

USER GUIDE

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Mammalian Expression System with Gateway® Technology

Gateway®-adapted destination vectors for cloning and high-level expression of native or tagged recombinant proteins in mammalian cells

Catalog numbers 11826-021, 12489-019, 11809-019, 11812-013

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Kit Contents and Storage

Types of Products This manual is supplied with the following products.

Product	Cat. no.
Mammalian Expression System with Gateway® Technology	11826-021
Gateway® pcDNA™3.2/V5-DEST Vector	12489-019
Gateway® pDEST™26 Vector	11809-019
Gateway® pDEST™27 Vector	11812-013



Important

The pcDNA™3.2/V5-DEST Vector has been renamed from pcDNA™3.2-DEST to be more descriptive and to better reflect the functionality of the vector.

Kit Components Each product contains the following components. For a detailed description of the contents of each component, see the next page.

Component	Cat. no.			
	11826-021	12489-019	11809-019	11812-013
pcDNA™3.2/V5-DEST Vector	√	√		
pDEST™26 Vector	√		√	
pDEST™27 Vector	√			√
Gateway® LR Clonase® II Enzyme Mix	√			
Library Efficiency® DH5α™ Competent <i>E. coli</i>	√			

Shipping and Storage

The Mammalian Expression System with Gateway® Technology is shipped as described below. Upon receipt, store each item as detailed below.

Box	Item	Shipping	Storage
1	Destination Vectors	Room temperature	-20°C
2	Gateway® LR Clonase® II Enzyme Mix	Dry ice	-20°C
3	Library Efficiency® DH5α™ Competent <i>E. coli</i>	Dry ice	-80°C

Note: The individual Gateway® destination vectors (Cat. nos. 12489-019, 11809-019, and 11812-013) are shipped at room temperature. **Upon receipt, store at -20°C.**

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Kit Contents and Storage, Continued

Destination Vectors

The following destination vectors (Box 1) are supplied with the Mammalian Expression System with Gateway® Technology. **Store the vectors at –20°C.**

Note: Cat. nos. 12489-019, 11809-019, and 11812-013 contain 6 µg of the appropriate destination vector **only**.

Reagent	Composition	Amount
pcDNA™3.2/V5-DEST Vector	150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL	6 µg
pDEST™26 Vector	150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL	6 µg
pDEST™27 Vector	150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL	6 µg

LR Clonase® II Enzyme Mix

The following reagents are included with the Gateway® LR Clonase® II Enzyme Mix (Box 2). **Store Box 2 at –80°C for up to 6 months.** For long-term storage, store at –80°C.

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

DH5α™ Competent *E. coli*

The Library Efficiency® DH5α™ Competent *E. coli* kit (Box 3) includes the following items. Transformation efficiency is $\geq 1 \times 10^8$ cfu/µg DNA. **Store Box 3 at –80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or 4°C)	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl ₂ 10 mM MgSO ₄ 20 mM glucose	2 × 6 mL
Library Efficiency® Chemically Competent DH5α™	–	5 × 200 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

Genotype of DH5α™

F⁻ *recA1 endA1 hsdR17(r_k⁻, m_k⁺) supE44 λ⁻ thi-1 gyrA96 relA1*

Introduction

Product Overview

Description of the System

The Mammalian Expression System with Gateway® Technology contains a series of Gateway®-adapted destination vectors designed to facilitate high-level expression of recombinant proteins in mammalian cells. Depending on the vector chosen, the destination vectors allow production of native, N-terminal, or C-terminal-tagged recombinant proteins (see table below).

Vector	Fusion Peptide	Fusion Tag
pcDNA™3.2/V5-DEST	C-terminal	V5 epitope (Southern <i>et al.</i> , 1991)
pDEST™26	N-terminal	6xHis
pDEST™27	N-terminal	Glutathione S-transferase (GST) (Smith <i>et al.</i> , 1986)

For more information about the Gateway® Technology, see the next page.

Features of the Vectors

pcDNA™3.2/V5-DEST, pDEST™26, and pDEST™27 contain the following elements:

- The human cytomegalovirus (CMV) immediate early enhancer/promoter for high-level, constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
 - N-terminal or C-terminal fusion tags for detection and purification of recombinant fusion proteins (choice of tag depends on the particular vector; see above)
 - Two recombination sites, *attR1* and *attR2*, downstream of the CMV promoter for recombinational cloning of the gene 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
 - Neomycin resistance gene for selection of stable cells lines using Geneticin® (Southern and Berg, 1982)
 - Ampicillin resistance gene for selection in *E. coli*
 - pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*
-

Product 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 the Gateway[®] Technology, simply:

1. Clone your gene of interest into a Gateway[®] entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction between the entry clone and a Gateway[®] destination vector (*e.g.* pcDNA[™]3.2/V5-DEST, pDEST[™]26, or pDEST[™]27).
3. Transform Library Efficiency[®] DH5 α [™] *E. coli* and select for an expression clone.
4. Purify plasmid and transfect your expression clone into the mammalian cell line of choice.

For detailed information about the Gateway[®] Technology, refer to the Gateway[®] Technology with Clonase[®] II manual. To generate an entry clone, refer to the manual for the entry vector you are using. The Gateway[®] Technology with Clonase[®] II manual and entry vector manuals are available for downloading from www.lifetechnologies.com/support or by contacting Technical Support (see page 25).

LR Recombination Reaction

You will perform an LR recombination reaction between the entry clone and your destination vector of choice to generate an expression clone. The LR recombination reaction is mediated by LR Clonase[®] II enzyme mix, a mixture of the bacteriophage λ Integrase (Int) and Excisionase (Xis) proteins, and the *E. coli* Integration Host Factor (IHF) protein. For more information about the LR recombination reaction, see the Gateway[®] Technology with Clonase[®] II manual.

Experimental Outline

Experimental Outline

The table below outlines the steps required to express your gene of interest in mammalian cells from pcDNA[™]3.2/V5-DEST, pDEST[™]26, or pDEST[™]27.

Step	Action	Page
1	Design an appropriate scheme and clone your gene of interest into the Gateway [®] entry vector of choice to generate an entry clone.	4–8
2	Perform an LR recombination reaction by mixing the entry clone and the appropriate destination vector with Gateway [®] LR Clonase [®] II enzyme mix.	9–10
3	Transform the recombination reaction into competent Library Efficiency [®] DH5 α [™] cells and select for expression clones.	11
4	Analyze transformants for the presence of insert by restriction enzyme digestion or colony PCR.	12
5	Optional: Sequence to confirm that the gene of interest is cloned in frame with the appropriate N-terminal or C-terminal tag	12
6	Prepare purified plasmid DNA and transfect the mammalian cell line of choice. Generate a stable cell line, if desired.	13, 15
7	Assay for expression of your recombinant protein.	14
8	Purify your recombinant protein, if desired.	16

Methods

Generating an Entry Clone

Introduction

To recombine your gene of interest into pcDNA™ 3.2/V5-DEST, pDEST™26, or pDEST™27, you will need an entry clone containing the gene of interest. Many entry vectors are available to facilitate generation of entry clones (see table below). For more information about each vector, see www.lifetechnologies.com/support or contact Technical Support (see page 25).

Entry Vector	Cat. no.
pENTR™/D-TOPO®	K2400-20
pENTR™/SD/D-TOPO®	K2420-20
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 www.lifetechnologies.com/support or by contacting Technical Support (see page 25).



If you wish to express a human gene of interest in pcDNA™ 3.2/V5-DEST, pDEST™26, or pDEST™27, you may want to use an Ultimate™ Human ORF (hORF) Clone available for ordering. The Ultimate™ hORF Clones are fully-sequenced clones provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with the destination vector. For more information about the Ultimate™ hORF Clones available, see www.lifetechnologies.com or contact Technical Support (see page 25).

Continued on next page

Generating an Entry Clone, Continued

Points to Consider Before Recombining into pcDNA™ 3.2/V5-DEST

pcDNA™ 3.2/V5-DEST is a C-terminal fusion vector; however, you may use this vector to express native proteins or C-terminal fusion proteins.

- Your gene of interest in the entry clone must contain an ATG initiation codon within the context of a Kozak consensus sequence for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak sequence is (G/A)NNATGG. The ATG initiation codon is underlined. Note that 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).
- 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 pcDNA™ 3.2/V5-DEST on page 7 to help you design a strategy to generate your entry clone.

Points to Consider Before Recombining into pDEST™ 26 and pDEST™ 27

pDEST™ 26 and pDEST™ 27 are N-terminal fusion vectors and contain an ATG initiation codon within the context of a Kozak consensus sequence upstream of the 6xHis and GST tags, respectively. Your gene of interest in the entry clone must:

- Be in frame with the N-terminal tag after recombination.
- Contain a stop codon.

Refer to the diagram of the pDEST™ 26 or pDEST™ 27 recombination region on page 7 or 8, respectively, to help you design a strategy to generate your entry clone.

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 the pcDNA™3.2/V5-DEST, pDEST™26, or pDEST™27 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 9–10) before beginning.



Important

The pcDNA™3.2/V5-DEST, pDEST™26, and pDEST™27 vectors are supplied as supercoiled plasmids. Although we have previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of the destination vector is **NOT** required to obtain optimal results for any downstream application.

Propagating the Vectors

To propagate and maintain the pcDNA™3.2/V5-DEST, pDEST™26, and pDEST™27 vectors prior to recombination, use the supplied 150 ng/mL stock solution in TE, pH 8.0 to transform One Shot® *ccdB* Survival™ T1^R Chemically Competent *E. coli* (see page 23 for ordering). The *ccdB* Survival™ T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccdB* 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 as these strains are sensitive to CcdB effects.

Continued on next page

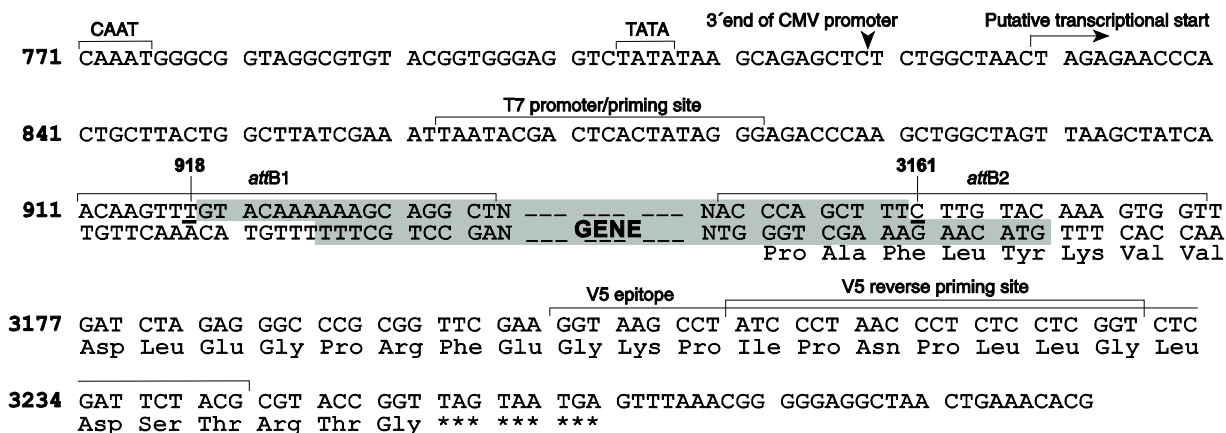
Creating an Expression Clone, Continued

Recombination Region of pcDNA™ 3.2/V5-DEST

The recombination region of the expression clone resulting from pcDNA™ 3.2/V5-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pcDNA™ 3.2/V5-DEST vector by recombination. Non-shaded regions are derived from the pcDNA™ 3.2/V5-DEST vector.
- The underlined nucleotides flanking the shaded region correspond to bases 918 and 3161, respectively, of the pcDNA™ 3.2/V5-DEST vector sequence.

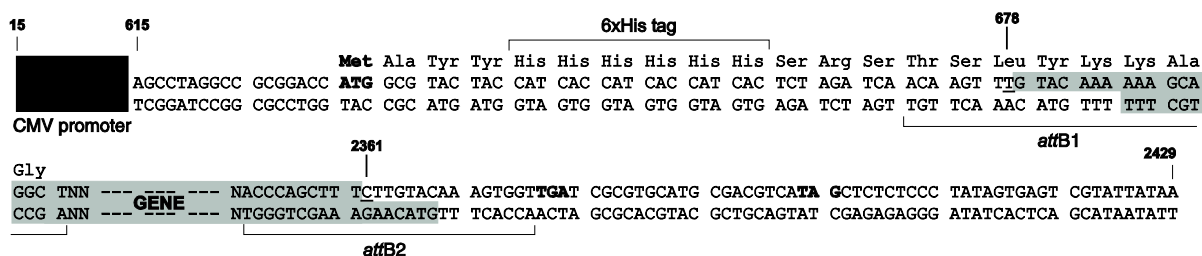


Recombination Region of pDEST™ 26

The recombination region of the expression clone resulting from pDEST™ 26 × entry clone is shown below.

Features of the Recombination Region:

- The location of the 6xHis tag is indicated to help you determine if your gene will be in frame with the 6xHis tag after recombination.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pDEST™ 26 vector by recombination. Non-shaded regions are derived from the pDEST™ 26 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 678 and 2361, respectively, of the pDEST™ 26 vector sequence.
- Potential stop codons are indicated in bold.



Continued on next page

Performing the LR Recombination Reaction

Introduction

Once you have produced an entry clone containing your gene of interest, you are ready to perform an LR recombination reaction between the entry clone and the appropriate destination vector, and to transform the reaction mixture into Library Efficiency[®] DH5 α [™] to select for an expression clone. We recommend that you include a positive control (see below) and a negative control (no LR Clonase[®] II) in your experiment to help you evaluate your results.

Positive Control

The pENTR[™]-gus plasmid is included in the Mammalian Expression System with Gateway[®] Technology for use as a positive control for LR recombination and expression. Using the pENTR[™]-gus entry clone in an LR recombination reaction with a destination vector will allow you to generate an expression clone containing the gene encoding β -glucuronidase (*gus*).

LR Clonase[®] II Enzyme Mix

LR Clonase[®] II enzyme mix is supplied with the kit (Cat. no. 11826-021 only) or available separately to catalyze the LR recombination reaction (see page 23). 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.

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/ μ L in TE, pH 8.0)
 - Destination vector (150 ng/ μ L in TE, pH 8.0)
 - LR Clonase[®] II enzyme mix (supplied with the kit, Box 2; keep at -20°C until immediately before use)
 - TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
 - Proteinase K solution (supplied with kit, Box 2; thaw and keep on ice until use)
 - pENTR[™]-gus positive control (50 ng/ μ L in TE, pH 8.0; supplied with the kit, Box 2)
-

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Performing the LR Recombination Reaction, Continued

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between your entry clone and the destination vector. If you want to include a negative control, set up a separate reaction but omit the LR Clonase® II enzyme mix.

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

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: For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times will yield more colonies.

6. Add 1 µL of Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming Library Efficiency® DH5α™ Cells**, next page.

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

Transforming Library Efficiency[®] DH5 α [™] Cells

Introduction

Once you have performed the LR recombination reaction, you will transform competent *E. coli*. Library Efficiency[®] DH5 α [™] chemically competent *E. coli* (Box 2) are included with the Mammalian Expression System for use in transformation.

Materials Needed

- LR recombination reaction (from Step 7, previous page)
 - Library Efficiency[®] DH5 α [™] chemically competent cells (supplied with the kit, Box 3; thaw on ice before use)
 - S.O.C. Medium (supplied with the kit, Box 3; warm to room temperature)
 - pUC19 control (supplied with the kit, Box 3; use as a control for transformation if desired)
 - LB plates containing 100 μ g/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes)
 - 42°C water bath
 - 37°C shaking and non-shaking incubator
-



Note

Library Efficiency[®] DH5 α [™] competent cells are supplied in 5 tubes containing 0.2 mL of competent cells each. Each tube contains enough competent cells to perform 4 transformations using 50 μ L of cells per transformation. Once you have thawed a tube of competent cells, discard any unused cells. **Do not** re-freeze cells as freezing and thawing of cells will result in the loss of transformation efficiency.

Transformation Procedure

1. For each transformation, aliquot 50 μ L of Library Efficiency[®] DH5 α [™] competent cells into a sterile microcentrifuge tube.
 2. Add 1 μ L of the LR recombination reaction (from **Setting Up the LR Recombination Reaction**, Step 7, previous page) into the tube containing 50 μ L of Library Efficiency[®] DH5 α [™] competent cells and mix gently. **Do not mix by pipetting up and down.**
 3. Incubate on ice for 30 minutes.
 4. Heat-shock the cells for 30 seconds at 42°C without shaking.
 5. Immediately transfer the tubes to ice.
 6. Add 450 μ L of room temperature S.O.C. Medium.
 7. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
 8. Spread 20 μ L and 100 μ L from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We generally plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies.
 9. An efficient LR recombination reaction should produce >5000 colonies if the entire LR reaction is transformed and plated.
-

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 100 µg/mL ampicillin.
 2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink® HQ Mini Plasmid Purification Kit (see page 23 for ordering).
 3. Analyze the plasmids by restriction analysis to confirm the presence of the insert.
-

Analyzing Transformants by PCR

You may also analyze positive transformants using PCR. For PCR primers, use a primer that hybridizes within the vector (*e.g.* T7 Promoter Primer; see page 23 for ordering) and one that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, you may want to perform restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed:

PCR SuperMix High Fidelity (see page 23 for ordering)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µL of PCR SuperMix High Fidelity into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
 2. Pick 5 colonies and resuspend them individually in 48 µL of the PCR SuperMix (remember to make a patch plate to preserve the colonies for further analysis).
 3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
 4. Amplify for 20 to 30 cycles.
 5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
 6. Visualize by agarose gel electrophoresis.
-

Confirming the Expression Clone

The *ccdB* 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 *ccdB* gene will be 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

Optional: To confirm that your gene of interest is in frame with the appropriate tag (if any), you may sequence your expression construct.

Transfection

Introduction

Once you have generated your expression clone, you are ready to transfect the plasmid into the mammalian cell line of choice. You may perform transient transfection experiments or use Geneticin[®] selection to generate stable cell lines. We recommend that you include a positive control (see below) and a negative control (mock transfection) in your experiment to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection in eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt may interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating DNA using the PureLink[®] HiPure Miniprep Kit or the PureLink[®] HiPure Midiprep Kit (see page 23 for ordering information), or CsCl gradient centrifugation.

Positive Control

If you used the pENTR[™]-gus control vector in an LR recombination reaction with a destination vector, you can use the resultant expression clone as a positive control for mammalian cell transfection and expression. A successful transfection will result in β -glucuronidase expression that can be detected by western blot or functional assay.

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 follow exactly the protocol for your cell line. 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 cells, use Lipofectamine[®] 2000 Reagent available for ordering (see page 23). For more information on Lipofectamine[®] 2000 and other transfection reagents, visit www.lifetechnologies.com or contact Technical Support (page 25).

Expression and Analysis

Introduction

Expression of your gene of interest from the expression clone can be performed in either transiently transfected cells or stable cell lines (see the next page for guidelines to create stable cell lines). You may use a functional assay or Western blot analysis to detect your recombinant protein.

Detection of Recombinant Protein

To detect expression of your recombinant fusion protein by western blot analysis, you may use an antibody to the appropriate fusion tag (V5 epitope, 6xHis, or GST) or an antibody to your protein of interest. Anti-V5 antibodies are available for ordering (see page 23) to facilitate detection of fusion proteins expressed from pcDNA™3.2/V5-DEST.



Note

Expression of your protein with the N-terminal or C-terminal tag will increase the size of your recombinant protein. The table below lists the increase in the molecular weight of your recombinant fusion protein that you should expect from the tag in each destination vector. Be sure to account for any additional amino acids between the fusion tag and the start of your protein.

Vector	Fusion Tag	Expected Size Increase (kDa)
pcDNA™3.2/V5-DEST	C-terminal V5	4.0
pDEST™26	N-terminal 6xHis	2.5
pDEST™27	N-terminal GST	27.3

Generating Stable Cell Lines

Introduction

The pcDNA™3.2/V5-DEST, pDEST™26, and pDEST™27 vectors contain the neomycin resistance gene to allow selection of stable cell lines using Geneticin®. General information and guidelines are provided below to generate stable cell lines.



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

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function, and is an aminoglycoside similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (*APH*), derived from Tn5, results in detoxification of Geneticin® (Southern and Berg, 1982).

Determining Geneticin® Sensitivity

To successfully generate a stable cell line, you need to determine the minimum concentration of Geneticin® required to kill your untransfected mammalian cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
 2. The next day, substitute culture medium with medium containing varying concentrations of Geneticin® (0, 50, 125, 250, 500, 750, and 1000 µg/mL Geneticin®).
 3. Replenish the selective medium 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 Geneticin® that kills the cells within 1–3 weeks after addition of Geneticin®.
-

Geneticin® Selection Guidelines

Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line with your expression construct. Geneticin® is available separately (see page 23 for ordering information). Use as follows:

1. Prepare Geneticin® in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
 2. Use the predetermined concentration of Geneticin® in complete medium.
 3. Calculate concentration based on the amount of active drug.
 4. Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 3 weeks of growth in selective medium.
-

Purifying the Recombinant Protein

Introduction

The presence of the N-terminal 6xHis tag in pDEST™26 allows purification of your recombinant fusion protein using a nickel-chelating resin such as ProBond™ or Ni-NTA, while the presence of the N-terminal GST tag in pDEST™27 allows purification of the recombinant fusion protein using glutathione agarose.

Purification of 6xHis-Tagged Recombinant Proteins

If you express your recombinant protein as a fusion to the 6xHis tag in pDEST™26, you may affinity purify your protein using a nickel-chelating resin such as ProBond™ or Ni-NTA. ProBond™ and Ni-NTA resin are available separately (see page 23 for ordering information). Other metal-chelating resins are suitable.

- To purify your fusion protein using ProBond™ or Ni-NTA, refer to the ProBond™ Purification System or Ni-NTA Purification System manuals as appropriate. Both manuals are available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 25).
 - To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.
-

Purification Using Glutathione Agarose

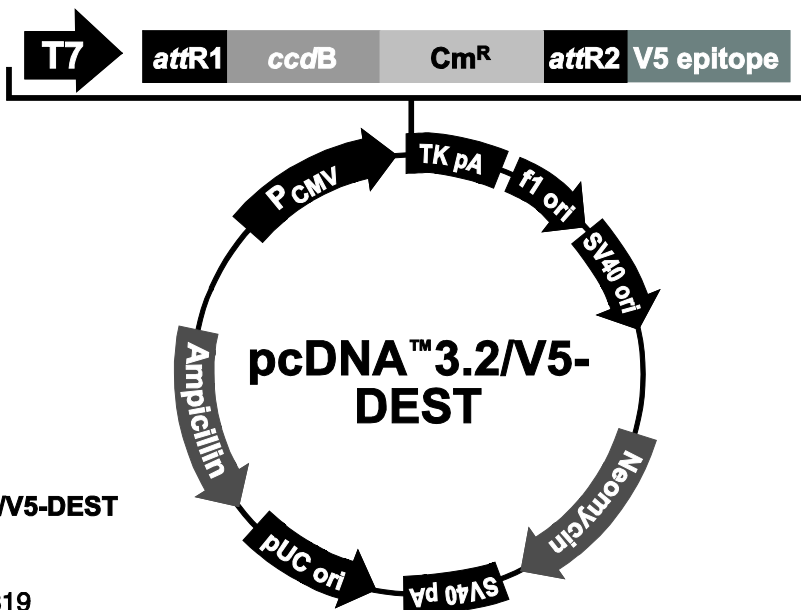
If you express your recombinant protein as a fusion to the GST tag in pDEST™27, you may affinity purify your protein using glutathione agarose. Refer to the manufacturer's instructions to purify your recombinant fusion protein.

Appendix

Map and Features of pcDNA™ 3.2/V5-DEST

pcDNA™ 3.2/V5-DEST Map

The map below shows the elements of pcDNA™ 3.2/V5-DEST. DNA from the entry clone replaces the region between bases 918 and 3161. The vector sequence for pcDNA™ 3.2/V5-DEST is available from www.lifetechnologies.com or by contacting Technical Support (see page 25).



Comments for pcDNA™ 3.2/V5-DEST 7711 nucleotides

CMV promoter: bases 232-819

T7 promoter/priming site: bases 863-882

attR1 site: bases 911-1035

ccdB gene (C): bases 1464-1769

Chloramphenicol resistance gene (Cm^R) (C): bases 2111-2770

attR2 site: bases 3051-3175

V5 epitope: bases 3201-3242

V5 reverse priming site: bases 3210-3230

TK polyadenylation signal: bases 3269-3540

f1 origin: bases 3576-4004

SV40 early promoter and origin: bases 4031-4339

Neomycin resistance gene: bases 4414-5208

SV40 early polyadenylation signal: bases 5384-5514

pUC origin (C): bases 5897-6570

Ampicillin (*bla*) resistance gene (C): bases 6715-7575

bla promoter (C): bases 7576-7674

(C) = complementary strand

Continued on next page

Map and Features of pcDNA™ 3.2/V5-DEST, Continued

Features of pcDNA™ 3.2/V5-DEST

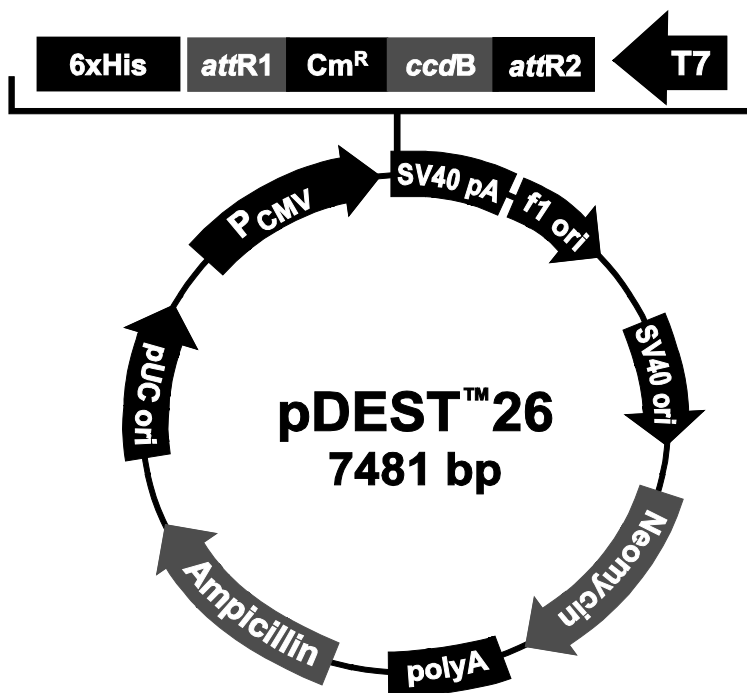
pcDNA™ 3.2/V5-DEST (7711 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson <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	Allows recombinational cloning of the gene of interest from an entry clone (Landy, 1989).
<i>ccdB</i> gene	Allows negative selection of plasmid.
Chloramphenicol resistance gene	Allows counterselection of plasmid.
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern <i>et al.</i> , 1991).
V5 reverse priming site	Allows sequencing of the insert.
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 neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen.
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982).
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin	Allows high-copy number replication and growth in <i>E. coli</i> .
Ampicillin (<i>bla</i>) resistance gene (β-lactamase)	Allows selection of transformants in <i>E. coli</i> .

Map and Features of the pDEST™ 26 and pDEST™ 27 Vectors

pDEST™ 26 Map

The map below shows the elements of pDEST™ 26. DNA from the entry clone replaces the region between bases 678 and 2361. The vector sequence for pDEST™ 26 is available from www.lifetechnologies.com or by contacting Technical Support (see page 25).



Comments for pDEST™ 26: 7481 nucleotides

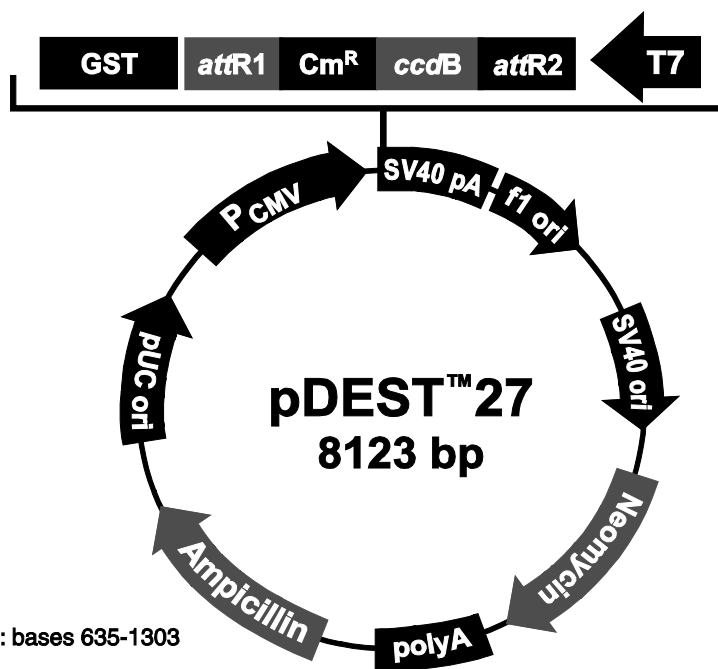
CMV promoter: bases 15-608
 6xHis tag: bases 644-661
 attR1 site: bases 671-795
 Chloramphenicol resistance gene (Cm^R): bases 904-1563
 ccdB gene: bases 1905-2210
 attR2 site: bases 2251-2375
 T7 promoter: bases 2407-2426 (C)
 SV40 early polyadenylation signal: bases 2726-2992
 f1 intergenic region: bases 3118-3573
 SV40 early promoter and origin: bases 3733-4041
 Neomycin resistance gene: bases 4100-4894
 Synthetic polyadenylation signal: bases 4958-5006
 bla promoter: bases 5318-5416
 Ampicillin (bla) resistance gene: bases 5417-6277
 pUC origin: bases 6422-7095
 (C) = complementary strand

Continued on next page

Map and Features of the pDEST™ 26 and pDEST™ 27 Vectors, Continued

pDEST™ 27 Map

The map below shows the elements of pDEST™ 27. DNA from the entry clone replaces the region between bases 1320 and 3003. The vector sequence for pDEST™ 27 is available from www.lifetechnologies.com/support or by contacting Technical Support (see page 25).



Comments for pDEST™ 27: 8123 nucleotides

CMV promoter: bases 15-608
 Glutathione S-transferase (GST) tag: bases 635-1303
 attR1 site: bases 1313-1437
 Chloramphenicol resistance gene (Cm^R): bases 1546-2205
 ccdB gene: bases 2547-2852
 attR2 site: bases 2893-3017
 T7 promoter: bases 3049-3068 (C)
 SV40 early polyadenylation signal: bases 3368-3634
 f1 intergenic region: bases 3760-4215
 SV40 early promoter and origin: bases 4375-4683
 Neomycin resistance gene: bases 4742-5536
 Synthetic polyadenylation signal: bases 5600-5648
 b/a promoter: bases 5960-6058
 Ampicillin (b/a) resistance gene: bases 6059-6919
 pUC origin: bases 7064-7737
 (C) = complementary strand

Continued on next page

Map and Features of the pDEST™ 26 and pDEST™ 27 Vectors, Continued

Features of the Vectors

pDEST™ 26 (7481bp) and pDEST™ 27 (8123 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 <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987).
N-terminal 6xHis tag (in pDEST™ 26 only)	Allows affinity purification of recombinant fusion protein using a metal-chelating resin such as ProBond™ or Ni-NTA.
N-terminal glutathione S-transferase (GST) tag (in pDEST™ 27 only)	Allows detection and purification of recombinant fusion protein.
<i>attR1</i> and <i>attR2</i> sites	Bacteriophage λ-derived DNA recombination sequences that permits recombinational cloning of the gene 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.
T7 promoter	Allows <i>in vitro</i> transcription.
SV40 polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
f1 intergenic region	Allows rescue of single-stranded DNA.
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen.
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982).
Synthetic polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
<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> .
pUC origin of replication (<i>ori</i>)	Allows high-copy replication and maintenance in <i>E. coli</i> .

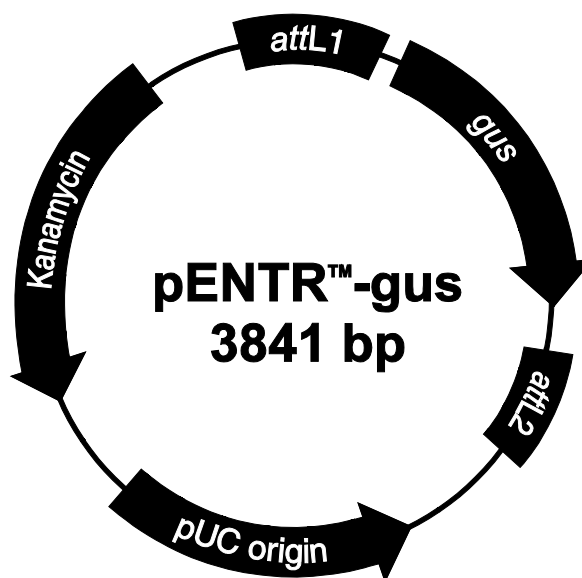
Map of pENTR™-gus

Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The *gus* gene was amplified using PCR primers containing *attB* recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR™201 to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase® II manual.

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. The vector sequence for pENTR™-gus is available from www.lifetechnologies.com or by contacting Technical Support (see page 25).



Comments for pENTR™-gus 3841 nucleotides

attL1: bases 99-198

gus gene: bases 228-2039

attL2: bases 2041-2140

pUC origin: bases 2200-2873 (C)

Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Accessory Products

Additional Products

Many of the reagents supplied in the Mammalian Expression System with Gateway® Technology as well as other products suitable for use with the kit are available separately. Ordering information for these reagents is provided below.

Item	Quantity	Cat. no.
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
One Shot® <i>ccdB</i> Survival™ T1 ^R Chemically Competent <i>E. coli</i>	10 reactions	C7510-03
Library Efficiency® DH5α™ Competent Cells	5 × 0.2 mL	18263-012
PureLink® HQ Mini Plasmid Purification Kit	100 preps	K2100-01
T7 Promoter Primer	2 µg	N560-02
PCR SuperMix High Fidelity	100 reactions	10790-020
Lipofectamine® 2000	0.75 mL	11668-027
	1.5 mL	11668-019
Geneticin®	1 g	11811-023
	5 g	11811-031
	20 mL (50 mg/mL)	10131-035
	100 mL (50 mg/mL)	10131-027

Detection of V5 Recombinant Fusion Proteins

You can detect expression of your recombinant fusion protein from pcDNA™3.2/V5-DEST using the Anti-V5 antibodies available for ordering. The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibody only).

Item	Epitope	Cat. no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern <i>et al.</i> , 1991). GKPIPPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25

Continued on next page

Accessory Products, Continued

Purification of Recombinant Protein

The presence of the polyhistidine (6xHis) tag in pDEST™26 allows purification of your recombinant fusion protein using a nickel-charged agarose resin such as ProBond™ or Ni-NTA. Ordering information is provided below.

Item	Quantity	Cat. no.
ProBond™ Nickel-Chelating Resin	50 mL	R801-01
	150 mL	R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 mL	R901-01
	25 mL	R901-15
	100 mL	R901-10
Ni-NTA Purification System	6 purifications	K950-01

Technical Support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

Certificate of Analysis

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

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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 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 licensing department at 760-603-7200.

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