invitrogen™
by life technologies™

E. coli Expression System with Gateway® Technology

Gateway®-adapted destination vectors for cloning and high-level expression of native or tagged recombinant proteins in *E. coli*

Catalog nos. 11824-026, 11801-016, 11802-014, 11803-012, 12216-016

Rev. Date: 7 June 2010
Manual part no. 25-0517

MAN0000278

User Manual

Table of Contents

Kit Contents and Storage

Types of Products

This manual is supplied with the following products listed below.

Product	Catalog no.
E. coli Expression System with Gateway® Technology	11824-026
Gateway® pDEST™14 Vector	11801-016
Gateway® pDEST™15 Vector	11802-014
Gateway® pDEST™17 Vector	11803-012
Gateway® pDEST™24 Vector	12216-016

Kit Components

Each product contains the following components. For a detailed description of the contents of each component, see pages 5-6.

	Catalog no.				
Component	**11824-026**	**11801-016**	**11802-014**	**11803-012**	**12216-016**
pDEST™14 Vector	√	√			
pDEST™15 Vector	√		√		
pDEST™17 Vector	√			√	
pDEST™24 Vector	√				√
Gateway® LR Clonase® II Enzyme Mix	√				
Library Efficiency® DH5α Competent *E. coli*	√				
BL21-AI™ One Shot® Chemically Competent *E. coli*	√				

Shipping/Storage

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

Box	Item	Shipping	Storage
1	pDEST™ Vectors	Wet ice	–20°C
2	Gateway® LR Clonase® II Enzyme Mix	Dry ice	–20°C
3	Library Efficiency® DH5α™ Competent *E. coli* Kit	Dry ice	–80°C
4	BL21-AI™ One Shot® Chemically Competent *E. coli* Kit	Dry ice	–80°C

Note: The individual Gateway® pDEST™ vectors (Catalog nos. 11801-016, 11802-014, 11803-012, 12216-016) are shipped on wet ice. **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 *E. coli* Expression System with Gateway® Technology. **Store the vectors at –20°C.**

Note: Catalog nos. 11801-016, 11802-014, 11803-012, and 12216-016 contain 40 µl of the appropriate pDEST™ vector at 150 ng/µl concentration in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 **only**.

Reagent	Composition	Amount
pDEST™14 Vector	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	20 µg
pDEST™15 Vector	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	20 µg
pDEST™17 Vector	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	20 µg
pDEST™24 Vector	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	20 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 –20°C for up to 6 months.** For long-term, store at –80°C.

Reagent	Composition	Amount
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 ≥ 1 x 10^8 cfu/µg DNA. **Store Box 3 at –80°C.**

Item	Composition	Amount
Library Efficiency® Chemically Competent DH5α	–	5 × 200 µl
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
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

continued on next page

Kit Contents and Storage, continued

Genotype of DH5α™

Use this strain to propagate and maintain your expression clone.

Genotype: F$^-$ *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1

BL21-AI™ One Shot® Competent *E. coli*

The BL21-AI™ One Shot® Chemically Competent *E. coli* Kit (Box 4) includes the following items. Transformation efficiency is ≥ 1 x 10^8 cfu/µg DNA.
Store Box 4 at –80°C.

Item	Composition	Amount
BL21-AI™ chemically competent cells	–	21 × 50 µl
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	6 ml
20% L-arabinose	20% L-arabinose in sterile water	1 ml
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Genotype of BL21-AI™

Note: Use this strain for expression only. Do not use these cells to propagate or maintain your expression clone.

Genotype: F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm araB*::*T7RNAP-tetA*

The BL21-AI™ strain is an *E. coli* B/r strain and does not contain the *lon* protease. It is also deficient in the outer membrane protease, OmpT. The lack of these proteases reduces degradation of heterologous proteins expressed in this strain.

The strain carries a chromosomal insertion of a cassette containing the T7 RNA polymerase (T7 RNAP) gene in the *ara*B locus, allowing expression of the T7 RNAP to be regulated by the *araBAD* promoter (see page 29 for more information). The presence of the *tet*A gene confers resistance to tetracycline and permits verification of strain identity using tetracycline.

Accessory Products

Introduction

The products listed in this section may be used with the *E. coli* Expression System with Gateway® Technology. For more information, refer to www.invitrogen.com or call Technical Support (see page 37).

Additional Products

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

Item	Quantity	Catalog no.
LR Clonase® II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Library Efficiency® DH5α Competent Cells	5 × 0.2 ml	18263-012
BL21-AI™ One Shot® Chemically Competent *E. coli*	20 × 50 µl	C6070-03
Gateway® pDEST™14 Vector	20 µg	11801-016
Gateway® pDEST™15 Vector	20 µg	11802-014
Gateway® pDEST™17 Vector	20 µg	11803-012
Gateway® pDEST™24 Vector	20 µg	12216-016
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
Carbenicillin, Disodium Salt	5 g	10177-012

Purification of Recombinant Protein

The presence of the polyhistidine (6×His) tag in pDEST™17 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	Catalog no.
ProBond™ Nickel-Chelating Resin	50 ml 150 ml	R801-01 R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 ml 25 ml 100 ml	R901-01 R901-15 R901-10
Ni-NTA Purification System	6 purifications	K950-01

Introduction

Overview

Introduction

The *E. coli* Expression System with Gateway® Technology contains a series of Gateway®-adapted destination vectors designed to facilitate high-level, inducible expression of recombinant proteins in *E. coli* using the pET system. Depending on the vector chosen, the pDEST™ vectors allow production of native, N-terminal, or C-terminal-tagged recombinant proteins (see table below).

Vector	Fusion Peptide	Fusion Tag
pDEST™14	–	–
pDEST™15	N-terminal	Glutathione *S*-transferase (GST) (Smith *et al.*, 1986)
pDEST™17	N-terminal	6×His
pDEST™24	C-terminal	Glutathione *S*-transferase (GST) (Smith *et al.*, 1986)

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

The pET Expression System

The pET system was originally developed by Studier and colleagues and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in *E. coli* from the T7 promoter (Rosenberg *et al.*, 1987; Studier and Moffatt, 1986; Studier *et al.*, 1990). For more information about T7-regulated expression, see the next page.

Features of the Vectors

pDEST™14, pDEST™15, pDEST™17, and pDEST™24 contain the following elements:

- T7 promoter for high-level, T7 RNA polymerase regulated expression of the gene of interest in *E. coli* (Studier and Moffatt, 1986; Studier *et al.*, 1990)
- N- 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, *att*R1 and *att*R2, downstream of the T7 promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- The *ccd*B gene located between the *att*R sites for negative selection
- Ampicillin resistance gene for selection in *E. coli*
- pBR322 origin for low-copy replication and maintenance of the plasmid in *E. coli*

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 *E. coli* 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.* pDEST™14, pDEST™15, pDEST™17, pDEST™24).
3. Transform Library Efficiency® DH5α *E. coli* and select for an expression clone.
4. Purify plasmid and transform your expression construct into BL21-AI™. Induce expression of your recombinant protein with L-arabinose.

For more detailed information about 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 our website (www.invitrogen.com) or by contacting Technical Support (see page 37).

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.

The Basis of T7-Regulated Expression

The pET expression system uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. In the pDEST™14, pDEST™15, pDEST™17, and pDEST™24 vectors, expression of the gene of interest is controlled by a strong bacteriophage T7 promoter. In bacteriophage T7, the T7 promoter drives expression of gene 10 (ϕ10). T7 RNA polymerase specifically recognizes this promoter. To express the gene of interest, it is necessary to deliver T7 RNA polymerase to the cells by inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the *E. coli* Expression System with Gateway® Technology, T7 RNA polymerase is supplied by the BL21-AI™ host *E. coli* strain in a regulated manner (see the next page for more information about the strain).

The BL21-AI™ *E. coli* Strain

Description of the Strain

The BL21-AI™ *E. coli* strain is included in the kit and is intended for use as a host for expression of T7 RNA polymerase-regulated genes. The BL21-AI™ strain is derived from the BL21 strain (Grodberg and Dunn, 1988; Studier and Moffatt, 1986) and contains a chromosomal insertion of the gene encoding T7 RNA polymerase (T7 RNAP) into the *ara*B locus of the *araBAD* operon, placing regulation of the T7 RNAP gene under the control of the *araBAD* promoter. The *ara*B gene is deleted in this strain.

Regulating Expression of T7 RNA Polymerase

Because the T7 RNAP gene is inserted into the *ara*B locus of the *araBAD* operon, expression of T7 RNA polymerase can be regulated by the sugars, L-arabinose and glucose.

- To induce expression from the *araBAD* promoter, use L-arabinose (Lee, 1980; Lee *et al.*, 1987). To modulate expression, simply vary the concentration of L-arabinose added.
- To repress basal expression from the *araBAD* promoter, use glucose.

  Note: In the absence of glucose, basal expression from the *araBAD* promoter is generally low (Lee, 1980; Lee *et al.*, 1987). Adding glucose further represses expression from the *araBAD* promoter by reducing the levels of 3´, 5´-cyclic AMP (Miyada *et al.*, 1984).

For more information on the mechanism of expression and repression of the *ara* regulon, see the **Appendix**, page 29 or refer to Schleif, 1992.

Experimental Outline

Experimental Outline

The table below outlines the steps required to express your gene of interest in *E. coli* from pDEST™14, pDEST™15, pDEST™17, or pDEST™24.

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.	12–13
2	Perform an LR recombination reaction by mixing the entry clone and the appropriate pDEST™ vector with Gateway® LR Clonase® II Enzyme Mix.	14–20
3	Transform the recombination reaction into competent Library Efficiency® DH5α™ cells and select for expression clones.	21
4	Analyze transformants for the presence of insert by restriction enzyme digestion or colony PCR.	22
5	**Optional:** Sequence to confirm that the gene of interest is cloned in frame with the appropriate N-terminal or C-terminal tag	22
6	Prepare purified plasmid DNA of the expression clone and transform into BL21-AI™ One Shot® cells.	23–24
7	Pick a transformant and perform a pilot expression study. Add L-arabinose to induce expression of your recombinant protein.	25–26
8	Purify your recombinant protein, if desired.	27

Methods

Generating an Entry Clone

Introduction

To recombine your gene of interest into pDEST™14, pDEST™15, pDEST™17, or pDEST™24, you will need an entry clone containing the gene of interest. Many entry vectors are available from Invitrogen to facilitate generation of entry clones (see table below). For more information about each vector, see our website or contact Technical Support (see page 37).

Entry Vector	Catalog 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 our website or by contacting Technical Support (see page 37).

Points to Consider Before Recombining into pDEST™14

Your gene of interest in the entry clone must:

- Contain an ATG initiation codon and a Shine-Dalgarno sequence (RBS) with optimal spacing for proper translation initiation in *E. coli* (Shine and Dalgarno, 1975).

  Note: If you clone your gene of interest into an entry vector that supplies a Shine-Dalgarno RBS (*e.g.* pENTR/SD/D-TOPO® or pENTR™11), then your gene of interest need only include an ATG initiation codon.

- Contain a stop codon.

Refer to the diagram of the recombination region of pDEST™14 on page 15 to help you design a strategy to generate your entry clone.

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Generating an Entry Clone, continued

Points to Consider Before Recombining into pDEST™15 and pDEST™17

pDEST™15 and pDEST™17 are N-terminal fusion vectors and contain an ATG initiation codon upstream of the GST and 6´His tags, respectively. In each vector, a Shine-Dalgarno RBS is included upstream of the initiation ATG to ensure optimal spacing for proper translation initiation in *E. coli*. 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 recombination region of pDEST™15 or pDEST™17 on pages 16 and 17, respectively to help you design a strategy to generate your entry clone.

Points to Consider Before Recombining into pDEST™24

pDEST™24 is a C-terminal fusion vector. Your gene of interest in the entry clone must:

- Contain an ATG initiation codon and a Shine-Dalgarno RBS with optimal spacing for proper translation initiation in *E. coli.*

  Note: If you clone your gene of interest into an entry vector that supplies a Shine-Dalgarno RBS (*e.g.* pENTR/SD/D-TOPO® or pENTR™11), then your gene of interest need only include an ATG initiation codon.
- Be in frame with the C-terminal GST tag after recombination.
- **NOT** contain a stop codon.

Refer to the diagram of the recombination region of pDEST™24 on page 18 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 pDEST™ 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 19–20) 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 pDEST™ vector. **Note:** Both the entry clone and the destination vector should be supercoiled (see **Important** below).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 21).
3. Select for expression clones (see pages 15–18 for illustrations of the recombination region of expression clones in pDEST™14, pDEST™15, pDEST™17, or pDEST™24.

Important

The pDEST™14, pDEST™15, pDEST™17, and pDEST™24 vectors are supplied as supercoiled plasmids. Although we have previously recommended using a linearized destination vector for more efficient recombination, further testing at Invitrogen has found that linearization of the destination vector is **NOT** required to obtain optimal results for any downstream application.

Propagating the Vectors

If you wish to propagate and maintain the pDEST™14, pDEST™15, pDEST™17, or pDEST™24 vectors prior to recombination, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (Cat. no. A10460) from Invitrogen. The *ccd*B Survival™ 2 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 g/ml ampicillin and 15–30 g/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5a™ for propagation and maintenance as these strains are sensitive to CcdB effects.

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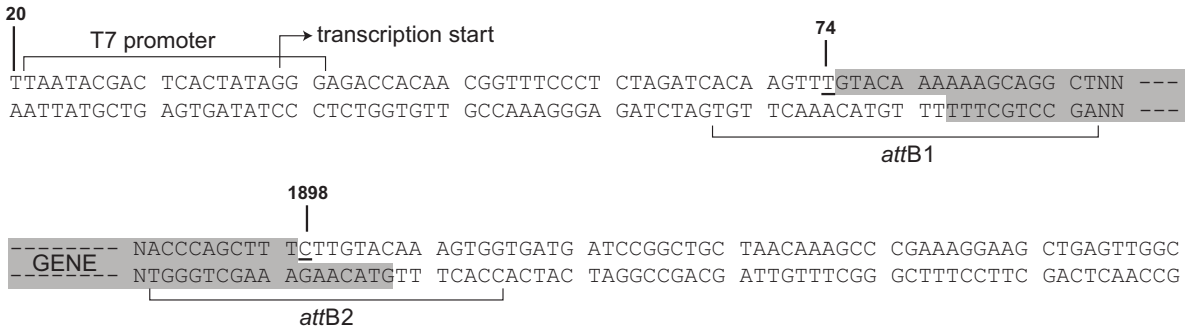
Creating an Expression Clone, continued

Recombination Region of pDEST™14

The recombination region of the expression clone resulting from pDEST™14 × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pDEST™14 vector by recombination. Non-shaded regions are derived from the pDEST™14 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 74 and 1898, respectively, of the pDEST™14 vector sequence.



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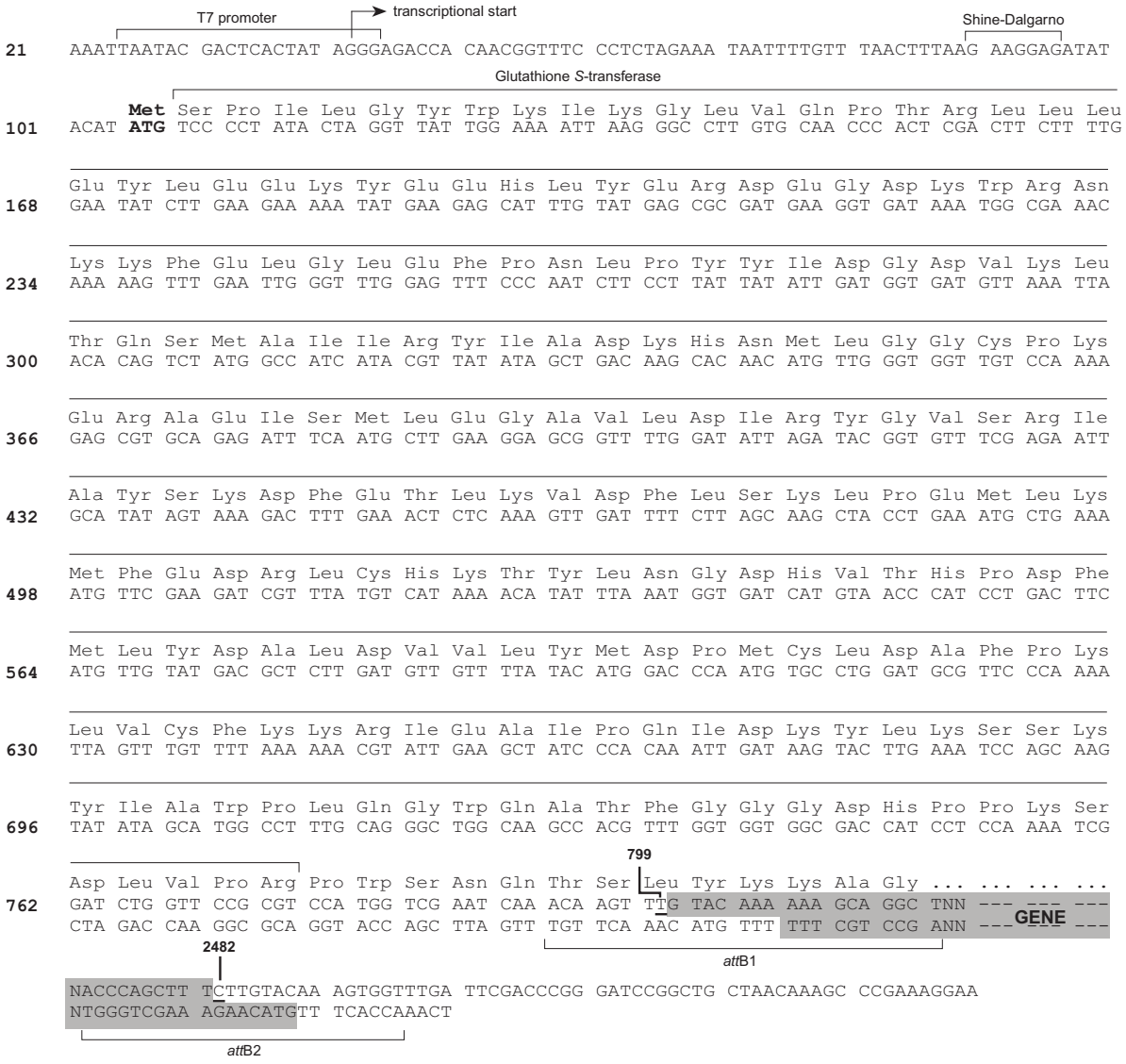
Creating an Expression Clone, continued

Recombination Region of pDEST™15

The recombination region of the expression clone resulting from pDEST™15 × entry clone is shown below.

Features of the Recombination Region:

- The glutathione S-transferase (GST) gene is marked to help you determine if your gene will be in frame with the GST tag after recombination.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pDEST™15 vector by recombination. Non-shaded regions are derived from the pDEST™15 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 799 and 2482, respectively, of the pDEST™15 vector sequence.



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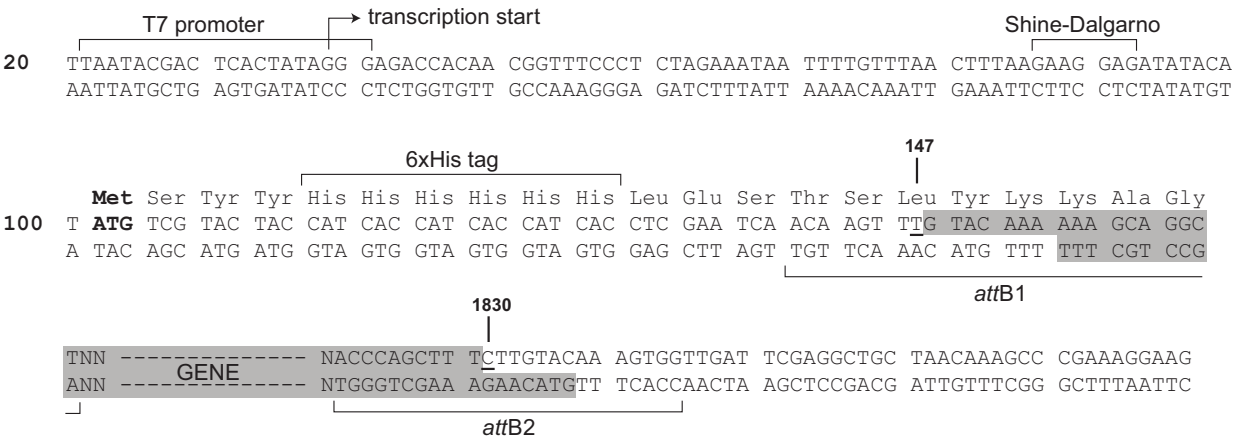
Creating an Expression Clone, continued

Recombination Region of pDEST™17

The recombination region of the expression clone resulting from pDEST™17 × entry clone is shown below.

Features of the Recombination Region:

- The location of the 6×His tag is indicated to help you determine if your gene will be in frame with the 6×His tag after recombination.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pDEST™17 vector by recombination. Non-shaded regions are derived from the pDEST™17 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 147 and 1830, respectively, of the pDEST™17 vector sequence.



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Recombination Region of pDEST™24

The recombination region of the expression clone resulting from pDEST™24 × entry clone is shown below.

Features of the Recombination Region:

- The glutathione S-transferase (GST) gene is marked to help you determine if your gene will be in frame with the GST tag after recombination.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pDEST™24 vector by recombination. Non-shaded regions are derived from the pDEST™24 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 78 and 1761, respectively, of the pDEST™24 vector sequence.

```
              T7 promoter      transcriptional start                                78
21    AAATTAATAC GACTCACTAT AGGGAGACCA CAACGGTTTC CCTCTAGATC ACAAGTTTGT ACAAAAAAGC AGGC TNN---
                                                  GGAGATCTCG TGTTCAAACA TGTTTTTTCG TCCG ANN---
                                                                                    attB1
                  1761                                    Glutathione S-transferase
                 Pro Ala Phe Leu Tyr Lys Val Val Ile Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile
      ------ NAC CCA GCT TTC TTG TAC AAA GTG GTG ATT ATG TCC CCT ATA CTA GGT TAT TGG AAA ATT
      GENE
      ------ NTG GGT CGA AAG AAC ATG TTT CAC CAC TAA TAC
                        attB2

      Lys Gly Leu Val Gln Pro Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
1810  AAG GGC CTT GTG CAA CCC ACT CGA CTT CTT TTG GAA TAT CTT GAA GAA AAA TAT GAA GAG CAT TTG

      Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu Gly Leu Glu Phe Pro Asn
1876  TAT GAG CGC GAT GAA GGT GAT AAA TGG CGA AAC AAA AAG TTT GAA TTG GGT TTG GAG TTT CCC AAT

      Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala
1942  CTT CCT TAT TAT ATT GAT GGT GAT GTT AAA TTA ACA CAG TCT ATG GCC ATC ATA CGT TAT ATA GCT

      Asp Lys His Asn Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu Gly Ala
2008  GAC AAG CAC AAC ATG TTG GGT GGT TGT CCA AAA GAG CGT GCA GAG ATT TCA ATG CTT GAA GGA GCG

      Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser Lys Asp Phe Glu Thr Leu Lys Val
2074  GTT TTG GAT ATT AGA TAC GGT GTT TCG AGA ATT GCA TAT AGT AAA GAC TTT GAA ACT CTC AAA GTT

      Asp Phe Leu Ser Lys Leu Pro Glu Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr
2140  GAT TTT CTT AGC AAG CTA CCT GAA ATG CTG AAA ATG TTC GAA GAT CGT TTA TGT CAT AAA ACA TAT

      Leu Asn Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp Val Val Leu Tyr
2206  TTA AAT GGT GAT CAT GTA ACC CAT CCT GAC TTC ATG TTG TAT GAC GCT CTT GAT GTT GTT TTA TAC

      Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu Val Cys Phe Lys Lys Arg Ile Glu Ala Ile
2272  ATG GAC CCA ATG TGC CTG GAT GCG TTC CCA AAA TTA GTT TGT TTT AAA AAA CGT ATT GAA GCT ATC

      Pro Gln Ile Asp Lys Tyr Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
2338  CCA CAA ATT GAT AAG TAC TTG AAA TCC AGC AAG TAT ATA GCA TGG CCT TTG CAG GGC TGG CAA GCC

      Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Val Pro Arg Pro Trp Gly Ser Gly Cys
2404  ACG TTT GGT GGT GGC GAC CAT CCT CCA AAA TCG GAT CTG GTT CCG CGT CCA TGG GGA TCC GGC TGC

      ***
2470  TAA CAAAGCCC GAAAGGAAGC TGAGTTGGCT GCTGCCACCG CTGAGCAATA
```

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 pDEST™ vector, and to transform the reaction mixture into Library Efficiency® DH5α™ to select for an expression clone. It is important to have everything you need set up and ready to use to ensure that you obtain the best results. We suggest that you read this section and the one entitled **Transforming Library Efficiency® DH5α™ Cells**, page 21 before beginning. We also recommend that you include a positive control (see below) and a negative control (no LR Clonase®) in your experiment.

Positive Control

The pENTR™-gus plasmid is included in the *E. coli* Expression System with Gateway® Technology for use as a positive control for LR recombination and expression. Use of the pENTR™-gus entry clone in an LR recombination reaction with a pDEST™ 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. 11824-026 only) or available separately from Invitrogen 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 20 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/µl in TE, pH 8.0)
- pDEST™ vector (Box 1, 150 ng/µl in TE, pH 8.0)
- LR Clonase® II Enzyme Mix (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 the LR Clonase® II Enzyme Mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µl in TE, pH 8.0)

continued on next page

Performing the LR Recombination Reaction, continued

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR recombination 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® DH5α™ Cells

Introduction

Once you have performed the LR recombination reaction, you will transform competent *E. coli*. Library Efficiency® DH5α™ chemically competent *E. coli* (Box 3) are included with the *E. coli* Expression System to facilitate transformation.

Materials Needed

- LR recombination reaction (from Step 7, previous page)
- Library Efficiency® DH5α™ 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 µg/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® DH5α™ 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 µl 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 Protocol

1. For each transformation, aliquot 50 µl of Library Efficiency® DH5α™ competent cells into a sterile microcentrifuge tube.
2. Add 1 µl of the LR recombination reaction (from **Setting Up the LR Recombination Reaction**, Step 7, previous page) into the tube containing 50 µl of Library Efficiency® DH5α™ 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 µl 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 µl and 100 µl 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 hundreds of colonies (> 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 S.N.A.P.™ MidiPrep Kit (Cat. no. K1910-01) or the PureLink™ HQ Mini Plasmid Purification Kit (Cat. no. K2100-01) available from Invitrogen.

   Note: Since pDEST™14, pDEST™15, pDEST™17, and pDEST™24 are low-copy number plasmids, you may need to increase the amount of bacterial culture to obtain enough plasmid DNA for sequencing or analysis purposes. Use extra care during purification to obtain plasmid DNA of sufficiently pure quality for sequencing (see below).
3. Analyze 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; Invitrogen, Cat. no. N560-02) 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 (Invitrogen, Cat. no. 10790-020)

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 50 µl of the PCR SuperMix containing primers (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 *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 *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 tags (if any), you may sequence your expression construct.

General Guidelines for Expression

Introduction

BL21-AI™ One Shot® *E. coli* are included with the *E. coli* Expression System with Gateway® Technology (Box 4) for use as the host for expression. You will need purified plasmid DNA of your pDEST™ expression construct to transform into BL21-AI™. Since each recombinant protein has different characteristics that may affect expression, we recommend performing a time course of expression to determine the best conditions to express your protein.

BL21-AI™ Strain

The BL21-AI™ *E. coli* strain is specifically designed for recombinant protein expression from any T7-based expression vector. Because T7 RNA polymerase levels can be tightly regulated by L-arabinose, the BL21-AI™ strain is especially suited to express genes that may be toxic to other BL21 strains where basal expression of T7 RNA polymerase is leakier.

Each time you perform an expression experiment, you will transform your plasmid into BL21-AI™. **Do not use this strain for propagation and maintenance of your plasmid. Use a general cloning strain (*e.g.* DH5α™) instead.**

Plasmid Preparation

Prepare plasmid DNA using your method of choice. We recommend using the S.N.A.P.™ MidiPrep Kit (Cat. no. K1910-01) or the PureLink™ HQ Mini Plasmid Purification Kit (Cat. no. K2100-01) for isolation of plasmid DNA. Note that since you are purifying a low-copy number plasmid, you should increase the amount of bacterial culture used to prepare your plasmid construct.

Choosing a Selection Agent

For most purposes, ampicillin works well for selection of transformants and expression experiments. However, if you find that your expression level is low, you may want to use carbenicillin instead. The resistance gene for ampicillin encodes a protein called β-lactamase. This protein is secreted into the medium where it hydrolyzes ampicillin, inactivating the antibiotic. Since β-lactamase is catalytic, ampicillin is rapidly removed from the medium, resulting in non-selective conditions. If your plasmid is unstable, this may result in the loss of plasmid and low expression levels.

Using Carbenicillin

Carbenicillin is generally more stable than ampicillin, and studies have shown that using carbenicillin in place of ampicillin may help to increase expression levels by preventing loss of the pDEST™ expression plasmid. If you wish to use carbenicillin, perform your transformation and expression experiments in LB containing 50 µg/ml carbenicillin.

Note: If your gene is highly toxic, increasing the concentration of carbenicillin used from 50 µg/ml to 200 µg/ml may help to increase expression levels.

Transforming BL21-AI™ One Shot® Cells

Modulating Gene Expression

To modulate expression of your gene of interest in BL21-AI™ cells, use:

- L-arabinose to induce expression of T7 RNA polymerase. L-arabinose is supplied with the BL21-AI™ cells, but is also available from Sigma (Cat. no. A3256).
- Glucose to repress **basal** transcription of T7 RNA polymerase and thereby, your gene of interest (optional). Add to plates and/or media (to a final concentration of 0.1% glucose), if needed.

Materials Needed

- Purified DNA of your pDEST™ expression clone (1–10 ng/µl)
- BL21-AI™ One Shot® chemically competent cells (supplied with the kit, Box 4; use one vial per transformation)
- pUC19 control (supplied with the kit, Box 4; use as a control for transformation if desired)
- S.O.C. Medium (supplied with the kit, Box 4; warm to room temperature)
- LB plates containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin (2 plates for each transformation; prewarm to 37°C for 30 minutes
- 37°C incubator (shaking and non-shaking)
- 42°C water bath

BL21-AI™ One Shot® Transformation Procedure

Follow the instructions below to transform your expression construct into BL21-AI™ One Shot® cells. If you are including the pUC19 control, transform 10 pg of DNA. You will need one vial of cells per transformation.

1. Thaw on ice, one vial of BL21-AI™ One Shot® cells per transformation.
2. Add 5-10 ng DNA, in a volume of 1–5 µl, into each vial of BL21-AI™ One Shot® cells and mix by tapping gently. **Do not mix cells 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 250 µl of room temperature S.O.C. Medium.
7. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 30 minutes.
8. Spread 20 µl and 100 µl 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. Select a transformant and proceed to **Pilot Expression**, next page. **Note:** Expression can vary between clones. You may wish to characterize additional transformants.

continued on next page

Expressing Your Recombinant Protein

Materials Needed

- LB media containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin
- 37°C shaking incubator
- 20% L-arabinose (supplied with the kit, Box 4)
- 20% glucose (if needed; prepare in sterile, deionized water)
- Lysis Buffer (see page 36 for a recipe)
- Liquid nitrogen
- 1X and 2X SDS-PAGE sample buffer (see page 36 for a recipe)
- Reagents and apparatus for SDS-PAGE gel (see the next page)
- Boiling water bath
- Sterile water

Pilot Expression

1. Pick 3 or 4 transformants from **BL21-AI™ One Shot® Transformation Procedure**, Step 8, page 24 and culture them in 5 ml of LB medium containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin. Grow at 37°C with shaking until the OD_{600} reaches 0.6 to 1.0.
2. Use these cultures to inoculate fresh LB medium containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin to an OD600 of 0.05–0.1 (~1:20 dilution of the initial culture). This dilution allows the cells to quickly return to logarithmic growth and reach the appropriate cell density. Use a volume appropriate for taking time points, if desired.
3. Grow the cultures until they reach mid-log phase (OD_{600}=~0.4, 2 to 3 hours).
4. Split each culture into two cultures. Add L-arabinose to a final concentration of 0.2% to one of the cultures. You will now have two cultures: one induced, one uninduced.
5. Remove a 500 µl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
6. Freeze the cell pellets at –20°C. These are the zero time point samples.
7. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 2 to 4 hours.
8. For each time point, remove 500 µl from the induced and uninduced cultures and process as described in Steps 5 and 6. Proceed to the next section.

continued on next page

Expressing Your Recombinant Protein, continued

Preparing Samples

Before starting this procedure, make sure that you have an appropriate gel for your protein size, or use one of the pre-cast polyacrylamide gels available from Invitrogen (see below and next page). If you wish to analyze your samples for soluble protein, see the next section.

1. When all the samples have been collected from Steps 5 and 7, previous page, resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5–10 µl of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing them at –20°C.

Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µl of Lysis Buffer (see **Recipes**, page 36).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times. **Note:** To facilitate lysis, you may need to add lysozyme or sonicate the cells.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE gel and electrophorese.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion 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 also carries a large selection of molecular weight protein standards and staining kits (see next page). For more information, refer to www.invitrogen.com or call Technical Support (see page 37).

Analyzing Samples

To determine the success of your expression experiment, you may want to perform the following types of analyses:

1. Stain the polyacrylamide gel and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control. See next page for recommended protein standards and stains.
2. Perform a western blot to confirm that the overexpressed band is your desired protein. You will need to have an antibody to your protein of interest. **Note:** If you are expressing your protein from pDEST™15 or pDEST™24, you may use an antibody to GST to detect your protein.

continued on next page

Recommended Electrophoresis Accessory Products

In addition to the pre-cast polyacrylamide gel systems, Invitrogen offers a wide range of pre-mixed buffers, protein standards, and stains, each with its own advantages. For more information, refer to www.invitrogen.com or contact Technical Support (page 37).

Product	Quantity	Cat. no.
NuPAGE® LDS Sample Buffer (4X)	10 ml 250 ml	NP0007 NP0008
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 ml	LC2676
SimplyBlue™ SafeStain	1 L	LC6060
SilverQuest™ Silver Staining Kit	1 kit	LC6070
SilverXpress® Silver Staining Kit	1 kit	LC6100
Colloidal Blue Staining Kit	1 kit	LC6025
Novex® Sharp Protein Standard Pre-stained Unstained	2 × 250 µl 2 × 250 µl	LC5800 LC5801
SeeBlue® Plus2 Pre-Stained Standard	500 µl	LC5925
UltraPure™ Sodium Dodecyl Sulfate (SDS)	500 g	15525-017

Note

Expression of your protein with the N- or C-terminal tags 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 pDEST™ 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)
pDEST™15	N-terminal	27.7
pDEST™17	N-terminal	2.6
pDEST™24	C-terminal	27.9

Purifying Recombinant Protein

- The presence of the N-terminal 6×His tag in pDEST™17 allows affinity purification of recombinant fusion protein using a nickel-chelating resin such as ProBond™ or Ni-NTA. ProBond™ and Ni-NTA resin are available separately from Invitrogen (see page 7 for ordering information). Refer to the ProBond™ or Ni-NTA manual, as appropriate, for guidelines to purify your protein. Both manuals are available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).
- The presence of the N-terminal or C-terminal GST tag in pDEST™15 and pDEST™24, respectively allows purification of recombinant fusion protein using glutathione agarose. Refer to the manufacturer's instructions to purify your protein.

Troubleshooting Expression

Introduction

Use the information below to troubleshoot your expression experiment.

No Expression

Sequence your construct and make sure it is in frame with the N-terminal or C-terminal tag, as appropriate.

Low Expression Due to Plasmid Instability

If you are using ampicillin for selection in your expression experiments and see low levels of expression, you may be experiencing plasmid instability due to the absence of selective conditions. This occurs as the ampicillin is destroyed by β-lactamase or hydrolyzed under the acidic media conditions generated by bacterial metabolism. You may want to substitute carbenicillin for ampicillin in your transformation and expression experiments (see page 23 for more information).

Low Expression Due to Toxicity

When expressing recombinant proteins in the BL21-AI™ strain, one can generally assume that the recombinant protein is toxic to bacterial cells when any of the following occurs:

- No transformants are obtained after following the **BL21-AI™ One Shot® Transformation Procedure**, page 24 **or** a combination of large and small, irregular colonies appears on the plate
- The initial culture does not grow (see Step 1 of **Pilot Expression**, page 25)
- It takes longer than 5 hours after a 1:20 dilution of the initial culture for the fresh culture to reach an $OD_{600} = 0.4$ (see Steps 2 and 3 of **Pilot Expression**, page 25)
- The cells lyse after induction with L-arabinose (see Step 4 of **Pilot Expression**, page 25)

Precautions

Several precautions may be taken to prevent problems resulting from basal level expression of a toxic gene of interest (see below). These methods all assume that the T7-based expression plasmid has been correctly designed and created.

- Propagate and maintain your expression plasmid in a strain that does not contain T7 RNA polymerase (*i.e.* DH5α™).
- Perform a fresh transformation of BL21-AI™ cells before each expression experiment.
- After following the transformation protocol on page 24, plate the transformation reaction on LB plates containing 100 µg/ml ampicillin and 0.1% glucose. The presence of glucose represses basal expression of T7 RNA polymerase.
- Following transformation of BL21-AI™ cells using the protocol on page 24, pick 3 or 4 transformants and inoculate directly into fresh LB medium containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin (and 0.1% glucose, if desired). When the culture reaches $OD_{600} = 0.4$, induce expression of the recombinant protein by adding L-arabinose to a final concentration of 0.2%.
- When performing expression experiments, supplement the growth medium with 0.1% glucose in addition to 0.2% arabinose.

Appendix

Regulation by L-Arabinose

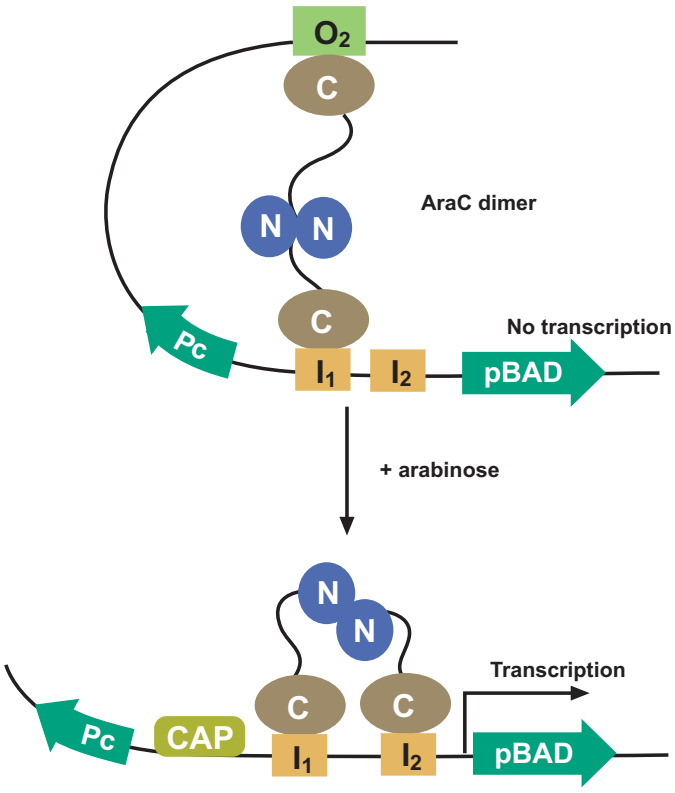
Introduction

The L-arabinose regulatory circuit is briefly described below.

Regulation of the *araBAD* (P_{BAD}) Promoter

The *ara*BAD promoter (P_{BAD}) used to control expression of T7 RNA polymerase in BL21-AI™ is both positively and negatively regulated by the product of the *ara*C gene (Ogden *et al.*, 1980; Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of L-arabinose the AraC dimer contacts the O_2 and I_1 half sites of the *ara*BAD operon, forming a 210 bp DNA loop (see figure below). For maximum transcriptional activation two events are required.

- L-Arabinose binds to AraC and causes the protein to release the O_2 site and bind the I_2 site that is adjacent to the I_1 site. This releases the DNA loop and allows transcription to begin.
- The cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.



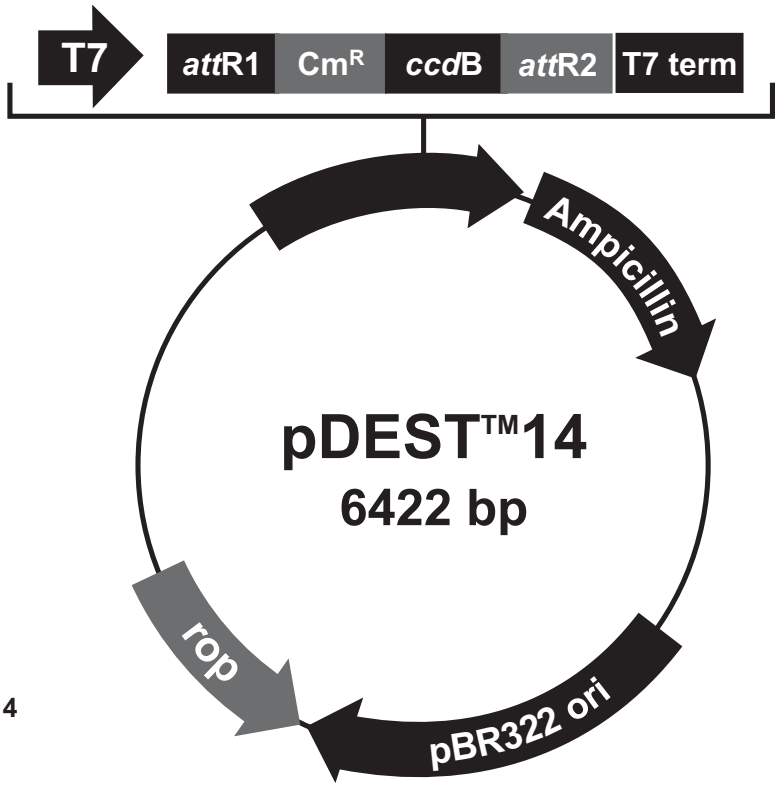
Glucose Repression

Basal expression levels can be repressed by introducing glucose to the growth medium. Glucose acts by lowering cAMP levels, which in turn decreases the binding of CAP. As cAMP levels are lowered, transcriptional activation is decreased.

Map and Features of the pDEST™ Vectors

pDEST™14 Map

The map below shows the elements of pDEST™14. DNA from the entry clone replaces the region between bases 74 and 1898. **The complete sequence for pDEST™14 is available from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).**



Comments for pDEST™14
6422 nucleotides

T7 promoter: bases 21-40
*att*R1: bases 67-191
Chloramphenicol resistance gene (Cm^R): bases 441-1100
*ccd*B gene: bases 1442-1747
*att*R2: bases 1788-1912
T7 transcription termination region: bases 1923-2051
bla promoter: bases 2539-2637
Ampicillin (*bla*) resistance gene: bases 2638-3498
pBR322 origin: bases 3643-4316
ROP ORF: bases 4687-4878 (C)

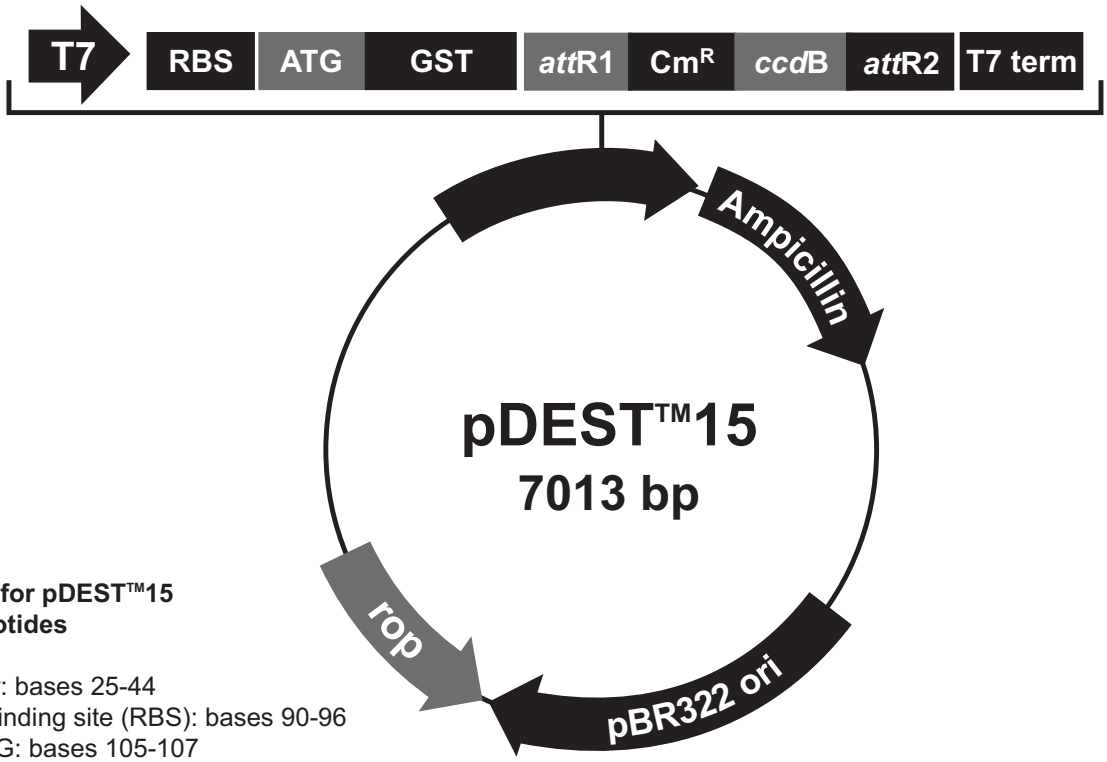
C=complementary strand

continued on next page

Map and Features of the pDEST™ Vectors, continued

pDEST™15 Map

The map below shows the elements of pDEST™15. DNA from the entry clone replaces the region between bases 799 and 2482. **The complete sequence for pDEST™15 is available from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).**



Comments for pDEST™15
7013 nucleotides

T7 promoter: bases 25-44
Ribosome binding site (RBS): bases 90-96
Initiation ATG: bases 105-107
GST tag: bases 108-776
*att*R1: bases 792-916
Chloramphenicol resistance gene (Cm^R): bases 1025-1684
*ccd*B gene: bases 2026-2331
*att*R2: bases 2372-2496
T7 transcription termination region: bases 2518-2646
bla promoter: bases 3134-3232
Ampicillin (*bla*) resistance gene: bases 3233-4093
pBR322 origin: bases 4238-4911
ROP ORF: bases 5282-5473 (C)

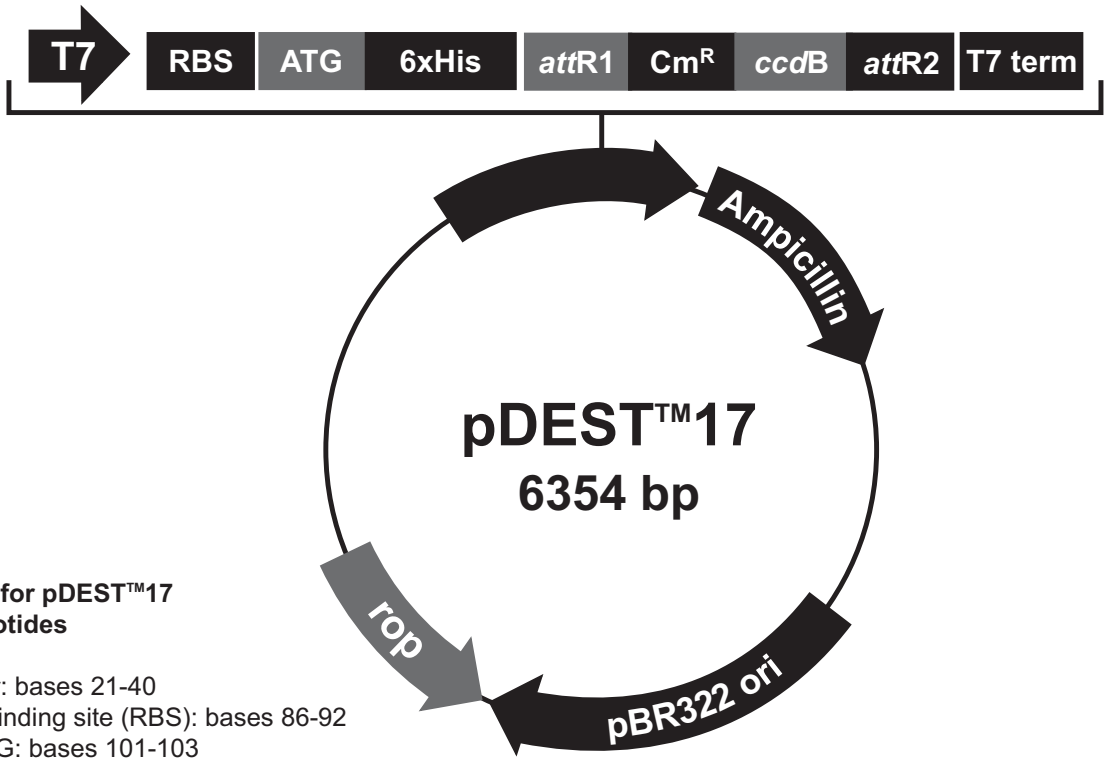
C=complementary strand

continued on next page

Map and Features of the pDEST™ Vectors, continued

pDEST™17 Map

The map below shows the elements of pDEST™17. DNA from the entry clone replaces the region between bases 147 and 1830. **The complete sequence for pDEST™17 is available from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).**



Comments for pDEST™17
6354 nucleotides

T7 promoter: bases 21-40
Ribosome binding site (RBS): bases 86-92
Initiation ATG: bases 101-103
6xHis tag: bases 113-130
*att*R1: bases 140-264
Chloramphenicol resistance gene (Cm^R): bases 373-1032
*ccd*B gene: bases 1374-1679
*att*R2: bases 1720-1844
T7 transcription termination region: bases 1855-1983
bla promoter: bases 2471-2569
Ampicillin (*bla*) resistance gene: bases 2570-3430
pBR322 origin: bases 3575-4248
ROP ORF: bases 4619-4810 (C)

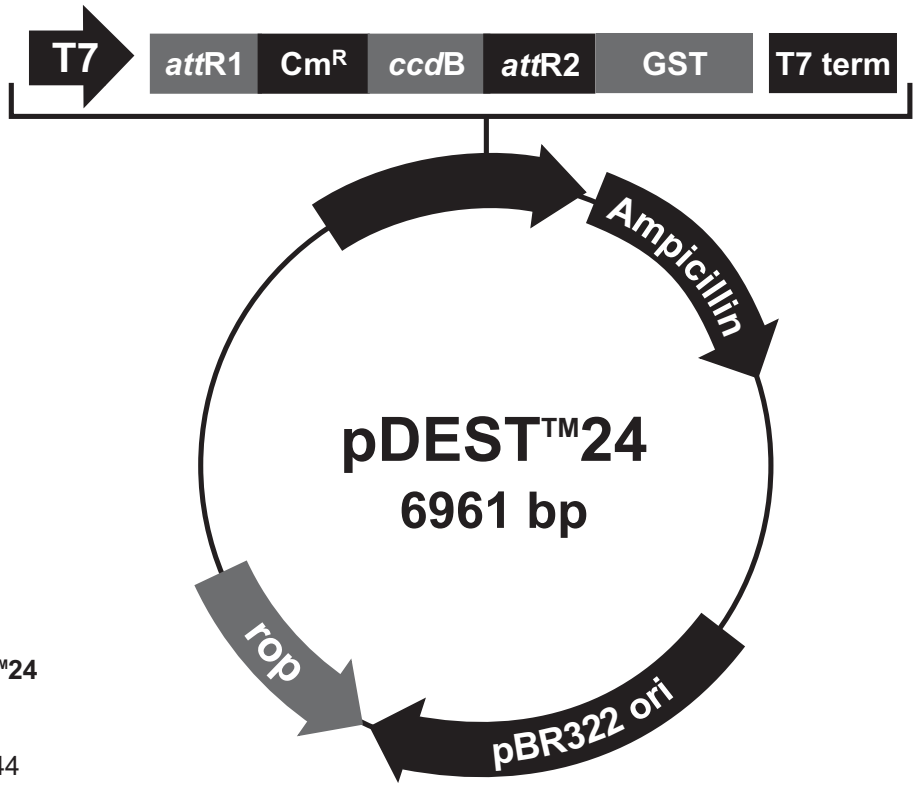
C=complementary strand

continued on next page

Map and Features of the pDEST™ Vectors, continued

pDEST™24 Map

The map below shows the elements of pDEST™24. DNA from the entry clone replaces the region between bases 78 and 1761. **The complete sequence for pDEST™24 is available from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).**



Comments for pDEST™24
6961 nucleotides

T7 promoter: bases 25-44
*att*R1: bases 71-195
Chloramphenicol resistance gene (Cm^R): bases 304-963
*ccd*B gene: bases 1305-1610
*att*R2: bases 1651-1775
GST tag: bases 1783-2451
T7 transcription termination region: bases 2466-2594
bla promoter: bases 3082-3180
Ampicillin (*bla*) resistance gene: bases 3181-4041
pBR322 origin: bases 4186-4859
ROP ORF: bases 5230-5421 (C)

C=complementary strand

continued on next page

Map and Features of the pDEST™ Vectors, continued

Features of the Vectors

pDEST™14 (6422 bp), pDEST™15 (7013 bp), pDEST™17 (6354 bp), and pDEST™24 (6961 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
Ribosome binding site (*i.e.* Shine-Dalgarno sequence) **(in pDEST™15 and pDEST™17 only)**	Optimally spaced from the initiation ATG in the N-terminal tag for efficient translation of the PCR product.
N-terminal glutathione *S*-transferase (GST) tag **(in pDEST™15 only)**	Allows affinity purification of recombinant fusion protein using glutathione agarose
N-terminal 6×His tag **(in pDEST™17 only)**	Permits affinity purification of recombinant fusion protein using a metal-chelating resin such as ProBond™ or Ni-NTA
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
C-terminal glutathione *S*-transferase (GST) tag **(in pDEST™24 only)**	Allows affinity purification of recombinant fusion protein using glutathione agarose
T7 transcription termination region	Sequence from bacteriophage T7 that permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*

Map of pENTR™-gus

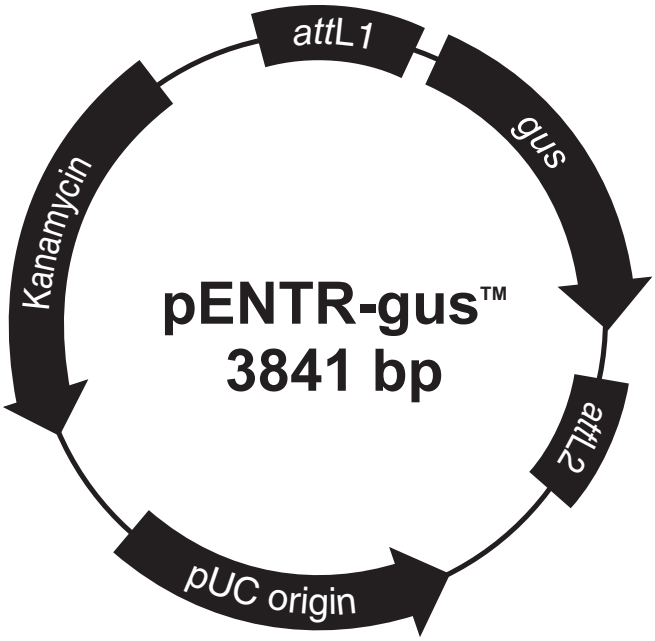
Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR201™ to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase® II manual.

Note: The molecular weight of GUS is 68.4 kDa.

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The complete sequence for pENTR™-gus is available from our website (www.invitrogen.com) or by contacting Technical Support (see page 37).**



Comments for pENTR-gus™
3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Recipes

Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   0.3 ml KH_2PO_4
   4.7 ml K_2HPO_4
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available on our website at www.invitrogen.com/cofa, and is searchable by product lot number, which is printed on each box.

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Purchaser Notification

Introduction

Use of the *E. coli* Expression System with Gateway® Technology is covered under the licenses detailed below.

Information for European Customers

The BL21-AI™ *E. coli* strain is genetically modified and carries a chromosomal insertion of a cassette containing the T7 RNA polymerase (T7 RNAP) gene. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Limited Use Label License No. 19: Gateway® Cloning Products

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Life Technologies Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Life Technologies under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200.

continued on next page

Purchaser Notification, continued

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 40.

Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

Limited Use Label License No. 23: GUS Control Vector

The GUS positive control vector in these products is claimed in patents and patent applications (See U.S. Patent No. 5,599,670 and Great Britain Patent No. 2,197,653) licensed to Life Technologies by Cambia Biosystems, L.L.C. ("CBL"). Use of the GUS gene is restricted to use as a positive control. Any other use may require a license from CBL.

Limited Use Label License No. 30: T7 Expression System

The composition and/or use of this product may be claimed in U.S. Patent No. 5,693,489 licensed to Life Technologies Corporation by Brookhaven Science Associates, LLC. The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy, and is the subject of patents and patent applications assigned to Brookhaven Science Associates, LLC (BSA,). By provisions of the Distribution License Agreement granted to Life Technologies covering said patents and patent applications, Life Technologies grants you a non-exclusive sub-license under patents assigned to BSA for the use of this technology, including the enclosed materials, based upon the following conditions: 1 – these materials are to be used for non-commercial research purposes only. A separate license under patents owned by BSA is required for any commercial use, including the use of these materials for research purposes or production purposes by any commercial entity. Information about commercial license may be obtained from The Office of Technology Transfer, Brookhaven National Laboratory, Bldg. 475D, P.O. Box 5000, Upton, New York 11973-5000. Phone (516) 344-7134. 2 - No materials that contain the cloned copy of the T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this sub-license and agrees to be bound by its terms. This limitation applies to strains BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE, CE6, BL21-SI Competent Cells and any derivatives that are made of them. You may refuse this sub-license by returning this product unused in which case Life Technologies accept return of the product with a full refund. By keeping or using this product, you agree to be bound by the terms of this license.

Limited Use Label License No. 54: ULB ccdB Selection Technology

This product is the subject of one or more of U.S. Patent Numbers 5,910,438, 6,180,407, and 7,176,029 and corresponding foreign patents and is sold under license from the Université Libre de Bruxelles for research purposes only. ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: Out Licensing, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail.

Limited Use Label License No. 125: GST

This product is the subject of U.S. Patent No. 5,654,176 and/or foreign equivalents and is to be used for scientific investigation and research and for no other purpose whatsoever. Licenses for commercial use of the above mentioned patents must be negotiated directly with Amrad Corporation, 576 Swan Street, Richmond, Victoria Australia 3121, Telephone: 61 3 9208 4000.

Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

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