Invitrogen™
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Instruction Manual

# Mammalian Lumio™ Gateway® Vectors with Lumio™ In-Cell Labeling Kits

## Gateway® destination vectors for site-specific fluorescence labeling and detection of proteins in live mammalian cells

**Catalog nos. 12589-016, 12589-024, and 12589-032**

**Version D**
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*25-0699*

# Table of Contents

# Kit Contents and Storage

## Types of Kits

This manual is supplied with the following products. All products are also supplied with the Lumio™ In-Cell Labeling Kits manual.

| Product | Catalog no. |
|---|---|
| Mammalian Lumio™ Gateway® Vectors with Lumio™ Green In-Cell Labeling Kit | 12589-016 |
| Mammalian Lumio™ Gateway® Vectors with Lumio™ Red In-Cell Labeling Kit | 12589-024 |
| Mammalian Lumio™ Gateway® Vectors with Lumio™ Dual Green and Red In-Cell Labeling Kits | 12589-032 |

## Kit Components

The Mammalian Lumio™ Gateway® Vectors with Lumio™ In-Cell Labeling Kits include the following components. For a detailed description of the contents of each component, see the next page.

| Component | Catalog no. | | |
|---|---|---|---|
| | 12589-016 | 12589-024 | 12589-032 |
| Mammalian Lumio™ Gateway® Vectors | √ | √ | √ |
| Lumio™ Green In-Cell Labeling Kit | √ | | √ |
| Lumio™ Red In-Cell Labeling Kit | | √ | √ |

## Shipping and Storage

The Mammalian Lumio™ Gateway® Vectors with Lumio™ In-Cell Labeling Kits are shipped on blue ice. Upon receipt, store as detailed below.

| Box | Storage |
|---|---|
| Mammalian Lumio™ Gateway® Vectors | -20ºC |
| Lumio™ Green In-Cell Labeling Kit | -20ºC, protected from light |
| Lumio™ Red In-Cell Labeling Kit | -20ºC, protected from light |

## Lumio™ Gateway® Vectors

The Lumio™ Gateway® Vectors are listed below. **Store vectors at -20°C.**

| Item | Concentration | Volume |
|---|---|---|
| pcDNA™6.2/cLumio™-DEST | Lyophilized in TE, pH 8.0 | 6 µg |
| pcDNA™6.2/nLumio™-DEST | Lyophilized in TE, pH 8.0 | 6 µg |
| pcDNA™6.2/nLumio™-GW/p64 Control Plasmid | Lyophilized in TE, pH 8.0 | 10 µg |

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# Kit Contents and Storage, continued

## Lumio™ In-Cell Labeling Kits

The Mammalian Lumio™ Gateway® Vectors are supplied with either the Lumio™ Green, the Lumio™ Red, or both the Lumio™ Green and Lumio™ Red In-Cell Labeling Kits. Refer to the Lumio™ In-Cell Labeling Kits manual for detailed information pertaining to each kit and a description of the reagents provided in each kit.

## Product Qualification

pcDNA™6.2/cLumio™-DEST, pcDNA™6.2/nLumio™-DEST, and pcDNA™6.2/nLumio™-GW/p64 are qualified by restriction endonuclease digestion. The pcDNA™6.2/cLumio™-DEST and pcDNA™6.2/nLumio™-DEST vectors are further 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.

For information about how the components of the Lumio™ In-Cell Labeling Kits are qualified, refer to the Lumio™ In-Cell Labeling Kits manual.

## Accessory Products

Additional products that may be used with the Mammalian Lumio™ Gateway® Vectors are available from Invitrogen. Ordering information is provided below.

| Product | Amount | Catalog no. |
|---|---|---|
| Lumio™ Green In-Cell Labeling Kit | 1 kit | 12589-057 |
| Lumio™ Red In-Cell Labeling Kit | 1 kit | 12589-040 |
| Lumio™ Green Detection Kit | 100 reactions | LC6090 |
| BenchMark™ Fluorescent Protein Standard | 100 reactions | LC5928 |
| Gateway® LR Clonase™ II Enzyme Mix | 20 reactions | 11791-020 |
| | 100 reactions | 11791-100 |
| One Shot® TOP10 Chemically Competent Cells | 10 reactions | C4040-10 |
| | 20 reactions | C4040-03 |
| One Shot® TOP10 Electrocompetent Cells | 10 reactions | C4040-50 |
| | 20 reactions | C4040-52 |
| PureLink™ HQ Plasmid Purification Kit | 100 reactions | K2100-01 |
| Lipofectamine™ 2000 | 1.5 ml | 11668-019 |
| | 0.75 ml | 11668-027 |
| Blasticidin | 50 mg | R210-01 |

# Introduction

## Overview

### Description

The Mammalian Lumio™ Gateway® Vector Kits contain Gateway®-adapted destination vectors designed for use with the Lumio™ Technology. The pcDNA™6.2/Lumio™-DEST vectors supplied with each kit facilitate *in vivo* fluorescence labeling and detection of recombinant proteins when used with a Lumio™ In-Cell Labeling Kit.

### Features

The pcDNA™6.2/cLumio™-DEST and pcDNA™6.2/nLumio™-DEST vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
- Lumio™ tag for C-terminal (pcDNA™6.2/cLumio™-DEST) or N-terminal (pcDNA™6.2/nLumio™-DEST) fusion to the gene of interest for fluorescence detection
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the Blasticidin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Blasticidin resistance gene for selection of stable cell lines
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- The ampicillin resistance gene for selection in *E. coli*

For a map of pcDNA™6.2/cLumio™-DEST and pcDNA™6.2/nLumio™-DEST, refer to pages 20 and 22, respectively.

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# Overview, continued

## The 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 gene of interest into multiple vector systems. To express your gene of interest in mammalian cells using Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector to create an entry clone.
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pcDNA™6.2/cLumio™-DEST or pcDNA™6.2/nLumio™-DEST).
3. Transfect your expression clone into the cell line of choice for transient or stable expression of your gene of interest.

For more information on Gateway®, refer to the Gateway® Technology with Clonase™ II manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).

## Advantages of the Lumio™ Technology

The Lumio™ System is based on the FlAsH (Fluorescein Arsenical Hairpin) technology which uses biarsenical labeling reagents to bind and detect proteins containing a tetracysteine motif (*i.e.* Lumio™ tag) (Griffin *et al.*, 1998). Using the Lumio™ Technology and the Lumio™ In-Cell Labeling Kits for fluorescence labeling of recombinant proteins provides the following advantages:

- Small size of the Lumio™ tag (6 amino acids, 585 Da) is less likely to interfere with the structure or biological activity of the protein of interest
- Lumio™ Labeling Reagents are membrane-permeable and readily cross the cell membrane, allowing labeling and detection of recombinant proteins in live mammalian cells
- Lumio™ Labeling Reagents bind the Lumio™ tag with high specificity and high affinity (nanomolar or lower dissociation constant), allowing targeted labeling of the protein of interest
- Lumio™ Labeling Reagents become strongly fluorescent only upon binding the Lumio™ tag, allowing specific detection of Lumio™-tagged proteins

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# Overview, continued

## Components of the Lumio™ System

The Lumio™ System consists of two major components:

- The tetracysteine Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys) in the pcDNA™6.2/Lumio™-DEST vector. When fused to a gene of interest, the Lumio™ tag allows the expressed fusion protein to be specifically recognized by a biarsenical labeling reagent. For more information on the tetracysteine motif, see below.
- A biarsenical labeling reagent, Lumio™ Green or Lumio™ Red, which becomes fluorescent upon binding to recombinant proteins containing the Lumio™ tag. The Lumio™ Green and Lumio™ Red Labeling Reagents are supplied pre-complexed to the dithiol EDT (1,2-ethanedithiol) which stabilizes and solubilizes the biarsenic reagents.

## Tetracysteine Motif

The Lumio™ Reagents bind a tetracysteine motif consisting of Cys-Cys-Xaa-Xaa-Cys-Cys where Cys equals cysteine and Xaa equals any amino acid other than cysteine. This motif is rarely seen in naturally occurring proteins allowing specific fluorescence labeling of recombinant proteins fused to the Lumio™ tag. In the Lumio™ System, the optimized Cys-Cys-Pro-Gly-Cys-Cys tetracysteine motif is used as this motif has been shown to have a higher affinity for and more rapid binding to biarsenic compounds as well as enhanced stability compared to other characterized motifs (Adams *et al.*, 2002).

## Lumio™ Green Detection Kit

For sensitive and specific in-gel detection of Lumio™-tagged fusion proteins, we recommend the Lumio™ Green Detection Kit available from Invitrogen (Catalog no. LC6090). The Lumio™ Green Detection Kit enables immediate visualization of Lumio™-tagged proteins in polyacrylamide gels using a UV transilluminator or a visible light laser-based scanner and without the need for staining or western blotting. In addition, the BenchMark™ Fluorescent Protein Standard (Catalog no. LC5928) allows you to easily visualize molecular weight ranges of proteins labeled with Lumio™ Green Detection Reagent. For more information on these products and other products that may be used with the Lumio™ Technology, visit our Web site (www.invitrogen.com) or contact Technical Service (page 25).

# Methods

## Generating an Entry Clone

### Introduction

To recombine your gene of interest into pcDNA™6.2/cLumio™-DEST or pcDNA™6.2/nLumio™-DEST, you will need an entry clone containing the gene of interest. Many entry vectors including pENTR/D-TOPO® (Catalog no. K2400-20) are available from Invitrogen to facilitate generation of entry clones. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

### Tag-On-Demand™ System

The pcDNA™6.2/cLumio™-DEST vector is compatible with the Tag-On-Demand™ System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues (Capone *et al.*, 1985) and consists of a recombinant adenovirus expressing a $tRNA^{ser}$ suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand™ Suppressor Supernatant manual. This manual is available for downloading from our Web site (www.invitrogen.com) or contact Technical Service (page 25).

**Note**

If you wish to express a human or mouse gene of interest, we recommend using an Ultimate™ Human ORF (hORF) or Ultimate™ Mouse ORF (mORF) Clone available from Invitrogen. Each Ultimate™ ORF Clone is a fully sequenced clone provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with pcDNA™6.2/cLumio™-DEST. In addition, each clone contains a **TAG** stop codon, making it fully compatible for use in the Tag-On-Demand™ System. For more information about the Ultimate™ ORF Clones available, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

### Kozak Consensus Sequence

If you will be expressing your protein from pcDNA™6.2/cLumio™-DEST, your insert in the entry clone should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

**(G/A)**NN<u>ATG</u>**G**

Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold).

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# Generating an Entry Clone, continued

### Points to Consider for pcDNA™6.2/ cLumio™-DEST

pcDNA™6.2/cLumio™-DEST allows expression of recombinant proteins with a C-terminal peptide containing the V5 epitope and the Lumio™ tag; however, you may use this vector to express a native protein, if desired. You may also use this vector in the Tag-On-Demand™ System (see previous page). Consider the following when generating your entry clone.

| If you wish to... | Then your insert... |
|---|---|
| include the V5 epitope and Lumio™ tag | • should contain a Kozak initiation sequence (see previous page)<br>• should **not** contain a stop codon<br>• should be in frame with the V5 epitope and Lumio™ tag after recombination (see page 7 for a diagram) |
| include the V5 epitope and Lumio™ tag for use in the Tag-On-Demand™ System | • should contain a Kozak initiation sequence (see previous page)<br>• should contain a **TAG** stop codon<br>• should be in frame with the V5 epitope and Lumio™ tag after recombination (see page 7 for a diagram) |
| **not** include the V5 epitope and Lumio™ tag | • should contain a Kozak initiation sequence (see previous page)<br>• should contain a stop codon |

### Points to Consider for pcDNA™6.2/ nLumio™-DEST

pcDNA™6.2/nLumio™-DEST allows expression of recombinant proteins with an N-terminal peptide containing the Lumio™ and V5 epitope tags and contains an ATG initiation codon within the context of a Kozak consensus sequence (see previous page). To include the Lumio™ and V5 epitope tags, your insert in the entry clone should:

- **not** contain a Kozak initiation sequence
- be in frame with the Lumio™ and V5 epitope tags after recombination (see page 8 for a diagram)
- contain a stop codon

# Creating an Expression Clone

## Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into your pcDNA™6.2/Lumio™-DEST vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction** (pages 9-11) before beginning.

## Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pcDNA™6.2/Lumio™-DEST vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (refer to pages 7-8 for diagrams of the recombination region of the resulting expression clones).

## Resuspending the Vectors

The pcDNA™6.2/Lumio™-DEST vectors are supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, simply resuspend the destination vector in 40 µl of sterile water to a final concentration of 150 ng/µl.

## Propagating the Vectors

If you wish to propagate and maintain the pcDNA™6.2/Lumio™-DEST vectors, we recommend using One Shot® *ccd*B Survival T1$^R$ Chemically Competent *E. coli* (Catalog no. C7510-03) from Invitrogen for transformation. The *ccd*B Survival T1$^R$ *E. coli* strain is 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 50-100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol.

**Note: Do not** use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance of the pcDNA™6.2/Lumio™-DEST vectors as these strains are sensitive to CcdB effects.

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# Creating an Expression Clone, continued

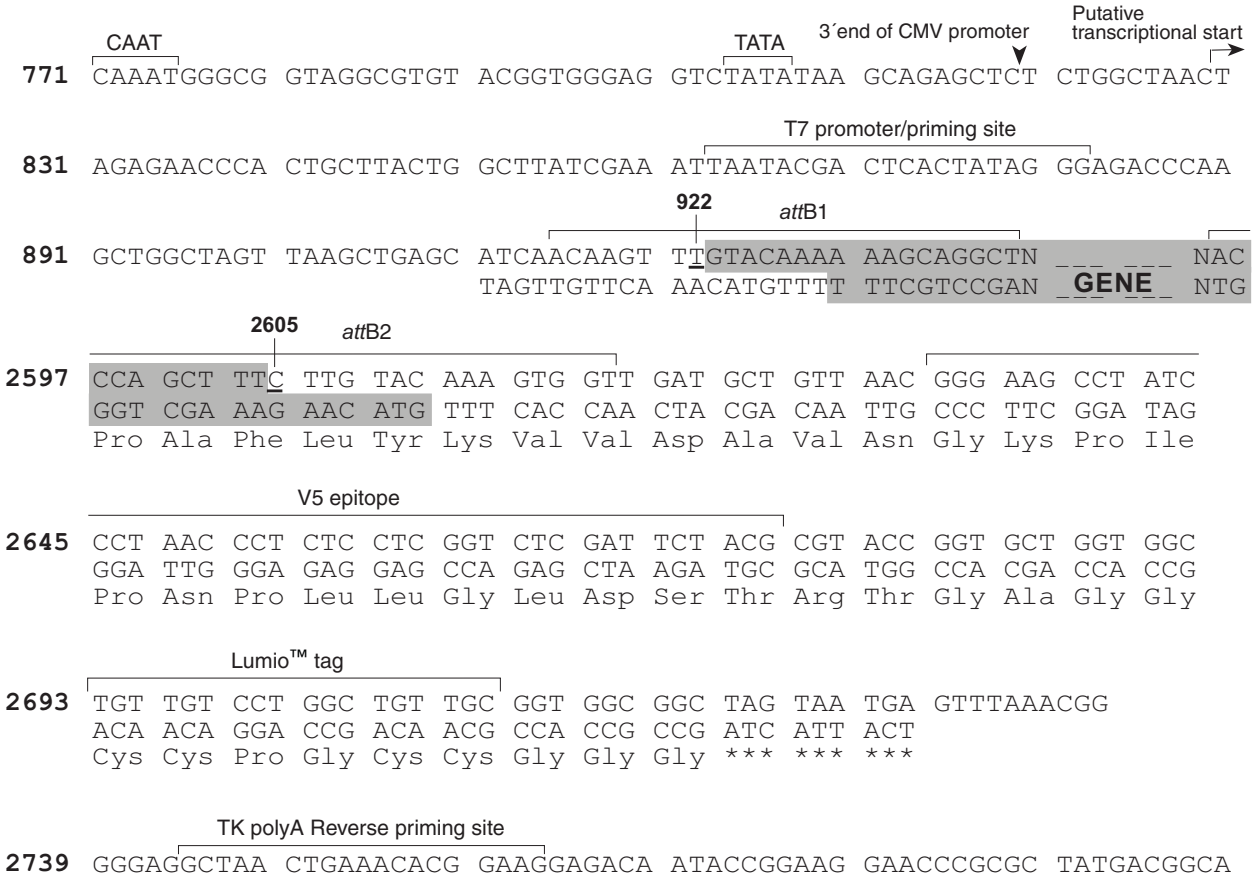
## Recombination Region for pcDNA™6.2/ cLumio™-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ cLumio™-DEST × entry clone is shown below.

**Note:** If you are using pcDNA™6.2/cLumio™-DEST in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page 4 for more information).

**Features of the Recombination Region:**

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/cLumio™-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/cLumio™-DEST vector.
- Bases 922 and 2605 of the pcDNA™6.2/cLumio™-DEST vector sequence are marked.

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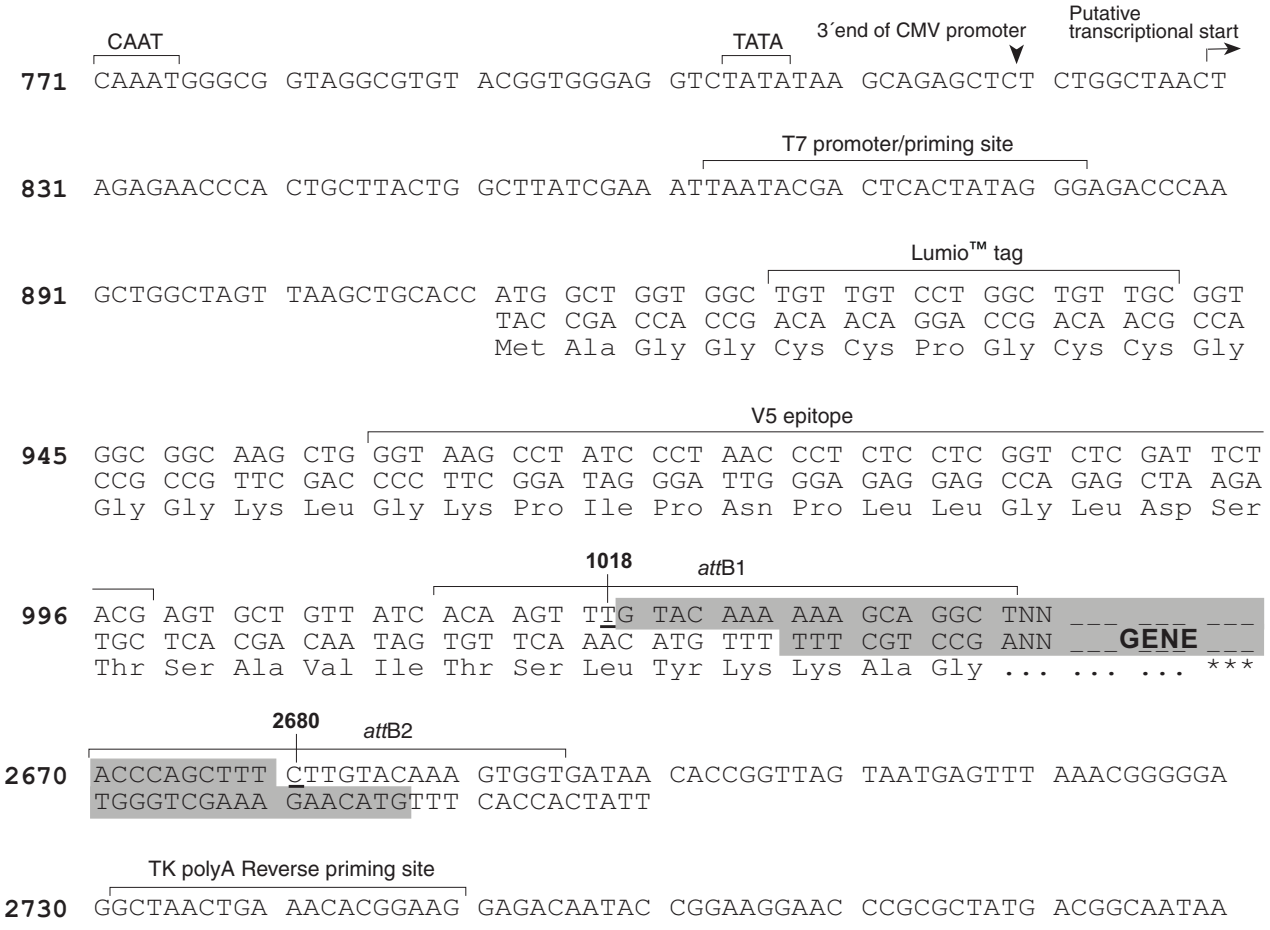
# Creating an Expression Clone, continued

## Recombination Region for pcDNA™6.2/ nLumio™-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ nLumio™-DEST × entry clone is shown below.

**Features of the Recombination Region:**

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/nLumio™-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/nLumio™-DEST vector.
- Bases 1018 and 2680 of the pcDNA™6.2/nLumio™-DEST vector sequence are marked.



# Performing the LR Recombination Reaction

## Introduction

Once you have obtained an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and your pcDNA™6.2/Lumio™-DEST vector, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for an expression clone. We recommend including the pENTR™-gus positive control supplied with the LR Clonase™ II enzyme mix in your experiments to help you evaluate your results.

## *E. coli* Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation. **Do not** transform the LR reaction mixture 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.

**Note**

The presence of the EM7 promoter and the Blasticidin resistance gene in the pcDNA™6.2/Lumio™-DEST vectors allows for selection of *E. coli* transformants using Blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/ml Blasticidin (see the **Appendix**, page 18 for a recipe). For Blasticidin to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.0.

Blasticidin is available separately from Invitrogen (see page vi for ordering information). Refer to the **Appendix**, page 19 for instructions on how to prepare and store Blasticidin.

## LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is available separately from Invitrogen (Catalog no. 11791-020) to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 10 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

**Note:** You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided in this manual as reaction conditions differ.

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# Performing the LR Recombination Reaction, continued

## Materials Needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- pcDNA™6.2/cLumio™-DEST or pcDNA™6.2/nLumio™-DEST vector (150 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Invitrogen, Catalog no. 11791-020; keep at -20°C until immediately before use)
- pENTR™-gus positive control, optional (50 ng/µl in TE, pH 8.0; supplied with the LR Clonase™ II enzyme mix)
- 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)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic to select for expression clones

## Setting Up the LR Recombination Reaction

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

   **Note:** To include a negative control, set up a second sample reaction and substitute TE Buffer, pH 8.0 for 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. To each sample above, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

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

   **Note:** Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µl of the LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

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

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# Performing the LR Recombination Reaction, continued

## 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 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 ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 μg/ml chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

## Sequencing

You may sequence your construct to confirm that your gene is in frame with the appropriate N-terminal or C-terminal fusion tag, if desired. We suggest using the T7 Promoter and TK polyA Reverse primer sequences (see below). Refer to the diagram on page 7 or page 8 for the location of the primer binding sites.

| Primer | Sequence |
|---|---|
| T7 Promoter Primer | 5´-TAATACGACTCACTATAGGG-3´ |
| TK polyA Reverse Primer | 5´-CTTCCGTGTTTCAGTTAGC-3´ |

# Transfecting Cells

## Introduction

This section provides general information for transfecting your expression clone into the mammalian cell line of choice. We recommend that you include the pcDNA™6.2/nLumio™-GW/p64 positive control vector and a non-Lumio™ control in your transfection experiment to help you evaluate your results. The non-Lumio™ control should contain cells transfected with a vector that does not encode the Lumio™ tag. Using untransfected cells (no transfection reagent, no DNA) as a control is not optimal since transfection may introduce fluorescent artifacts or alter cell morphology that could affect the labeling and detection of your protein.

## Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01). Other plasmid purification kits or methods are suitable.

## Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and the other transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

## Cell Density

If you plan to label your recombinant protein using the Lumio™ In-Cell Labeling Kit, we recommend designing your transfection experiment so that cells will be at optimal density at the time of labeling. Suspension cells typically label most efficiently at a density of 1-2 x $10^6$ cells/ml. Adherent cells label most efficiently when they are 60-90% confluent at the time of labeling.

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# Transfecting Cells, continued

## Positive Control

pcDNA™6.2/nLumio™-GW/p64 is provided as a positive control vector for mammalian cell transfection and expression (see page 24 for a map) and may be used to optimize recombinant protein expression levels in your cell line. This vector allows expression of the human c-myc (p64) protein with an N-terminal fusion to the Lumio™ and V5 epitope tags.

**To propagate and maintain the plasmid:**

1. Resuspend the vector in 10 µl sterile water to prepare a 1 µg/µl stock solution. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, or equivalent.
2. Select transformants on LB agar plates containing 50-100 µg/ml ampicillin.
3. Prepare a glycerol stock of a positive transformant for long-term storage.

# Creating Stable Cell Lines

## Introduction

The pcDNA™6.2/Lumio™-DEST vectors contain the Blasticidin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Blasticidin. General information and guidelines are provided below.

**Note**

The Lumio™ Technology works best for labeling proteins that are expressed at high levels or are concentrated in a subcellular region. If you are expressing proteins at low levels (*e.g.* from a weak promoter) or if you are expressing a cytoplasmic protein, we recommend conducting initial labeling and detection studies using the Lumio™ In-Cell Labeling Kits in transiently transfected cells where protein expression levels are higher. Once labeling conditions are optimized, you may analyze your protein in stable cell lines.


To obtain stable transfectants, we recommend that you linearize your pcDNA™6.2/Lumio™-DEST construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, cut at a unique site that is not located within a critical element or within your gene of interest.

## Determining Blasticidin Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line by performing a kill curve experiment (see below). Typically, concentrations ranging from 2.5 to 10 µg/ml Blasticidin are sufficient to kill most untransfected mammalian cell lines. Blasticidin is available separately from Invitrogen (Catalog no. R210-01). Refer to the **Appendix**, page 19 for instructions on how to prepare and store Blasticidin.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates.
2. On the following day, replace the growth medium with fresh growth medium containing varying concentrations of Blasticidin (*e.g.* 0, 1, 3, 5, 7.5, and 10 µg/ml Blasticidin).
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Blasticidin that prevents growth within 10-14 days after addition of Blasticidin.

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# Creating Stable Cell Lines, continued

## Generating Stable Cell Lines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your pcDNA™6.2/Lumio™-DEST construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/ cLumio™-DEST or pcDNA™6.2/nLumio™-DEST expression construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium.
3. 48 hours after transfection, split the cells into fresh growth medium such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2-3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Blasticidin at the predetermined concentration required for your cell line.
6. Feed the cells with selective media every 3-4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 10 Blasticidin-resistant colonies and expand them to assay for recombinant protein expression.

# Detecting Lumio™ Fusion Proteins

## Introduction

Once you have transfected your expression clone into mammalian cells, you may:

- Detect protein expression and localization in live cells by fluorescence microscopy using the Lumio™ In-Cell Labeling Kit. For detailed guidelines and protocols, refer to the Lumio™ In-Cell Labeling Kits manual.
- Detect protein expression directly in polyacrylamide gels using the Lumio™ Green Detection Kit (see below).
- Detect protein expression by Western blot analysis using Anti-V5 Antibodies available from Invitrogen (see below).

## Lumio™ Green Detection Kit

The Lumio™ Green Detection Kit (Catalog no. LC6090) enables immediate and specific visualization of Lumio™-tagged proteins in polyacrylamide gels using a UV transilluminator or a visible light laser-based scanner and without the need for staining or western blotting. Refer to the Lumio™ Green Detection Kit manual for detailed protocols to prepare lysate samples specifically for detection with the Lumio™ Green Detection Reagent. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).

## Western Blotting

You may detect expression of your recombinant fusion protein using the Anti-V5 Antibody (Catalog no. R960-25), Anti-V5-HRP Antibody (Catalog no. R961-25), or Anti-V5-AP Antibody (Catalog no. R962-25) available from Invitrogen. You may use any method of choice to prepare your mammalian cell lysates for Western blot analysis. We recommend the following guidelines:

- If you plan to analyze your samples using the Lumio™ Green Detection Kit (see above) in addition to Western blotting, you will need to prepare your samples using lysis buffer. Lysates containing standard Laemmli SDS-PAGE sample buffer will not be suitable for in-gel detection with the Lumio™ Green Detection Kit. Refer to the Lumio™ Green Detection Kit manual for a protocol to prepare cell lysates that are compatible with both in-gel detection and Western blot analysis.
- For cells transfected with the pcDNA™6.2/nLumio™-GW/p64 positive control vector, you will need to prepare lysates using RIPA or SDS-PAGE sample buffer to adequately release p64 from the nucleoli. If you are preparing samples using lysis buffer, you may sonicate your samples to release p64.
- To detect p64 (human c-myc) expression, you may use any of the Anti-V5 Antibodies or the Anti-*myc* Antibodies available from Invitrogen.

  **Note:** The *c-myc* gene encodes a protein with an expected molecular weight of 48 kDa, however, the native protein actually runs at a range of 55-64 kDa on an SDS-PAGE gel.

*continued on next page*

# Detecting Lumio™ Fusion Proteins, continued

### Polyacrylamide Gels Available from Invitrogen

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

**Note**

Expression of your protein fused to the Lumio™ and V5 epitope tags will increase the size of your recombinant protein. The table below lists the expected size increase in molecular weight for your recombinant protein. Note that the expected sizes take into account any additional amino acids between the gene of interest and the fusion peptide (see pages 7 and 8 for a diagram).

| Vector | Fusion | Expected Size Increase |
|---|---|---|
| pcDNA™6.2/cLumio™-DEST | C-terminal | 4 kDa |
| pcDNA™6.2/nLumio™-DEST | N-terminal | 4 kDa |

# Appendix

## Recipes

### LB (Luria-Bertani) Medium and Plates

**Composition:**

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (100 µg/ml ampicillin) if needed.
4. Store at room temperature or at +4°C.

**LB agar plates**

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (100 µg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

### Low Salt LB Medium with Blasticidin

**Low Salt LB Medium:**

10 g Tryptone
**5 g NaCl**
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 µg/ml final concentration.
4. Store plates at +4°C in the dark. Plates containing Blasticidin are stable for up to 2 weeks.

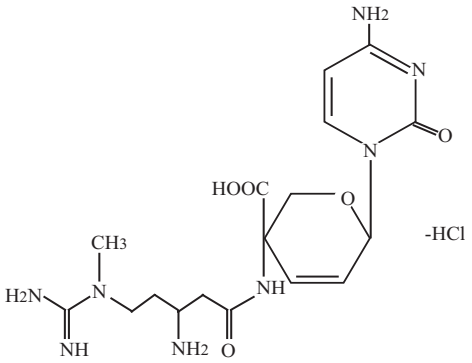
# Blasticidin

## Blasticidin

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

## Molecular Weight, Formula, and Structure

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




## Handling Blasticidin

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

## Preparing and Storing Stock Solutions

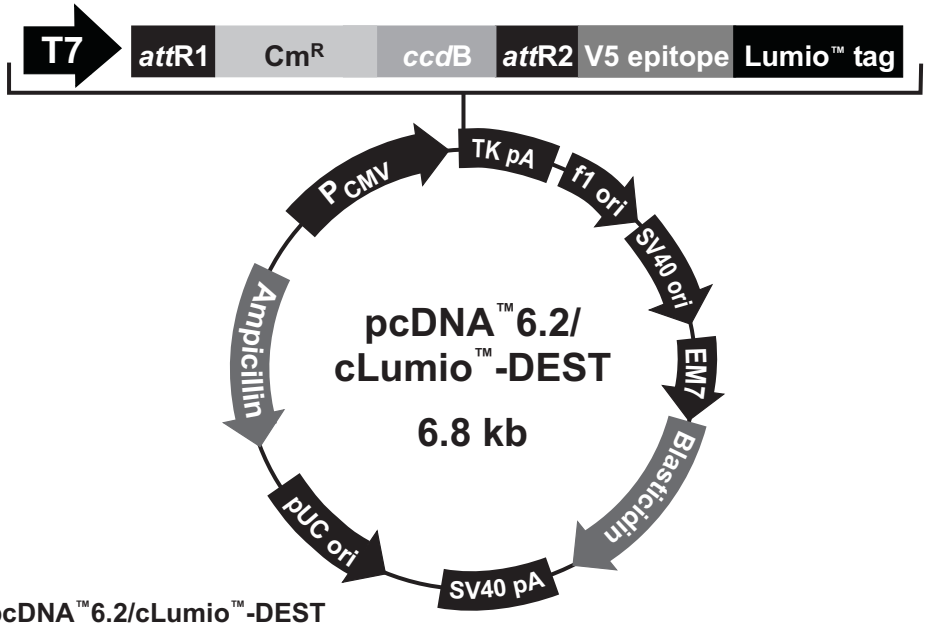
Blasticidin may be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

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

# Map and Features of pcDNA™6.2/cLumio™-DEST

## Map

The map below shows the elements of pcDNA™6.2/cLumio™-DEST. DNA from the entry clone replaces the region between bases 922 and 2605. **The complete sequence of this vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**




**Comments for pcDNA™6.2/cLumio™-DEST**
**6809 nucleotides**

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
*att*R1 site: bases 915-1039
Chloramphenicol resistance gene: bases 1148-1807
*ccd*B gene: bases 2149-2454
*att*R2 site: bases 2495-2619
V5 epitope: bases 2633-2674
Lumio™ tag: bases 2693-2710
TK polyadenylation signal: bases 2737-3008
TK polyA reverse priming site: bases 2744-2762
f1 origin: bases 3044-3472
SV40 early promoter and origin: bases 3499-3807
EM7 promoter: bases 3862-3928
Blasticidin resistance gene: bases 3929-4327
SV40 early polyadenylation signal: bases 4485-4615
pUC origin (c): bases 4998-5668
Ampicillin (*bla*) resistance gene (c): bases 5813-6673
*bla* promoter (c): bases 6674-6772

(c) = complementary strand

*continued on next page*

# Map and Features of pcDNA™6.2/cLumio™-DEST, continued

## Features

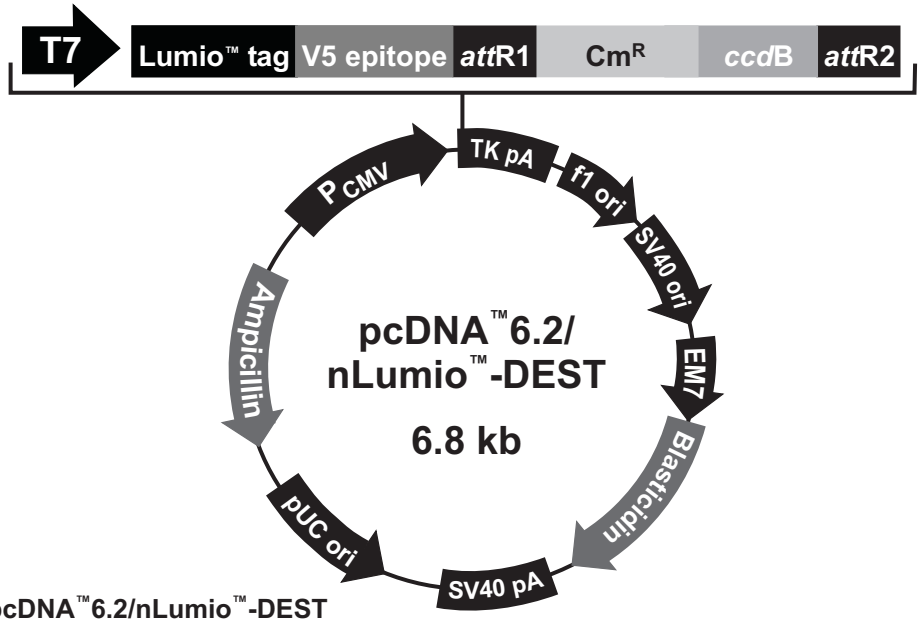
pcDNA™6.2/cLumio™-DEST (6809 bp) contains the following elements. All features have been functionally tested.

| Feature | Benefit |
|---|---|
| Human cytomegalovirus (CMV) immediate-early promoter/enhancer | Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) |
| T7 promoter/priming site | Allows *in vitro* transcription in the sense orientation and sequencing through the insert |
| *att*R1 and *att*R2 sites | Allows recombinational cloning of the gene of interest from an entry clone |
| Chloramphenicol resistance gene | Allows counterselection of plasmid |
| *ccd*B gene | Allows negative selection of plasmid |
| V5 epitope | Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern *et al.*, 1991) |
| Lumio™ tag | Allows binding of the Lumio™ Labeling Reagents to facilitate *in vivo* fluorescence detection of the recombinant fusion protein (Adams *et al.*, 2002) |
| Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985) |
| f1 origin | Allows rescue of single-stranded DNA |
| SV40 early promoter and origin | Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen |
| EM7 promoter | Allows expression of the Blasticidin resistance gene in *E. coli* |
| Blasticidin (*bsd*) resistance gene | Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994) |
| SV40 early polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA |
| pUC origin | Allows high-copy number replication and growth in *E. coli* |
| Ampicillin resistance gene | Allows selection of transformants in *E. coli* |

# Map and Features of pcDNA™6.2/nLumio™-DEST

## Map

The map below shows the elements of pcDNA™6.2/nLumio™-DEST. DNA from the entry clone replaces the region between bases 1018 and 2680. **The complete sequence of this vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**




**Comments for pcDNA™6.2/nLumio™-DEST**
**6796 nucleotides**

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Lumio™ tag: bases 924-941
V5 epitope: bases 957-998
*att*R1 site: bases 1011-1135
Chloramphenicol resistance gene: bases 1244-1903
*ccd*B gene: bases 2224-2529
*att*R2 site: bases 2570-2694
TK polyadenylation signal: bases 2724-2995
TK polyA reverse priming site: bases 2731-2749
f1 origin: bases 3031-3459
SV40 early promoter and origin: bases 3486-3794
EM7 promoter: bases 3849-3915
Blasticidin resistance gene: bases 3916-4314
SV40 early polyadenylation signal: bases 4472-4602
pUC origin (c): bases 4985-5655
Ampicillin (*bla*) resistance gene (c): bases 5800-6660
*bla* promoter (c): bases 6661-6759

(c) = complementary strand

*continued on next page*

# Map and Features of pcDNA™6.2/nLumio™-DEST, continued

## Features

pcDNA™6.2/nLumio™-DEST (6796 bp) contain the following elements. All features have been functionally tested.

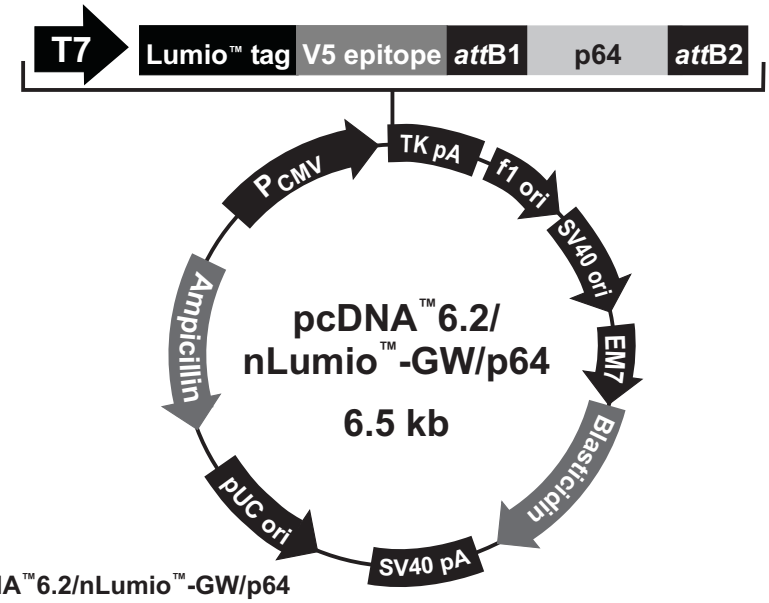
| Feature | Benefit |
|---|---|
| Human cytomegalovirus (CMV) immediate-early promoter/enhancer | Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) |
| T7 promoter | Allows *in vitro* transcription in the sense orientation |
| Lumio™ tag | Allows binding of the Lumio™ Labeling Reagents to facilitate *in vivo* fluorescence detection of the recombinant fusion protein (Adams *et al.*, 2002) |
| V5 epitope | Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern *et al.*, 1991) |
| *att*R1 and *att*R2 sites | Allows recombinational cloning of the gene of interest from an entry clone |
| Chloramphenicol resistance gene | Allows counterselection of plasmid |
| *ccd*B gene | Allows negative selection of plasmid |
| Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985) |
| TK polyA reverse priming site | Allows sequencing through the insert |
| f1 origin | Allows rescue of single-stranded DNA |
| SV40 early promoter and origin | Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen |
| EM7 promoter | Allows expression of the Blasticidin resistance gene in *E. coli* |
| Blasticidin (*bsd*) resistance gene | Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994) |
| SV40 early polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA |
| pUC origin | Allows high-copy number replication and growth in *E. coli* |
| Ampicillin resistance gene | Allows selection of transformants in *E. coli* |

# Map of pcDNA™6.2/nLumio™-GW/p64

## Description

pcDNA™6.2/nLumio™-GW/p64 (6485 bp) is a control vector expressing the p64 gene, and was generated using the Gateway® LR recombination reaction between an entry clone containing the human *c-myc* gene (Bernard *et al.*, 1983; Colby *et al.*, 1983; Watt *et al.*, 1983) and pcDNA™6.2/nLumio™-DEST. **The complete sequence of this vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**

**Note:** The *c-myc* gene encodes a protein with an expected molecular weight of 48 kDa, however, the native protein actually runs at a range of 55-64 kDa on an SDS-PAGE gel.




**Comments for pcDNA™6.2/nLumio™-GW/p64**
**6485 nucleotides**

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Lumio™ tag: bases 924-941
V5 epitope: bases 957-998
*att*B1 site: bases 1011-1035
p64 gene: bases 1038-2357
*att*B2 site: bases 2359-2383
TK polyadenylation signal: bases 2413-2684
TK polyA reverse priming site: bases 2420-2438
f1 origin: bases 2720-3148
SV40 early promoter and origin: bases 3175-3483
EM7 promoter: bases 3538-3604
Blasticidin resistance gene: bases 3605-4003
SV40 early polyadenylation signal: bases 4161-4291
pUC origin (c): bases 4674-5344
Ampicillin (*bla*) resistance gene (c): bases 5489-6349
*bla* promoter (c): bases 6350-6448

(c) = complementary strand

# Technical Service

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Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

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- View and download vector maps and sequences
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- Obtain citations for Invitrogen products
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...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

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# Technical Service, continued

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# Purchaser Notification

## Introduction

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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 29.

*continued on next page*

# Purchaser Notification, continued

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**Limited Use Label License No. 167: Target Sequences for Synthetic Molecules**

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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.

# References


Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002). New Biarsenical Ligands and Tetracysteine Motifs for Protein Labeling in Vitro and in Vivo: Synthesis and Biological Applications. J. Am. Chem. Soc. *124*, 6063-6076.

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Bernard, O., Cory, S., Gerondakis, S., Webb, E., and Adams, J. M. (1983). Sequence of the Murine and Human Cellular myc Oncogenes and Two Modes of myc Transcription Resulting from Chromosome Translocation in B Lymphoid Tumors. EMBO J. *2*, 2375-2383.

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Capone, J. P., Sharp, P. A., and RajBhandary, U. L. (1985). Amber, Ochre and Opal Suppressor tRNA Genes Derived from a Human Serine tRNA Gene. EMBO J. *4*, 213-221.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Colby, W. W., Chen, E. Y., Smith, D. H., and Levinson, A. D. (1983). Identification and Nucleotide Sequence of a Human Locus Homologous to the v-myc Oncogene of Avian Myelocytomatosis Virus MC29. Nature *301*, 722-725.

Cole, C. N., and Stacy, T. P. (1985). Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Griffin, B. A., Adams, S. R., and Tsien, R. Y. (1998). Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells. Science *281*, 269-272.

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233.


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# References, continued

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659.

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5.

Watt, R., Stanton, L. W., Marcu, K. B., Gallo, R. C., Croce, C. M., and Rovera, G. (1983). Nucleotide Sequence of Cloned cDNA of Human c-myc Oncogene. Nature *303*, 725-728.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677.


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