

USER GUIDE

invitrogen™
by *life* technologies™

pAd/CMV/V5-DEST™ and pAd/PL-DEST™ Gateway® Vectors

Gateway®-adapted destination vectors for cloning and high-level, transient expression in mammalian cells using the ViraPower™ Adenoviral Expression System

Catalog numbers V493-20 and V494-20

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Table of Contents

| | |
|---|-----------|
| Table of Contents | 3 |
| Kit Contents and Storage | 4 |
| Accessory Products..... | 5 |
| Introduction | 6 |
| Overview | 6 |
| Methods | 9 |
| Generating an Entry Clone | 9 |
| Creating Expression Clones..... | 11 |
| Performing the LR Recombination Reaction..... | 13 |
| Expression and Analysis..... | 16 |
| Appendix..... | 20 |
| Map and Features of pAd/CMV/V5-DEST™ | 20 |
| Map and Features of pAd/PL-DEST™ | 22 |
| Map of pAd/CMV/V5-GW/lacZ..... | 24 |
| Technical Support | 25 |
| Purchaser Notification | 26 |
| Gateway® Clone Distribution Policy | 27 |
| References | 28 |

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products. Catalog numbers K4930-00 and K4940-00 are also supplied with the 293A Cell Line, the 293A Cell Line manual, and the ViraPower™ Adenoviral Expression System manual.

Note: The 293A Cell Line and ViraPower™ Adenoviral Expression System manuals are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).

| Product | Cat. no. |
|--|----------|
| ViraPower™ Adenoviral Gateway® Expression Kit | K4930-00 |
| ViraPower™ Adenoviral Promoterless Gateway® Expression Kit | K4940-00 |
| pAd/CMV/V5-DEST™ Gateway® Vector | V493-20 |
| pAd/PL-DEST™ Gateway® Vector | V494-20 |

Shipping/Storage

The pAd/CMV/V5-DEST™ and pAd/PL-DEST™ Gateway® Vectors are shipped on dry ice. Upon receipt, store at -20°C.

Contents

The following reagents are supplied with the pAd/CMV/V5-DEST™ and pAd/PL-DEST™ Gateway® Vectors. **Store at -20°C.**

| Vector | Composition | Amount |
|------------------------------------|--------------------------------|--------|
| pAd/CMV/V5-DEST™ or pAd/PL-DEST™ | 150 ng/μl in TE Buffer, pH 8.0 | 40 μl |
| pAd/CMV/V5-GW/lacZ control plasmid | 1 μg/μl in TE Buffer, pH 8.0 | 10 μl |

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

Product Use

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

Accessory Products

Additional Products

The products below may be used with the pAd/CMV/V5-DEST™ and pAd/PL-DEST™ vectors. For more information, refer to our website (www.lifetechnologies.com) or call Technical Support (see page 25).

| Item | Amount | Cat. no. |
|---|-------------------------------|------------------------|
| ViraPower™ Adenoviral Gateway® Expression Kit | 1 kit | K4930-00 |
| ViraPower™ Adenoviral Promoterless Gateway® Expression Kit | 1 kit | K4940-00 |
| 293A Cell Line | 3 × 10 ⁶ cells | R705-07 |
| Gateway® LR Clonase® II Enzyme Mix | 20 reactions 100 reactions | 11791-020 11791-100 |
| Library Efficiency® DB3.1™ Competent Cells | 1 ml (5 × 0.2 ml) | 11782-018 |
| One Shot® TOP10 Chemically Competent <i>E. coli</i> | 20 × 50 µl 40 × 50 µl | C4040-03 C4040-06 |
| Library Efficiency DH5 Chemically Competent <i>E. coli</i> | 5 × 0.2 ml | 18263-012 |
| One Shot® <i>ccdB</i> Survival™ 2 T1 ^R Chemically Competent <i>E. coli</i> | 10 × 50 µl | A10460 |
| PureLink® HiPure Plasmid Miniprep Kit | 25 preps | K2100-02 |
| PureLink® HiPure Plasmid Midiprep Kit | 25 preps | K2100-04 |
| Lipofectamine® 2000 | 0.75 ml 1.5 ml | 11668-027 11668-019 |
| β-Gal Antiserum | 50 µl* | R901-25 |
| β-Gal Assay Kit | 80 ml | K1455-01 |
| β-Gal Staining Kit | 1 kit | K1465-01 |
| Ampicillin Sodium Salt, irradiated | 200 mg | 11593-027 |

Detection of Recombinant Protein

If you express your recombinant protein from pAd/CMV/V5-DEST™, you can use an antibody to the V5 epitope for detection (see table below). Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. A fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments.

| Item | Quantity | Cat. no. |
|-----------------------|----------|----------|
| Anti-V5 Antibody | 50 µl* | R960-25 |
| Anti-V5-HRP Antibody | 50 µl* | R961-25 |
| Anti-V5-AP Antibody | 125 µl* | R962-25 |
| Anti-V5-FITC Antibody | 50 µl* | R963-25 |

*Amount supplied is sufficient for 25 western blots or 25 immunostaining reactions, as appropriate.

Introduction

Overview

Introduction

pAd/CMV/V5-DEST™ (36.7 kb) and pAd/PL-DEST™ (34.9 kb) are destination vectors adapted for use with the Gateway® Technology, and are designed to allow high-level, transient expression of recombinant fusion proteins in dividing and non-dividing mammalian cells using Life Technologies' ViraPower™ Adenoviral Expression System.

A choice of vectors allows you to generate an adenovirus expressing your recombinant protein of interest under the following conditions (see table below).

| Vector | Feature | Benefit |
|------------------|-----------------------|---|
| pAd/CMV/V5-DEST™ | CMV promoter | For high-level, constitutive expression of the gene of interest |
| | C-terminal V5 epitope | For detection of recombinant protein using the Anti-V5 antibodies |
| pAd/PL-DEST™ | No promoter | Allows expression of the gene or sequence of interest using your promoter of choice |
| | No 3' sequences | Allows addition of a C-terminal epitope tag (if desired) and a polyadenylation signal of choice |

For more information about the Gateway® Technology and the ViraPower™ Adenoviral Expression System, see page 8.

Continued on next page

Overview, continued

Features of the Vectors

The pAd/CMV/V5-DEST™ and pAd/PL-DEST™ vectors contain the following elements:

- Human adenovirus type 5 sequences (Ad 1-458 and 3513-35935) encoding genes and elements (*e.g.* Left and Right Inverted Terminal Repeats (ITRs), encapsidation signal sequence, late genes) required for proper packaging and production of adenovirus (Hitt *et al.*, 1999; Russell, 2000)
- Human cytomegalovirus (CMV) immediate early promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (**in pAd/CMV/V5-DEST™ only**) (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
- Two recombination sites, *attR1* and *attR2* for recombinational cloning of the DNA sequence of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *attR* sites for counterselection
- The *ccdB* gene located between the *attR* sites for negative selection
- C-terminal V5 epitope for detection of the recombinant protein of interest (**in pAd/CMV/V5-DEST™ only**) (Southern *et al.*, 1991)
- Herpes Simplex Virus thymidine kinase (TK) polyadenylation sequence for efficient transcription termination and polyadenylation of mRNA (**in pAd/CMV/V5-DEST™ only**) (Cole & Stacy, 1985)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

The control plasmid, pAd/CMV/V5-GW/*lacZ*, is included for use as a positive expression control in the mammalian cell line of choice.

Continued on next page

Overview, continued

The Gateway® Technology

Gateway® is a universal cloning technology 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 gene of interest in mammalian cells using the Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone. **Note:** If you are using pAd/PL-DEST™, your insert will need to include a promoter of choice, the gene or sequence of interest, and a polyadenylation signal.
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (e.g. pAd/PL-DEST™ or pAd/CMV/V5-DEST™).
3. Use your expression clone in the ViraPower™ Adenoviral Expression System (see below).

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual. This manual is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).

The ViraPower™ Adenoviral Expression System

The ViraPower™ Adenoviral Expression System facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene to dividing and non-dividing mammalian cells using a replication-incompetent adenovirus. The System utilizes Gateway®-adapted destination vectors to allow highly efficient and rapid creation of adenoviral vectors that circumvent the need for traditional, homologous recombination and the use of *recA*⁺ bacteria or extensive DNA manipulation and ligation protocols to construct the recombinant adenovirus genome. To express your gene of interest in mammalian cells using the ViraPower™ Adenoviral Expression System, you will:

1. Create an expression clone in pAd/CMV/V5-DEST™ or pAd/PL-DEST™ using Gateway® Technology (see the previous section).
2. Digest the expression clone with *Pac* I to expose the viral inverted terminal repeats (ITRs).
3. Transfect your *Pac* I-digested expression clone into the 293A Cell Line to produce a crude adenoviral stock. Amplify the adenovirus by infecting 293A cells.
4. Titer the adenoviral stock and use it to transduce the mammalian cell line of choice.
5. Assay for “transient” expression of the recombinant protein.

For more information about the ViraPower™ Adenoviral Expression System, refer to the ViraPower™ Adenoviral Expression System manual. For more information about the 293A Cell Line, refer to the 293A Cell Line manual. Both manuals are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25)

Methods

Generating an Entry Clone

Introduction

To recombine your DNA sequence of interest into pAd/CMV/V5-DEST™ or pAd/PL-DEST™, you will need an entry clone containing the DNA sequence of interest. Many entry vectors are available from Life Technologies to facilitate generation of entry clones (see table below). For more information about each vector, see our website (www.lifetechnologies.com) or contact Technical Support (see page 25).

| Entry Vector | Cat. no. |
|--------------------|-----------|
| pENTR™/D-TOPO® | K2400-20 |
| pENTR™/SD/D-TOPO® | K2420-20 |
| pENTR™/TEV/D-TOPO® | K2525-20 |
| pENTR™1A | 11813-011 |
| pENTR™2B | 11816-014 |
| pENTR™3C | 11817-012 |
| pENTR™4 | 11818-010 |
| pENTR™11 | 11819-018 |

Once you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions to construct an entry clone. All entry vector manuals are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).

Insert Size Limitations

The size of the wild-type adenovirus type 5 genome is approximately 35.9 kb. Studies have demonstrated that recombinant adenovirus can efficiently package up to 108% of the wild-type virus size from E1 and E3-deleted vectors (Bett *et al.*, 1994). Taking into account the size of the elements required for expression from each pAd-DEST vector, we recommend that your DNA sequence or gene of interest **not** exceed the size indicated for efficient packaging (see table below).

| Vector | Insert Size Limit |
|------------------|-------------------|
| pAd/CMV/V5-DEST™ | 6.0 kb |
| pAd/PL-DEST™ | 7.5 kb |

Continued on next page

Generating an Entry Clone, continued

Points to Consider Before Recombining into pAd/CMV/V5-DEST™

pAd/CMV/V5-DEST™ is a C-terminal fusion vector; however, you may use this vector to express native proteins or C-terminal fusion proteins. Consider the following when generating your entry clone.

- If you will be recombining your entry clone with a destination vector for mammalian expression, your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position -3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NNATGG

- If you wish to include the V5 epitope tag, your gene in the entry clone **should not** contain a stop codon. In addition, the gene should be in frame with the V5 epitope tag after recombination.
- If you **do not** wish to include the V5 epitope tag, make sure that your gene contains a stop codon in the entry clone.

Refer to the diagram of the recombination region of pAd/CMV/V5-DEST™ on page 12 to help you design a strategy to generate your entry clone.

Points to Consider Before Recombining into pAd/PL-DEST™

pAd/PL-DEST™ allows generation of an adenovirus that contains a gene of interest whose expression is controlled by a promoter of choice. Alternatively, the vector may also be used to express small RNA molecules from their appropriate promoters. To facilitate proper expression of your gene or sequence of interest from pAd/PL-DEST™, you will need to generate an entry clone containing the following:

- A promoter of choice to control expression of the gene or sequence of interest in mammalian cells.
- The gene or sequence of interest. Note that the gene of interest should contain an ATG initiation codon in the context of a Kozak consensus sequence for proper initiation of translation in mammalian cells (Kozak, 1987; Kozak, 1990; Kozak, 1991) and a stop codon.
- A polyadenylation signal sequence of choice for proper transcription termination and polyadenylation of mRNA.

Note: You may also include an N-terminal or C-terminal fusion tag, if desired.

Refer to the diagram of the recombination region of pAd/PL-DEST™ on page 12 to help you design a strategy to generate your entry clone.

Creating Expression Clones

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into the pAd/CMV/V5-DEST™ or pAd/PL-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 13–15) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *attL*-containing entry clone and the *attR*-containing pAd/CMV/V5-DEST™ or pAd/PL-DEST™ vector.
Note: Both the entry clone and the destination vector should be supercoiled (see **Important Note** below).
 2. Transform the reaction mixture into a suitable *E. coli* host (see page 13).
 3. Select for expression clones (see the next page for illustrations of the recombination region of expression clones in pAd/CMV/V5-DEST™ or pAd/PL-DEST™).
-

Important

The pAd/CMV/V5-DEST™ and pAd/PL-DEST™ vectors are supplied as supercoiled plasmids. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient recombination, further testing at Life Technologies has found that linearization of pAd/CMV/V5-DEST™ and pAd/PL-DEST™ is **not** required to obtain optimal results for any downstream application.

Destination Vectors

Each destination vector is supplied in solution at a concentration of 150 ng/μl in TE Buffer, pH 8.0, and is ready-to-use in the LR recombination reaction.

Propagating the Destination Vectors

If you wish to propagate and maintain the pAd/CMV/V5-DEST™ and pAd/PL-DEST™ vectors, we recommend using One Shot® *ccdB* Survival™ 2 T1^R Chemically Competent *E. coli* Cells from Life Technologies (see page 5) for transformation. The *ccdB* Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccdB* gene. To maintain integrity of the vector, select for transformants in media containing 50–100 μg/ml ampicillin or carbenicillin and 15–30 μg/ml chloramphenicol.

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

Continued on next page

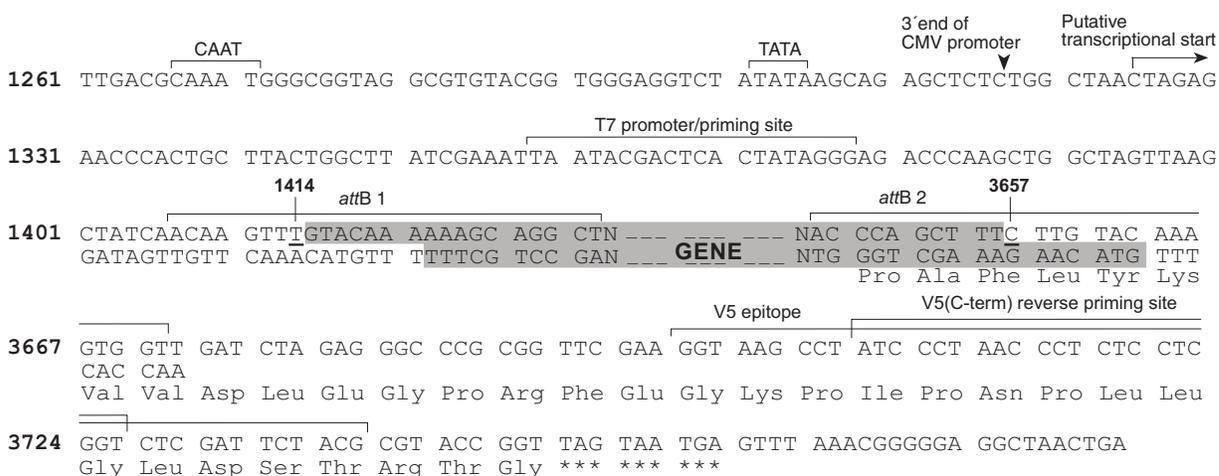
Creating Expression Clones, continued

Recombination Region of pAd/CMV/V5-DEST™

The recombination region of the expression clone resulting from pAd/CMV/V5-DEST™ x entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pAd/CMV/V5-DEST™ vector by recombination. Non-shaded regions are derived from the pAd/CMV/V5-DEST™ vector.
- Bases 1414 and 3657 of the pAd/CMV/V5-DEST™ sequence are marked.

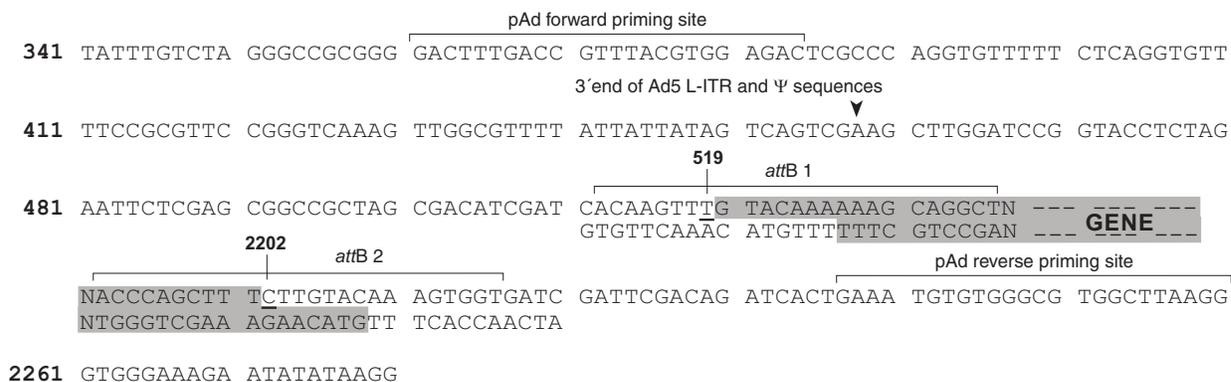


Recombination Region of pAd/PL-DEST™

The recombination region of the expression clone resulting from pAd/PL-DEST™ x entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pAd/PL-DEST™ vector by recombination. Non-shaded regions are derived from the pAd/PL-DEST™ vector.
- Bases 519 and 2202 of the pAd/PL-DEST™ 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 pAd/CMV/V5-DEST™ or pAd/PL-DEST™, and transform the reaction mixture into a suitable *E. coli* host (see *E. coli* Host) to select for an expression clone. We recommend including a negative control (no LR Clonase® II) in your experiment to help you evaluate your results.

E. coli Host

You may use any *recA*, *endA* *E. coli* strain including TOP10, DH5α™, or equivalent for transformation (see page 5 for ordering information). **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F' episome (e.g. TOP10F'). These strains contain the *ccdA* gene and will prevent negative selection with the *ccdB* gene.

LR Clonase® II Enzyme Mix

To catalyze the LR recombination reaction, you will use LR Clonase® II enzyme mix, which is available separately from Life Technologies (see page 5). 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 14 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.

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 Buffer, pH 8.0)
 - pAd/CMV/V5-DEST™ or pAd/PL-DEST™ vector (150 ng/μl in TE Buffer, pH 8.0)
 - LR Clonase® II enzyme mix (see page 5); 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 (supplied with the LR Clonase® II enzyme mix)
 - Appropriate competent *E. coli* host and growth media for expression
 - S.O.C. Medium
 - LB agar plates containing 100 μg/ml ampicillin or carbenicillin to select for expression clones
-

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Performing the LR Recombination Reaction, continued

Important

Use care when handling the pAd/CMV/V5-DEST™ or pAd/PL-DEST™ plasmid DNA. The pAd-DEST plasmids are large (> 34 kb in size) and excessive manipulations can shear the DNA, resulting in reduced LR recombination efficiency. When working with the pAd-DEST plasmids, **do not vortex or pipet the solution vigorously**.

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between the pAd/CMV/V5-DEST™ or pAd/PL-DEST™ vector and your entry clone. To include a negative control, set up a separate reaction but omit the LR Clonase® II enzyme mix.

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

| Component | Sample | Positive Control |
|--------------------------------------|---------|------------------|
| Entry clone (50–150 ng/reaction) | 1-7 µl | -- |
| pENTR™-gus (50 ng/µl) | -- | 2 µl |
| Destination vector (300 ng/reaction) | 2 µl | 2 µl |
| TE Buffer, pH 8.0 | to 8 µl | 4 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 2–3 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, if desired.
-

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, you should see >5000 colonies if the entire LR reaction is transformed and plated.

Continued on next page

Performing the LR Recombination Reaction, continued

Confirming the Expression Clone

The *ccdB* gene mutates at a low frequency, resulting in a low number of false positives. True expression clones will be ampicillin/carbenicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be ampicillin/carbenicillin- and chloramphenicol-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

To confirm that your gene of interest is in the correct orientation and in frame with a fusion tag (if present), you may sequence your expression construct. We recommend using the following primer binding sites to help you sequence your expression construct. Refer to the diagrams on page 12 for the location of the primer binding sites in each vector.

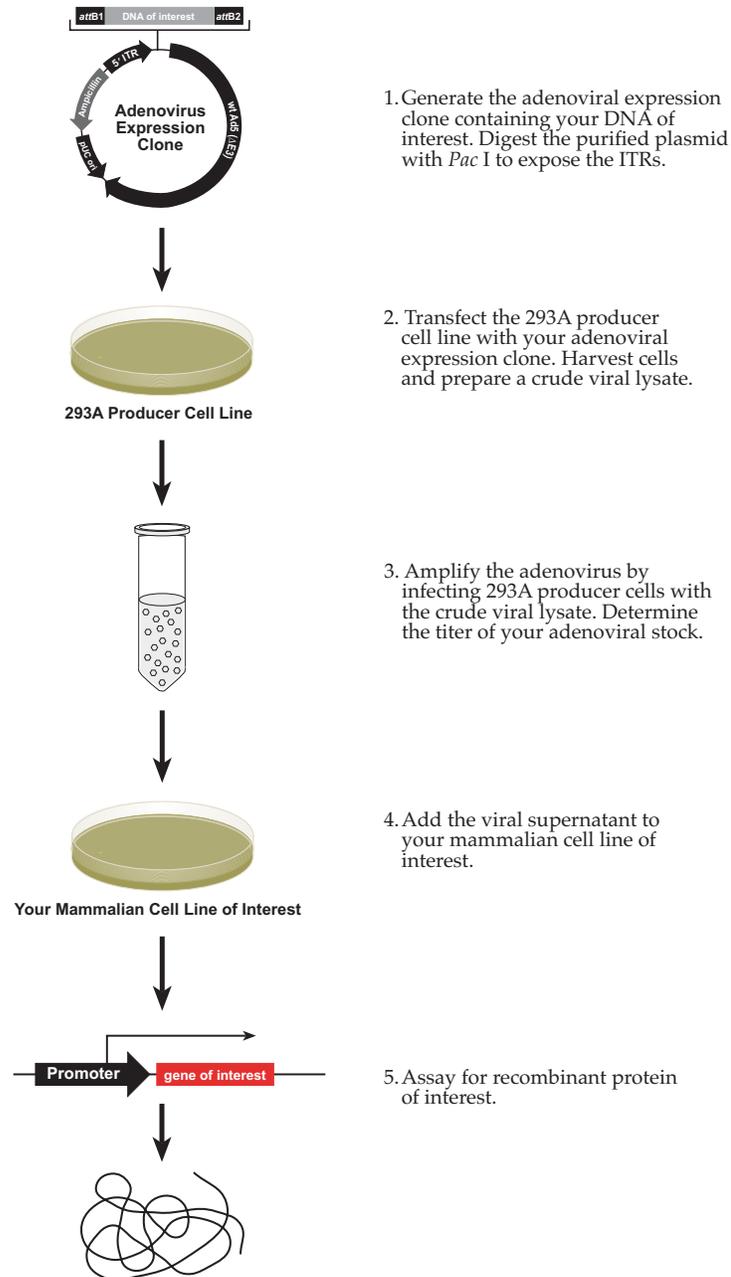
Note: For your convenience, Life Technologies has a custom primer synthesis service. For more information, see our website (www.lifetechnologies.com) or call Technical Support (see page 25).

| Vector | Primer | Sequence |
|------------------|---------------------------------|--------------------------------|
| pAd/CMV/V5-DEST™ | T7 promoter/priming site | 5'-TAATACGACTCACTATAGGG-3' |
| | V5(C-term) reverse priming site | 5'-ACCGAGGAGAGGGTTAGGGAT-3' |
| pAd/PL-DEST™ | pAd forward priming site | 5'-GACTTTGACCGTTTACGTGGAGAC-3' |
| | pAd reverse priming site | 5'-CCTTAAGCCACGCCACACATTTTC-3' |

Expression and Analysis

Introduction

Once you have obtained purified plasmid DNA of your pAd/CMV/V5-DEST™ or pAd/PL-DEST™ expression construct, you will prepare the vector for use in Life Technologies' ViraPower™ Adenoviral Expression System by digesting with *Pac* I. The *Pac* I-digested vector is used to transfect 293A cells to produce an adenoviral stock. After amplification, this adenoviral stock may be used to transduce your mammalian cell line of choice to express your recombinant protein (see experimental outline below).



Continued on next page

Expression and Analysis, continued

Plasmid Preparation

Once you have generated your pAd/CMV/V5-DEST™ or pAd/PL-DEST™ expression clone, prepare purified plasmid DNA. You may use any method of choice to prepare purified plasmid DNA. We recommend isolating plasmid DNA using the PureLink® HiPure Plasmid Midiprep Kit (see page 5) or CsCl gradient centrifugation.

Note: We recommend performing restriction analysis to verify the integrity of your expression construct after plasmid preparation.

Pac I Digestion

Before you can transfect your expression clone into 293A cells, you must expose the left and right viral ITRs on the vector to allow proper viral replication and packaging. Both pAd/CMV/V5-DEST™ and pAd/PL-DEST™ vectors contain *Pac* I restriction sites (see maps on pages 20 and 22, respectively for the location of the *Pac* I sites). Digestion of the vector with *Pac* I allows exposure of the left and right viral ITRs and removal of the bacterial sequences (*i.e.* pUC origin and ampicillin resistance gene). **Note:** Make sure that your DNA sequence of interest does not contain any *Pac* I restriction sites.

1. Digest at least 5 µg of purified plasmid DNA of your pAd/CMV/V5-DEST™ or pAd/PL-DEST™ expression construct with *Pac* I (New England Biolabs, Cat. no. R0547S). Follow the manufacturer's instructions.
 2. Purify the digested plasmid DNA using phenol/chloroform extraction followed by ethanol precipitation or a DNA purification kit (*e.g.* Life Technologies' PureLink® HiPure Plasmid Miniprep Kit; see page 5). **Note:** Gel purification is not required.
 3. Resuspend or elute the purified plasmid, as appropriate in sterile water or TE Buffer, pH 8.0 to a final concentration of 0.1–3.0 µg/µl.
-

Positive Control

pAd/CMV/V5-GW/*lacZ* is included with the each kit for use as a positive control for expression in the ViraPower™ Adenoviral Expression System. In pAd/CMV/V5-GW/*lacZ*, β-galactosidase is expressed as a C-terminally tagged fusion protein that may be easily detected by western blot or functional assay. To use pAd/CMV/V5-GW/*lacZ* as a positive control, you will need to digest the vector with *Pac* I using the protocol above. The *Pac* I-digested plasmid may then be used in your transfection experiment to generate an adenoviral stock.

For details about the vector, see page 24. To propagate and maintain the plasmid:

1. Use the 1 µg/µl stock solution provided to transform a *recA*, *endA* *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent. Use 10 ng of plasmid for transformation.
 2. Select transformants on LB agar plates containing 50–100 µg/ml ampicillin or carbenicillin.
 3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.
-

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Expression and Analysis, continued

Important

Reminder: Use care when handling your pAd-DEST expression clone and pAd/CMV/V5-GW/*lacZ* plasmid DNA. The adenoviral plasmids are large (>34 kb in size) and excessive manipulations can shear the DNA, resulting in reduced transfection efficiency and lower viral titers. When working with the plasmids, **do not vortex or pipet the solution vigorously.**

Materials to Have on Hand

To express your gene of interest from pAd/CMV/V5-DEST™ or pAd/PL-DEST™ using Life Technologies' ViraPower™ Adenoviral Expression System, you will need to have the following reagents:

- A cell line that stably expresses the E1 proteins (E1a and E1b) for producing viral stocks. We recommend using the 293A Cell Line. This cell line, a subclone of the 293 cell line, supplies the E1 proteins required for production of replication-competent adenovirus and exhibits a flattened morphology to enhance visualization of plaques.
- Transfection reagent for efficient delivery of the pAd/CMV/V5-DEST™ or pAd/PL-DEST™ expression construct to 293A cells. We recommend using Lipofectamine® 2000 Reagent for optimal transfection efficiency.

For more information about the 293A Cell Line and Lipofectamine® 2000 Reagent, see the 293A Cell Line manual and the Lipofectamine® 2000 Reagent manual, respectively. Both manuals are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).

Obtaining Reagents

The 293A Cell Line and Lipofectamine® 2000 Reagent are available separately from Life Technologies (see page 5 for ordering information). The 293A Cell Line is also supplied with each ViraPower™ Adenoviral Expression System (Cat. nos. K4930-00 and K4940-00).

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Expression and Analysis, continued

Producing Viral Stocks and Transducing Mammalian Cells

Refer to the ViraPower™ Adenoviral Expression System manual for detailed guidelines and protocols to:

- Transfect your *Pac* I-digested pAd/CMV/V5-DEST™ or pAd/PL-DEST™ expression construct into the 293A Cell Line to generate an adenoviral stock. Remember to generate an adenoviral stock of the pAd/CMV/V5-GW/*lacZ* positive control.
 - Amplify the adenovirus by infecting 293A cells.
 - Determine the titer of your adenoviral stock.
 - Transduce your Ad/CMV/V5-DEST™ or Ad/PL-DEST™ adenoviral construct into the mammalian cell line of interest at the appropriate multiplicity of infection (MOI).
-

Detecting Recombinant Protein

To detect expression of your recombinant fusion protein from pAd/CMV/V5-DEST™ or pAd/PL-DEST™, you may perform:

- Western blot analysis using an antibody to your protein or the Anti-V5, Anti-V5-HRP, or Anti-V5-AP antibodies available from Life Technologies (pAd/CMV/V5-DEST™ only).
- Immunofluorescence using an antibody to your protein or the Anti-V5-FITC antibody available from Life Technologies (pAd/CMV/V5-DEST™ only).
- Functional analysis

For more information about the Anti-V5 antibodies, refer to our website (www.lifetechnologies.com) or call Technical Support (see page 25). Ordering information is provided on page 5.

Note

The C-terminal peptide containing the V5 epitope and the *attB2* site will add approximately 4.3 kDa to the size of your protein.

Assay for β -galactosidase Activity

The β -galactosidase protein expressed from the pAd/CMV/V5-GW/*lacZ* control, adenoviral construct is approximately 120 kDa in size. You may assay for β -galactosidase expression by western blot analysis, activity assay using cell-free lysates (Miller, 1972), or by staining the cells for activity. Life Technologies offers the β -Gal Antiserum, β -Gal Assay Kit, and the β -Gal Staining Kit for fast and easy detection of β -galactosidase expression (see page 5 for ordering information).

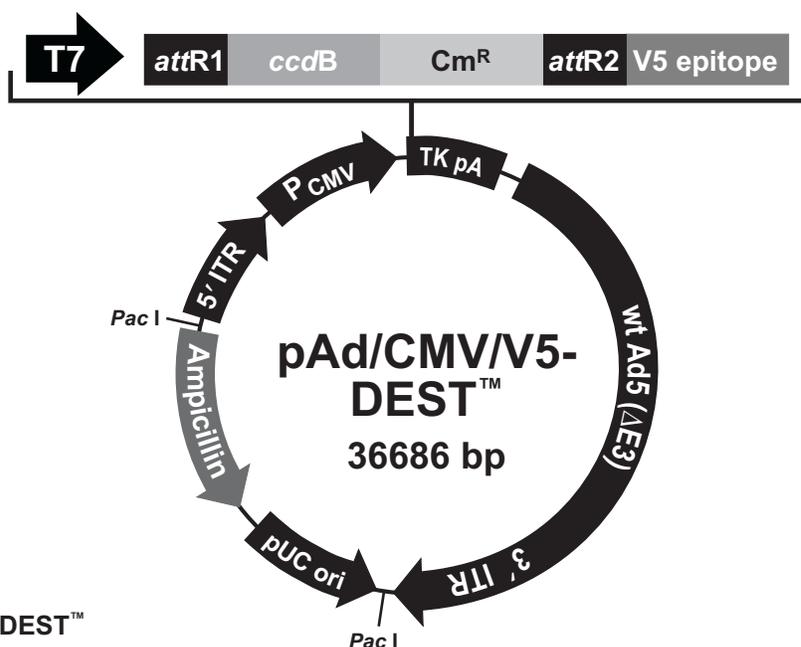
Note: You may also detect β -galactosidase expression using the Anti-V5 antibodies.

Appendix

Map and Features of pAd/CMV/V5-DEST™

Map of pAd/CMV/V5-DEST™

The map below shows the elements of pAd/CMV/V5-DEST™. DNA from the entry clone replaces the region between bases 1414 and 3657. The vector sequence of pAd/CMV/V5-DEST™ is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).



Comments for pAd/CMV/V5-DEST™ 36686 nucleotides

Human Ad5 sequences (wt 1-458; includes 5' L-ITR and packaging signal): 1-458

pAd forward priming site: bases 361-384

CMV promoter: bases 728-1315

T7 promoter/priming site: bases 1359-1378

attR1 site: bases 1407-1531

ccdB gene: bases 1960-2265 (C)

Chloramphenicol resistance gene (Cm^R): bases 2607-3266 (C)

attR2 site: bases 3547-3671

V5 epitope: bases 3697-3738

TK polyadenylation signal: bases 3765-4036

Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3' R-ITR): bases 4056-34604

pAd reverse priming site: bases 4059-4082

pUC origin: bases 34781-35442 (C)

Ampicillin (*bla*) resistance gene: bases 35568-36428 (C)

bla promoter: bases 36429-36527 (C)

Pac I restrictions sites: bases 34610 and 36684

(C) = complementary strand

Continued on next page

Map and Features of pAd/CMV/V5-DEST™, continued

Features of the Vector

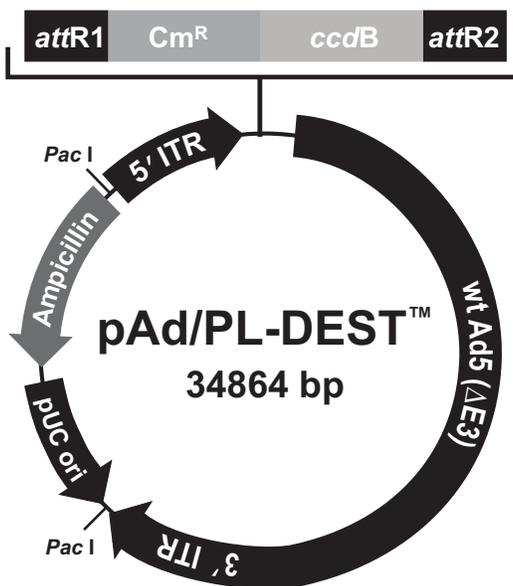
The pAd/CMV/V5-DEST™ vector (36686 bp) contains the following elements. All features have been functionally tested.

| Feature | Benefit |
|---|---|
| Human adenovirus type 5 sequences (corresponds to wild-type 1–458 and 3513–35935 sequence) Note: The E1 and E3 regions are deleted. | Encodes all elements (except E1 and E3 proteins) required to produce replication-incompetent adenovirus (Russell, 2000) including: <ul style="list-style-type: none"> • Left and right ITRs • Encapsidation signal for packaging • E2 and E4 regions • Late genes |
| pAd forward priming site | Allows sequencing of the insert. |
| CMV promoter | Allows high-level expression of the gene of interest (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987). |
| T7 promoter/priming site | Allows <i>in vitro</i> transcription in the sense orientation and sequencing through the insert. |
| <i>attR1</i> and <i>attR2</i> sites | Bacteriophage λ -derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989). |
| <i>ccdB</i> gene | Allows negative selection of the plasmid. |
| Chloramphenicol resistance gene (Cm ^R) | Allows counterselection of the plasmid. |
| V5 epitope | Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern <i>et al.</i> , 1991). |
| Herpes Simplex Virus thymidine kinase (TK) polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985). |
| pAd reverse priming site | Allows sequencing of the insert in the anti-sense orientation. |
| pUC origin | Allows high-copy replication and maintenance in <i>E. coli</i> . |
| <i>bla</i> promoter | Allows expression of the ampicillin resistance gene. |
| Ampicillin resistance gene (β -lactamase) | Allows selection of the plasmid in <i>E. coli</i> . |
| <i>Pac</i> I restriction sites (positions 34610 and 36684) | Allows exposure of the left and right ITRs required for viral replication and packaging. |

Map and Features of pAd/PL-DEST™

Map of pAd/PL-DEST™

The map below shows the elements of pAd/PL-DEST™. DNA from the entry clone replaces the region between bases 519 and 2202. The vector sequence of pAd/PL-DEST™ is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).



Comments for pAd/PL-DEST™ 34864 nucleotides

Human Ad5 sequences (wt 1-458; includes 5' L-ITR and packaging signal): 1-458

pAd forward priming site: bases 361-384

attR1 site: bases 512-636

Chloramphenicol resistance gene (Cm^R): bases 745-1404

ccdB gene: bases 1746-2051

attR2 site: bases 2092-2216

Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3' R-ITR): bases 2234-32782

pAd reverse priming site: bases 2237-2260

pUC origin: bases 32959-33620 (C)

Ampicillin (*bla*) resistance gene: bases 33746-34606 (C)

bla promoter: bases 34607-34705 (C)

Pac I restriction sites: bases 32788 and 34862

(C) = complementary strand

Continued on next page

Map and Features of pAd/PL-DEST™, continued

Features of the Vector

The pAd/PL-DEST™ vector (34864 bp) contains the following elements. All features have been functionally tested.

| Feature | Benefit |
|---|---|
| Human adenovirus type 5 sequences (corresponds to wild-type 1–458 and 3513–35935 sequence) Note: The E1 and E3 regions are deleted. | Encodes all elements (except E1 and E3 proteins) required to produce replication-incompetent adenovirus (Russell, 2000) including: <ul style="list-style-type: none"> • Left and right ITRs • Encapsidation signal for packaging • E2 and E4 regions • Late genes |
| pAd forward priming site | Allows sequencing of the insert. |
| <i>attR1</i> and <i>attR2</i> sites | Bacteriophage λ -derived DNA recombination sequences that permit recombinational cloning of the DNA sequence of interest from a Gateway® entry clone (Landy, 1989). |
| Chloramphenicol resistance gene (Cm ^R) | Allows counterselection of the plasmid. |
| <i>ccdB</i> gene | Allows negative selection of the plasmid. |
| pAd reverse priming site | Allows sequencing of the insert in the anti-sense orientation. |
| pUC origin | Allows high-copy replication and maintenance in <i>E. coli</i> . |
| <i>bla</i> promoter | Allows expression of the ampicillin resistance gene. |
| Ampicillin resistance gene (β -lactamase) | Allows selection of the plasmid in <i>E. coli</i> . |
| <i>Pac</i> I restriction sites (positions 32788 and 34862) | Allows exposure of the left and right ITRs required for viral replication and packaging. |

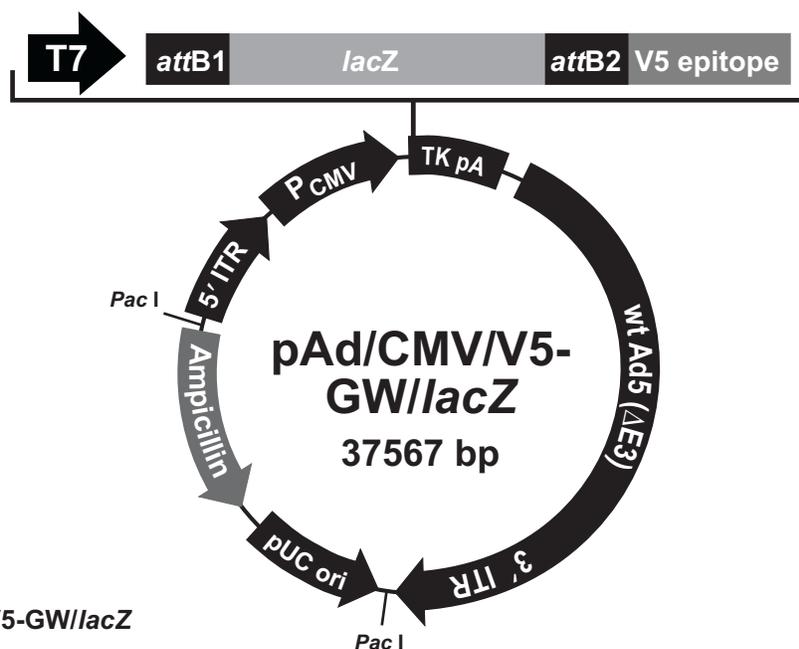
Map of pAd/CMV/V5-GW/lacZ

Description

pAd/CMV/V5-GW/lacZ is a 37567 bp control vector expressing β -galactosidase, and was generated using the Gateway[®] LR recombination reaction between an entry clone containing the *lacZ* gene and pAd/CMV/V5-DEST[™]. β -galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 120 kDa.

Map of pAd/CMV/V5-GW/lacZ

The map below shows the elements of pAd/CMV/V5-GW/lacZ. The vector sequence of the vector is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 25).



Comments for pAd/CMV/V5-GW/lacZ 37567 nucleotides

Human Ad5 sequences (wt 1-458; includes 5' L-ITR and packaging signal): 1-458

pAd forward priming site: bases 361-384

CMV promoter: bases 728-1315

T7 promoter/priming site: bases 1359-1378

attB1 site: bases 1407-1431

lacZ ORF: bases 1452-4508

attB2 site: bases 4528-4552

V5 epitope: bases 4578-4619

TK polyadenylation signal: bases 4646-4917

Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3' R-ITR): bases 4937-35485

pAd reverse priming site: bases 4940-4963

pUC origin: bases 35662-36323 (C)

Ampicillin (*bla*) resistance gene: bases 36449-37309 (C)

bla promoter: bases 37310-37408 (C)

Pac I restriction sites: bases 35491 and 37565

(C) = complementary strand

Technical Support

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

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 - Obtain information about customer training
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Gateway® Clone Distribution Policy

For additional information about Life Technologies' policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 27.

Continued on next page

Gateway[®] Clone Distribution Policy

Introduction

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

Gateway[®] Entry Clones

Life Technologies understands that Gateway[®] entry clones, containing *attL1* and *attL2* sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway[®] Expression Clones

Life Technologies also understands that Gateway[®] expression clones, containing *attB1* and *attB2* sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway[®] expression clones for a nominal fee (\$10 per clone) payable to Life Technologies.

Additional Terms and Conditions

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

References

- 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
- Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (1994) An Efficient and Flexible System for Construction of Adenovirus Vectors with Insertions or Deletions in Early Regions 1 and 3. *Proc. Natl. Acad. Sci. USA* *91*, 8802-8806
- 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
- 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
- Hitt, M. M., Parks, R. J., and Graham, F. L. (1999) in *The Development of Human Gene Therapy* (Friedmann, T., ed), pp. 61-86, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Kozak, M. (1987) An Analysis of 5'-Noncoding Sequences from 699 Vertebrate Messenger RNAs. *Nucleic Acids Res.* *15*, 8125-8148
- Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. *Proc. Natl. Acad. Sci. USA* *87*, 8301-8305
- Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. *J. Cell Biology* *115*, 887-903
- Landy, A. (1989) Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Ann. Rev. Biochem.* *58*, 913-949
- Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- 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
- Russell, W. C. (2000) Update on Adenovirus and its Vectors. *J. Gen. Virol.* *81*, 2573-2604
- 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

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Notes

Notes

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