDNA™6.2-GW/EmGFP-miR-1 with 10 units *Bam*H I and 10 units *Xho* I for 2 hours at 37º C.
2. **Backbone:** Digest 1 µg pcDNA™6.2-GW/EmGFP-miR-2 with 10 units *Bgl* II and 10 units *Xho* I for 2 hours at 37º C.
3. Run fragments on 2% E-Gel® or other high percentage agarose gels.
4. Excise the backbone and insert fragments from the gel. Purify the fragments using the PureLink™ Quick Gel Extraction Kit from Invitrogen or equivalent.
5. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase from Invitrogen or equivalent.
6. Transform competent cells, such as *E. coli*. One Shot® TOP10 as described on page 53.
7. Analyze resulting clones as described on pages 54-56
8. Test construct for both miRNAs by transfecting cells as described on page 60.

# Removing EmGFP Coding Sequence

## Introduction

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

## Materials Needed

You will need the following materials:

- Appropriate restriction enzymes
- PureLink™ Quick Gel Extraction Kit (page 160)
- One Shot® TOP10 Competent Cells (page 160)

## Procedure for Removing EmGFP

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

1. Digest 1 μg pcDNA™6.2-GW/EmGFP-miR with 10 units *Dra* I for 2 hours at 37ºC.
2. Run fragments on 0.8% E-Gel® or other low percentage agarose gels.
3. Excise the vector fragment from the gel and purify the fragment using the PureLink™ Quick Gel Extraction Kit from Invitrogen or equivalent.
4. Ligate the purified backbone and insert fragment at a 1:4 molar ratio, using T4 DNA ligase.
5. Transform competent cells, such as *E. coli*. One Shot® TOP10 as described on page 53.
6. Analyze resulting clones by restriction analysis with *Dra* I; no 750 bp fragment should be visible.

# Transfection and RNAi Analysis

## Experimental Outline

The experimental steps necessary to transfect the pcDNA™6.2-GW/EmGFP-miR expression clone into a mammalian cell line of choice and perform RNAi analysis are outlined below. We recommend performing the steps as described below to achieve the best results.

| Step | Action | Page no. |
|---|---|---|
| 1 | Purify plasmid DNA from the verified expression clone using PureLink™ HQ Mini Plasmid DNA Purification Kit. | 61 |
| 2 | Culture the mammalian cell of choice to the required cell density. | 64 |
| 3 | Perform transient transfection using Lipofectamine™ 2000 Reagent. | 67 |
| 4 | Perform analysis of the EmGFP fluorescent protein from the expression clone using fluorescent microscopy to determine the expression of your miRNA. | 69 |
| 5 | Assay for target gene knockdown using qPCR, Western analysis, immunohistochemistry, or any other functional assay. | -- |
| 6 | **Optional:** | |
| | Generate stable cell lines that constitutively express your miRNA. | 70 |
| | Transfer the pre-miRNA expression cassette into appropriate Gateway® destination vectors to allow for expression of the miRNA in viral systems or using tissue-specific promoters. | 74 |

# Plasmid DNA Purification

## Introduction

Once you have obtained your expression clone, isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol or sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency.

The PureLink™ HQ Mini Plasmid Purification Kit (Box 3) is included with each BioModule™ BLOCK-iT™ RNAi Units for isolating high quality plasmid DNA.

**Note:** If you are using the BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System, do not use the S.N.A.P.™ MidiPrep Kit for plasmid DNA purification at this step. Use the PureLink™ HQ Mini Plasmid Purification Kit.

## Experimental Outline

To isolate plasmid DNA, you will:

1. Grow *E. coli* cells.
2. Prepare bacterial lysates.
3. Bind the plasmid DNA from the lysate to the PureLink™ Spin Column.
4. Perform washing to remove impurities.
5. Elute the plasmid DNA in Elution Buffer.

## Materials Needed

You will need the following materials:

- PureLink™ HQ Mini Plasmid Purification Kit (Box 3, supplied with the kit)
- 64 ml 100% ethanol
- Sterile microcentrifuge tubes
- Microcentrifuge

## Before Starting

- Prepare the Resuspension Solution with the RNase A supplied in the kit. Resuspend the lyophilized RNase A (12 mg) in 200 µl of Resuspension Solution, and then add the resuspended mixture to the remaining Resuspension Solution for a final concentration of 0.1 mg/ml RNase A.

  After mixing, store the Resuspension Solution with RNase A at +4°C. Stable for up to 6 months.
- Prepare the Wash Buffer with ethanol. Add 64 ml of 96–100% ethanol to the entire volume of Wash Buffer (16 ml) to obtain a total volume of 80 ml.
- Before each use, check the Neutralization/Binding Buffer and Lysis Buffer for a white salt precipitate. If present, place each buffer in a 37°C water bath for 5 minutes or until the salts redissolve and the solution clears.

  Do not shake the Lysis Buffer, as this can lead to foaming.

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# Plasmid DNA Purification, Continued

## Column Capacity

Each column has a DNA binding capacity of up to 60 µg plasmid DNA.

For cell volumes >$2 \times 10^9$ cells, prepare cells as separate lysates of ≤$1–2 \times 10^9$ cells each as described below, and load lysates consecutively on the same column as described in **Binding DNA**, Step 3, below.

## Preparing Bacterial Cell Lysates

To prepare the bacterial cell lysate:

1. In a microcentrifuge tube, pellet 1–3 ml ($1–2 \times 10^9$) of *E. coli* cells from overnight cultures by centrifugation in a tabletop centrifuge at 1,500 × g for 15 minutes. Remove the culture media completely.
2. Completely resuspend the pellet in 240 µl Resuspension Solution, prepared with RNase A as described on the previous page.
3. Add 240 µl Lysis Buffer to the above solution. Mix gently by inverting the tube 4–8 times.
4. Incubate for 3-5 minutes at room temperature. Do not exceed 5 minutes.
5. Add 340 µl Neutralization/Binding Buffer, and immediately mix gently by inverting the tube 4-8 times.
6. Centrifuge for 10 minutes at maximum speed in a tabletop centrifuge to clarify the cell lysate.
7. Proceed to **Binding DNA,** below.

## Binding DNA

1. Place a PureLink™ spin column inside a 2-ml collection tube.
2. Pipette or decant the supernatant from Step 6, above, into the spin column.
3. Centrifuge the column at room temperature at 10,000–14,000 × g for 1 minute. Discard the flowthrough, and place the column back in the tube.

   If you are loading multiple samples on the same column, repeat Steps 2–3 for each lysate preparation.
4. Add 650 µl Wash Buffer, prepared with ethanol as described on the previous page, to the column.
5. Centrifuge the column at room temperature at 10,000–14,000 × g for 1 minute. Discard the flowthrough from the collection tube, and place the column back in the tube.
6. Centrifuge the column at maximum speed for 1–3 minutes to remove the residual wash buffer.
7. Proceed to **Eluting DNA**, next page.

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## Eluting DNA

1. Place the spin column in a clean 1.7-ml elution tube supplied with the kit.
2. Add the following volume of Elution Buffer (10 mM Tris-HCl, pH 8.5) or sterile, distilled water (pH >7.0):
   - Add 50 µl Elution Buffer or water to the center of the column if the expected DNA yield is <30 µg.
   - Add 100 µl Elution Buffer or water to the center of the column if the expected DNA yield is >30 µg.
3. Incubate the column at room temperature for 1 minute, then centrifuge at maximum speed for 1 minute.
4. The elution tube contains your purified DNA. Remove and discard the column. Store the plasmid DNA at -20ºC.

Determine the quantity and quality of the DNA as described below.

## Estimating DNA Yield and Quality

**DNA Yield**

You can estimate the quantity of the purified plasmid DNA using UV absorbance at 260 nm or Quant-iT™ DNA Assay Kits.

*UV Absorbance*

1. Dilute an aliquot of the plasmid DNA sample in 10 mM Tris-HCl, pH 7.5. Mix well. Transfer to a cuvette (1-cm optical path length).
2. Measure the $A_{260}$ of the solution using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.5.
3. Calculate the amount of DNA using the following formula:

DNA (µg) = $A_{260}$ × 50 µg/(1 $A_{260}$ x 1 ml) x dilution factor x total sample volume (ml)

Assumption: For dsDNA, $A_{260}$ = 1 for a 50 µg/ml solution measured in a cuvette with an optical path length of 1 cm.

*Quant-iT™ DNA Assay Kits*

The Quant-iT DNA Assay Kits (page 160) provide a rapid, sensitive, and specific method for DNA quantitation with minimal interference from RNA, protein, or other common contaminants that affect UV absorbance readings.

The kit contains a state-of-the-art quantitation reagent, pre-diluted standards for standard curve, and a ready-to-use buffer. The assay is performed in a microtiter plate format and is designed for reading in standard fluorescent microplate readers. Follow manufacturer's recommendations to perform the assay.

**DNA Purity**

Typically, DNA isolated using the PureLink™ HQ Mini Plasmid Purification Kit has an $A_{260}/A_{280}$ >1.80 when samples are diluted in Tris-HCl (pH 7.5). An $A_{260}/A_{280}$ of >1.80 indicates that the DNA is reasonably clean of proteins that could interfere with downstream applications. Absence of contaminating genomic DNA and RNA may be confirmed by agarose gel electrophoresis.

# Transfecting Cells

## Introduction

This section provides general guidelines to transfect your pcDNA™6.2-GW/EmGFP-miR expression construct into the mammalian cell line of interest to perform transient RNAi analysis. Performing transient RNAi analysis is useful to:

- Quickly test multiple miRNA sequences to a particular target gene
- Quickly screen for an RNAi response in your mammalian cell line
- Test the effect of gene knockdown on your particular transient assay

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

## Experimental Outline

To perform transfection, you will:

1. Grow the mammalian cell line of choice.
2. Transfect the plasmid DNA into mammalian cells using Lipofectamine™ 2000.
3. Harvest cells after 18-48 hours to perform transient knockdown experiments.

## Factors Affecting Gene Knockdown Levels

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

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

Take these factors into account when designing your RNAi experiments.

## Lipofectamine™ 2000 Reagent

Lipofectamine™ 2000 Reagent (Ciccarone *et al.*, 1999) is included with BioModule™ Units to transfect plasmid DNA into eukaryotic cells and offers the following advantages:

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

An optimized transfection protocol for mammalian cells is described on page 67. However, depending on your cell type, you may need to optimize the transfection conditions such as cell number, amount of the transfection reagent and plasmid DNA, and the time period to assay for target gene knockdown to obtain the best results.

Cell-type specific transfection protocols are available at www.invitrogen.com/rnai.

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### Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine™ 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium supplied with the kit.

### Positive and Negative Controls

If you have performed the positive control reaction and have cloned the miR-*lacZ* positive ds control oligo supplied with the kit into pcDNA™6.2-GW/EmGFP-miR, we recommend using the resulting pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression construct as a positive control to assess the RNAi response in your cell line. Simply co-transfect the pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression construct and the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid supplied with the kit into your mammalian cells and assay for knockdown of β-galactosidase expression 24-48 hours post-transfection using Western blot analysis or activity assay. For more information about the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid, recommendations for transfection, and methods to assay for β-galactosidase activity, see below.

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

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

The pcDNA™1.2/V5-GW/*lacZ* reporter plasmid is supplied with the kit for use as a positive control to assay for the RNAi response in your mammalian cell line. In this vector, β-galactosidase is expressed as a C-terminally tagged fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). See page 146 for more information.

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

### Transfecting the *LacZ*-Containing Reagents

To perform RNAi analysis using the *lacZ* control reagents, you will co-transfect the pcDNA™1.2/V5-GW/*lacZ* reporter plasmid and pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression construct that you have generated into your mammalian cell line. For optimal results, we recommend using 6-fold more expression construct DNA than reporter plasmid DNA in the co-transfection.

For example, use 600 ng of pcDNA™6.2-GW/miR-*lacZ* DNA and 100 ng of pcDNA™1.2/V5-GW/*lacZ* DNA when transfecting cells plated in a 24-well format.

### Assaying for β-galactosidase Expression

If you perform RNAi analysis using the control expression clone containing the *lacZ* ds oligo (pcDNA™6.2-GW/EmGFP-miR-*lacZ*), you may assay for β-galactosidase expression by western blot analysis using β-gal Antiserum (Cat. no. R901-25), by activity assay using cell-free lysates (Miller, 1972) and FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Cat. no. F-2905), or by staining the cells for activity using the β-Gal Staining Kit (Cat. no. K1465-01) for fast and easy detection of β-galactosidase expression. For an example of results, see page 72.

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The β-galactosidase protein expressed from the pcDNA™1.2/V5-GW/*lacZ* control plasmid is fused to a V5 epitope and is approximately 119 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (*e.g.* Anti-V5-HRP Antibody; Cat. no. R961-25 or Anti-V5-AP Antibody, Cat. no. R962-25) for detection. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (page 163).


- We recommend using Opti-MEM® I Reduced Serum Medium to dilute Lipofectamine™ 2000 and plasmid DNA before complexing.
- **Do not** add antibiotics to media during transfection as this causes cell death.
- Use low-passage cells, and make sure that cells are healthy and greater than 90% viable before transfection.
- Maintain the same seeding conditions between experiments.
- **Transfect cells at 90-95% confluence.** Transfecting cells at a lower density allows a longer time interval to elapse between transfection and assay time, and minimizes the loss of cell viability due to cell overgrowth. Depending on the nature of the target gene, transfecting cells at higher densities may be suitable with optimization of conditions.
- Test serum-free media for compatibility with Lipofectamine™ 2000 Reagent since some serum-free formulations (*e.g.* CD293, SFM II, VP-SFM) may inhibit cationic lipid-mediated transfection.

## Materials Needed

You will need the following materials:

- Mammalian cell line of interest cultured in the appropriate growth medium
- Lipofectamine™ 2000 Reagent (supplied with the kit, store at 4ºC until use)
- Opti-MEM® I Reduced Serum Medium (supplied with the kit, pre-warm to 37ºC before use)
- pcDNA™6.2-GW/EmGFP-miR plasmid DNA
- Appropriate tissue culture plates and supplies
- Appropriate controls

*Continued on next page*

# Transfecting Cells, Continued

## Transfection Protocol

Use the following procedure to transfect plasmid DNA into mammalian cells in a **24-well format**. All amounts and volumes are given on a per well basis.

For other formats, see **Scaling Up or Down Transfections** (next page). Prepare complexes using a DNA (µg) to Lipofectamine™ 2000 (µl) ratio of 1:2 to 1:3 for most cell lines. Transfect cells at high cell density for high efficiency, high expression levels, and to minimize cytotoxicity.

Use this procedure as a starting point; optimize transfections as described in **Optimizing Transfection** (see below), especially if you are transfecting a mammalian cell line for the first time.

1. **Adherent cells:** One day before transfection, plate 0.5-2 x $10^5$ cells in 500 µl growth medium without antibiotics such that cells will be **90-95% confluent** at the time of transfection.

   **Suspension cells:** Just prior to preparing complexes, plate 4-8 x $10^5$ cells in 500 µl of growth medium without antibiotics.
2. **For each transfection sample**, prepare complexes as follows:
   a. Dilute DNA (0.8 µg) in 50 µl Opti-MEM® I Reduced Serum Medium without serum. Mix gently.
   b. Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount (2 µl) in 50 µl Opti-MEM® I Medium. Incubate for 5 minutes at room temperature.

      **Note:** Combine diluted Lipofectamine™ 2000 with diluted DNA within 30 minutes.
   c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine™ 2000 (total volume = 100 µl). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).

   **Note:** Complexes are stable for 6 hours at room temperature.
3. Add 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4. Incubate cells at 37°C in a $CO_2$ incubator for 18-48 hours prior to testing for expression. Medium may be changed after 4-6 hours.

## Optimizing Transfection

To obtain the highest transfection efficiency and low cytotoxicity, optimize transfection conditions by varying cell density as well as DNA and Lipofectamine™ 2000 concentrations.

- Make sure that cells are greater than 90% confluent
- Vary DNA (µg): Lipofectamine™ 2000 (µl) ratios from 1:0.5 to 1:5
- Depending on the nature of the target gene, transfecting cells at higher densities may also be considered when optimizing conditions.

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### Scaling Up or Down Transfections

To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine™ 2000, DNA, cells, and medium used in proportion to the relative surface area, as shown in the table.

With automated, high-throughput systems, a complexing volume of 50 µl is recommended for transfections in 96-well plates.

**Note:** You may perform rapid 96-well plate transfections by plating cells directly into the transfection mix. Prepare complexes in the plate and directly add cells at twice the cell density as in the basic protocol in a 100 µl volume. Cells will adhere as usual in the presence of complexes.

| Culture vessel | Surf. Area per well[1] | Vol. of plating medium | DNA (µg) in media volume (µl) | Lipofectamine™ 2000 (µl) in media volume (µl) |
|---|---|---|---|---|
| 96-well | 0.3 $cm^2$ | 100 µl | 0.2 µg in 25 µl | 0.5 µl in 25 µl |
| 24-well | 2 $cm^2$ | 500 µl | 0.8 µg in 50 µl | 2.0 µl in 50 µl |
| 12-well | 4 $cm^2$ | 1 ml | 1.6 µg in 100 µl | 4.0 µl in 100 µl |
| 6-well | 10 $cm^2$ | 2 ml | 4.0 µg in 250 µl | 10 µl in 250 µl |
| 60-mm | 20 $cm^2$ | 5 ml | 8.0 µg in 0.5 ml | 20 µl in 0.5 ml |
| 10-cm | 60 $cm^2$ | 15 ml | 24 µg in 1.5 ml | 60 µl in 1.5 ml |

[1] Surface areas may vary depending on the manufacturer.

# 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 (see next page). Once you have transfected your expression clone into mammalian cells, you may detect EmGFP protein expression directly in cells by fluorescence microscopy or other methods that use light excitation and detection of emission. See below for recommended fluorescence microscopy filter sets.

The EmGFP expression is essentially 100% correlated with the expression of your miRNA.

## Filters for Use with EmGFP

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

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

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

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

## Fluorescence Microscope

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

## Color Camera

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

## Detecting Transfected Cells

After transfection, allow the cells to recover for 24-48 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 appear brightly labeled and emit a green fluorescence signal that should be easy to detect above the background fluorescence. **Note:** The fluorescence signal of EmGFP from miRNA-containing vectors is reduced due to processing of the transcripts when compared to non-miRNA containing vectors.

Cells with bright fluorescence demonstrate the highest knockdown with a functional miRNA. However, cells with reduced fluorescence may still express the miRNA and demonstrate knockdown since the expression levels required to observe gene knockdown are generally lower than that required to detect EmGFP expression. See the **Expected Results**, page 72.

# Generating a Stable Cell Line

## Introduction

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

## Blasticidin Selection

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

For more information about how to prepare and handle Blasticidin, see page 134.

## Experimental Outline

To generate stable cell lines, you will:

1. Grow the mammalian cell line of choice.
2. Determine antibiotic sensitivity for your mammalian cell line.
3. Perform transfection using Lipofectamine™ 2000 Reagent.
4. On the next day, replace the medium with fresh, complete medium containing Blasticidin.
5. Continue to replace the medium with fresh, complete medium containing Blasticidin every 3-4 days until Blasticidin-resistant colonies are visible.

## Determining Antibiotic Sensitivity

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

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

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# Generating a Stable Cell Line, continued

## Materials Needed

You will need the following materials:

- Mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)
- pcDNA™6.2-GW/EmGFP-miR expression clone
- pcDNA™6.2-GW/EmGFP-miR-neg control plasmid
- Lipofectamine™ 2000 Reagent (supplied with the kit, store at 4ºC until use)
- Opti-MEM® I Reduced Serum Medium (supplied with the kit, pre-warm to 37ºC before use)
- Blasticidin (5 to 10 mg/ml)
- Appropriate tissue culture dishes and supplies

## Guidelines for Transfection and Selection

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

1. Perform transfection of your pcDNA™6.2-GW/EmGFP-miR expression construct and pcDNA™6.2-GW/EmGFP-miR-neg control plasmid into cells using the transfection protocol on page 67. Use separate wells for separate constructs.
2. Four to six hours after transfection, remove the medium and replace with fresh growth medium. Incubate the cells overnight at 37°C.
3. The following day (Day 2), trypsinize and replate cells into a larger-sized tissue culture format in fresh complete medium containing the appropriate concentrations of Blasticidin.

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

## The Next Step

Once you have assessed the expression of your miRNA of interest using EmGFP fluorescence, we recommend that you assay for gene knockdown using qPCR, Western analysis, immunohistochemistry, or any other functional assay.

A variety of BioModule™ Units that include qualified reagents and validated protocols are available from Invitrogen to perform validation experiments (page 160).

# Expected Results for miRNA Expression

## Introduction

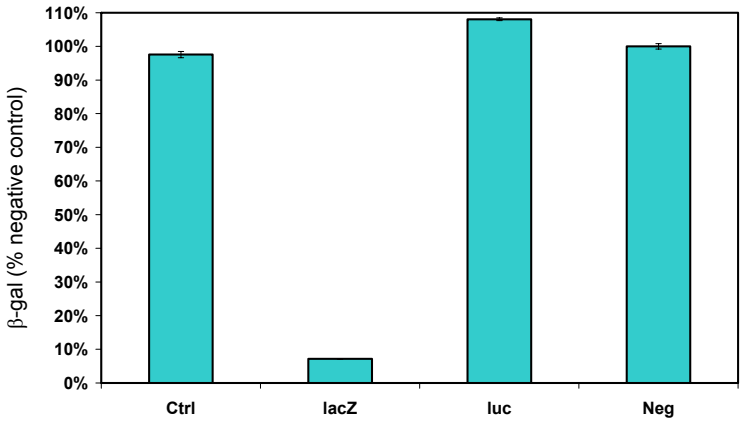
Examples of results obtained with pcDNA™6.2-GW/ EmGFP-miR expression vector for miRNA expression are shown below.

## Knockdown of Reporter Gene

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

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

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



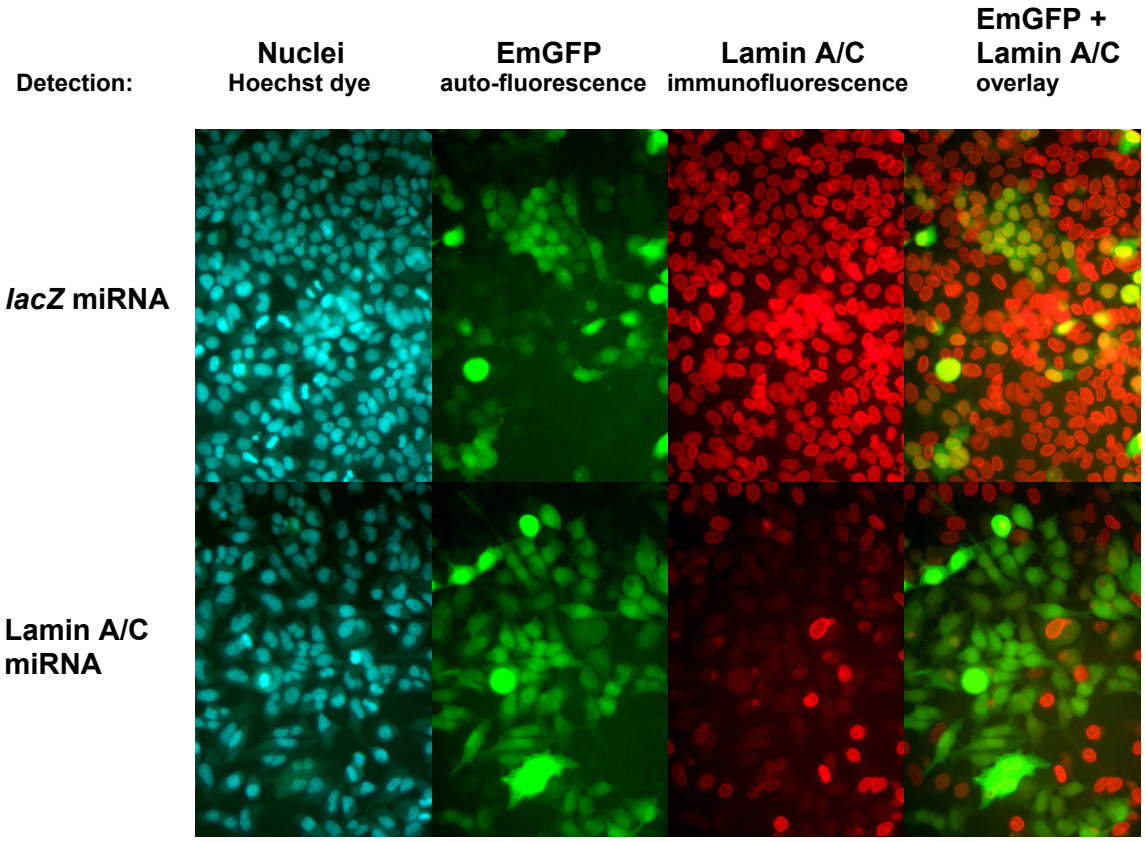

*Continued on next page*

# Expected Results for miRNA Expression, Continued

**Knockdown of Endogenous Lamin A/C**

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

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



# Transferring the Pre-miRNA Expression Cassette to Destination Vectors

## Experimental Outline

The experimental steps necessary to transfer the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR expression clone into other Gateway® destination vectors are outlined below. We recommend performing the steps as described below to achieve the best results.

| Step | Action | Page no. |
|---|---|---|
| 1 | Generate an expression clone. | 36 |
| 2 | Perform the Rapid BP/LR Gateway® recombination reactions to transfer the pre-miRNA expression cassette from an expression clone into a destination vector. | 80 |
| 3 | Transform the recombination reaction mix into One Shot® TOP10 Chemically Competent *E. coli* or One Shot® Stbl3™ Chemically Competent *E. coli* (for lentiviral destination vectors). | 87 |
| 4 | Analyze transformants and purify plasmid DNA for transfection. | 54 |
| 5 | Perform transient transfection using Lipofectamine™ 2000 Reagent into a mammalian cell line of choice. | 67 |
| 6 | Perform analysis of the EmGFP fluorescent protein from the expression clone using fluorescent microscopy to determine the expression of your miRNA. | 69 |
| 7 | Assay for target gene knockdown using qPCR, Western analysis, immunohistochemistry, or any other functional assay. | -- |
| 8 | **Optional:** | |
| | Perform lentiviral expression of your miRNA, if you used a lentiviral destination vector. | 92 |

# Creating Entry Clones for Use with Destination Vectors

## Introduction

The pcDNA™6.2-GW/EmGFP-miR expression vector contains *att* sites to facilitate the transfer of the pre-miRNA expression cassette into appropriate Gateway® destination vectors to allow for expression of the miRNA in viral systems or using tissue-specific promoters.

The pre-miRNA is transcribed by RNA Polymerase II (Pol II); the pre-miRNA expression cassette can be transferred to other Gateway® adapted destination vectors utilizing Pol II promoters to control expression of the pre-miRNA.

**Important**

Since the pcDNA™6.2-GW/EmGFP-miR expression vector contains *att*B sites, the expression vector containing the pre-miRNA expression cassette **cannot** be used directly with a destination vector to perform the LR recombination reaction.

## Transferring the Cassette

To transfer your pre-miRNA expression cassette into other destination vectors, you need to first generate an entry clone containing *att*L sites by performing a BP recombination reaction, then use the resulting entry clone in an LR recombination reaction with a destination vector to generate a new miRNA expression clone.

The transfer of the miRNA sequence into the destination vector can be performed using the standard BP and LR recombination reactions or Rapid BP/LR recombination reactions as described on the next page.

See below for an overview of the Gateway® recombination reactions and page 79 for the recombination region.

## Gateway® Recombination Reactions

Two recombination reactions constitute the basis of the Gateway® Technology as described below. You will perform both Gateway® recombination reactions to transfer the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR expression vector to a new destination vector as outlined below.

**BP Reaction**

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

You will recombine pcDNA™6.2-GW/EmGFP-miR expression clone (*att*B substrate) with an *att*P substrate (pDONR™221 donor vector) first to form an entry clone.

**LR Reaction**

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

The resulting entry clone (*att*L substrate) from the BP reaction is then recombined with the destination vector (*att*R substrate) to form a new miRNA expression clone.

*Continued on next page*

# Creating Entry Clones for Use with Destination Vectors,
Continued

## Choosing a Suitable Protocol

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

| If You Wish to…. | Then Choose….. | Described |
|---|---|---|
| Generate the expression clone using a fast protocol but obtain fewer (~10% of the total number of clones) expression clones than the standard protocol | Rapid BP/LR Recombination Protocol | On page 80. |
| Maximize the number of expression clones generated and isolate entry clones for future use | Standard BP and LR Protocols | On page 135. |

## Substrates for the Recombination Reactions

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

- Linearized *att*B-containing expression clones (see page 78 for guidelines to linearize *att*B expression clones)
- *att*P-containing donor (pDONR™221) vector (see below)

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

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

## Donor Vector

A large variety of donor vectors are available from Invitrogen. We recommend using the pDONR™221 vector. The pDONR™221 vector is supplied with the BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System (Cat. no. WFGE08).

*Continued on next page*

# Creating Entry Clones for Use with Destination Vectors, Continued

## Appropriate Destination Vectors

A large selection of Gateway® destination vectors are available from Invitrogen. The various Gateway® vectors have widely different transcriptional and technical properties, which can be used to express the pre-miRNA. They offer custom promoter cloning, tissue-specific expression, regulated expression, and lentiviral transduction of the pre-miRNA. In addition, destination vectors providing N-terminal reporter genes can be used after removal of EmGFP.

A list of Gateway® destination vectors that are compatible with the pcDNA™6.2-GW/EmGFP-miR expression vector is shown below. For more information on these destination vectors, visit www.invitrogen.com or contact Technical Service (page 163).

| Destination Vector | Catalog No. |
|---|---|
| **Standard Destination Vectors** | |
| pLenti6/V5-DEST (included with WFGE08) | V496-10 |
| pLenti6/UbC/V5-DEST | V499-10 |
| pEF-DEST51 | 12285-011 |
| pT-REx™-DEST30 | 12301-016 |
| pEF5/FRT/V5-DEST™ (Flp-In™) | V6020-20 |
| N-terminal reporter tag vectors, *e.g.*: | |
| pcDNA™6.2/nGeneBLAzer™-DEST | 12578-068, 12578-050 |
| pcDNA™6.2/N-YFP-DEST | V358-20 |
| **MultiSite Gateway® Destination Vectors** | |
| pDEST™/R4-R3 | 12567-023 |
| pLenti6/R4R2/V5-DEST | K591-10 |

**Important**

Transferring the pre-miRNA expression cassette from the pcDNA™6.2-GW/EmGFP-miR to the pLenti6/BLOCK-iT™-DEST destination vector will not yield a functional miRNA expression vector because this vector does not carry a Pol II promoter upstream of the *att*R1 site. **Expression of the pre-miRNA requires the destination vector to supply a Pol II promoter.**

For lentiviral expression, transfer to pLenti6/V5-DEST as described on page 80.

## Resuspending the Donor and Destination Vector

The donor and destination vectors are supplied as 6 µg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, simply resuspend each plasmid DNA in 40 µl sterile water to a final concentration of 150 ng/µl.

*Continued on next page*

# Creating Entry Clones for Use with Destination Vectors, Continued

## Propagating the Donor and Destination Vectors

**Donor Vector**

If you wish to propagate and maintain the pDONR™221 vector supplied with cat. no WFGE08, we recommend using One Shot® *ccd*B Survival T1$^R$ Chemically Competent *E. coli* or Library Efficiency® DB3.1™ Competent *E. coli* from Invitrogen (page 160) for transformation. The *ccd*B Survival T1$^R$ and DB3.1™ *E. coli* strains are resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50 µg/ml kanamycin and 15 µg/ml chloramphenicol.

**Destination Vector**

If you wish to propagate and maintain the pLenti6/V5-DEST vector supplied with Cat. no WFGE08, we recommend using Library Efficiency® DB3.1™ Competent *E. coli* from Invitrogen (page 160) for transformation. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain integrity of the vector, select for transformants in media containing 50-100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol. Do not use One Shot® *ccd*B Survival T1$^R$ Chemically Competent *E. coli* to propagate lentiviral vectors as it is more susceptible to recombination and results in lower yields.

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

## Linearizing Expression Clones

We recommend that you linearize the pcDNA™6.2-GW-EmGFP-miR expression clone using *Eag* I or *Bsr*D I.

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 pre-miRNA region of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50-150 ng/µl.

## Recombination Region

The recombination region of the lentiviral expression clone resulting from pLenti6/V5-DEST x pENTR™221/EmGFP-miR entry clone is shown on the next page.

For details on the recombination region of other destination vectors, refer to the manual supplied with each destination vector.

*Continued on next page*

# Creating Entry Clones for Use with Destination Vectors,
## Continued

### Recombination Region of pLenti6/V5-DEST

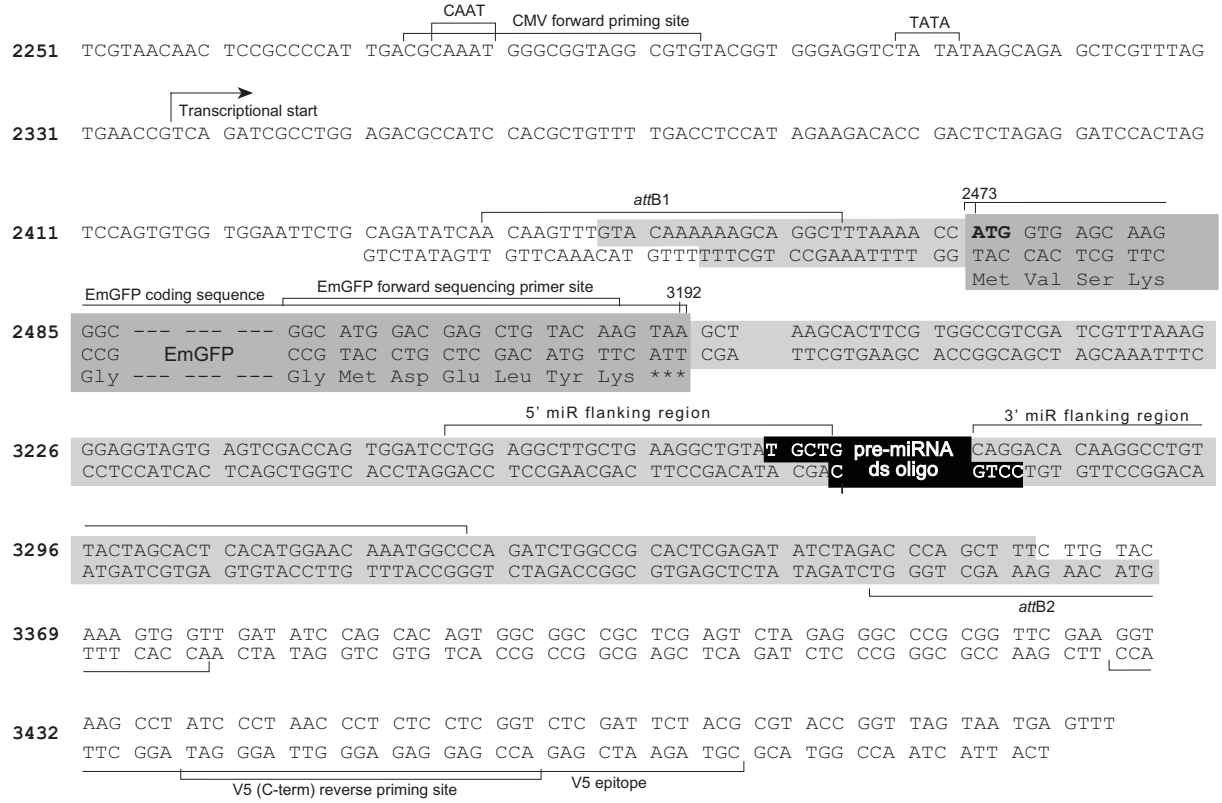
The recombination region of the lentiviral expression clone resulting from pLenti6/V5-DEST x pENTR™221/EmGFP-miR entry clone is shown below.

The pENTR™221/EmGFP-miR entry clone is obtained by transferring the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR into pDONR™221.

**Features of the Recombination Region:**

Shaded regions correspond to those DNA sequences transferred from the pENTR™221/EmGFP-miR entry clone into the pLenti6/V5-DEST vector by recombination. Non-shaded regions are derived from the pLenti6/V5-DEST vector. Bases 2473 and 3192 indicate the coding sequence of EmGFP.

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




**Note**

Since the pLenti6-V5-GW/EmGFP-miR expression construct 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 pcDNA™6.2-GW/EmGFP-miR vector containing the pre-miRNA expression cassette, pDONR™221, and a destination vector. See below for details on the Rapid protocol.

If you wish to perform the standard BP recombination reaction followed by the standard LR recombination reaction, see page 135.

## Rapid BP/LR Protocol

The Rapid BP/LR protocol is used to transfer a pre-miRNA expression cassette from an expression clone into a destination vector in 2 steps - a BP reaction using a donor vector followed by an LR reaction using a destination vector without purification of the intermediate entry clone.

**Note:** Using this protocol allows you to generate expression clones more rapidly than the standard BP and LR protocols provided in the Gateway® Technology with Clonase™ II manual. 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 protocols on page 135.

**Note**

This protocol is for experienced users of the Gateway® Technology. If you are unfamiliar with the Gateway® system, refer to the Gateway® Technology with Clonase™ II manual available at www.invitrogen.com.

## 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.
2. Use a small aliquot of the BP reaction mix to perform the LR recombination reaction using the destination vector to generate the new miRNA expression clone.
3. Perform Proteinase K treatment.
4. Transform the reaction mixture into a suitable *E. coli* host.
5. Select for expression clones.

**Important**

Based on the destination vector that you are using, you need to perform the appropriate LR recombination reaction as described below.

If you are using standard destination vectors (see page 77), perform the LR recombination reaction with **LR Clonase™ II enzyme mix** as described on page 84,

If you are using MultiSite Gateway® destination vectors (see page 77), perform the LR recombination reaction with **LR Clonase™ Plus enzyme mix** as described on page 85.

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

## *E. coli* Host

Once you have performed the Rapid BP/LR recombination reaction, you will transform the recombination reaction into competent *E. coli* and select for the appropriate transformants. You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation.

**DO NOT** transform the LR recombination reaction into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene. See page 160 for ordering information on competent cells.

**Important:** When performing the LR recombination reaction with the lentiviral destination vectors, transformation into the Stbl3™ *E. coli* strain is recommended for optimal results (see below).

## Recommended *E. coli* Host for pLenti6/V5-DEST

**For optimal results with pLenti6/V5-DEST, 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 with Cat. no WFGE08 for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 87.

## Positive Control

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

## Converting Femtomoles (fmol) to Nanograms (ng)

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

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

where N is the size of the DNA in bp. An example using *att*B-PCR product is described below.

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

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

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

## Materials Needed

You will need the following materials:

- Linearized expression clone (50-150 ng/µl in TE Buffer, pH 8.0, see page 78)
- pDONR™221 vector (supplied with Cat. no WFGE08, resuspend to 150 ng/µl in sterile water)
- Destination vector including pLenti6/V5-DEST (150 ng/µl in TE Buffer, pH 8.0)
- BP Clonase™ II enzyme mix (supplied with the kit, store at -20°C until immediately before use)
- LR Clonase™ II enzyme mix (supplied with the kit for standard destination vectors) or LR Clonase™ Plus enzyme mix (for MultiSite Gateway® destination vectors); 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)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate competent cells (One Shot® Stbl3™ Competent *E. coli* are supplied with Cat. no. WFGE08 for transforming the pLenti6/V5-DEST constructs)
- Sterile 0.5 ml microcentrifuge tubes

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction,
Continued

### 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, and the destination vector.

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

| Component | Sample | Positive Control |
|---|---|---|
| Linearized *att*B expression clone from page 78, (75-180 ng) | 1-7 µl | -- |
| pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid (50 ng/µl) | -- | 2 µl |
| pDONR™221 vector (150 ng/µl) | 1 µl | 1 µl |
| TE Buffer, pH 8.0 | to 8 µl | 5 µl |

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

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

   **Important:** Unlike the standard BP reaction, **do not** add Proteinase K to the samples. Instead, proceed immediately to the next step.
6. Depending on the destination vector that you will use, perform the appropriate LR reaction as described on the following pages using an aliquot of the BP reaction mix that contains the resulting **entry clone**.

   **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* to check the efficiency of the BP reaction. This also allows you to isolate entry clones for future use.

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

## LR Reaction for Standard Destination Vectors

Use this LR recombination reaction for standard destination vectors. For LR recombination reaction with MultiSite Gateway® destination vectors, see next page.

1. Transfer 3 µl from each of the BP reaction from Step 5, previous page to clean, sterile 0.5 ml microcentrifuge tubes.
2. Add the following components to the microcentrifuge tubes containing 3 µl BP reaction at room temperature and mix.

| Component | Sample | Positive Control |
|---|---|---|
| Standard destination vector (150 ng/µl) | 1 µl | 1 µl |
| TE Buffer, pH 8.0 | 4 µl | 4 µl |

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

   **Reminder:** Return LR Clonase™ II enzyme mix to -20°C immediately after use.
6. Incubate the reaction at 25°C for 2-4 hours.

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

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

   For **pLenti6/V5-DEST**, proceed to **Transforming One Shot® Stbl3™ Competent *E. coli***, next page.
9. Pick transformants and isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kit. Perform restriction analysis to select a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

## LR Reaction for MultiSite Gateway® Destination Vectors

Use this LR recombination reaction for MultiSite Gateway® destination vectors. For LR recombination reaction with standard destination vectors, see previous page.

1. Transfer 6 µl from each of the BP reaction from Step 5, page 83, to clean, sterile 0.5 ml microcentrifuge tubes.
2. Add the following components to the microcentrifuge tubes containing 6 µl BP reaction at room temperature and mix.

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

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

   **Reminder:** Return LR Clonase™ Plus enzyme mix to -20°C immediately after use.
6. Incubate the reaction at 25°C **overnight.**
7. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37ºC.
8. Transform an appropriate *E. coli* strain as recommended for your destination vector.

   **Note:** You may store the reaction at -20°C for up to 1 week before transformation, if desired.
9. Pick transformants and isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kit. Perform restriction analysis to select a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

## What You Should See

When using One Shot® Stbl3™ or TOP10 Chemically Competent *E. coli* for transformation, the LR recombination reaction should result in greater than 4,000 colonies if the entire LR reaction is transformed and plated.

*Continued on next page*

# Performing the Rapid BP/LR Recombination Reaction, Continued

## Confirming the Expression Clone

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

## Sequencing

Sequencing the expression construct is not required as transfer of the miRNA cassette from pcDNA™6.2-GW/EmGFP-miR miRNA vector into the destination vector preserves the orientation of the cassette. However, you may sequence your expression construct using appropriate sequencing primers, if desired.

## Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the expression clone in LB medium containing the appropriate antibiotic.

## The Next Step

Once you have obtained your new expression clone, we recommend that you test the clone by transfecting an appropriate mammalian cell line to perform transient RNAi analysis. Refer to the manual supplied with the destination vector to take advantage of the features of the new expression clone.

Once you have assessed the gene knockdown, we recommend that you validate the results using additional methods such as qPCR, Western analysis, immunohistochemistry, or any other functional assay.

A variety of BioModule™ Units that include qualified reagents and validated protocols are available from Invitrogen to perform validation experiments (page 160).

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

## Introduction

Follow the instructions in this section to transform the LR recombination reaction from a **lentiviral destination vector** into One Shot® Stbl3™ Chemically Competent *E. coli* (Box 19) included with the kit. The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is 1 x $10^8$ cfu/µg plasmid DNA.

## Materials Needed

You will need the following materials:

- LR recombination reaction (page 84)
- One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with the kit, Box 19; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (supplied with the kit, Box 19; warm to room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency; supplied with the kit, Box 19)
- 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

## 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 2-3 µl of the LR recombination reaction (from Step 7, page 84 or Step 7, page 141) 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 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 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*

# Transforming One Shot® Stbl3™ Competent *E. coli*, Continued

## What You Should See

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.

# Analyzing Transformants

## Introduction

We recommend analyzing the transformants using restriction digestion as described below, even if you have observed a successful LR recombination. This allows you to confirm the presence of the insert as well as ensure the absence of any aberrant lentiviral vector recombination (between the LTRs). Depending on the sequences, lentiviral vectors have a tendency to undergo aberrant recombination (recombine or delete within regions such as between the LTRs), which can occur from time to time, even during vector propagation.

## Materials Needed

You will need the following materials:

- Appropriate restriction enzymes
- LB medium containing 100 µg/ml ampicillin (page 133 for recipe)
- S.N.A.P.™ MidiPrep Kit or PureLink™ HQ Mini Plasmid Kit to isolate plasmid DNA (supplied with the kit)
- 1.2% E-Gel® agarose gels (supplied with the kit)
- TrackIt™ 1 Kb Plus DNA Ladder (supplied with the kit)
- TE Buffer

## Restriction Digest Analysis

This protocol allows you to quickly analyze plasmid DNA isolated using S.N.A.P.™ MidiPrep Kit from 2 transformants. If you wish to use a mini-prep kit for plasmid DNA isolation or screen more transformants, an alternate protocol is included on the next page.

1. Grow 2 ampicillin- and Blasticidin-resistant colonies overnight in LB medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using S.N.A.P.™ MidiPrep Kit (see page 93 for protocol).
3. Perform restriction analysis to confirm the presence of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.
4. Analyze the digests on 1.2% E-Gel® agarose gels (supplied with the kit) as follows:
   - To 10 µl of the digest, add 10 µl TE buffer and mix well.
   - Load 20 µl sample on a pre-run 1.2% E-Gel® agarose gels (see page 48 for pre-running protocol).
   - Mix 2 µl TrackIt™ 1 Kb Plus DNA Ladder with 18 µl deionized water and load 20 µl of the diluted ladder. Loading undiluted TrackIt™ DNA Ladder on an E-Gel® will result in loss of resolution.
   - Perform electrophoresis for 30 minutes using a E-Gel® PowerBase™ v.4 as described on page 48.
   - At the end of the run, remove the gel cassette from the power unit and analyze your results using a UV transilluminator. Example of results are shown on page 91.
5. Use the plasmid DNA from the positive clone for producing the lentivirus (page 105).

*Continued on next page*

# Analyzing Transformants, Continued

## Alternate Protocol

This alternate protocol allows you to screen more transformants using a mini-prep kit for plasmid DNA isolation.

1. Isolate plasmid DNA using PureLink™ HQ Mini Plasmid Kit (page 61).
2. Perform restriction digest analysis as described on the previous page.
3. Dilute 1 µl of the plasmid DNA from the positive clone to 1:500 in TE. Use 1 µl of this diluted DNA to retransform into One Shot® Stbl3™ Chemically Competent Cells as described on page 87.Plate approximately one-tenth of the transformation on LB plates containing 100 µg/ml ampicillin.
4. Pick colonies, grow starter culture, and isolate plasmid DNA using S.N.A.P.™ MidiPrep Kit as described on page 93 for use in lentivirus production (page 105).

## Sequencing

Sequencing the expression construct is not required as transfer of the miRNA cassette from pcDNA™6.2-GW/EmGFP-miR into the pLenti6/V5-DEST vector preserves the orientation of the cassette.

If you wish to sequence your pLenti6/V5 expression construct, we recommend using the following primers. Refer to the diagram on page 79 for the location of the primer binding sites in the expression vector.

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

**Note:** For your convenience, Invitrogen has a custom primer synthesis service. For more information, see our web site (www.invitrogen.com) or call Technical Service (page 160).

## 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.

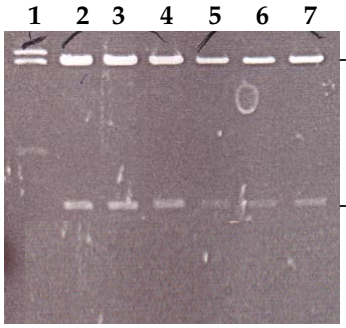
*Continued on next page*

### Example of Expected Results with pLenti6/V5-DEST

In this experiment, MAP2 (microtubule-associated protein 2) microRNA was cloned into pcDNA™6.2-GW/EmGFP-miR as described in this manual. The resulting expression clone and the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid (supplied with the kit) were each recombined with pLenti6/V5-DEST using the Rapid BP/LR Gateway® recombination reaction and transformed into One Shot® Stbl3™ Chemically Competent cells as described in this manual to produce lentiviral expression clones.

The transformants were subjected to restriction digestion using *Xho* I and *Spe* I, followed by analysis on a 1.2% E-Gel® agarose gel as described in this section.

**Results:** The gel analysis shows the correct digestion pattern indicating proper LR recombination with the lentiviral vector. No aberrant lentiviral vector recombination is observed.




**Lane 1**
Digest from pLenti6/V5-DEST

**Lanes 2-4**
Digest from pcDNA™6.2-GW/EmGFP-miR-neg control lentiviral expression clone

**Lanes 5-7**
Digest from pcDNA™6.2-GW/EmGFP-miR-MAP2 lentiviral expression clone

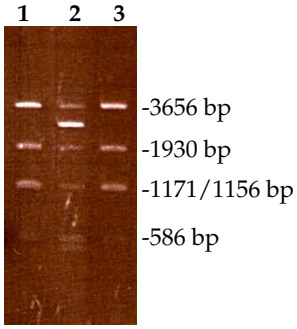
### Example of Expected Results with pLenti6/R4R2/V5-DEST

In this experiment, MAP2 (microtubule-associated protein 2) microRNA was cloned into pcDNA™6.2-GW/EmGFP-miR as described in this manual. The resulting expression clone and the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid (supplied with the kit) were each recombined with MultiSite pLenti6/R4R2/V5-DEST using the Rapid BP/LR Gateway® recombination reaction and transformed into One Shot® Stbl3™ Chemically Competent cells as described in this manual to produce lentiviral expression clones.

The transformants were analyzed by restriction digestion using *Xho* I and *Afl* II. The digests were analyzed on a 1.2% E-Gel® agarose gel as described in this section.

**Results:** The gel analysis shows the correct digestion pattern indicating proper LR recombination with the lentiviral vector for lanes 1 and 3. Lane 2 shows additional bands indicating aberrant recombination of the lentiviral vector.

**Lanes 1-3:** Digest from pcDNA™6.2-GW/EmGFP-miR-MAP2 lentiviral expression clone



# Lentivirus Production, Transduction, and RNAi Analysis

## Experimental Outline

The experimental steps necessary to generate replication-incompetent lentivirus that delivers the miRNA sequence of interest into dividing or non-dividing mammalian cells for RNAi analysis are outlined below. We recommend performing the steps as described below to achieve the best results.

| Step | Action | Page no. |
|---|---|---|
| 1 | Purify plasmid DNA from the lentiviral expression clone using the S.N.A.P.™ MidiPrep Kit. | 93 |
| 2 | Thaw and culture 239FT Cells to the required density. | 96 |
| 3 | Produce a lentiviral stock (containing the packaged pLenti6/V5 expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti6/V5-GW/miR expression construct into the 293FT Producer Cell Line. | 101 |
| 4 | Determine the titer of the lentiviral stock using Blasticidin selection or EmGFP detection. | 108 |
| 5 | Transduce the lentiviral construct into your mammalian cell to express the miRNA of interest. | 114 |
| 6 | Assay for target gene knockdown using qPCR, Western analysis, immunohistochemistry, or any other functional assay. | -- |

# Lentiviral Plasmid DNA Purification

## Introduction

Once you have generated your expression clone using pLenti6/V5-DEST, 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 lentiviral plasmid DNA using the S.N.A.P.™ MidiPrep Kit supplied with Cat. no. WFGE08.

**Important:** Do not use mini-prep plasmid DNA for transfection or PureLink™ HQ Mini Plasmid DNA Purification Kit for lentiviral plasmid DNA isolation.

## Experimental Outline

To purify plasmid DNA from pLenti6/V5-DEST construct, you will:

1. Lyse cells using the Lysis Buffer.
2. Precipitate protein and genomic DNA. Filter the lysate/precipitate through Column A.
3. Add Binding Buffer to the flowthrough and apply to Column B.
4. Wash the bound plasmid and dry the resin by centrifugation.
5. Elute the plasmid DNA with TE buffer.


The Binding Buffer and Wash Buffer contain a chaotropic salt. Use gloves and protective eye wear when handling these solutions.

## Materials Needed

You will need the following materials:

- S.N.A.P.™ MidiPrep Kit (Box 23 supplied with Cat. no WFGE08)
- 100 ml overnight culture
- 240 ml 95% ethanol for diluting 4X Final Wash
- 500 ml sterile bottle for diluting 4X Final Wash
- Sterile 50 ml conical tubes
- Centrifuge with rotor and rotor adapters for 50 ml conical tubes
- TE Buffer

## Before Starting

- Resuspend the entire contents of the RNase A tube (6 mg) in 1 ml of Resuspension Buffer. Add the resulting solution to the remaining Resuspension Buffer. Resuspension Buffer containing RNase A should now be stored at +4°C.
- Add the 80 ml 4X Final Wash to 240 ml 95% ethanol to make 320 ml 1X Final Wash. Store in a 500 ml sterilized bottle.
- Check the Lysis Buffer for a white precipitate. If present, place the buffer in a 37°C water bath for 5 minutes or until the solution clears.

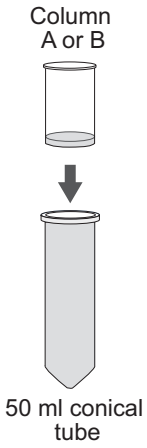
*Continued on next page*

# Lentiviral Plasmid DNA Purification, Continued

**Important**

Use the plasmid DNA isolation protocol described below to maximize the yield from lentiviral plasmids. We recommend using 50-100 ml overnight culture and process the 100 ml bacterial culture as two samples until DNA binding and then process the column as a single sample to obtain the best results.

## Lysis and Removal of Precipitate




All centrifugations are at room temperature except as noted.

1. Pick 2 colonies and culture in LB medium containing 100 μg/ml ampicillin for 6-8 hours to obtain a starter culture. Transfer the starter culture to 100 ml LB medium containing 100 μg/ml ampicillin and culture overnight.
2. Transfer 100 ml overnight bacterial culture into two sterile 50 ml conical tubes.
3. Centrifuge the culture at 4000 x g for 5-10 minutes at 4°C to pellet the cells. Discard the medium. Process each tubes as two samples.
4. Resuspend the cell pellet in each tube in 4 ml Resuspension Buffer with RNase by vortexing or gently pipetting up and down.
5. Add 4 ml Lysis Solution to each tube and mix by inverting gently 5-6 times. Incubate for 3 minutes at room temperature.
6. Add 4 ml Precipitation Salt to each tube and invert gently 6-8 times. Incubate 5 minutes in ice. Invert tubes twice during incubation to ensure even formation of precipitate within the solution. Do not centrifuge.
7. During the incubation, label two, sterile 50 ml conical tubes "A" and one sterile 50 ml tube "B". Place one S.N.A.P.™ MidiPrep Column A (Filtering) into each 50 ml conical tube labeled "A", and one S.N.A.P.™ MidiPrep Column B (Binding) into the 50 ml conical tube labeled "B" (see adjacent figure).
8. After incubation, transfer each solution from Step 6 onto each Column A and centrifuge for 5 minutes at 3,000 x g. Discard Column A.

   *Save the filtrate containing the plasmid DNA.*
9. Proceed to **Plasmid Binding,** next page.

*Continued on next page*

# Lentiviral Plasmid DNA Purification, Continued

## Plasmid Binding

1. To each filtrate from Step 8, previous page, add 12 ml Binding Buffer and mix by gently inverting twice.
2. Transfer the solution from one filtrate to Column B in a 50 ml conical tube.
3. Centrifuge for 2 minutes at 1,000 x g. Discard the flowthrough.

   *The plasmid DNA from one sample is now bound to Column B.*
4. Transfer the solution from the second filtrate to Column B in a 50 ml conical tube from Step 3.
5. Centrifuge for 2 minutes at 1,000 x g. Discard the flowthrough.

   *The plasmid DNA from both samples is now bound to Column B.*
6. Add 5 ml Wash Buffer to the column and centrifuge for 1 minute at 2,000 x g. Discard the flowthrough.
7. Add 5 ml 1X Final Wash Buffer to the column and centrifuge for 2 minutes at 2,000 x g.
8. Add 10 ml 1X Final Wash Buffer to the column and centrifuge for 2 minutes at 2,000 x g. Discard the flowthrough.
9. Centrifuge the Column B at >4,000 x g for 5 minutes to dry the resin.

## Plasmid Elution

1. To elute the plasmid DNA, transfer Column B to a new, sterile 50 ml conical tube and add 750 µl TE buffer directly to column resin.
2. Incubate for 3 minutes at room temperature.

   **Note:** Do not elute in less than 750 µl. Plasmid recovery will decrease.
3. Centrifuge for 5 minutes at >4,000 x g. The plasmid DNA is now eluted from Column B.
4. Remove and discard the column.
5. Determine plasmid DNA yield and purity as described on page 63. Ensure that the $A_{260}/A_{280}$ of the purified plasmid DNA is >1.8 to obtain good transfection efficiency.

# Growth and Maintenance of the 293FT Cell Line

## Introduction

The human 293FT Cell Line is supplied with the BioModule™ BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin®.

General guidelines for handling the cells and protocols for thawing, sub culturing, and freezing the cells are included in this section.


Handle as potentially biohazardous material under at least Biosafety Level 2 containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Material Safety Data Sheet before handling.

## General Cell Handling

Follow the general guidelines below to grow and maintain 293FT cells.

- **Make sure that all solutions and equipment that come in contact with the cells are sterile.** Always use proper sterile technique and work in a laminar flow hood.
- Before starting experiments, be sure to establish the cells and have some frozen stocks. We recommend using early-passage cells for your experiments.
- For general maintenance of cells, pass 293FT cells when they are > 80% confluent. Avoid overgrowing cells before passaging.
- Maintain 293FT cells in complete medium containing 500 µg/ml Geneticin®.
- Use trypan blue exclusion to determine cell viability. Log phase cultures should be >90% viable.
- When thawing or subculturing cells, transfer cells into medium warmed to room temperature.
- Cells should be at the appropriate confluence and at greater than 90% viability prior to transfection.

## Materials Needed

You will need the following materials:

- 15 ml sterile, conical tubes
- Appropriate sized tissue culture flasks and pipettes
- Complete medium (next page)
- 50 mg/ml Geneticin®
- Phosphate-Buffered Saline (PBS; Invitrogen, Catalog no. 10010-023)
- Reagents for counting cells
- Trypsin/versene (EDTA) solution or other trypsin solution
- Freezing Medium (next page)
- Table-top centrifuge
- Cryovials (if needed)

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# Growth and Maintenance of the 293FT Cell Line, Continued

## Media for 293FT Cells

The table below lists the recommended complete medium, freezing medium, and antibiotic concentration required to maintain and culture the 293FT Cell Line.

**Note:** FBS does not need to be heat-inactivated for use with the 293FT Cell Line.

The cell culture media are available from Invitrogen individually or you may purchase the BioModule™ Lentiviral 293 Unit from Invitrogen (page 160) that contains all necessary media, antibiotics, serum, and Trypan Blue for growth, maintenance, and checking cell viability of 293 FT cells.

| Complete Medium | [Antibiotic] | Freezing Medium |
|---|---|---|
| D-MEM (high glucose)<br>10% fetal bovine serum (FBS)<br>0.1 mM MEM Non-Essential Amino Acids (NEAA)<br>2 mM L-glutamine<br>1 mM MEM Sodium Pyruvate<br>1% Pen-Strep (optional) | 500 µg/ml Geneticin® | 90% complete medium<br>10% DMSO |

## Preparing Medium

Prepare the complete D-MEM medium containing 10% FBS supplemented with 0.1 mM MEM Non-Essential Amino Acids and 2 mM L-glutamine as described below using the BioModule™ Lentiviral 293 Unit from Invitrogen (page 160):

Perform all steps in a tissue culture hood under sterile conditions.

1. Remove 100 ml D-MEM from 1 L D-MEM bottle and replace with 100 ml FBS.
2. To the bottle of medium, add the following:

| | |
|---|---|
| 200 mM L-Glutamine (100X) | 10 ml |
| 10 mM MEM Non-Essential Amino Acids (100X) | 10 ml |
| 100 mM MEM Sodium Pyruvate (100X) | 10 ml |
| *Optional:* Penicillin-Streptomycin (100X) | 10 ml |

3. Filter sterilize the medium using 0.45 µm filtration device.
4. Store the complete medium at 4°C until use. The medium is stable for 6 months at 4°C (avoid introducing any contamination into the medium).
5. To an aliquot of the complete medium, add Geneticin® to prepare complete medium with 500 µg/ml Geneticin®.

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# Growth and Maintenance of the 293FT Cell Line, Continued

## Thawing Cells

The 293FT Cell Line is supplied in a vial containing 3 x $10^6$ cells in 1 ml of Freezing Medium. Store frozen 293FT cells in liquid nitrogen until ready to use.

Use the following procedure to thaw 293FT cells to initiate cell culture. Thaw cells in prewarmed, complete medium **without** Geneticin®.

1. Remove the vial of frozen cells from liquid nitrogen and thaw quickly in a 37°C water bath.
2. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol, and transfer the cells to a sterile 15 ml tube containing PBS. Briefly centrifuge the cells at 150-200 x g and resuspend the cells in 2 ml complete medium without Geneticin®.
3. Transfer the cells to T-75 $cm^2$ flask containing 10 ml of complete medium without Geneticin®.
4. Incubate the flask overnight at 37°C for allowing the cells to attach to the bottom of the flask.
5. The next day, aspirate off the medium and replace with fresh, complete medium containing 500 µg/ml Geneticin®.
6. Incubate the cells and check them daily until the cells are 80-90% confluent.
7. Proceed to **Subculturing Cells**, next page.


We recommend subculturing cells for a minimum of 3 passages after thawing before use in other applications.

## Subculturing Conditions

Use the following recommended conditions to subculture 293FT cells. For a procedure to subculture cells, see below.

| Parameter | Recommended Condition |
|---|---|
| Cell density | > 5 x $10^5$ viable cells/ml (> 80% confluent) |
| Culture vessel | T-75 $cm^2$ to T-162 $cm^2$ disposable sterile T-flasks. Dilute cells in a total working volume of 15-20 ml for T-75 $cm^2$ flasks and 40-50 ml for T-162 $cm^2$ flasks |
| Seeding density | 2-5 x $10^4$ viable cells/$cm^2$ |
| Incubation conditions | 37°C incubator with a humidified atmosphere of 5-10% $CO_2$ in air; loosen caps to allow for oxygenation/aeration |

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# Growth and Maintenance of the 293FT Cell Line, Continued

## Determining Viability and Cell Density

Follow the procedure below to determine viable and total cell counts using the trypan blue exclusion method. Trypan Blue Stain (0.4%) is included with the BioModule™ Lentiviral 293 Unit (page 160).

Trypan blue is a vital dye. The chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, cells that exclude the dye are viable while cells that absorb the dye (blue cells) are non-viable.

1. Transfer a small aliquot of the cell suspension to a microcentrifuge tube and dilute the cells such that the total number of cells counted will not be less than 100 or more than 1,000.
2. To 1 ml of the diluted cell suspension, add 100 µl Trypan Blue Stain (0.4%) solution. Gently aspirate to mix.
3. Record the dilution factor. The dilution factor equals the total volume (amount of cell suspension and amount of trypan blue) divided by the amount of cell suspension.
4. Incubate the cells with the trypan blue solution for 1-2 minutes.
5. Count all cells (including the blue cells) using a Coulter Counter or manually using a hemocytometer chamber.
6. To calculate the total cells per ml in suspension, multiply the total count by the dilution factor.
7. To determine the viability, count only the blue cells. Calculate the % viability:

   [1.00 - (Number of blue cells ÷ Number of total cells)] x 100

Cell viability should be at least 95% for healthy log-phase cultures.

## Subculturing Cells

Use this procedure to subculture 293FT cells grown in a T-75 $cm^2$ flask. If you are using other-sized flasks, scale the reagent volumes accordingly.

1. Remove all medium from the flask and wash the cells once with 10 ml PBS to remove excess medium and serum. Serum contains inhibitors of trypsin.
2. Add 2 ml of trypsin/versene (EDTA) solution to the monolayer and incubate 1-5 minutes at room temperature until cells detach. Check the cells under a microscope and confirm that most of the cells have detached. If cells are still attached, incubate a little longer until most of the cells have detached.
3. Add 8 ml complete medium containing Geneticin® and transfer the cell suspension to a 15 ml sterile, conical tube.
4. Determine viable and total cell counts (see procedure above).
5. Seed cells at the recommended density (see table on previous page), diluting in pre-warmed complete medium containing 500 µg/ml Geneticin®. Incubate flasks as recommended (see table on previous page).
6. Maintain cells as adherent monolayer cultures in complete medium containing 500 µg/ml Geneticin®.
7. For the transfection protocol, you will need 6 x $10^6$ 293FT cells for each sample (page 105).

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# Growth and Maintenance of the 293FT Cell Line, Continued

## Freezing Cells

Once you have established the cells, we recommend freezing some cells for future use as described below.

- Freeze cells at a density of **at least** 3 x $10^6$ viable cells/ml.
- Use a freezing medium composed of 90% complete medium and 10% DMSO. Prepare freezing medium **immediately before use**. Filter-sterilize the freezing medium and store at +4°C until use. Discard any remaining freezing medium after use.

Before starting, label cryovials and prepare freezing medium (see above). Keep the freezing medium on ice.

1. Culture the desired quantity of 293FT cells to 70-90% confluency.
2. Remove the cells from the tissue culture flask(s) following Steps 1-3, **Subculturing Cells**, page 99.
3. Determine viable and total cell counts (see procedure on previous page) and calculate the volume of freezing medium required to yield a final cell density of ≥ 3 x $10^6$ cells/ml.
4. Prepare the required volume of freezing medium (see above).
5. Centrifuge the cells suspension (from Step 2) at 250 x g for 5 minutes in a table top centrifuge at room temperature. Carefully aspirate off the medium and resuspend the cell pellet in the pre-determined volume of chilled freezing medium.
6. Dispense aliquots of this suspension (frequently mixing to maintain a homogeneous cell suspension) into cryovials according to manufacturer's specifications.
7. Freeze cells in an automated, controlled-rate freezing apparatus or using a manual method following standard procedures. For ideal cryopreservation, the freezing rate should be decrease at 1°C per minute.
8. Transfer vials to liquid nitrogen storage.

**Note:** You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in **Thawing Cells**, page 98.

## Transfection Methods

The 293FT Cell Line is generally amenable to transfection using standard methods including lipid-mediated transfection with Lipofectamine™ 2000. See page 105 for transfection protocol.

## Transient or Stable Transfection

The 293FT Cell Line may be transiently transfected with any plasmid. Make sure that cells are healthy at the time of plating. Overgrowth of cells prior to passaging can compromise their transfection efficiency.

293FT cells can be used as hosts to generate a stable cell line expressing your gene of interest from most plasmids. The introduced plasmid must contain a selection marker other than neomycin resistance. Stable cell lines can then be generated by transfection and dual selection with Geneticin® and the appropriate selection agent.

**Note:** Since 293FT cells stably express the SV40 large T antigen, we **do not** recommend generating stable cell lines with plasmids that contain the SV40 origin of replication.

# Producing Lentivirus in 293FT Cells

## Introduction

Before you can create a stably transduced cell line expressing your miRNA, you need to produce a lentiviral stock (containing the packaged pLenti6/V5 expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti6/V5-GW/miR expression construct into the 293FT Producer Cell Line. The following section provides protocols and instructions to generate a lentiviral stock.

## Experimental Outline

To produce lentivirus in 293FT Cells, you will:

1. Grow the 293FT Cells to obtain 6 x $10^6$ 293FT cells for each sample (page 96).
2. Prepare plasmid DNA of your expression clone (page 93).
3. Cotransfect the ViraPower™ Packaging Mix and pLenti6/V5-GW/miR expression plasmid DNA into 293FT Cells using Lipofectamine™ 2000.
4. Harvest virus-containing supernatants 48-72 hours post-transfection.


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

- Make sure that cells are greater than 90% viable.
- Subculture and maintain cells as recommended on page 96. Do not allow cells to overgrow before passaging. You will need 6 x $10^6$ 293FT cells for each sample.
- Use cells that have been subcultured for less than 20 passages.

## ViraPower™ Packaging Mix

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

The amount of the packaging mix (195 µg) and Lipofectamine™ 2000 Reagent (0.75 ml) supplied in the BioModule™ BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System is sufficient to perform 20 cotransfections in 10 cm plates using the recommended protocol on page 105.

To use the ViraPower™ Packaging Mix, resuspend the contents of one tube (195 µg) in 195 µl of sterile water to obtain a 1 µg/µl stock.

**Note:** ViraPower™ Packaging Mix is available separately from Invitrogen (page 160) or as part of the ViraPower™ Bsd Lentiviral Support Kit (page 160).

*Continued on next page*

### 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 on page 36.
- Use the pcDNA™6.2-GW/EmGFP-miR-*lacZ* expression control to generate the lentiviral construct with pLenti6-V5-DEST vector using the Rapid BP/LR recombination reaction as described in this manual.
- Use the resulting lentiviral expression construct, pLenti6/V5-GW/EmGFP-miR-*lacZ,* to generate a miR control lentiviral stock (*lacZ* targeting miRNA).

Once generated, the miR control lentivirus may be transduced into mammalian cell lines (page 117) to express an miRNA targeted to the human *lacZ* gene, and may be used as a control for the RNAi response in these cell lines.

### pLenti6/V5-GW/*lacZ* Positive Control

A pLenti6/V5-GW/*lacZ* positive control vector is included with the pLenti6/V5-DEST vector for use as an expression control in the ViraPower™ Lentiviral Expression System. The β-galactosidase is expressed as a C-terminally tagged fusion protein that may be easily detected by western blot or functional assay. For details on the vector, see page 149.

To propagate and maintain the control plasmid:

1. Resuspend the vector in 10 µl of sterile water to prepare a 1 µg/µl stock solution.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like Stbl3™, TOP10, DH5α™-T1$^R$, or equivalent. Use 10 ng of plasmid for transformation.
3. Select transformants on LB agar plates containing 100 µg/ml ampicillin (for **Stbl3™** cells) or LB agar plates containing 100 µg/ml ampicillin and 50 µg/ml Blasticidin (for **TOP10 or DH5α**).
4. Prepare a glycerol stock of a transformant containing plasmid for long-term storage. Propagate the plasmid in LB containing 100 µg/ml ampicillin.
5. Use the pLenti6/V5-GW/*lacZ* positive control to generate a control lentiviral stock (expressing the LacZ protein).
6. Use the pLenti6/V5-GW/*lacZ* lentiviral control and the pLenti6/V5-GW/EmGFP-miR-*lacZ* lentiviral control in cotransduction experiments as a positive control for lentiviral induced RNAi analysis in your system (see page 115 for details).

*Continued on next page*

# Producing Lentivirus in 293FT Cells, Continued

## Materials Needed

You will need the following materials:

- pLenti6/V5-GW/miR expression construct (0.1-3.0 µg/µl in TE Buffer, pH 8.0). Resuspend the purified pLenti6/V5-GW/miR expression plasmid in 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.
- Positive controls (see previous page to generate positive controls; resuspend in sterile water to a concentration of 1 µg/µl)
- ViraPower™ Packaging Mix (supplied with the kits; resuspend in 195 µl of sterile water to a concentration of 1 µg/µl)
- 293FT cells (page 96), you will need 6 x $10^6$ 293FT cells for each sample.
- Lipofectamine™ 2000 transfection reagent (supplied with the kit; store at +4°C and mix gently before use)
- Opti-MEM® I Reduced Serum Medium (supplied with the kit, pre-warmed; see page 65)
- Fetal bovine serum (FBS)
- Complete growth medium for 239FT Cell (see page 97 for a recipe).
- Sterile, 10 cm tissue culture plates (one each for lentiviral construct and controls)
- Sterile, tissue culture supplies
- 5 and 15 ml sterile, capped, conical tubes
- Cryovials

## Recommended Transfection Conditions

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

| Condition | Amount |
|---|---|
| Tissue culture plate size | 10 cm (one per lentiviral construct) |
| Number of 293FT cells to transfect | 6 x $10^6$ cells (see **Recommendation** on page 101 to prepare cells for transfection) |
| Amount of ViraPower™ Packaging Mix | 9 µg (9 µl of 1 µg/µl stock) |
| Amount of pLenti6/V5-GW/miR expression plasmid | 3 µg |
| Amount of Lipofectamine™ 2000 Reagent to use | 36 µl |

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

*Continued on next page*


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, allow the cells to attach, and transfect overnight (see details on the next page).

Using this procedure, we consistently obtain lentiviral stocks with titers that are 3 to 4-fold higher than lentiviral stocks generated using the traditional Lipofectamine™ 2000 transfection procedure (i.e. plating cells first followed by transfection with DNA:Lipofectamine™ 2000 complexes). You may use the traditional Lipofectamine™ 2000 transfection procedure, if desired, but keep in mind that the viral titer obtained may be lower (see **Alternative Transfection Procedure**, page 106).

*Continued on next page*

## Transfection Procedure

Follow the procedure below to cotransfect 293FT cells. We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help evaluate your results.

1. **For each transfection sample,** prepare DNA-Lipofectamine™ 2000 complexes as follows:
   a. In a sterile 5 ml tube, dilute 9 µg ViraPower™ Packaging Mix and 3 µg pLenti6/V5-GW/miR expression plasmid DNA (12 µg total) in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 5 ml tube, mix Lipofectamine™ 2000 gently before use, then dilute 36 µl in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine™ 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipid 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 include antibiotics in 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 media containing the DNA-Lipofectamine™ 2000 complexes and replace with complete culture medium containing sodium pyruvate (*i.e.* D-MEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate).

   **Note:** Expression of the VSV glycoprotein causes 293FT cells to fuse, resulting in the appearance of multinucleated syncitia which is normal and does not affect lentivirus production. See page 107 for an example of expected results after transfection.
6. Harvest virus-containing supernatants 48-72 hours post-transfection by removing medium to a 15 ml sterile, capped, conical tube.

   **Note:** Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours post-transfection.

   **Caution:** Remember that you are working with infectious virus at this stage. See pages 28 and 110.
7. Centrifuge at 3000 rpm for 5 minutes at +4°C to pellet cell debris. Perform filtration step, if desired (see **Note** on the next page).
8. Pipet viral supernatants into cryovials in 1 ml aliquots. Store viral stocks at -80°C.

*Continued on next page*

## Alternative Transfection Procedure

An alternative transfection procedure is provided below to cotransfect 293FT cells. Note that use of this procedure generally results in production of lentiviral stocks with a slightly lower titer that those produced when using the **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 x $10^6$ cells in 10 ml of growth medium containing serum).
2. On the day of transfection, remove the culture medium from the 293FT cells and replace with 5 ml of growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not include antibiotics in the medium.**
3. Prepare DNA-Lipofectamine™ 2000 complexes as instructed in the **Transfection Procedure**, Step 1, previous page.
4. Add the DNA-Lipofectamine™ 2000 complexes dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a $CO_2$ incubator.
5. Follow Steps 5-8 as instructed in the **Transfection Procedure**, previous page.

**Note**

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (see Step 7, previous page) to remove any remaining cellular debris. We recommend using Millex-HV 0.45 µm PVDF filters (Millipore, Catalog no. SLHVR25LS) for filtration.

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

## Long-Term Storage

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

## Scaling Up Virus Production

It is possible to scale up the cotransfection experiment to produce a larger volume of lentivirus, if desired. For example, we have scaled up the cotransfection experiment from a 10 cm plate to a T-175 $cm^2$ flask and harvested up to 30 ml of viral supernatant. If you wish to scale up your cotransfection, remember that you will need to 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.

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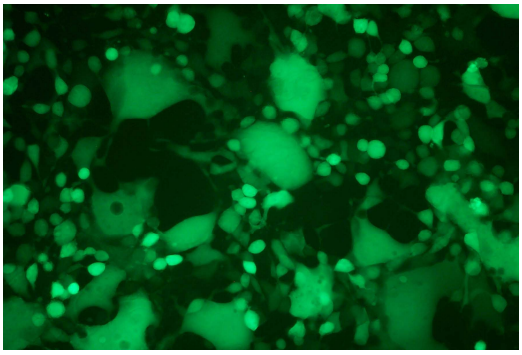
## Example of Expected Results

In this experiment, 293FT Cells were co-transfected with ViraPower™ Packaging Mix and a lentiviral expression plasmid expressing the cycle 3 GFP gene (pLenti6/V5-GW/cycle 3 GFP) using the transfection protocol on page 105 with Lipofectamine™ 2000 Reagent.

48 hours post-transfection, the cells were subjected to fluorescence microscopy to visualize cycle GFP fluorescence using suitable filters.

**Result:** Cycle 3 GFP expression is observed in most cells indicating an efficient transfection. Multinucleated syncitia (fused producer cells) are seen due to VSV glycoprotein expression.



# Titering Your Lentiviral Stock

## Introduction

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

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

Guidelines and protocols are provided in this section.

## Titering Methods

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

- Blasticidin selection (usually takes 2 weeks to determine the titer)
- EmGFP detection (usually takes 4 days post-transduction to determine the titer)

## Experimental Outline

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

1. Prepare 10-fold serial dilutions of your lentiviral stock.
2. Transduce the different dilutions of lentivirus into the mammalian cell line of 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 the 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, we recommend titering or re-titering your lentiviral stock prior to use in an RNAi experiment.
- Number of freeze/thaw cycles. Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- Improper storage of your lentiviral stock. Lentiviral stocks should be aliquotted and stored at -80°C.

*Continued on next page*

### Selecting a Cell Line

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

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

We generally use the HT1080 human fibrosarcoma cell line (ATCC, Catalog 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. If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line.

### Blasticidin Selection

The pLenti6/V5-GW/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.

If you are using the BioModule™ BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System, Blasticidin is supplied with the kit. Blasticidin is also available separately from Invitrogen or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 161 for ordering information). For more information about how to prepare and handle Blasticidin, and determine the Blasticidin sensitivity, refer to page 134.

### Using Polybrene® During Transduction

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

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# Titering Your Lentiviral Stock, Continued

## Preparing and Storing Polybrene®

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

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

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

## Materials Needed

You will need the following materials:

- pLenit6/V5-GW/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
- Blasticidin (10 mg/ml stock) and crystal violet (Sigma, Catalog 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 27 for filters), if you are using EmGFP titering method
- PBS (Cat. no. 10010-023)

## Preparing Mammalian Cells

Initiate your mammalian cell line of choice that will be used for titering. Grow 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.
- Treat used pipets, pipette tips, and other tissue culture supplies with bleach and dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

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# Titering Your Lentiviral Stock, Continued

## Transduction and Titering Procedure

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

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

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

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

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

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

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

*Continued on next page*

### Preparing Cells for Flow Cytometry

If you have used EmGFP titering method, prepare cells for flow cytometry according to the established protocols in use at your flow cytometry facility. Refer to page 27 for spectral characteristics of EmGFP. The steps below provide general guidelines, and other methods may be suitable.

1. At day 4 post-transduction, dissociate the cells from the plate by using trypsin or cell dissociation buffer.
2. Spin the cells at low speed to remove residual media components and resuspend the cell pellet in flow cytometry buffer such as calcium/magnesium free PBS with 1% FBS at the required density for analysis on your flow cytometer. Fixing the cells is not necessary for analysis, but may be done, if desired. **Note:** To fix your cells before flow cytometry, use 2% formaldehyde or paraformaldehyde in calcium/magnesium free PBS. However, these fixatives may increase autofluorescence of cells, thus it is critical to include fixed, mock-transduced cells as a negative control for flow cytometry.
3. Use the mock-transduced cells and the lowest dilution of virus (*i.e.* $10^{-2}$) as the negative and positive samples, respectively, to set up the parameters of your flow cytometer.

### Calculating Lentiviral Titer

Calculate the EmGFP lentivirus titers from the dilutions at which the percentage of EmGFP-positive cells fall within the range of 1-30% (Sastry *et al.*, 2002; White *et al.*, 1999). 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:

**[F × C/V] × D**

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

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

V = volume of inoculum in ml

D = lentivirus dilution

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

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

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

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

The lentiviral titer for this example is $8.8 \times 10^7$ TU/ml.

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# Titering Your Lentiviral Stock, Continued

## What You Should See

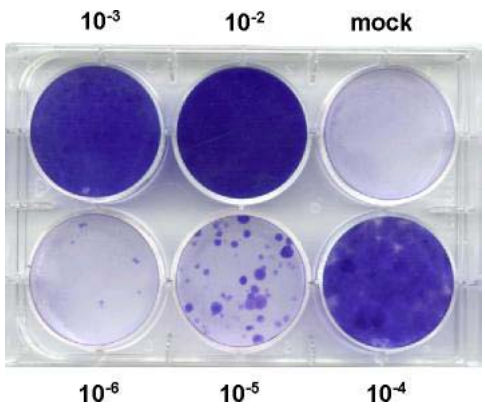
When titering pLenti6/V5 lentiviral stocks using HT1080 cells, we generally obtain titers ranging from $5 \times 10^5$ to $2 \times 10^7$ transducing units (TU)/ml.

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

**Note:** If the titer of your lentiviral stock is less than $1 \times 10^5$ TU/ml, we recommend producing a new lentiviral stock. See page 101 and the **Troubleshooting** section, page 121 for more tips and guidelines to optimize your viral yield.

## Example of Expected Results Using Blasticidin Titering Method

In this experiment, a pLenti6 lentiviral stock was generated using the protocol on page 105. 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

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral construct into your mammalian cell line to express the miRNA 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 miRNA in transient transfections

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

## Transient vs. Stable Expression

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

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

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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 134. If you titered your lentiviral construct in the same mammalian cell line that you are using to generate a stable cell line, then you may use the same concentration of Blasticidin for selection that you used for titering.

### Multiplicity of Infection (MOI)

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

### Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type; see **Note** below), its transduction efficiency, and the nature of your target gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, we recommend using 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 that will be used for transduction. Grow the cells in the appropriate medium. Cells should be >95% viable.

### Positive Controls

If you have generated two positive control lentiviral constructs (pLenti6/V5-GW/EmGFP-miR-*lacZ* control and pLenti6/V5-GW/*lacZ* control constructs) as described on page 102, you may use the controls in cotransduction experiments to verify the lentiviral induced RNAi response in mammalian cells.

For cotransductions, use a 3:1 MOI ratio of pLenti6/V5-GW/miR-*lacZ* to pLenti6/V5-GW/*lacZ* expression clone. For expected results, see page 119.

The β-galactosidase protein expressed from the pLenti6/V5-GW/*lacZ* control lentiviral construct is approximately 121 kDa in size. You may assay for β-galactosidase expression by western blot analysis using β-gal Antiserum (Catalog no. R901-25), activity assay FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Catalog no. F-2905), or by staining the cells for activity using the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

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**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

You will need the following materials before starting:

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

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## Transduction Procedure

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

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

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

*Continued on next page*

**Note**

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of target gene knockdown from different Blasticidin-resistant clones. We recommend testing at least 5 Blasticidin-resistant clones and selecting 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 our Web site (www.invitrogen.com/lux). A variety of BioModule™ Units that include qualified reagents and validated protocols are available from Invitrogen to perform validation experiments (page 160).

You can also detect EmGFP fluorescence using fluorescence microscopy or flow cytometry (see page 69).

### What You Should See

When performing RNAi studies using pLenti6/V5 lentiviral constructs, we generally observe inhibition of gene expression within 48-120 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 114. Note that 100% gene knockdown is generally not observed, but > 80% is possible with optimized conditions

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

# Expected Results for Lentiviral Expression

## Example of Expected Results

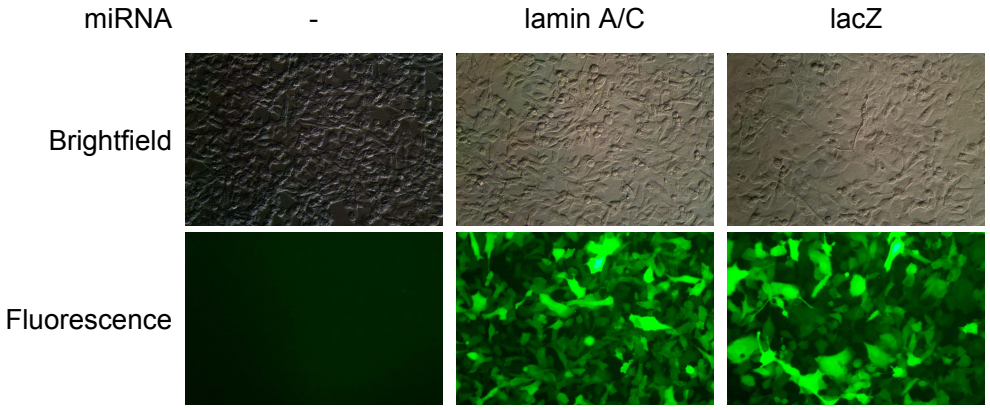
In this experiment, a double-stranded miR-*lac*Z control oligo (directed towards exogenous LacZ) and a double-stranded miR-lamin oligo (directed towards endogenous lamin) were each cloned into pcDNA6.2™-GW/EmGFP-miR expression vector using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit. The miR-*lac*Z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

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

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

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

**Figure A**

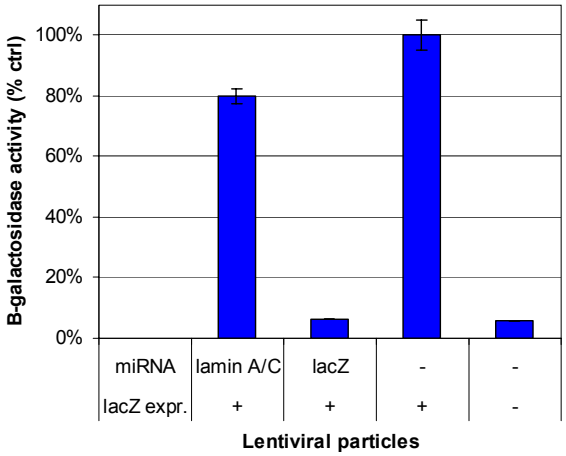



Cell lysates were then prepared from duplicate wells 48 hours (*i.e.* 2 days) after transduction. β-galactosidase activity was determined from equivalent amounts of cell lysate using the FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Cat. no. F-2905) from Invitrogen. Results are shown in figure B, next page.

*Continued on next page*

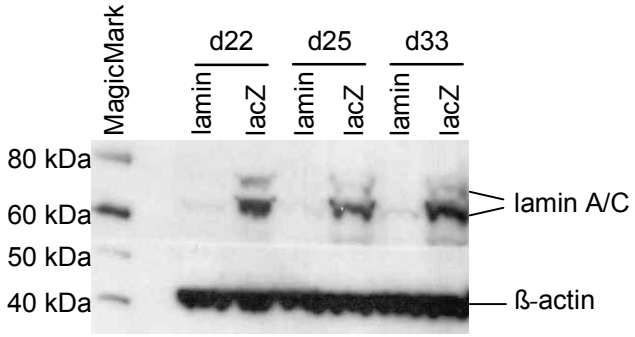
**Example of Expected Results,** continued

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




HeLa cells were transduced with the *lacZ* and lamin miRNA lentiviruses and stable cell lines were generated using Blasticidin selection as described in this manual. Cell lysates were prepared from stably transduced cells after 22-33 days and subjected to western blot analysis. The western blot was cut in half and one half was probed with Anti-Lamin A/C Antibody (1:1000 dilution, BD Biosciences, Cat. no. 612162) and the other half was probed with Anti-β-Actin Antibody (1:5000 dilution, Abcam, Cat. no. ab6276). The blot was developed using WesternBreeze® Chemiluminescent Kit available from Invitrogen. Western blot results are shown in figure C, below. The results show substantial decrease in the lamin A/C protein isoforms (molecular weight of lamin A is 70 kDa and lamin C is 65 kDa) even 33 days post-transduction indicating the reliable and stable transduction of lamin-directed miRNA in lentiviral system.

**Figure C**



# Troubleshooting

## Introduction

Review the information in this section to troubleshoot your expression, lentiviral expression, and RNAi experiments.

## Annealing Reaction

The table below lists some potential problems and possible solutions that may help you troubleshoot the annealing reaction.

| Problem | Reason | Solution |
|---|---|---|
| Weak band representing ds oligo observed on an agarose gel | Single-stranded oligos designed incorrectly | Verify that the sequence of the bottom strand oligo is complementary to the sequence of the top strand oligo. If not, re-synthesize the bottom strand oligo. |
| | Allowed oligos to cool at +4°C instead of room temperature during annealing procedure | After heating to 95°C, anneal the oligos by setting the microcentrifuge tube at room temperature for 5-10 minutes (see the procedure on page 44). |
| | Did not anneal equal amounts of top and bottom strand oligo | Anneal equal amounts of the top and bottom strand oligo using the procedure on page 44. |
| No band representing ds oligo observed on an agarose gel | Single-stranded oligos designed incorrectly | Verify that the sequence of the bottom strand oligo is complementary to the sequence of the top strand oligo. If not, re-synthesize the bottom strand oligo. |
| | Used the wrong single-stranded oligos | Make sure that you mix single-stranded oligos with complementary sequence. |

## Ligation and Transformation Reactions

The table below lists some potential problems and possible solutions that may help you troubleshoot the ligation and transformation procedures.

| Problem | Reason | Solution |
|---|---|---|
| Few spectinomycin-resistant colonies obtained on the selective plate | Single-stranded oligos designed incorrectly | Make sure that each single-stranded oligo contains the 4 nucleotides on the 5′ end required for cloning into pcDNA™6.2-GW/EmGFP-miR:<br>Top strand oligo: include TGCT on the 5′ end.<br>Bottom strand oligo: include CCTG on the 5′ end. |
| | ds oligos were degraded | Store the 10 nM ds oligo stock in 1X Oligo Annealing Buffer.<br>Avoid repeated freeze/thaw cycles. Aliquot the 10 nM ds oligo stock and store at -20°C. |

*Continued on next page*

### Ligation and Transformation Reactions, continued

| Problem | Reason | Solution |
|---|---|---|
| Few spectinomycin-resistant colonies obtained on the selective plate, continued | ds oligos stored incorrectly | Store the ds oligo stocks at -20°C. |
| | 500 nM ds oligo stock solution diluted into water instead of 1X Oligo Annealing Buffer | To dilute the 50 µM ds oligo reaction:<br>Dilute the 50 µM stock 100-fold into DNase/RNase-free water to generate a 500 nM stock.<br>Dilute the 500 nM stock 50-fold into 1X Oligo Annealing Buffer to generate a 10 nM stock. Use the 10 nM stock for cloning. |
| | 10 nM ds oligo stock solution heated above room temperature prior to use | Thaw ds oligo stock solution on ice or at +4°C prior to use.<br>**Important:** Diluted ds oligos will melt and form intramolecular hairpins if heated above room temperature. These hairpins will not clone into pcDNA™6.2-GW/EmGFP-miR. |
| | Incorrect vector: insert ratio used in ligation reaction<br>Forgot to dilute annealed ds oligo 1:5,000 before use<br>Annealed ds oligo diluted incorrectly | Dilute the 50 µM ds oligo mixture as instructed on page 45 to generate a 10 nM stock. Use the 10 nM ds oligo stock for cloning. |
| | Ligation reaction not adequately mixed or incorrectly mixed prior to incubation | Mix the ligation reaction well by pipetting up and down.<br>**Note:** Flicking the tube is not adequate to mix the reagents.<br>Do not vortex the ligation reaction. |
| | Did not use the 5X Ligation Buffer supplied with the kit | Use the T4 DNA Ligase and 5X Ligation Buffer supplied with the kit for ligation as these reagents have been optimized to facilitate<br>5-minute ligation at room temperature.<br>**Important:** Other T4 DNA Ligase and ligation buffers may not support 5-minute, room temperature ligation. |
| | Ligation reaction not incubated for long enough | Extend the incubation time of the ligation reaction up to 2 hours at room temperature. |
| | Ligation reaction incubated overnight at 16°C | The ligation conditions used to clone the ds oligo into pcDNA™6.2-GW/EmGFP-miR differ from traditional ligation conditions. Incubate the ligation reaction at room temperature for 5 minutes. |

*Continued on next page*

### Ligation and Transformation Reactions, continued

| Problem | Reason | Solution |
|---|---|---|
| Few spectinomycin-resistant colonies obtained on the selective plate, continued | Not enough transformation mixture plated | Increase the amount of the transformation mixture plated. |
| | Selective plates contained too much spectinomycin | Use LB agar plates containing 50 µg/ml spectinomycin for selection. |
| | Did not use the competent cells supplied with the kit | Use the One Shot® TOP10 Chemically Competent *E. coli* supplied with the kit; transformation efficiency is > 1 x $10^9$ cfu/µg DNA. |
| | Not enough of the ligation reaction transformed | Increase the amount of ligation reaction transformed. |
| | Did not perform the 1 hour grow-out period before plating the transformation mixture | After the heat-shock step, add S.O.C. Medium and incubate the bacterial culture for 1 hour at 37°C with shaking before plating. |
| Many clones contain inserts with sequence mutations | Poor quality single-stranded oligos used | Use high-quality single-stranded oligos. |
| | Oligo preparation contains mutated sequences | Use mass spectrometry to check for peaks of the wrong mass. |
| | Oligo preparation contains contaminants | Order HPLC or polyacrylamide gel (PAGE)-purified oligos.<br>Order oligos from Invitrogen's custom primer synthesis service (see www.invitrogen.com for details). |
| | Did not use the competent cells supplied with the kit | Use One Shot® TOP10 Chemically Competent *E. coli* supplied with the kit; transformation efficiency is > 1 x $10^9$ cfu/µg DNA. |
| Poor sequencing results | Loss of sequencing signal in the hairpin region due to secondary structure formation | Use high-quality, purified plasmid DNA for sequencing.<br>Add DMSO to the sequencing reaction to a final concentration of 5%.<br>Increase the amount of template used for sequencing (up to twice the normal amount).<br>Use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction.<br>Cut your construct with *Msc* I and purify before sequencing (see page 55). |
| No colonies obtained on the selective plate | Used the wrong antibiotic for selection | Select for transformants on LB agar plates containing 50 µg/ml spectinomycin. |

*Continued on next page*

### Transfection and RNAi Analysis

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

| Problem | Reason | Solution |
|---|---|---|
| Low levels of gene knockdown observed due to low transfection efficiency | Antibiotics added to the media during transfection | Do not add antibiotics to the media during transfection. |
| | Cells not healthy or too sparse at the time of transfection | Use healthy cells. Plate cells such that they will be 90-95% confluent at the time of transfection. |
| | Not enough plasmid DNA transfected | Increase the amount of plasmid DNA transfected. |
| | Not enough Lipofectamine™ 2000 Reagent used | Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used. |
| Low levels of gene knockdown observed (other causes) | Didn't wait long enough after transfection before assaying for gene knockdown | Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown. |
| | | Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs. |
| | ds oligo insert in your pcDNA™6.2-GW/EmGFP-miR construct contains mutations | When analyzing spectinomycin-resistant transformants, sequence the ds oligo insert to verify its sequence. Select constructs containing the correct ds oligo insert for use in RNAi analysis. |
| | miRNA sequence not optimal due to the target region selected | Select a different target region. |

*Continued on next page*

### Transfection and RNAi Analysis, continued

| Problem | Reason | Solution |
|---|---|---|
| Cytotoxic effects observed after transfection | Too much Lipofectamine™ 2000 Reagent used | Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used. |
| | Plasmid DNA not pure | Prepare purified plasmid DNA for transfection. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit as described on page 61. |
| | Targets an essential gene | Make sure that your target gene is not essential for cell viability or growth. |
| No gene knockdown observed | miRNA with no activity chosen | Select a different target region. |
| | pre-miRNA designed incorrectly | Follow the guidelines on pages 37 to select the target sequence and design the single-stranded oligos. |
| Non-specific off-target gene knockdown observed | Target sequence contains strong homology to other genes | Select a different target region. |
| 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 and use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for additional days before assaying for fluorescence.<br>**Note:** We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable. |

*Continued on next page*

# Troubleshooting, Continued

## Rapid BP/LR Reaction and Transformation

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

| Problem | Reason | Solution |
|---|---|---|
| Few or no colonies obtained from sample reaction **and** the transformation control gave colonies | Incorrect antibiotic used to select for transformants | Select for transformants on LB agar plates containing 100 µg/ml ampicillin. |
| | Rapid BP/LR reaction may not work for your insert | Use the standard BP and LR recombination reactions as described on page 135. |
| | BP recombination reaction is treated with Proteinase K | **Do not** treat the BP reaction with Proteinase K before the LR reaction. |
| | Did not use the suggested Clonase™ II enzyme mixes or Clonase™ II enzyme mixes were inactive | • Make sure to store the BP and LR Clonase™ II enzyme mix at -20°C or -80°C.<br>• Do not freeze/thaw the BP and LR Clonase™ II enzyme mix more than 10 times.<br>• Use the recommended amount of BP and LR Clonase™ II enzyme mix (page 80).<br>• Test another aliquot of the Clonase™ II enzyme mix. |
| | Not enough LR reaction transformed | Transform 2-3 µl of the LR reaction into One Shot® Stbl3™ Chemically Competent *E. coli*. |
| | Not enough transformation mixture plated | Increase the amount of *E. coli* plated. |
| | Did not perform the 1 hour grow-out period before plating the transformation mixture | After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating. |
| | Too much BP reaction used in the LR reaction | Use the recommended amount of BP reaction for the LR reaction. |
| 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 of lentiviral constructs. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally give rise to fewer unwanted recombinants. |

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

| Problem | Reason | Solution |
|---|---|---|
| Few or no colonies obtained from the transformation control | Competent cells stored incorrectly | • Store the One Shot® 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 your co-transfection and titering experiments.

| Problem | 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 media 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 S.N.A.P.™ MidiPrep Kit to prepare plasmid DNA.<br>• Use healthy 293FT cells under passage 20; do not overgrow.<br>• **Do not** add Geneticin® in the media 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; (page 105). |
| | 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.** |

*Continued on next page*

### Generating the Lentiviral Stock, continued

| Problem | Reason | Solution |
|---|---|---|
| Low viral titer | Viral supernatant harvested too early | Viral supernatants can generally be collected 48-72 hours post-transfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant. |
| | Viral supernatant too dilute | Concentrate virus using any method of choice (Yee, 1999). |
| | Viral supernatant frozen and thawed multiple times | **Do not** freeze/thaw viral supernatant more than 3 times. |
| | Poor choice of titering cell line | Use HT1080 cells or another adherent cell line with the characteristics discussed on page 109. |
| | Target gene is essential for cell viability | Make sure that your target gene is not essential for cell viability or growth by performing a transient transfection with the entry construct containing the miRNA of interest. |
| | Polybrene® not included during titering procedure | Transduce the lentiviral construct into cells in the presence of Polybrene®. |
| No colonies obtained upon titering | Too much Blasticidin used for selection | Determine the Blasticidin sensitivity of your cell line by performing a kill curve experiment. Use the minimum Blasticidin concentration required to kill your untransduced cell line. |
| | Viral stocks stored incorrectly | Aliquot and store stocks at -80°C. Do not freeze/thaw more than 3 times. |
| | Polybrene® not included during transduction | Transduce the lentiviral construct into cells in the presence of Polybrene®. |
| Titer indeterminable; cells confluent | Too little Blasticidin used for selection | Increase amount of Blasticidin used for selection. |
| | Viral supernatant not diluted sufficiently | Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.* $10^{-2}$ to $10^{-8}$). |

*Continued on next page*

### Transduction and RNAi Analysis

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

| Problem | 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 111 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. |
| | miRNA with weak activity chosen | Select a different target region. If possible, screen miRNA first by transient transfection of the expression construct to verify its activity, then perform BP/LR recombination with the pLenti6/V5-DEST vector and proceed to generate lentivirus.<br>**Note:** Generally, transient transfection greatly overexpresses miRNA, so moderately active expression clones may be less active when expressed from a lentiviral construct. |

*Continued on next page*

### Transduction and RNAi Analysis, continued

| Problem | Reason | Solution |
|---|---|---|
| No gene knockdown observed | miRNA with no activity chosen | Select a different target region. If possible, screen miRNA first by transient transfection of the expression construct to verify its activity, then perform BP/LR recombination with the pLenti6/V5-DEST vector and proceed to generate lentivirus. |
| | Viral stocks stored incorrectly | Aliquot and store stocks at -80°C. Do not freeze/thaw more than 3 times. |
| | MOI too low | Transduce your lentiviral construct into cells using a higher MOI. |
| Cytotoxic effects observed after transduction | Target gene is essential for cell viability | Make sure that your target gene is not essential for cell viability or growth. |
| | Large volume of viral supernatant used for transduction | • Remove the "spent" media containing virus and replace with fresh, complete media.<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. 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 and 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. |

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# Troubleshooting, Continued

## E-Gel® Analysis

The table below provides solutions to some problems that you may encounter with E-Gel® agarose gels.

| Problem | Cause | Solution |
|---|---|---|
| No current | Copper contacts in the base are damaged due to improper use | Make sure the copper contacts in the base are intact. |
| | Expired or defective gel cassette | Use fresh gel cassette. Use properly stored gels before the specified expiration date. |
| | E-Gel® cassette is not inserted properly into a base | Remove cassette and reinsert; a steady red light illuminates on the base when the cassette is correctly inserted and power is on. |
| | Incorrect adaptor used | Use only UL Listed Class 2 Direct Plug-in Adaptor included with the E-Gel® PowerBase™. |
| Poor resolution or smearing of bands | Sample is overloaded | Load the recommended amount of sample on the gel (page 47). Less DNA is required since E-Gel® agarose gels are thinner. |
| | Very low volume of sample loaded or sample was not loaded properly | Avoid introducing bubbles while loading the samples. Bubbles will cause band distortion.<br>Load the recommended sample volume based on the gel type and loading method.<br>For proper band separation, we recommend keeping sample volumes uniform. Load deionized water or TE into any empty wells. |
| | Gel was not electrophoresed immediately after sample loading | For best results, run the gel within 15 minutes of sample loading.<br>If you cannot run the gel immediately after sample loading, use the Two-Step Loading method (refer to the E-Gel® Technical Guide available at www.invitrogen.com). |
| | Expired gel used | Use properly stored gels before the expiration date. |
| | Longer electrophoresis run time or high current during the run | Longer run times cause an increase in the current, resulting in poor band migration or a melted gel. Do not run the gel longer than the recommended time for each gel type. |
| Sample leaking from the wells | Sample is overloaded | Load the recommended sample volume per well. |
| | | Use the Two-Step Loading method. |
| | Wells damaged during comb removal | Remove the comb gently without damaging the wells. |
| Failure Mode indicated by a steady red light and continuous rapid beeping | Defective cassette | Disconnect the base and replace gel cassette with a fresh gel cassette. Press and release the power button to return to Ready Mode. |
| | Cold cassette or improper operating conditions | Use a cassette stored at room temperature. Avoid storing gel cassettes at 4°C. Use E-Gel® PowerBase™ and E-Gel® Base at room temperature (20-25°C). |

*Continued on next page*

## Plasmid Purification

Review the information provided in the table below to troubleshoot your plasmid purification experiments using the PureLink™ HQ Mini Plasmid Kit or S.N.A.P.™ MidiPrep Kit.

| Problem | Cause | Solution |
|---|---|---|
| Low yield | Incomplete lysis or too much cell lysate has clogged the column | Decrease cell culture volume used. Use up to 100 ml of an overnight culture, density 2 x $10^9$ cells/ml ($OD_{600}$ < 3.0 per ml). For more dense cultures, decrease volume of culture.<br>Decrease cell culture density. Use LB medium instead of TB (Terrific Broth).<br>Ensure complete resuspension of the bacterial cell pellet. |
| | Poor quality of starting material. | Check the growth conditions of the cell culture to ensure plasmid propagation. Use a high copy number plasmid if possible. |
| | Incorrect elution conditions | Ensure that the DNA elution was performed with the Elution Buffer supplied in the kit or sterile water (pH >7.0) |
| | Genomic DNA present | Perform all mixing steps very gently to avoid damaging DNA. Genomic DNA must be intact to be efficiently removed. |
| Plasmid resistant to restriction enzyme digestion | Denatured, supercoiled plasmid present (migrates below native, supercoiled plasmid) | Do not incubate longer than 3 minutes in Lysis Buffer. |

# Appendix I

# Recipes

## Preparing Antibiotic Stock Solutions

Prepare the following antibiotic stock solutions. Store all stock solutions at 4ºC for up to 2 weeks. For long-term storage, store at -20ºC in small aliquots to avoid repeated freezing and thawing.

**Spectinomycin (10 mg/ml)**

To 100 µl Spectinomycin solution (100 mg/ml) supplied with the kit, add 900 µl sterile deionized water to produce a 10 mg/ml stock solution. Filter-sterilize.

**Ampicillin (50 mg/ml)**

To a sterile microcentrifuge tube, add 50 mg Ampicillin powder supplied with the kit. Add 1 ml sterile deionized water to the tube and mix well to produce a 50 mg/ml stock solution. Filter sterilize.

**Kanamycin (25 mg/ml)**

Use the 25 mg/ml solution supplied with the kit as the stock solution.

**Blasticidin (10 mg/ml)**

See next page for details.

## Preparing LB Broth with Antibiotics

Prepare LB Broth containing the appropriate antibiotic as described in the table below using the LB Broth (1X) supplied with the kit and antibiotic stock solution prepared as above:

| Required Media | Recipe |
|---|---|
| LB medium with 100 µg/ml ampicillin | To 20 ml LB Broth (1X), add 40 µl ampicillin stock solution (50 mg/ml). |
| LB medium with 50 µg/ml kanamycin | To 20 ml LB Broth (1X), add 40 µl kanamycin stock solution (25 mg/ml). |
| LB medium with 50 µg/ml Spectinomycin | To 20 ml LB Broth (1X), add 100 µl Spectinomycin Stock solution (10 mg/ml). |
| LB medium with 100 µg/ml Blasticidin | To 20 ml LB Broth (1X), add 200 µl Blasticidin stock solution (10 mg/ml). |

## LB Agar Plates

Composition:

10 g Tryptone

5 g Yeast Extract

5 g NaCl

12 g Agar

pH 7.0

1. For 1 liter, dissolve 32 g of LB Agar (page 160) in 900 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring volume up to 1 L.
3. Autoclave on liquid cycle for 20 minutes at 15 psi.
4. Allow solution to cool to 55°C. Add appropriate antibiotic and pour into 10 cm plates.
5. Let harden, then invert and store at +4°C. Plates containing antibiotic are stable for up to 2 weeks.

# Blasticidin

## Handling Blasticidin

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

## Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Invitrogen (see page 160) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

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

## Determining Antibiotic Sensitivity

Since you will be selecting for stable cells using Blasticidin, 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. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

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

# 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 138).

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 78 for details on the donor vector, resuspending the donor vector, and propagating the donor vector. See page 78 for linearizing the expression clone.

## Positive Control

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

## *E. coli* Host Strain

You may use any *rec*A, *end*A *E. coli* strain including TOP10, OmniMAX™ 2-T1$^R$ or equivalent for transformation. **Do not** use *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

*Continued on next page*

# Performing the Standard BP Recombination Reaction,
Continued

## Materials Needed

You will need the following materials:

- Linearized *att*B expression clone (page 78)
- pDONR™221 vector (supplied with Cat. no. WFGE08; resuspend to 150 ng/µl with water)
- BP Clonase™ II enzyme mix (supplied with the kit; keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ II enzyme mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µl; supplied with the kit)

**For transformation**

- One Shot® TOP10 Chemically Competent *E. coli* (one vial per transformation; thaw on ice before use)
- S.O.C. Medium (supplied with the competent cells, warm to room temperature)
- Positive control (*e.g.* pUC19 supplied with the competent cells, use as a control for transformation if desired)
- 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

*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 BP Clonase™ II enzyme mix (see Step 4).

| Components | Sample | Positive Control |
|---|---|---|
| Linearized *att*B expression clone from Step 4, page 78 (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 BP Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Vortex the BP Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µl of BP Clonase™ II enzyme mix to the sample and positive control vials. Do not add BP Clonase™ II to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).

   **Reminder:** Return 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*

# Performing the Standard BP Recombination Reaction, Continued

## 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 cells, 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 (> 1500 colonies if the entire BP reaction is transformed and plated).

## Verifying Entry Clones

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

Once you have verified the entry clone, isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kits (page 160). For the LR recombination reaction, you will need purified plasmid DNA at a concentration of 50-150 ng/µl in TE, pH 8.0. Proceed to the LR recombination reaction, next page.

## Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your BP recombination reaction, you may transform One Shot® TOP10 Competent cells using the above protocol. Assess the efficiency of the BP reaction by streaking entry clones onto LB agar plates containing 20 µg/ml tetracycline. True entry clones should be tetracycline-resistant.

# Performing the Standard LR Recombination Reaction

## Introduction

Once you have obtained an entry clone containing the pre-miRNA expression cassette, you will perform an LR recombination reaction between the entry clone (previous page) and a destination vector or pLenti6/V5-DEST, and transform the reaction mixture into competent *E. coli* to select for expression clones (see page 87).

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

## Experimental Outline

To generate an expression clone, you will:

1. Perform a LR recombination reaction using the *att*L-containing entry clone (previous page) and *att*R-containing destination vector including pLenti6/V5-DEST.
2. Transform the reaction mixture into competent *E. coli*.
3. Select for expression clones.

## Destination Vector

See page 78 for details on the destination vector, resuspending the destination vector, and propagating the destination vector.

## 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 with Cat. no. WFGE08 for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 87.

You can use One Shot® TOP10 Competent Cells, if you are using non-lentiviral destination vectors.

## Positive Control

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

*Continued on next page*

# Performing the Standard LR Recombination Reaction,
Continued

**Materials Needed**

You will need the following materials:

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- Destination vector (150 ng/µl in TE, pH 8.0); pLenti6/V5-DEST is supplied with Cat. no. WFGE08
- LR Clonase™ II enzyme mix (supplied with the kit, keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µl; supplied with the LR Clonase™ II enzyme mix)
- Competent *E. coli* or One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with WFGE08, for use with pLenti6/V5-DEST)
- S.O.C. Medium (supplied with the competent cells, warm to room temperature)
- 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

**Note**

Depending on the destination vector that you will use, perform the appropriate LR reaction as described on the following pages using plasmid DNA from the resulting **entry clone**.

*Continued on next page*

# Performing the Standard LR Recombination Reaction, Continued

## LR Recombination Reaction for Standard Destination Vectors

Use this LR recombination reaction for standard destination vectors. For LR recombination reaction with MultiSite Gateway® destination vectors, see next page.

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 LR Clonase™ II enzyme mix (see Step 4).

| Component | Sample | Positive Control |
|---|---|---|
| Entry clone (50-150 ng/reaction) | 1-7 µl | -- |
| Destination vector (150 ng/µl) | 1 µl | 1 µl |
| pENTR™-gus (50 ng/µl) | -- | 2 µl |
| TE Buffer, pH 8.0 | to 8 µl | 5 µl |

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

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

   **Note:** For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times (*i.e.* overnight incubation) will yield more colonies and are recommended.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transformation using competent *E. coli* or **Transforming One Shot® Stbl3™ Competent *E. coli*** for transforming pLenti6/V5-DEST construct (page 87).

   **Note:** You may store the reaction at -20°C for up to 1 week before transformation, if desired.
8. Pick transformants and isolate plasmid DNA using PureLink™ HQ Mini Plasmid DNA Purification Kit (page 61). Perform restriction analysis to select a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

## What You Should See

If you use *E. coli* cells with a transformation efficiency of ≥ 1 x $10^8$ cfu/µg, the LR reaction should give > 5000 colonies if the entire LR reaction is transformed and plated. See page 88 for confirming the expression clone.

*Continued on next page*

# Performing the Standard LR Recombination Reaction, Continued

## LR Reaction for MultiSite Gateway® Destination Vectors

Use this LR recombination reaction for MultiSite Gateway® destination vectors. For LR recombination reaction with standard destination vectors, see previous page.

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 LR Clonase™ Plus enzyme mix (see Step 4).

| Component | Sample | Positive Control |
|---|---|---|
| Entry clone (60 ng/µl) | 1 µl | 1 µl |
| 5′ pENTR™ vector (60 ng/µl) | 1 µl | 1 µl |
| 3′ pENTR™ vector (60 ng/µl) | 1 µl | 1 µl |
| MultiSite Gateway® destination vector (60 ng/µl) | 1 µl | 1 µl |
| 5X LR Clonase™ Plus Buffer | 3 µl | 3 µl |
| TE Buffer, pH 8.0 | to 16 µl | to 16 µl |

2. Remove the LR Clonase™ Plus enzyme mix from -80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ Plus enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 4 µl of LR Clonase™ Plus enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   **Reminder:** Return LR Clonase™ Plus enzyme mix to -80°C immediately after use.
5. Incubate reactions at 25°C for 16 hours or overnight.
6. Add 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming One Shot® TOP10 Chemically Competent *E. coli*, follow the protocol on page 138. For transforming lentiviral construct, proceed to **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 87).

   **Note:** You may store the MultiSite Gateway® LR reaction at -20°C for up to 1 week before transformation, if desired.
8. Pick transformants and isolate plasmid DNA using PureLink™ HQ Mini Plasmid DNA Purification Kit (page 61). Perform restriction analysis to select a clone that has incorporated the pre-miRNA expression cassette (sequence analysis is not necessary).

## What You Should See

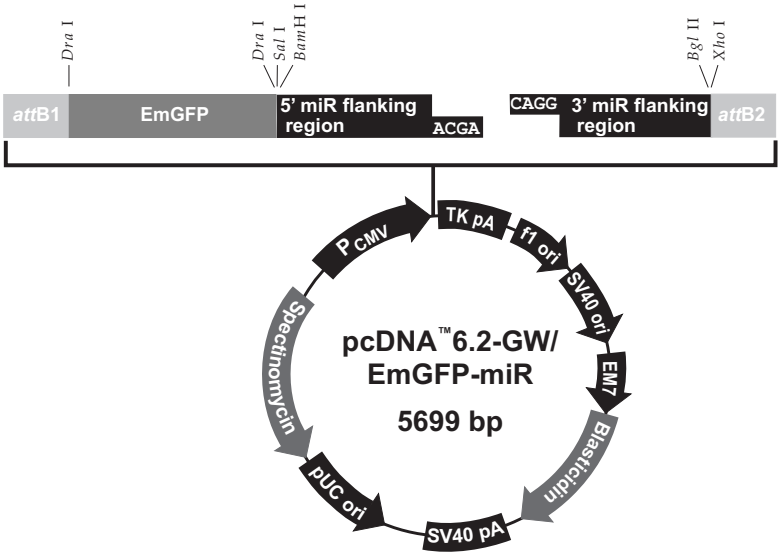
If you use *E. coli* cells with a transformation efficiency of 1 x $10^9$ cfu/µg, the MultiSite Gateway® LR reaction should give approximately 2,000-8,000 colonies if the entire reaction is transformed and plated.

# Map of pcDNA™6.2-GW/EmGFP-miR

## pcDNA™6.2-GW/EmGFP-miR

The figure below shows the features of the pcDNA™6.2-GW/EmGFP-miR vector. The vector is supplied linearized between nucleotides 1518 and 1519 with 4 base pair 5′ overhangs on each strand as indicated. **The complete sequence of pcDNA™6.2-GW/ EmGFP-miR is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 163).**

**Note:** For an explanation of the features see page 145.




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

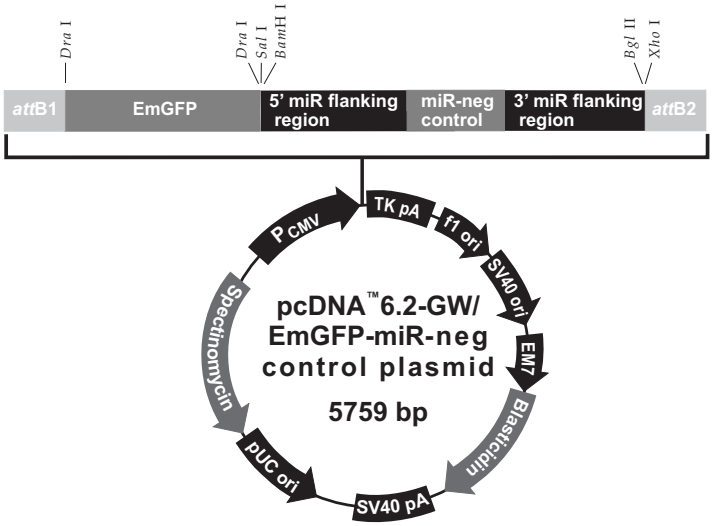
CMV promoter: bases 1-588
*att*B1 site: bases 680 - 704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
5' overhang (C): bases 1515-1518
5'overhang: bases 1519-1522
3' miR flanking region: bases 1519-1563
*att*B2 site (C): bases 1592-1616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

(C) = Complementary strand

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

## pcDNA™6.2-GW/EmGFP-miR-neg control plasmid

The figure below shows the features of the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid. The vector contains an insert between bases 1519 and 1578 that can form a hairpin structure just as a regular pre-miRNA, but is predicted not to target any known vertebrate gene. The insert has been cloned according to the instructions in this manual. Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with pcDNA™6.2-GW/EmGFP-miR expression vectors. **The complete sequence of pcDNA™6.2-GW/EmGFP-miR-neg control plasmid is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 163).**




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

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

(C) = Complementary strand

# Features of pcDNA™6.2-GW/EmGFP-miR

## Features of the Vectors

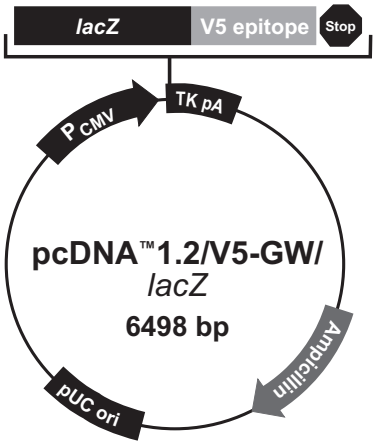
The pcDNA™6.2-GW/EmGFP-miR vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

| Feature | Benefit |
|---|---|
| CMV promoter | Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). |
| miRNA forward sequencing primer | Allows sequencing of the insert (for pcDNA™6.2-GW/miR vectors). |
| *att*B1 and *att*B2 sites | Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the expression construct with a Gateway® destination vector (Landy, 1989). |
| EmGFP coding sequence | Allows visual detection of transfected mammalian cells using fluorescence microscopy. |
| EmGFP forward sequencing primer | Allows sequencing of the insert. |
| 5′ miR flanking region | Allows formation of functional engineered pre-miRNA. |
| 5′ overhangs | Allows ligase-mediated directional cloning of the double-stranded oligonucleotide of interest. |
| miR-neg control | Allows formation of a pre-miRNA hairpin sequence predicted not to target any known vertebrate gene (only for pcDNA™6.2-GW/EmGFP-miR-neg control). Sequence without 5′ overhangs is shown below: `5′-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′` |
| 3′ miR flanking region | Allows formation of functional engineered pre-miRNA. |
| miRNA reverse sequencing primer | Allows sequencing of the insert. |
| TK polyadenylation signal | Allows transcription termination and polyadenylation of mRNA. |
| f1 origin | Allows rescue of single-stranded DNA. |
| SV40 early promoter and origin | Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen. |
| EM7 promoter | Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.* |
| Blasticidin (*bsd*) resistance gene | Permits selection of stably transfected mammalian cell lines (Kimura *et al.*, 1994). |
| SV40 polyadenylation signal | Allows transcription termination and polyadenylation of mRNA. |
| pUC origin | Permits high-copy replication and maintenance in *E. coli*. |
| Spectinomycin resistance gene (*aad*A1) | Allows selection of the plasmid in *E. coli* (Liebert *et al.*, 1999). |
| Spectinomycin promoter | Allows expression of the spectinomycin resistance gene in *E. coli*. |

# Map of pcDNA™1.2/V5-GW/*lacZ*

## Description

pcDNA™1.2/V5-GW/*lacZ* (6498 bp) is a control vector expressing a C-terminally-tagged β-galactosidase fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987), and was generated using the MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023). Briefly, a MultiSite Gateway® LR recombination reaction was performed with pDEST™R4-R3 and entry clones containing the CMV promoter, *lacZ* gene, and V5 epitope and TK polyadenylation signal (Cole & Stacy, 1985) to generate the pcDNA™1.2/V5-GW/*lacZ* vector. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 119 kDa. **The complete sequence of pcDNA™1.2/V5-GW/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 163).**




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

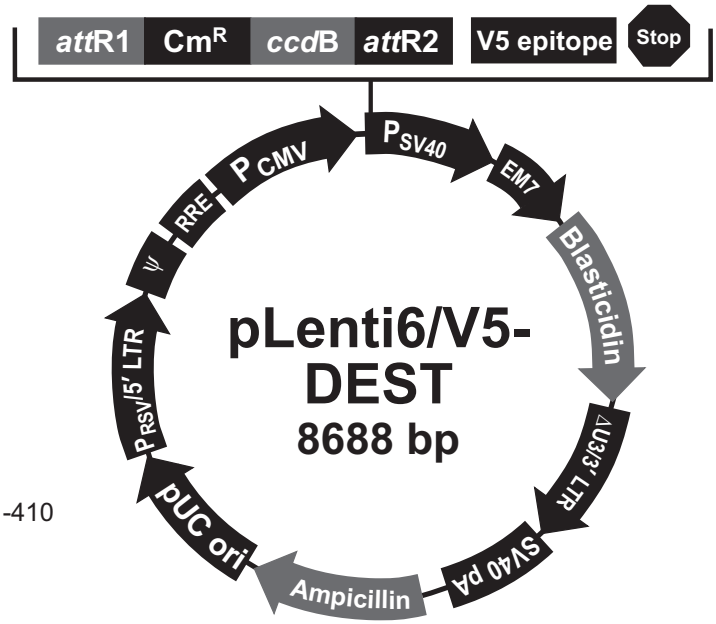
*att*B4: bases 5-25
CMV promoter: bases 137-724
*att*B1: bases 614-637
LacZ fusion protein: bases 643-3798
LacZ ORF: bases 643-3714
*att*B2: bases 3716-3739
V5 epitope: bases 3739-3780
lacZ forward 2 priming site: 840-859
lacZ reverse 2 priming site: 1820-1839 (C)
TK polyadenylation signal: bases 3807-4078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

(C) = complementary strand

# Map and Features of pLenti6/V5-DEST

## Map of pLenti6/V5-DEST

The map below shows the elements of pLenti6/V5-DEST. DNA from the entry clone replaces the region between bases 2447 and 4130. **The complete sequence for pLenti6/V5-DEST is available from our web site (www.invitrogen.com) or by contacting Technical Service (page 160).**




**Comments for pLenti6/V5-DEST**
**8688 nucleotides**

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

*Continued on next page*

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

## Features of the Vector

The pLenti6/V5-DEST (8688 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

| Feature | Benefit |
|---|---|
| Rous Sarcoma Virus (RSV) enhancer/promoter | Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998). |
| HIV-1 truncated 5′ LTR | Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996). |
| 5′ splice donor and 3′ acceptors | Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998). |
| HIV-1 psi (ψ) packaging signal | Allows viral packaging (Luciw, 1996). |
| HIV-1 Rev response element (RRE) | Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989). |
| CMV promoter | Permits high-level, constitutive expression of the gene or miRNA of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). |
| *att*R1 and *att*R2 sites | Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989). |
| Chloramphenicol resistance gene ($Cm^R$) | Allows counterselection of the plasmid. |
| *ccd*B gene | Permits negative selection of the plasmid. |
| V5 epitope | Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991). **This feature is not used when expressing miRNAs.** |
| SV40 early promoter and origin | Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen. |
| EM7 promoter | Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.* |
| Blasticidin (*bsd*) resistance gene | Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994). |
| ΔU3/HIV-1 truncated 3′ LTR | Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells. |
| SV40 polyadenylation signal | Allows transcription termination and polyadenylation of mRNA. |
| *bla* promoter | Allows expression of the ampicillin resistance gene. |
| Ampicillin resistance gene (β-lactamase) | Allows selection of the plasmid in *E. coli*. |
| pUC origin | Permits high-copy replication and maintenance in *E. coli*. |

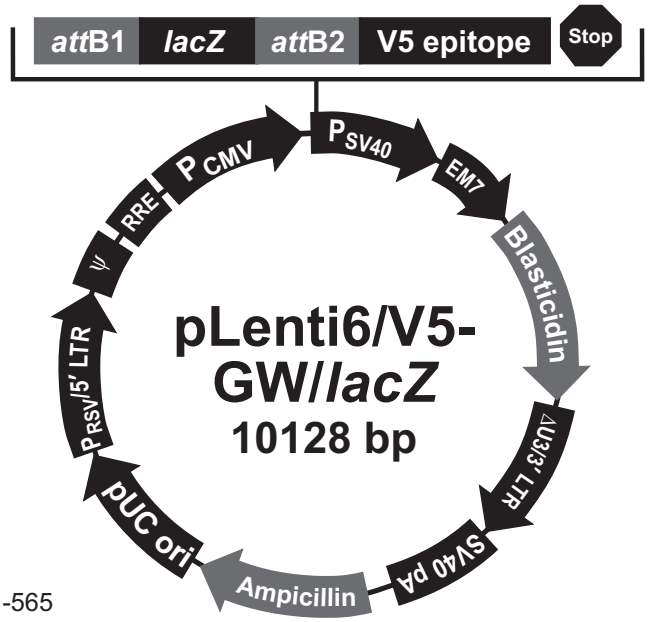
# Map of pLenti6/V5-GW/*lacZ*

## Description

pLenti6/V5-GW/*lacZ* is a 10128 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pLenti6/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

## Map of pLenti6/V5-GW/*lacZ*

The map below shows the elements of pLenti6/V5-GW/*lacZ*. **The complete sequence of the vector is available from our Web site (www.invitrogen.com) or by calling Technical Service (page 160).**



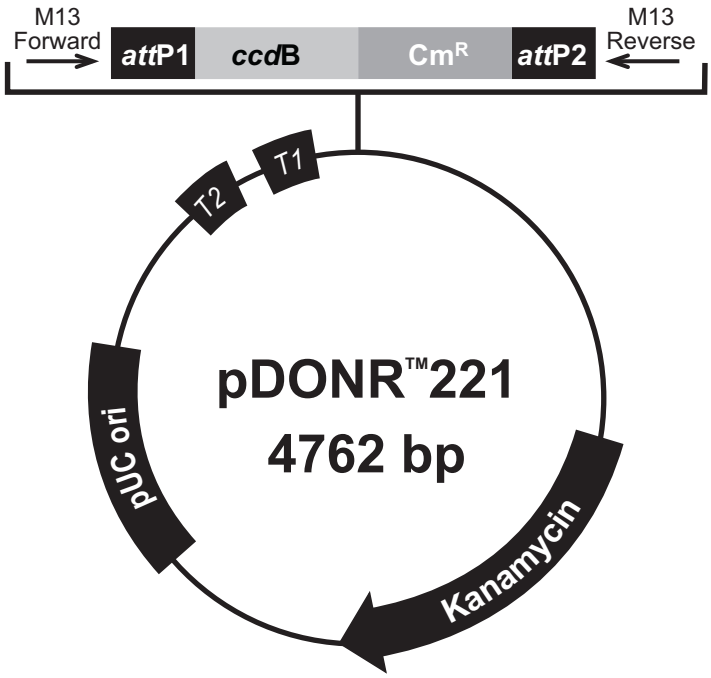

**Comments for pLenti6/V5-GW/*lacZ***
**10128 nucleotides**

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

# Map and Features of pDONR™221

## Map of pDONR™221

The map below shows the elements of pDONR™221 vector. **The complete sequences of pDONR™221 is available for downloading from our web site (www.invitrogen.com) or by contacting Technical Service (page 160).**




**Comments for pDONR™221**
**4762 nucleotides**

*rrn*B T2 transcription termination sequence: bases 268-295 (C)
rrnB 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 1847-2506 (C)
*att*P2: bases 2754-2985 (C)
M13 Reverse priming site: bases 3027-3043
Kanamycin resistance gene: bases 3156-3965
pUC origin: bases 4086-4759

(C) = complementary strand

*Continued on next page*

# Map and Features of pDONR™221, Continued

## Features of pDONR™221

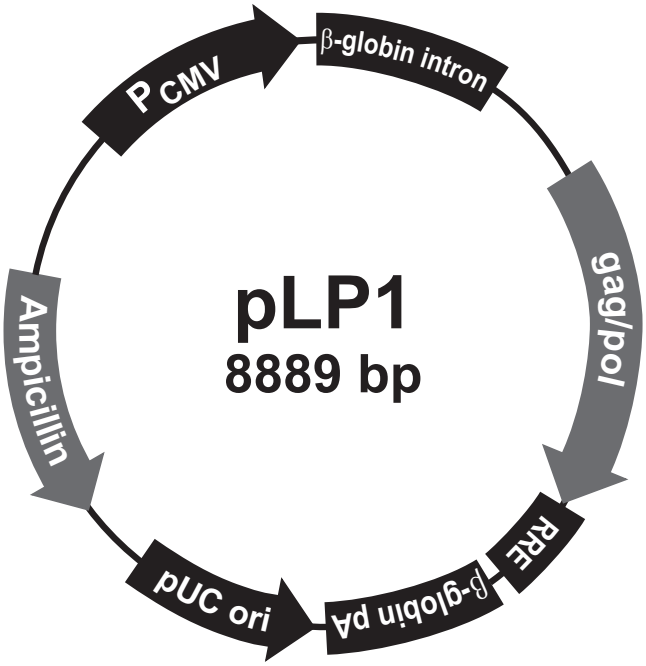
pDONR™221 (4762 bp) contains the following elements. All features have been functionally tested and the vectors fully sequenced.

| 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 then self-cleaved by the viral protease into individual Gag and Pol polyproteins. **The complete sequence of pLP1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 160).**




**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

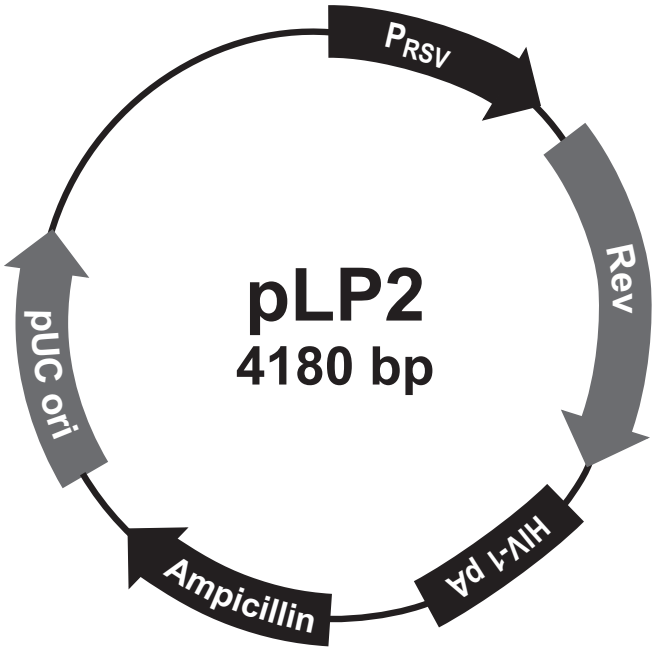
pLP1 (8889 bp) contains the following elements. All features have been functionally tested.

| 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 complete sequence of pLP2 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 160).**




**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

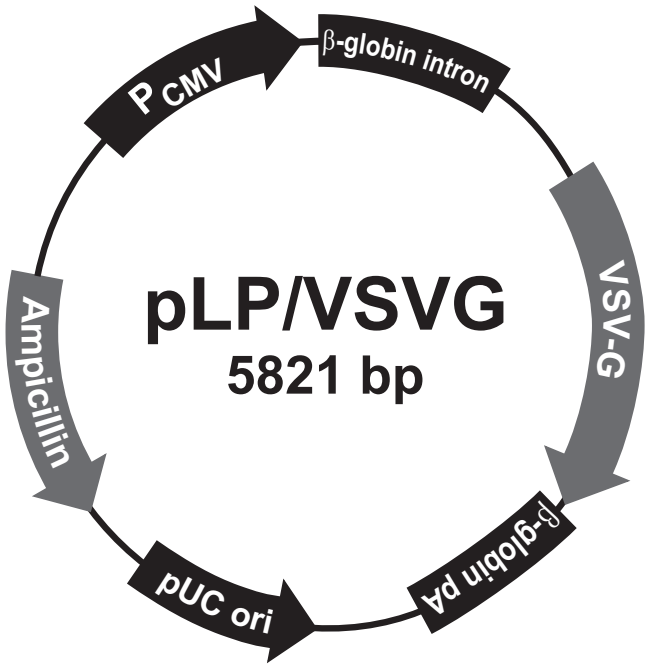
pLP2 (4180 bp) contains the following elements. All features have been functionally tested.

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

# Map and Features of pLP/VSVG

## pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. **The complete sequence of pLP/VSVG is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 160).**




**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 (5821 bp) contains the following elements. All features have been functionally tested.

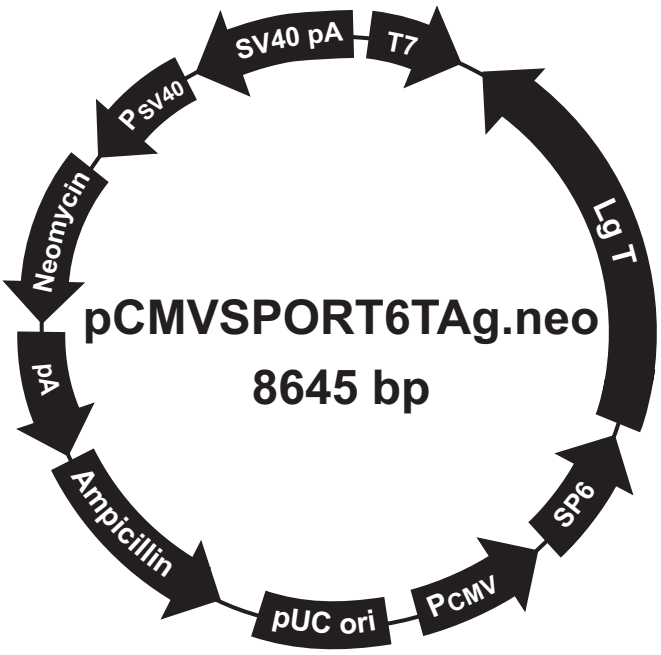
| Feature | Benefit |
|---|---|
| Human CMV promoter | Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). |
| Human β-globin intron | Enhances expression of the VSV-G gene in mammalian cells. |
| VSV G glycoprotein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

# Map of pCMVSPORT6TAg.neo

## Description

The pCMVSPORT6TAg.neo plasmid is derived from pCMVSPORT6, which has been modified to include the following features:

- The neomycin resistance gene for stable selection in mammalian cells (Southern & Berg, 1982). Expression of the neomycin resistance gene is controlled by the SV40 early enhancer/promoter from which the SV40 origin of replication has been removed.
- The gene encoding the SV40 large T antigen to facilitate optimal virus production (*e.g.* Invitrogen's ViraPower™ Lentiviral Expression System) and to permit episomal replication of plasmids containing the SV40 early promoter and origin. Expression of the SV40 large T antigen is controlled by the human cytomegalovirus (CMV) promoter.



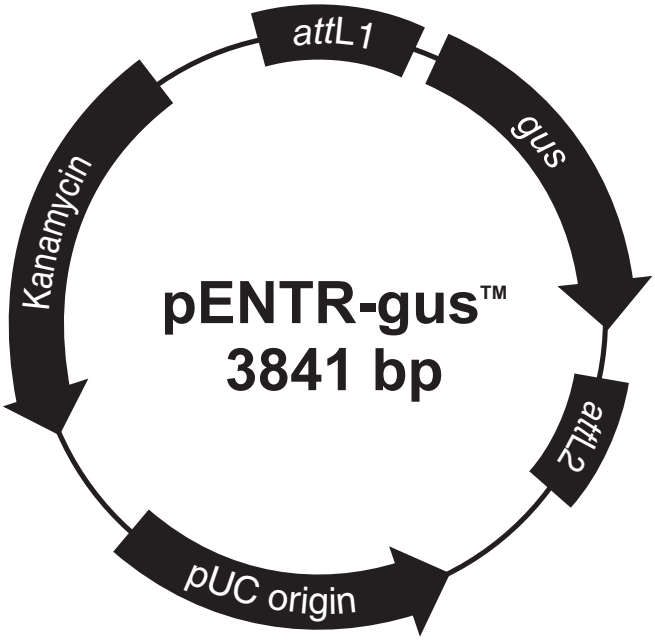
# Map of pENTR™-gus

## Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR201™ to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from our Web site or by contacting Technical Service.

## Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The complete sequence for pENTR™-gus is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 160).**




**Comments for pENTR-gus™**
**3841 nucleotides**

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

C = complementary strand

# Appendix II

# Accessory Products

## BioModule™ Units

Additional BioModule™ Units that can be used for validation experiments or gene expression profiling are available from Invitrogen. Ordering information is provided below. For more information, visit www.invitrogen.com or call Technical Service (page 163).

| Product | Amount | Catalog no. |
|---|---|---|
| BioModule™ Western Analysis Unit | | |
| *for chromogenic detection* | 20 transfers | WFGE09 |
| *for chemiluminescent detection* | 20 transfers | WFGE10 |
| BioModule™ qRT-PCR Unit | 100 reactions | WFGE01 |
| | 1000 reactions | WFGE02 |
| BioModule™ Immunohistochemical (IHC) Staining for Tissue | 150 slides | WFGE11 |
| BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology | 1 unit | WFGE06 |
| BioModule™ Microarray Analysis | | |
| *with indirect labeling* | 15 arrays | WFGE03 |
| *with direct labeling* | 15 arrays | WFGE04 |
| BioModule™ Lentiviral 293 Unit (for contents, see below) | 1 Unit | WFGE08-S |

## BioModule™ Lentiviral 293 Unit

The BioModule™ Lentiviral 293 Unit contains all necessary media, antibiotics, serum, and Trypan Blue for growth, maintenance, and checking cell viability of 293 FT cells.

| Product | Amount |
|---|---|
| Fetal Bovine Serum | 2 x 100 ml |
| 200 mM L-Glutamine (100X) | 100 ml |
| Penicillin-Streptomycin, liquid (5,000 units of penicillin and 5,000 µg of streptomycin) | 100 ml |
| Trypsin-EDTA | 100 ml |
| Dulbecco's Modified Eagle Medium | 2 x 1000 ml |
| 10 mM MEM Non-Essential Amino Acids Solution (100X) | 100 ml |
| MEM Sodium Pyruvate Solution (100X) | 100 ml |
| Phosphate-Buffered Saline, pH 7.4 | 500 ml |
| Opti-MEM® I Reduced Serum Medium | 500 ml |
| Geneticin® Selective Antibiotic (50 mg/ml) | 20 ml |
| Trypan Blue Stain | 100 ml |

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# Accessory Products, Continued

## Accessory Products

Many of the reagents supplied in the BioModule™ BLOCK-iT™ RNAi Units as well as other products suitable for use with the units are available separately from Invitrogen. Ordering information is provided below.

| Product | Amount | Catalog no. |
|---|---|---|
| Lipofectamine™ 2000 Reagent | 0.75 ml | 11668-027 |
| | 1.5 ml | 11668-019 |
| pDONR™221 Vector | 6 µg | 12536-017 |
| Opti-MEM® I Reduced Serum Medium | 100 ml | 31985-062 |
| Gateway® BP Clonase™ II Enzyme Mix | 20 reactions | 11789-020 |
| | 100 reactions | 11789-100 |
| Gateway® LR Clonase™ II Enzyme Mix | 20 reactions | 11791-020 |
| | 100 reactions | 11791-100 |
| Phosphate-Buffered Saline (PBS), pH 7.4 | 500 ml | 10010-023 |
| One Shot® Stbl3™ Chemically Competent *E. coli* | 20 x 50 µl | C7373-03 |
| One Shot® TOP10 Chemically Competent *E. coli* | 20 x 50 µl | C4040-03 |
| Library Efficiency® DB3.1™ Competent Cells | 5 x 200 µl | 11782-018 |
| One Shot® *ccd*B Survival™ T1$^R$ Chemically Competent Cells | 10 reactions | C7510-03 |
| ViraPower™ Lentiviral Packaging Mix | 60 reactions | K4975-00 |
| Blasticidin | 50 mg | R210-01 |
| Kanamycin Sulfate (100X) | 100 ml | 15160-054 |
| Ampicillin | 5 g | Q100-16 |
| Geneticin® Selective Antibiotic (50 mg/ml) | 20 ml | 10131-035 |
| LB Broth (1X) | 500 ml | 10855-021 |
| LB Agar (Lennox L Agar)® | 500 g | 22700-025 |
| PureLink™ HQ Mini Plasmid Purification Kit | 100 preps | K2100-01 |
| PureLink™ Quick Gel Extraction Kit | 50 preps | K2100-12 |
| BlueJuice™ Gel Loading Buffer | 3 x 1 ml | 10816-015 |
| TrackIt™ Cyan/Orange Loading Buffer | 3 x 0.5 ml | 10482-028 |
| TrackIt™ Cyan/Yellow Loading Buffer | 3 x 0.5 ml | 10482-035 |
| TrackIt™ 10 bp DNA Ladder | 20 applications | 10488-019 |
| TrackIt™ 1 Kb Plus DNA Ladder | 100 applications | 10488-085 |
| Quant-iT™ DNA Assay Kit, Broad-Range | 1000 assays | Q33130 |

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# Accessory Products, Continued

**BLOCK-iT™ RNAi Designer**

The BLOCK-iT™ RNAi Designer is an online tool (www.invitrogen.com/rnaidesigner) to help you design and order microRNA sequences for any target gene of interest. The RNAi Designer incorporates published rules on RNAi design into a proprietary algorithm to design most effective microRNA sequences to obtain high level gene knockdown.

**BLOCK-iT™ RNAi Products**

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

For details, visit the RNAi Central portal at www.invitrogen.com/rnai or contact Technical Service (page 163).

**Gateway® Destination Vectors**

A large selection of Gateway® destination vectors are available from Invitrogen to facilitate the transfer of the pre-miRNA sequence into a suitable destination vector to allow miRNA expression in multiple systems including viral expression systems and tissue-specific expression. See page 77 for a list of destination vectors compatible with the pcDNA™6.2-GW/EmGFP-miR Vector.

**Antibodies**

A large variety of high-quality antibodies including the Zymed® Antibodies is available from Invitrogen for use in Western immunodetection, immunohistochemistry, or ELISA assays. For details, visit www.invitrogen.com or contact Technical Service (page 163).

# Technical Service

## Web Resources

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

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

## Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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## Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on Technical Resources and follow instructions on the page to download the MSDS for your product.

## Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

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# Product Qualification

## Introduction

This section describes the criteria used to qualify the components of the BioModule™ BLOCK-iT™ RNAi Units.

## Vectors

The structure of each vector is verified by restriction enzyme digestion. Additional functional qualification is also performed as below.

**pcDNA™6.2-GW/EmGFP-miR Vector**

The miR-*lacZ* positive ds control oligo is annealed and cloned into each vector as described in this manual and the reaction is transformed into One Shot® TOP10 Chemically Competent *E. coli*. Sequence analysis is performed on 20 colonies using the appropriate forward and reverse sequencing primers. The qualification must meet the following criteria:

- Cloning efficiency of $\geq$95%
- Sequencing analysis must show $\geq$95% clones contain the insert in the correct orientation and $\geq$80% clones must have the correct sequence in both directions (forward and reverse)

The *att*B1 and *att*B2 sequences should be correct

**pLenti6/V5-DEST vector**

The pLenti6/V5-DEST vector is qualified in a recombination assay using Gateway® LR Clonase™ II Enzyme Mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

**pDONR™221 Vector**

pDONR™221 vector is qualified in a BP recombination reaction using the Gateway® BP Clonase™ II. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

## Lipofectamine™ 2000

Lipofectamine™ 2000 is tested for the absence of microbial contamination using blood agar plates, Sabaraud dextrose agar plates, and fluid thioglycolate medium, and functionally by transfection with a luciferase reporter-containing plasmid.

## Antibiotics

**Kanamycin, Ampicillin, and Spectinomycin**

Competent *E. coli* cells are transformed with an appropriate antibiotic-resistant plasmid and appropriate antibiotic-sensitive plasmid. Cells are plated on LB medium containing the appropriate antibiotic. Colony growth should only be observed on LB plates plated with antibiotic-resistant plasmid transformation mixture.

**Blasticidin**

Blasticidin is lot-qualified by performing a kill curve on Blasticidin-sensitive and resistant mammalian cell lines. Blasticidin-sensitive cells should be killed at all concentrations tested (2.5-10 μg/ml) within 10 days after addition of Blasticidin.

*Continued on next page*

# Product Qualification, Continued

| | |
|---|---|
| **Gateway® Clonase™ II Enzyme Mix** | Gateway® BP and LR Clonase™ II Enzyme Mixes are functionally tested in a one hour recombination reaction followed by a transformation assay. |
| **One Shot® Competent Cells** | Each lot of One Shot® Competent cells is tested for transformation efficiency using the pUC19 control plasmid included in the kit and following the procedure described in this manual. Test transformations are performed on 3 to 20 vials per lot, depending on batch size. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and incubated overnight. Transformation efficiency should be greater than 1 x $10^8$ cfu/µg plasmid DNA. In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and the absence of phage contamination. |
| **293FT Cell Line** | Each lot of cells is tested for cell growth and viability post-recovery from cryo-preservation. Master Cell Banks are screened for viruses, mycoplasma, and sterility, and expression of the SV40 large T antigen is confirmed by western blot. |
| **Lentivirus Production** | Using the reagents provided in the kit, the ViraPower™ Packaging Mix and control lentiviral construct are cotransfected into 293FT cells using the protocol on page 105. Lentiviral supernatants are harvested 48 hours post-transfection, and the titer is determined using HT1080 cells. The control lentiviral construct must demonstrate a titer of greater than 1 x $10^5$ TU/ml. |
| **miR-*lacZ* ds oligo** | The miR-*lacZ* positive ds control Oligo is functionally qualified by use in a ligation reaction as described in this manual. |
| **Sequencing Primers** | Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique. |
| **T4 DNA Ligase and 5X DNA Ligase Buffer** | T4 DNA Ligase is lot-qualified and must pass the following quality control assays:<br>• Functional absence of endonuclease and exonuclease activities<br>• Ligation/recut<br>• Ligation efficiency<br>5X DNA Ligase Buffer is functionally qualified with the enzyme and meets quality control specifications. |

*Continued on next page*

# Product Qualification, Continued

## PureLink™ HQ Mini Plasmid Purification Kit

The PureLink™ HQ Mini Plasmid Purification Kit is functionally qualified by isolating a high copy number plasmid DNA from 1–2 × $10^9$ *E. coli* cells as described in this manual. The kit must produce the following results:

- $A_{260}/A_{280} \geq 1.80$
- No detectable genomic DNA or RNA contamination on a 0.8% agarose gel

In addition, each kit component is sterile and is lot qualified for optimal performance.

## S.N.A.P.™ MidiPrep Kit

Each component of the S.N.A.P.™ MidiPrep is lot qualified for maximum performance in accordance with the S.N.A.P.™ MidiPrep protocol. A sample of 4 columns from each lot is qualified in the following manner:

**Binding Capacity**

300 µg of pre-purified control plasmid is applied to the column and eluted according to the protocol. The quantity of plasmid DNA eluted from the column must be >200 µg.

**Kit Performance**

50 ml of control plasmid is grown in TOP10F´ cells and plasmid DNA is isolated using the S.N.A.P.™ MidiPrep Kit.

Plasmid DNA is qualified in the following manner:

**Visual inspection**

Four samples are run on 0.8% agarose gel for a visual inspection. Each isolated plasmid must show supercoiled plasmid DNA with no RNA contamination.

**Restriction Digest**

Four 500 ng plasmid DNA samples are digested with *Apa*I restriction enzyme for 90 minutes. Restriction digest is run on a 0.8% agarose gel and must be >99% complete.

**Endonuclease Activity**

Four 500 ng plasmid DNA samples are incubated with 10 mM $Mg^{2+}$ for 4 hours and then run on a 0.8% agarose gel. Each sample must show no endonuclease contamination of supercoiled DNA present.

## DNA Ladders

Agarose gel analysis must show that bands are distinguishable after ethidium bromide staining.

## E-Gel® Agarose Gels

E-Gel® agarose gels are tested by running a mixture of DNA markers under standard running conditions described in this manual. Gels are visualized under UV light for proper staining, resolution, and migration of bands.

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### Opti-MEM® I Reduced Serum Medium

Opti-MEM® I Reduced Serum Medium is subjected to pH, osmolality, endotoxin, bacterial, fungal, and mycoplasma testing. The endotoxin level must be less than 1.0 EU/ml.

Each lot of Opti-MEM® I is evaluated utilizing sensitive quantitative assays for its ability to support cloning efficiency of a murine myeloma cell line, and growth over multiple subcultures of an adherent cell line. Test lots of Opti-MEM® I Reduced Serum Medium at 2% (CHO growth) and 4% (Sp2 cloning) serum supplementation are compared to a previously approved Opti-MEM® I Reduced Serum Medium control.

GIBCO® cell culture liquid products are prepared by an aseptic process for which each step has been validated to ensure that all products meet the industry standard sterility assurance level of $10^{-3}$; i.e., product that demonstrates a contamination level of no more than 1 of 1000 units during the manufacturing process. The highest level of sterility assurance (equal to or greater than $10^{-6}$) cannot be achieved without terminal sterilization which is harmful to the performance of cell culture products.

# Purchaser Notification

## Introduction

Use of the BioModule™ BLOCK-iT™ RNAi Units is covered under the licenses detailed below.

## 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. 19: Gateway® Cloning Products

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### Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 176.

### Limited Use Label License No. 23: GUS Control Vector

The GUS positive control vector in these products is claimed in patents and patent applications licensed to Invitrogen by Cambia Biosystems, L.L.C. ("CBL"). Use of the GUS gene is restricted to use as a positive control. Any other use may require a license from CBL.

### Limited Use Label License No. 27: Lipofectamine™ 2000

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# Purchaser Notification, Continued

**Limited Use Label License No. 51: Blasticidin and the Blasticidin Selection Marker**

Blasticidin and the blasticidin resistance gene (*bsd*) are the subject of U.S. patents sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

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# Purchaser Notification, Continued

## Limited Use Label License No. 108: Lentiviral Technology

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### Limited Use Label License No. 109: Retroviral Helper Lines

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### Limited Use Label License No. 181: Gateway®-adapted Viral Vectors

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## Limited Use Label License No. 267: Mutant GFP Products

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# Purchaser Notification, Continued

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**Limited Use Label License No. 272: Humanized GFP**

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# Gateway® Clone Distribution Policy

## Introduction

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

## Gateway® Entry Clones

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

## Gateway® Expression Clones

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

## Additional Terms and Conditions

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

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