SureFrame™ Allele Library Construction Kit

For efficient generation of full-length allele libraries

Catalog nos. K2005-01 and PQ10002-01

Version A
24 October 2005
25-0872

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Product	Catalog no.
SureFrame™ Allele Library Construction Kit	K2005-01
ProQuest™ Reverse Two-Hybrid System	PQ10002-01

Kit Components

The SureFrame™ Allele Library Construction Kit and ProQuest™ Reverse Two-Hybrid System contain the following components. For a detailed description of the contents of the SureFrame™ Allele Library Construction Kit, see page v. For a detailed description of the contents of the ProQuest™ Two-Hybrid System, see the ProQuest™ Two-Hybrid System manual.

Component	Catalog no.	
	K2005-01	PQ10002-01
SureFrame™ Allele Library Construction Kit	√	√
ProQuest™ Two-Hybrid System		√

The SureFrame™ Allele Library Construction Kit contains enough reagents for the generation of 5 allele libraries

Shipping/Storage

The SureFrame™ Allele Library Construction Kit is shipped as described below. Upon receipt, store each item as detailed below. For information on shipping and storage of the ProQuest™ Two-Hybrid System, refer to the ProQuest™ Two-Hybrid System manual.

Box	Component	Shipping	Storage
1	SureFrame™ Allele Library Construction Kit	Dry ice	-20°C
2-3	One Shot® TOP10 Electrocomp *E. coli* (2 boxes with 20 reactions/box)	Dry ice	-80°C
4	Gateway® BP Clonase™ II Enzyme Mix	Dry ice	-20°C
5	Gateway® LR Clonase™ II Enzyme Mix	Dry ice	-20°C

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Kit Contents and Storage, continued

SureFrame™ Allele Library Construction Kit

The following components are included in the SureFrame™ Allele Library Construction Kit (Box 1). **Store the reagents at -20°C.**

Component	Composition	Amount
pDONR™-Express	Lyophilized plasmid DNA	6 µg
pENTR™-Express/Fos-m1	Lyophilized plasmid DNA	10 µg
Kanamycin	25 mg/ml in dH_2O	12 x 1 ml
Spectinomycin	100 mg/ml in dH_2O	2 x 1 ml
IPTG	1 M in dH_2O	6 x 1 ml
SOB Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$	2 x 10 ml

One Shot® TOP10 Electrocomp Reagents

The following reagents are included in each One Shot® TOP10 Electrocomp *E. coli* kit (two kits are supplied; Boxes 2-3). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store the One Shot® TOP10 Electrocomp *E. coli* kit at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	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
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10 Cells

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lacX*74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

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Gateway® BP Clonase™ II Enzyme Mix

The following reagents are included with the Gateway® BP Clonase™ II Enzyme Mix (Box 4).

Store Box 4 at -20°C for up to 6 months. For long-term storage, store at -80°C.

Reagent	Composition	Amount
Gateway® BP 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
30% PEG 8000/30 mM $MgCl_2$ Solution	30% PEG 8000 30 mM $MgCl_2$	1 ml
pEXP7-tet Positive Control	50 ng/µl in TE Buffer, pH 8.0	20 µl

Gateway® LR Clonase™ II Enzyme Mix

The following reagents are included with the Gateway® LR Clonase™ II Enzyme Mix (Box 5).

Store Box 5 at -20°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

ProQuest™ Reverse Two-Hybrid System

In addition to the SureFrame™ Allele Library Construction Kit, the ProQuest™ Reverse Two-Hybrid System also includes the following boxes:

Box	Components	Storage
1	ProQuest™ Vectors	-80°C
2	ProQuest™ Control Vectors	-20°C

Refer to the ProQuest™ Two-Hybrid System manual supplied with Catalog no. PQ10002-01 for a detailed description of the two-hybrid reagents.

Accessory Products

Introduction

The products listed in this section may be used with the SureFrame™ Allele Library Construction Kit and ProQuest™ Reverse Two-Hybrid System. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 47).

Accessory Products

Some of the reagents supplied in the SureFrame™ Allele Library Construction Kit as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below.

Item	Amount	Catalog no.
ProQuest™ Two-Hybrid System	1 kit	PQ10001-01
Platinum® PCR SuperMix HiFi	100 reactions	12532-016
Platinum® PCR SuperMix	100 reactions	11306-016
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25
PureLink™ PCR Purification Kit	50 reactions	K3100-01
PureLink™ HiPure Plamid Midiprep Kit	25 preps	K2100-04
One Shot® TOP10 Electrocomp *E. coli*	20 reactions	C4040-52
One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli*	10 transformations	C7510-03
E-Shot™ Standard Electroporation Cuvettes	1 pack; 0.1 cm	P510-50
pCR®8/GW/TOPO® TA Cloning Kit	20 reactions	K2500-20
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
Gateway® BP Clonase™ II Enzyme Mix	20 reactions	11789-020
Proteinase K	100 mg	25530-015
10X REact® 4 Buffer	2 x 1 ml	16304-016
Dpn I	100 units	15242-019
Kanamycin Sulfate	5 g	11815-024
Ampicillin Sodium Salt	200 mg	11593-019
IPTG	1 g	15529-019
SOC Medium	10 x 10ml	15544-034
LB Agar, powder (Lennox L Agar)	500 g 2.5 kg	22700-025 22700-041
LB Broth Base, powder (Lennox L Broth Base)®	500 g 2.5 kg	12780-052 12780-029

Spectinomycin

For selection of pENTR™-Express transformants in *E. coli*, you will need spectinomycin. Spectinomycin Dihydrochloride is supplied with the kit, but is also available from Sigma (Catalog no. S4014).

Introduction

Overview

Introduction

The generation of an allele library can be achieved by polymerase chain reaction (PCR) of a gene of interest, using either standard or mutagenic reaction conditions. The SureFrame™ Allele Library Construction Kit is designed to select against PCR products containing nonsense or frameshift mutations and facilitate the isolation of full-length open reading frames following PCR amplification. This section provides an overview of the SureFrame™ Allele Library Construction Kit.

Use of the SureFrame™ Kit

Use the SureFrame™ Allele Library Construction Kit for the following applications:

- Generation of full-length enriched allele libraries for use in a reverse two-hybrid analysis of protein-protein, protein-DNA and protein-RNA interaction.
- Generation of Gateway® entry clones lacking a stop codon. SureFrame™ technology allows the elimination of ORF (open reading frame) clones containing undesirable frameshift and nonsense mutations.
- Generation of full-length enriched allele libraries for functional analysis of expressed proteins.
  Note: It is important to have a downstream assay that allows for the screening or selection of a large numbers of mutants easily.

SureFrame™ Technology

The SureFrame™ Technology consists of a modified Gateway® donor vector that allows easy cloning and expression of PCR products as N-terminal fusion to the kanamycin resistance gene, neomycin phosphotransferase. Following selection on kanamycin, the resulting library is enriched for full-length open reading frames. This library may be isolated and transferred to a Gateway® destination vector for downstream expression and analysis, thus losing the C-terminal fusion used for full-length selection.

Advantages of the SureFrame™ Kit

The SureFrame™ Allele Library Construction Kit has a number of clear advantages over traditional methods:

- The scheme selects against interaction defective truncated proteins prior to the downstream assay, eliminating almost all background normally associated with screens for loss-of-function mutations if using counterselection.
- Gateway® recombination combined with the efficiency of *E. coli* transformation allows for larger (10^6-10^7), more complex allele libraries.
- Clones lose the C-terminal kanamycin resistance gene fusion used for full-length selection before the downstream assay; therefore the proteins expressed in the downstream assay may be evaluated in the original context.

Continued on next page

Overview, continued

SureFrame™ System Components

The SureFrame™ Allele Library Construction Kit contains the following components (for details, see page 5):

- pDONR™-Express, a Gateway® adapted vector that is used for full-length fusion protein selection
- Reagents for performing Gateway® BP recombination reactions
- Reagents for performing Gateway® LR recombination reactions
- Reagents to transform and select for constructs in *E. coli*
- Positive controls to help with recombination, transformation and selection

Gateway® Technology

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

The SureFrame™ Allele Library Construction Kit contains the vector pDONR™-Express. This vector allows cloning of PCR products via a Gateway® BP recombination reaction to yield an entry clone, pENTR™-Express/ORF, which allows the ORF to be expressed as an N-terminal fusion to the kanamycin resistance gene (Kan^R). Following selection with kanamycin, full-length entry clones may be transferred to a destination vector via a Gateway® LR recombination reaction to yield an expression clone, pEXP/ORF.

See the **Appendix**, page on page 37 for an explanation of the Gateway® Technology. For more detailed information, refer to the Gateway® Technology with Clonase™ II manual which is available from our web site (www.invitrogen.com) or by contacting Technical Service (see page 47).

Purpose of this Manual

This manual provides the following information:

- An overview of selection strategy used in the SureFrame™ Allele Library Construction Kit
- Procedures to perform mutagenesis of your insert
- A protocol to determine the appropriate kanamycin concentration to use in the selection process
- A procedure to select for full-length fusion proteins
- Guidelines to transfer your allele library into the vector used in your downstream application

General Description of Allele Library Generation

Introduction

This section explains the basis of allele library generation and provides more detail about the advantages of the SureFrame™ Allele Library Construction Kit for generating allele libraries.

Random Mutagenesis

Allele library generation usually relies on a random mutagenesis step, typically error-prone PCR (for a good explanation of error-prone PCR, refer to Molecular Biology handbooks, such as *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994). Due to the unpredictable nature of the mutations generated, a proportion of mutated protein products will have premature stop codons, resulting in truncated proteins. Additionally, frameshift mutations can also easily result in truncated proteins if an out of frame stop codon is encountered.

Loss-of-Function Mutations

Several types of single nucleotide mutations introduced through PCR can interfere with protein function (loss-of-function mutations):

- Frameshift mutations. These result in failure to translate large regions of a protein properly or in truncated proteins.
- Point mutations introducing stop codons, resulting in truncated proteins.
- Point mutations resulting in missense mutations. These proteins are the most informative. For instance, in interaction assays, proteins containing point mutations can point to the role of single residues in the interaction surface.

Eliminating Truncated Proteins

If you are looking for loss-of-function mutations, it is desirable to be able to select for full-length proteins. Traditional methods rely on C-terminal tags that indicate whether a certain protein product is translated during or after the assay.

Continued on next page

General Description of Allele Library Generation, Continued

SureFrame™ Allele Library Construction Kit

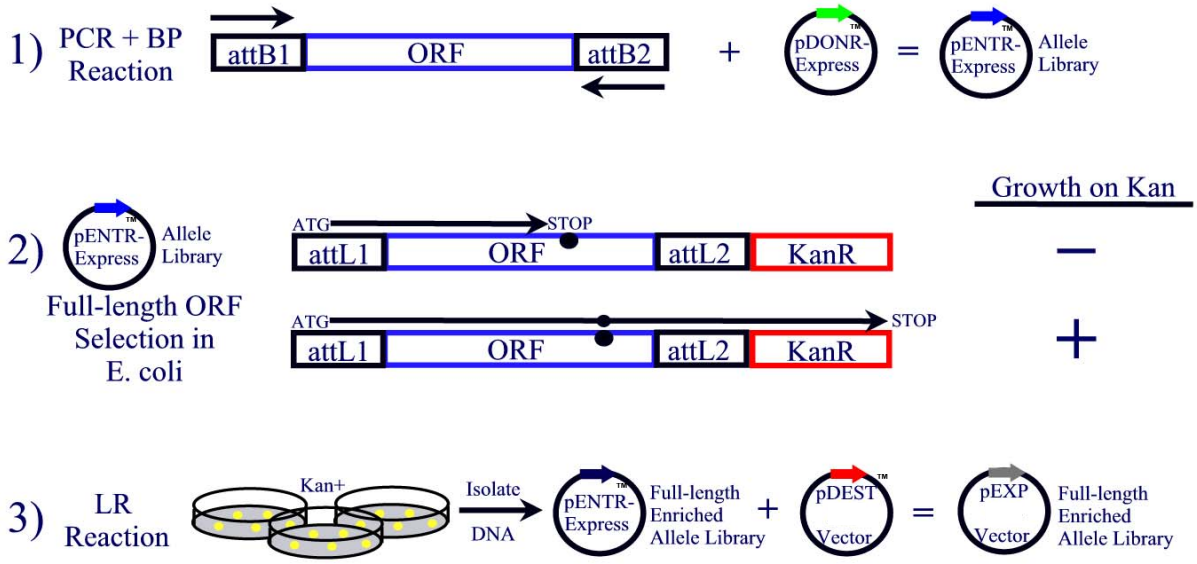
We have developed a new strategy to select against truncated proteins while generating allele libraries. This strategy includes producing allele libraries *in vitro* and selecting for full-length proteins in *E. coli* prior to analysis in a downstream system. The allele library is generated in a Gateway®-adapted vector, enabling rapid and efficient transfer of the allelic inserts into a suitable vector for downstream analysis. To use the SureFrame™ Allele Library Construction Kit, perform the following steps (see also figure below):

1. Perform PCR on an expression clone containing your insert and recombine the amplified products with the pDONR™-Express vector. This new clone facilitates the expression of entry clones as an N-terminal fusion to the kanamycin resistance gene (neomycin phosphotransferase).

   Note: As template, you need an expression clone containing your gene of interest as insert flanked by *att*B sites, without stop codon and in correct reading frame (see the methods section, page 8, for details).

2. Transform the allele library clones into *E. coli*. Only alleles coding for full-length proteins will confer kanamycin resistance and produce colonies for DNA (i.e. allele library) isolation.

3. Transfer the pENTR™–Express allele library to the vector used for the downstream assay through a second Gateway® recombination reaction. This yields a full-length enriched expression library in your expression vector.



SureFrame™ Allele Library Construction Kit

Description of the System

The SureFrame™ Allele Library Construction Kit is a Gateway® adapted method to select for full-length proteins after mutagenesis. It includes:

- pDONR™-Express; this vector facilitates the production of entry clones as an N-terminal fusion with the kanamycin resistance gene (neomycin phosphotransferase). The fusion allows selection of full-length proteins.
- Gateway® BP Clonase™ II Enzyme Mix to facilitate the BP recombination reaction to transfer inserts to pDONR™-Express.
- Gateway® LR Clonase™ II Enzyme Mix to facilitate the LR recombination reaction to transfer the library to destination vectors.
- One Shot® TOP10 Electrocomp *E. coli* cells for transformation of the allele library.
- Kanamycin, IPTG and S.O.B. medium to perform the selection with kanamycin for allele library clones that express a full-length fusion protein.
- Spectinomycin, to determine recombination efficiency.

Controls

The SureFrame™ Allele Library Construction Kit contains the following controls:

- pENTR™-Express/Fos-m1; positive control for the kanamycin titration
- pEXP7-tet; positive control for the BP recombination reaction
- pENTR™-gus; positive control for the LR recombination reaction
- pUC19; positive control for transformation

Generating a Full-length Allele Library

Generating a full-length allele library with the SureFrame™ Allele Library Construction Kit consists of the following steps:

1. PCR amplification of your ORF of interest
2. BP recombination reaction of PCR product with pDONR™-Express to yield pENTR™-Express/ORF
3. Transformation and selection for full-length proteins in *E. coli* with kanamycin
4. Isolation of allele library as entry clones
5. LR recombination reaction of allele library entry clones with destination vector to yield pEXP/ORF, where ORF = allele library

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Features of pDONR™-Express

The pDONR™-Express vector contains the following features:

- Prokaryotic EML promoter for inducible expression. This promoter is derived from the EM-7 promoter and made inducible with IPTG by insertion of a *lac* operator.
- Two recombination sites, *attP1* and *attP2*, downstream of the EML promoter and upstream of the Kan^R gene (neomycin phosphotransferase). The allele library will be introduced in between these sites upon a BP recombination reaction (which will form a library of entry clones, designated pENTR™-Express, containing your allele library).

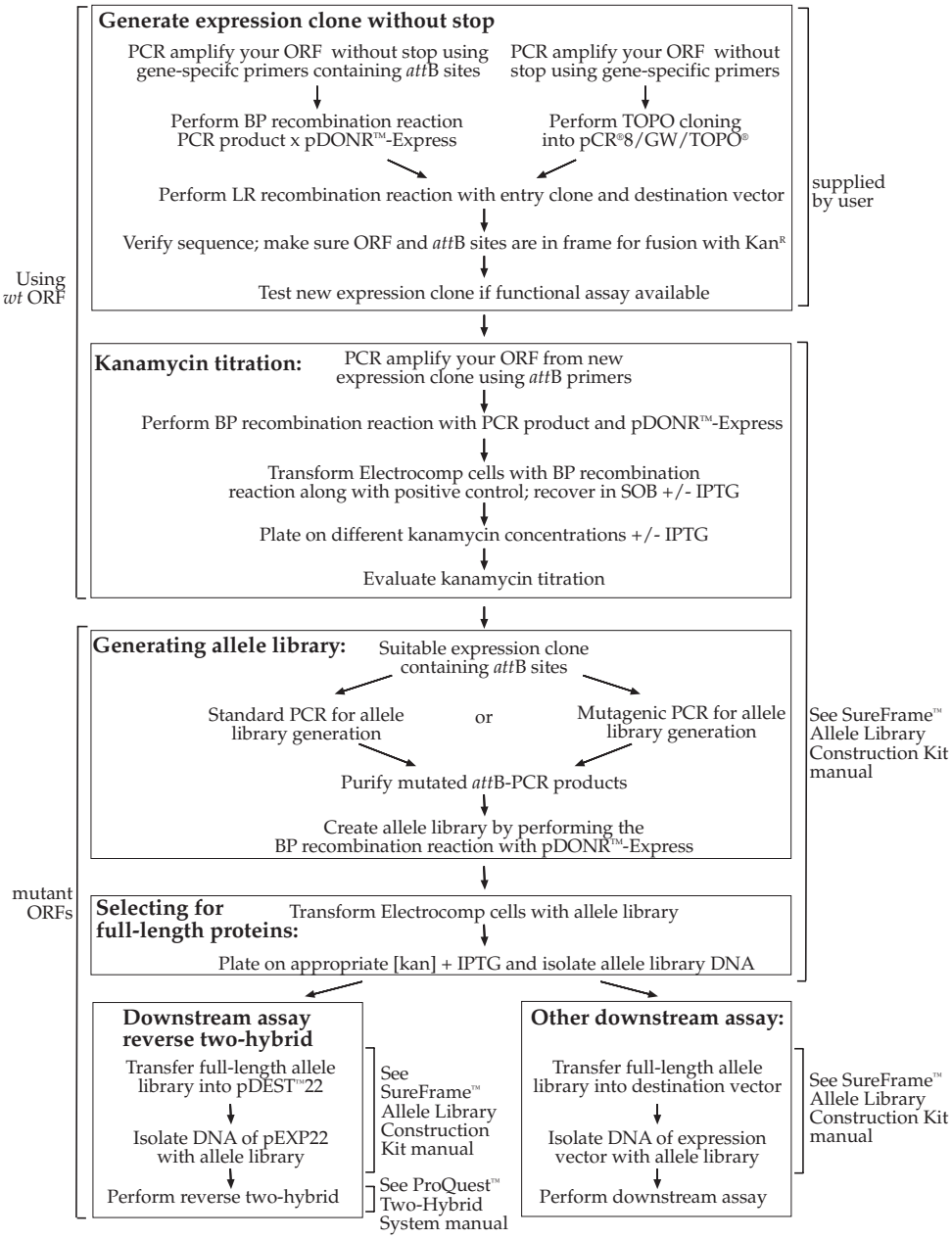
- A cassette containing *ccd*B and the Chloramphenicol Resistance gene. This cassette is spliced out upon BP recombination. Plasmids containing the cassette can be selected for using Chloramphenicol and selected against using most regular *E. coli* strains, which are sensitive to the CcdB effects.
- A kanamycin resistance gene, the neomycin phosphotransferase gene. In the entry clones (designated pENTR™-Express), this gene is expressed as fusion protein with the insert if the insert does not contain an in-frame stop codon and has the right frame. Selection with kanamycin therefore selects for full-length fusion proteins.
- Spectinomycin resistance gene for selection in *E. coli.*
- *Lac*I gene, which codes for the lac repressor protein, and helps in silencing the EML promoter in the absence of IPTG
- pUC origin for high-copy maintenance of the plasmid in *E. coli*.

For a map of pDONR™-Express, see the **Appendix**, page 43.

Experimental Outline

Flow Chart

The figure below illustrates the major steps necessary to produce a full-length allele library using the SureFrame™ Allele Library Construction Kit.



Methods

Required Expression Clone

Introduction

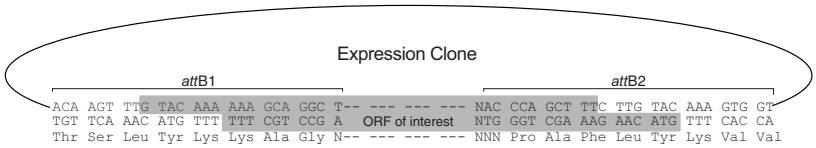
To generate allele libraries using the SureFrame™ Allele Library Construction Kit, you need to have a Gateway® expression clone to use as template, i.e. your ORF of interest flanked by *att*B sites. In this section we explain the requirements for a suitable expression clone.

Required Features of Expression Clone

The required features for a Gateway® expression clone to be used as template are:

- The plasmid should contain your gene of interest, flanked at the 5′ site by an *att*B1 site, and at the 3′ site by an *att*B2 site
- The forward PCR primer used in mutagenesis is 5′-ACA AGT TTG TAC AAA AAA GCA G-3′, which anneals to the *att*B1 site on your template. Your gene of interest should be in frame with the reading frame indicated in the figure below.
- The reverse PCR primer used in mutagenesis is 5′-AC CAC TTT GTA CAA GAA AGC-T-3′, which anneals to the *att*B2 site on your template. Your gene of interest should be in frame with the reading frame indicated in the figure below.
- There should be no stop codon in frame in between the *att* sites

Expression clone



After PCR and the BP recombination reaction, the shaded sequence in the figure will eventually transfer to pDONR™-Express, forming pENTR™-Express/ORF. See next page for the recombination region of the resulting entry clone and the reading frame required for expression of the downstream Kan^R gene.

continued on next page

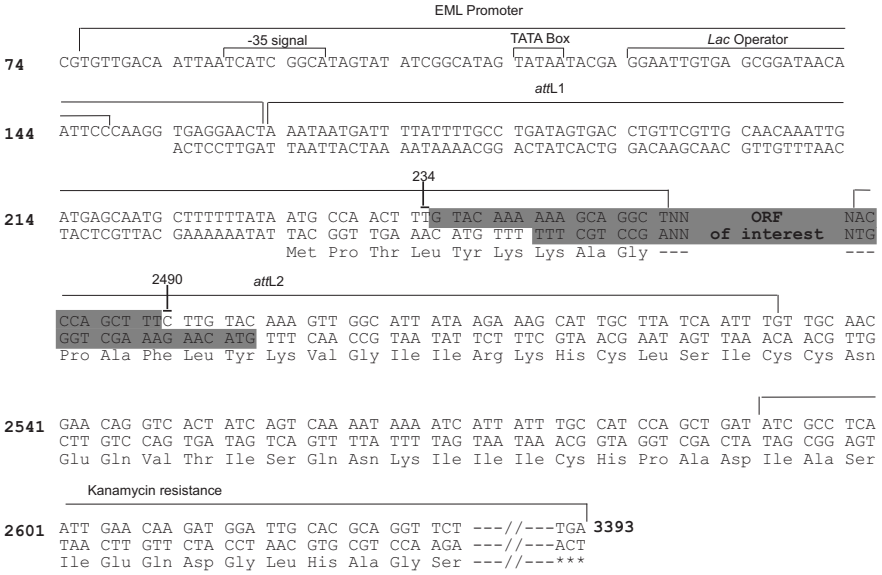
Required Expression Clone, continued

Recombination Region of pDONR™-Express

The recombination region of the expression clone resulting from pDONR™-Express × *att*B PCR product is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the *att*B substrate into pDONR™-Express by recombination. Non-shaded regions are derived from pDONR™-Express
- The reading frame indicated expresses the kanamycin resistance gene.



Generating Expression Clone without Stop

If you do not have an expression clone without a stop codon in the insert and in the correct frame, perform the following steps to make a Gateway® expression clone containing your ORF of interest:

1. Generate an entry clone:
   - PCR your ORF with gene-specific primers; exclude stop codons. Ensure the insert will be in the correct frame (see above). Clone the PCR product into a suitable entry vector. We recommend pCR®8/GW/TOPO® (K2500-20); see manual for instructions.
   - PCR your ORF with gene-specific primers flanked with *att*B sites (approximately 60 bp) and exclude stop codons. These are **not** the same as the *attB* primers described on page 11. Make sure the insert will be in the right reading frame (see above). Clone the PCR product into pDONR™-Express; for instructions see page 13.
2. Transfer insert into a suitable expression vector (pDEST™22 if performing a reverse yeast two-hybrid screen) using an LR recombination reaction.
3. If you have a downstream functional assay, test your new construct in the assay before generating the allele library.

Note: For other entry vector options; see the Gateway® portal at our website, http://www.invitrogen.com/Gateway, or contact Technical Service (page 47).

Kanamycin Titration

Overview

Introduction

SureFrame™ Technology facilitates the expression of a gene of interest as a fusion to the kanamycin resistance gene, neomycin phosphotransferase, following a BP reaction with pDONR™-Express. The resulting fusion consists of *att*L1-ORF-*att*L2-Kan^R. As a result, the activity of Kan^R will be influenced by the ORF under study. Some ORFs will not affect Kan^R function, allowing selection on high concentrations of kanamycin, while others will affect Kan^R function. Thus, ORFs that affect Kan^R activity must be selected for on lower concentrations of kanamycin.

In addition, a threshold concentration of kanamycin was found to exist for all ORFs tested with SureFrame™ where Kan$^+$ colonies appeared independent of IPTG induction when a kanamycin concentration below their respective threshold was used. The background growth is most likely due to cryptic promoter activity and internal ribosome binding sites, which will produce a Kan$^+$ phenotype in the absence of a complete *att*L1-ORF-*att*L2-Kan^R fusion protein.

To minimize this background, and identify the optimal kanamycin concentration specific to a particular ORF, it is necessary to perform a kanamycin titration step. This will determine the kanamycin concentration that allows for a maximum number of colonies in the presence of IPTG, while suppressing growth on kanamycin in the absence of IPTG

Outline

To perform a kanamycin titration, perform the following steps:

1. Amplify the *wt* ORF under non-mutagenic conditions **using attB primers**
2. Perform a BP recombination reaction between the PCR product and pDONR™-Express
3. Transform the recombination reaction into One Shot® TOP10 Electrocomp *E. coli* cells (along with a Kan$^+$ control, pENTR™-Express/Fos-m1)
4. Recover *E. coli* transformants in SOB +/- IPTG
5. Plate out on increasing concentrations of kanamycin +/- IPTG

The kanamycin concentration that yields the highest number of colonies in the presence of IPTG and minimal, or zero, colonies without IPTG is the optimal kanamycin concentration to use for full-length selection.

Note: Expression clones (*att*B flanked ORF expression plasmids) may be used in the BP recombination reaction. However we recommend performing the BP recombination using an *att*B-flanked PCR product because the BP recombination efficiency is much higher. This will also give you an idea how many colonies you can expect when performing the selection for full-length protein using your mutagenized PCR product.

Standard PCR with *att*B primers

Introduction

To carry out the kanamycin titration, you first need to generate an entry clone by performing a BP recombination reaction between pDONR™-Express and your *wt* ORF of interest. In this section, you will carry out a PCR on your expression vector using *att*B primers. This will be used in the next section for the BP recombination reaction to generate the pENTR™-Express entry clone containing your ORF of interest.

PCR Primers

We recommend using the following PCR primers; you can order them from Invitrogen's custom primer synthesis service (see www.invitrogen.com).

Primer Name	Primer Sequence
*att*B1-5′	5′- ACA AGT TTG TAC AAA AAA GCA G –3′
*att*B2-3′	5′- AC CAC TTT GTA CAA GAA AGC T –3′

Note: Primers specific to the vector backbone may be used as long as the *att*B1 and *att*B2 sites are included in the PCR product.

Important

Do not use gene specific primers flanked with *att*B sites for kanamycin titration or allele library generation. These primers are large (~60 bp) and are more prone to contain deletions and insertions in the *att*B sites. PCR products with such deletions (in the *att*B1 site) have been observed to slip through the kanamycin selection process (i.e. they grow on kanamycin even though the fusion is out-of-frame), presumably through ribosome slippage. Therefore, for the amplication of the ORF, only use *att*B primers, or primers specific to the backbone.

Primer Concentration

- 50 nmol of standard purity, desalted oligonucleotides are sufficient for most applications.
- Dissolve oligonucleotides to 20-50 mM in water or TE Buffer and verify the concentration before use.

Recommended Polymerases

We recommend Platinum® PCR SuperMix HiFi (Catalog no. 12532-016), which contains Platinum® *Taq* DNA Polymerase and the proofreading enzyme *Pyrococcus* species *GB-D* polymerase. If you are amplifying small inserts (≤1 kB), you can also use Platinum® PCR SuperMix (Catalog no. 11306-016), which contains Platinum® *Taq* DNA Polymerase. Other *Taq* Polymerases may be suitable.

Note: It is not necessary to use a SuperMix formulation; separate reagents and other suitable DNA polymerases can be used.

continued on next page

Standard PCR with *att*B primers, continued

Producing PCR Products

- Follow the manufacturer's instructions for the DNA polymerase you are using.
- Use 10 ng of an *att*B containing plasmid as template as described on page 8.
- If using *att*B1-5′ and *att*B2-3′ as forward and reverse primers, a good starting point is to use 100 ng of each primer at an annealing temperature of 55°C for 25 cycles. Adjust cycling conditions as necessary.

Checking the PCR Product

Remove 5 µl from each PCR reaction and use agarose gel electrophoresis to verify the quality and yield of your PCR product. If the PCR product is clearly visible, of the right size and constitutes a distinct band, proceed to purify the PCR product.

Removing Primers and Primer-Dimers

After you have generated your *att*B-PCR product, purify the PCR product to remove *att*B primers and any *att*B primer-dimers. Primers and primer-dimers can recombine efficiently with the donor vector in the BP reaction and may increase background after transformation into *E. coli*.

Note

If your PCR template is a plasmid that contains the kanamycin resistance gene, we suggest treating your PCR reaction mixture with *Dpn* I before purifying the *att*B-PCR product. This treatment degrades the plasmid (*i.e. Dpn* I recognizes methylated GATC sites) and helps to reduce background in the BP recombination reaction associated with template contamination.

Protocol:

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer (Invitrogen, Catalog no. 16304-016) and ≥5 units of *Dpn* I (Invitrogen, Catalog no. 15242-019).
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purifying *att*B-PCR Products**, below

Purifying *att*B-PCR Products

We recommend using the S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25). Other gel purification kits may also be suitable. Follow the manufacturer's instructions.

Alternatively, you may purify your PCR product without gel electrophoresis. Use the PureLink™ PCR Purification Kit (Catalog no. K3100-01) or the protocol provided in the **Appendix** (page 40; this protocol is not suitable if your PCR product is less than 300 bp).

Note: Store the purified PCR product at -20°C, since you may be able to use it to generate the allele library (page 20).

BP Recombination Reaction

Introduction

In this section, you will perform a BP recombination reaction between pDONR™-Express and your *wt* ORF of interest. This recombination reaction will then be transformed and plated in **Transformation of BP Recombination Reaction** (page 16). To ensure that you obtain the best possible results, we suggest that you read this section entirely before starting.

BP Recombination Reaction

The BP recombination reaction facilitates transfer of a gene of interest in the *att*B-PCR product to the *att*P-containing pDONR™-Express to create a pENTR™-Express clone. Once you have created your entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction.

Substrates for the BP Recombination Reaction

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

- *att*B-flanked PCR product
- *att*P-containing pDONR™-Express (see page 9 and 43)

Resuspending the Donor Vectors

pDONR™-Express is supplied as 6 µg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, simply resuspend the pDONR™-Express plasmid DNA in 40 µl of sterile water to a final concentration of 150 ng/µl.

Important

Do not use donor vectors other than pDONR™-Express for allele library generation. pDONR™-Express contains an in-frame kanamycin resistance gene that is essential for this method.

*ccd*B Gene

The presence of the *ccd*B gene in pDONR™-Express allows negative selection of the vector in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard & Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* OmniMAX™ 2-T1^R, DH5α™, TOP10). When recombination occurs (*i.e.* between a destination vector and an entry clone or between a donor vector and an *att*B-PCR product), the *ccd*B gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

continued on next page

BP Recombination Reaction, continued

Propagating Gateway® Vectors

Because of the lethal effects of the CcdB protein, all Gateway® vectors containing the *ccd*B gene **must** be propagated in an *E. coli* strain that is resistant to CcdB effects. If you wish to propagate pDONR™-Express, we recommend using the *ccd*B Survival T1^R *E. coli* strain which is resistant to CcdB effects (Bernard & Couturier, 1992; Bernard *et al.*, 1993; Miki *et al.*, 1992). One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* are available from Invitrogen (Catalog no. C7510-03) for transformation.

Positive Control

If desired, you can include a positive control (pEXP7-tet, see below) in your experiment to verify the efficiency of the BP recombination reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. Determine the efficiency of the BP recombination reaction by plating onto LB plates with 20 µg/ml tetracycline.

BP Clonase™ II Enzyme Mix

BP Clonase™ II enzyme mix is supplied with the SureFrame™ Allele Library Construction Kit to catalyze the BP recombination reaction. The BP Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X BP Clonase Reaction Buffer previously supplied as separate components in BP Clonase™ enzyme mix (Catalog no. 11789-019) into an optimized single tube format to allow easier set-up of the BP recombination reaction.

Note: You may perform the BP recombination reaction using BP Clonase™ enzyme mix, if desired. To use BP Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for BP Clonase™ II enzyme mix provided below.

Materials Needed

You should have the following materials on hand before beginning:

- *att*B-flanked PCR product
- pDONR™-Express (supplied with the kit; resuspend to 150 ng/µl with water)
- BP Clonase™ II enzyme mix (supplied with the kit; keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ II enzyme mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µl; supplied with the BP Clonase™ II enzyme mix), if desired. Substitute the *att*B-flanked PCR product with 2 µl pEXP7-tet for a positive recombination control

continued on next page

BP Recombination Reaction, continued

Setting Up the BP Recombination Reaction

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

Components	Sample	Positive Control
pDONR™ vector (150 ng/µl)	1 µl	1 µl
Purified *att*B flanked PCR product (100 ng)	1-7 µl	--
pEXP7-tet positive control	--	2 µl
TE Buffer, pH 8.0	to 8 µl	to 8 µl

Note: To include a negative control, set up a second sample reaction and omit the BP Clonase™ II enzyme mix (see Step 4).

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

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

   Note: This incubation time is longer than the time required in a standard BP recombination reaction, and is required to get a higher number of recombinants
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transformation of BP Recombination Reaction,** next page.

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

Transformation of BP Recombination Reaction

Introduction

In this section, you will transform the BP recombination reaction into One Shot® TOP10 Electrocomp *E. coli* cells to determine the optimal kanamycin concentration , as defined in the **Overview**, page 10. One Shot® TOP10 Electrocomp *E. coli* cells are supplied with the kit for this purpose.

pENTR™-Express/Fos-m1 Titration Control

Include the titration control, pENTR™-Express/Fos-m1, in your kanamycin titration (supplied with the kit). pENTR™-Express/Fos-m1 contains a mutated version of the transcription factor fos, isolated in an allele library screen. After transformation in *E. coli*, pENTR™-Express/Fos-m1 should yield colonies only on kanamycin plates containing 1 mM IPTG, but not without 1 mM IPTG.

pENTR™-Express/Fos-m1 is supplied as 10 µg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, simply resuspend the plasmid DNA in 100 µl of sterile water to a final concentration of 100 ng/µl. Dilute 1 µl of this stock in 9 µl sterile water to use in the transformation (final concentration of 10 ng/µ1).

One Shot® TOP10 Electrocomp *E. coli* cells

The SureFrame™ Allele Library Construction Kit includes One Shot® TOP10 Electrocomp *E. coli* cells for transformation. Since allele library generation requires a large number of colonies for good library representation, highly competent electrocomp cells must be used. Pool two separate vials of competent cells (50 µl per vial) for every allele library transfer or control; use 80 µl with 1 µl of transformation reaction; the remaining 20 µl can be used for controls. Dispose of any unused cells once thawed.

Note: Do not use other *E. coli* strains, such as Mach1 or DH10B. Of the strains tested, TOP10 cells were superior in the kanamycin selection procedure.

Plates Required

You will plate the transformations on the following plates:

- Plates containing IPTG and a range of concentrations of kanamycin, which will determine the threshold kanamycin concentration you should use to select for your allele library
- Plates containing a range of concentrations of kanamycin but no IPTG, to test for kanamycin resistance gene expression due to cryptic promoter activity in the insert
- Plates containing spectinomycin, to test the efficiency of the BP recombination

For a kanamycin titration for one allele library with control pENTR™-Express/Fos-m1 you need the following number of 10 cm diameter LB agar plates

	Spectinomycin	Kanamycin				Kanamycin + 1 mM IPTG			
Concentration antibiotic (µg/ml)	100	20	30	40	50	20	30	40	50
Number of Plates Needed	12	6	6	6	6	6	6	6	6

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Transformation of BP Recombination Reaction, continued

Materials Needed

You will need to provide the following items for transformation:

- 37°C shaking and **30°C** non-shaking incubator
- LB medium
- Ice bucket with ice
- Electroporator
- Cuvettes (0.1 cm)
- 15 ml snap-cap tubes (two for each transformation); sterile 1.5 ml tubes
- One Shot® TOP10 Electrocomp cells (supplied with the kit)
- A vial of S.O.B. Medium (supplied with the kit)
- pUC19, if checking for transformation efficiency (supplied with the kit)
- LB plates containing the reagents as described on the previous page (spectinomycin, kanamycin and IPTG are supplied with the kit)

Verifying pEXP7-tet Entry Clones

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

Preparation

- Thaw the vial of S.O.B medium and bring to room temperature. **Do not** use S.O.C. medium, as it interferes with the kanamycin selection (S.O.C. medium is supplied with the competent cells, but this is for regular transformations).
- Thaw IPTG, control vector pENTR™-Express/Fos-m1 (10 ng/µl dilution) and the allele library BP recombination reaction if frozen (from page 15).
- Warm selective plates at 30°C for 30 minutes.
- Place cuvettes on ice.
- Thaw **on ice** 4 vials of One Shot® TOP10 Electrocomp cells.
- Dilute 10 µl 1 M IPTG stock in 90 µl dH_2O (final concentration 100 mM).

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Transformation of BP Recombination Reaction, continued

Electroporation Procedure

Important: To avoid arcing, use **only** One Shot® TOP10 Electrocomp cells for electroporation (supplied with the kit). Do not use chemically competent cells.

1. Set up your electroporator for bacterial transformation. Follow the manufacturer's instructions. Generally, the settings are: 1700 V; 200Ω; 25µF.
2. Add 1 µl of the BP recombination reaction or the 10 ng/µl stock of pENTR™-Express/Fos-m1 to 80 µl of cells. Mix gently with pipette tip. **Do not mix by pipetting up and down.**

   Note: The remaining batch of 40 µl of competent cells can be used to transform recombination and transformation controls, if desired. Use 20 µl of electrocompetent cells for each control transformation.
3. Transfer the cells to the chilled electroporation cuvette on ice.
4. Electroporate the cells as per the manufacturer's recommended protocol.
5. Quickly add 920 µl room temperature S.O.B medium and mix gently. Do not use S.O.C. medium, as it interferes with the kanamycin selection.

   Note: To recover the 20 µl transformation reactions for the recombination and transformation controls, use 230 µl S.O.B. medium.
6. Transfer the solution to a 15 ml snap-cap tube; label the tube “induced”.
7. Take out 500 µl and transfer to a second 15 ml snap-cap tube; label “uninduced”.
8. Add 5 µl IPTG (100 mM) to the tube labeled “induced” to induce expression of the fusion protein containing the kanamycin resistance gene.

   Note: Do not add IPTG to transformation reactions for the recombination and transformation controls.
9. Shake both tubes for at least 1 hour at 37 °C to allow expression of the antibiotic resistance genes.

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Transformation of BP Recombination Reaction, continued

Plating Procedure

1. Fill eighteen 1.5 ml tubes with 450 µl LB medium.
2. Make serial dilutions of each sample transformation (both induced and uninduced) by transferring 50 µl from the 15 ml snap-cap tube to the first dilution tube, mix (10^{-1} dilution). Transfer 50 µl from the 10^{-1} dilution to the second dilution tube, mix (10^{-2} dilution), and so on until the final dilution is 10^{-4}.
3. Make serial dilutions of the control vector pENTR™-Express/Fos-m1 transformations (both induced and uninduced) by transferring 50 µl from the 15 ml snap-cap tube to the first dilution tube, mix (10^{-1} dilution). Transfer 50 µl from the 10^{-1} dilution to the second dilution tube, mix (10^{-2} dilution), and so on till the final dilution is 10^{-5}.
4. Spread 100 µl of the indicated diluted transformations on prewarmed LB agar plates containing the following additives:

	Uninduced Sample	Induced Sample (+IPTG)	Uninduced Control	Induced Control (+IPTG)
Dilutions to plate	10^{-2}, 10^{-3}, 10^{-4}	10^{-2}, 10^{-3}, 10^{-4}	10^{-3}, 10^{-4}, 10^{-5}	10^{-3}, 10^{-4}, 10^{-5}
Kanamycin (µg/ml) plates	20, 30, 40 and 50	20, 30, 40 and 50 + 1 mM IPTG	20, 30, 40 and 50	20, 30, 40 and 50 + 1 mM IPTG
Spectinomycin (µg/ml) plates	100	100	100	100

Note: The remaining transformation mix may be stored at +4°C and plated out the next day, if desired.

5. Incubate the plates 24-36 hours at **30°C** (which results in better expression of the fusion proteins) and count colonies.

Optimal Kanamycin Concentration

The kanamycin concentration that yields the highest number of colonies in the presence of IPTG and minimal, or zero, colonies without IPTG is the optimal kanamycin concentration to use for full-length selection. Choose this concentration in **Selecting for Full-length Protein**, page 26.

Example of Expected Results for Titration Control

Below is an example of the number of colonies that we typically obtain using the control pENTR™-Express/Fos-m1. If your results for the titration control differ greatly from those shown in the table, refer to **Troubleshooting** (see page 31).

Reaction	Dilution	# of colonies on LB/Spec	# of colonies on LB/Kan50	# of colonies on LB/Kan50 + 1mM IPTG
Uninduced Control	10^{-3}	~ 3000+	<10	N/A
	10^{-4}	~ 400+	0	N/A
	10^{-5}	≥ 40	0	N/A
Induced Control (+IPTG)	10^{-3}	~ 3000+	N/A	~ 400
	10^{-4}	~ 400+	N/A	~ 40
	10^{-5}	≥ 40	N/A	~ 4

Generating the Allele Library

Overview

Introduction

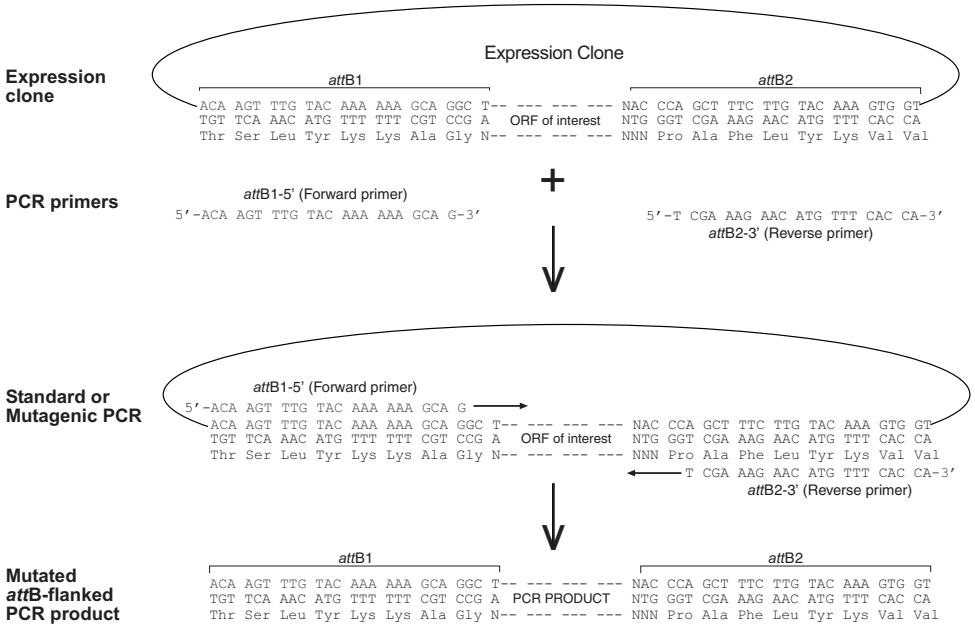
Once you have determined the optimal kanamycin concentration to use for selection, you are ready to generate the allele library. In this section you will generate the allele library in three steps.

1. Perform a PCR with *att*B primers on your gene of interest, to generate fragments with mutations.
2. Purify the *att*B-flanked PCR products.
3. Clone the mutated fragments into pDONR™-Express through a Gateway® BP recombination reaction to generate the allele library in pENTR™-Express.

Note: Your PCR reaction will consist of a pool of PCR products containing random point mutations throughout the DNA fragment, as well as products containing the *wt* allele.

Outline for PCR-based Mutagenesis

Below the outline for the PCR-based mutagenesis is shown:



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Desired Mutation Frequency

The desired mutation frequency depends on your downstream application and size of the insert. We suggest three different ways to generate mutations

- If you work with large inserts (>1 kB), want only single mutations, and generate the allele library for an assay that allows you to screen many clones (such as a Reverse Two-Hybrid), you don't want to generate mutations at a high frequency. Perform a standard PCR reaction using Platinum® PCR SuperMix HiFi (Catalog no. 12532-016). This method relies on the low inherent mutation rate of high fidelity *Taq* polymerases (10^{-5} mutations / bp polymerized). Refer to **Standard PCR with *att*B Primers,** page 11, for guidelines.
- If you work with small inserts (≤500 bp), want only single mutations, and generate the allele library for an assay that allows you to screen many clones (such as a Reverse Two-Hybrid), you want to generate mutations at a moderate frequency. Perform a standard PCR reaction using Platinum® PCR SuperMix (Catalog no. 11306-016). This method relies on the inherent mutation rate of *Taq* polymerases (10^{-6} mutations / bp polymerized). Refer to **Standard PCR with *att*B Primers,** page 11, for guidelines.
- If you cannot screen large numbers of clones (such as in transfection experiments), you want to generate mutations at a high frequency. Perform a mutagenic PCR as described on page 22. Following this protocol will result in a PCR product containing a mutation in roughly 1 out of every 60 bp.

Note: If you are going to perform a **Standard PCR with *att*B primers**, page 11, you may use the purified PCR product generated to create the entry clone for kanamycin titration, if the same polymerase and method is used.

Mutagenic PCR for Allele Library Generation

Introduction

Use the procedure described in this section to produce PCR products containing a high degree of mutations. See **Desired Mutation Frequency**, page 21 for explanation.

Note: The protocol described in this section will result in a mutation in roughly 1 out of every 60 bp. Refer to Molecular Biology handbooks, such as *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), for more background information on error-prone PCR.

PCR Primers

We recommend using the following PCR primers:

Primer Name	Primer Sequence
*att*B1-5′	5′- ACA AGT TTG TAC AAA AAA GCA G –3′
*att*B2-3′	5′- AC CAC TTT GTA CAA GAA AGC T –3′

Note: Primers specific to the vector backbone may be used as long as the *att*B1 and *att*B2 sites are included in the PCR product.

Important

Do not use gene specific primers flanked with *att*B sites for kanamycin titration or allele library generation. These primer are large (~60bp) and are more prone to contain deletions and insertions in the *att*B sites. PCR products with such deletions (in the *att*B1 site) have been observed to slip through the kanamycin selection process (i.e. they grow on kanamycin even though the fusion is out-of-frame), presumably through ribosome slippage. Therefore, for the amplication of the ORF, only use *att*B primers, or primers specific to the backbone.

Primer Concentration

- 50 nmol of standard purity, desalted oligonucleotides are sufficient for most applications.
- Dissolve oligonucleotides to 20-50 mM in water or TE Buffer and verify the concentration before use.

Recommended Polymerases

We recommend using Platinum® *Taq* DNA Polymerase (Catalog no. 10966-018). Other *Taq* Polymerases may also be used. **Do not** use DNA polymerases with lowered error frequency, such high-fidelity DNA polymerases, polymerases with proofreading capability or *pfx* polymerases, as these lower the mutation frequency.

continued on next page

Mutagenic PCR for Allele Library Generation, continued

Producing PCR Products

1. Set up reaction tubes/plates on ice.
2. Add the following components to each reaction vessel (if desired, a master mix can be prepared for multiple reactions, to minimize reagent loss and to enable accurate pipetting):

Components	Volume	Final Concentration
10X PCR Buffer, Minus Mg	5 µl	1X
100 mM dCTP	1 µl	2 mM
100 mM dGTP	1 µl	2 mM
100 mM dTTP	1 µl	2 mM
10 mM dATP	1 µl	0.2 mM
50 mM $MgCl_2$	15 µl	15 mM
5 mM $MnCl_2$	4 µl	0.4 mM
*att*B1-5′ primer	100 ng	2 ng/µl
*att*B1-3′ primer	100 ng	2 ng/µl
Template DNA	10 ng	0.2 ng/µl
5 U/µl Platinum® *Taq* DNA Polymerase	1 µl	5 units
Autoclaved, distilled water	to 50 µl	Not applicable

3. Mix contents of the tubes and overlay with 50 µl of mineral or silicone oil, if necessary.
4. Cap the tubes and centrifuge briefly to collect the contents.
5. Perform 30 cycles of PCR amplification as follows:

Step	Action	Time	Temperature
1	Denature	94°C	3 min.
2	Denature	94°C	45 s
3	Anneal	55°C	30 s
4	Extend	72°C	2 min.
5	Repeat 29 times steps 2-4		
6	Extend	72°C	10 min.
7	Store	4°C	

6. Maintain the reaction at 4°C after cycling. Alternatively, store the samples at -20°C until use.

Note: This protocol is a modification of one derived from the Powers lab, UC Davis, CA.

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Checking the PCR Product

Remove 5 µl from each PCR reaction and use agarose gel electrophoresis to verify the quality and yield of your PCR product. If the PCR product is clearly visible, of the right size and constitutes a distinct band, proceed to purify the PCR product.

Removing Primers and Primer-Dimers

After you have generated your *att*B-PCR product, purify the PCR product to remove *att*B primers and any *att*B primer-dimers. Primers and primer-dimers can recombine efficiently with the donor vector in the BP reaction and may increase background after transformation into *E. coli*.

Note

If your PCR template is a plasmid that contains the kanamycin resistance gene, we suggest treating your PCR reaction mixture with *Dpn* I before purifying the *att*B-PCR product. This treatment degrades the plasmid (*i.e. Dpn* I recognizes methylated GATC sites) and helps to reduce background in the BP recombination reaction associated with template contamination.

Protocol:

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer (Invitrogen, Catalog no. 16304-016) and ≥5 units of *Dpn* I. (Invitrogen, Catalog no. 15242-019)
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purifying *att*B-PCR Products**, below

Purifying *att*B-PCR Products

We recommend using the S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) to purify your *att*B PCR products. Other gel purification kits may also be suitable. Follow the manufacturer's instructions.

Alternatively, you may purify your PCR product without gel electrophoresis. Use the PureLink™ PCR Purification Kit (Catalog no. K3100-01) or the protocol provided in the **Appendix** (page 40; this protocol is not suitable if your PCR product is less than 300 bp).

Creating the Allele Library Using the BP Recombination Reaction

Introduction

Once you have purified your *att*B-flanked PCR product, you are ready to transfer the ORFs containing the mutations into pDONR™-Express to create your pENTR™-Express entry clones. Perform a BP recombination reaction as described in **BP Recombination Reaction**, page 13.

Substrates for the BP Recombination Reaction

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

- *att*B-flanked PCR products containing the mutations
- *att*P-containing pDONR™-Express (see page 9)

Important

Do not use donor vectors other than pDONR™-Express for allele library generation. pDONR™-Express contains an in-frame kanamycin resistance gene that is essential for this method.

Positive Control

If desired, you can include a positive control (pEXP7-tet, see below) in your experiment. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene; determine the efficiency of the BP recombination reaction by plating onto LB plates with 20 µg/ml tetracycline.

Performing the BP Recombination Reaction

Follow the protocol described in **BP Recombination Reaction** (page 13) to perform the BP recombination reaction with your *att*B PCR product and pDONR™-Express. When you have completed step 6, page 15, proceed to **Transforming Electrocomp Cells with Allele Library** using the guidelines in the next section, pages 26-28.

Selecting for Full-length Protein

Transforming Electrocomp Cells with Allele Library

Introduction

Once you have determined the appropriate kanamycin concentration to use for selection (see **Kanamycin Titration**, page 8), you may proceed to:

- Transform One Shot® TOP10 Electrocomp *E. coli* cells with the allele library recombination reaction.
- Determine titer of the transformation reaction.
- Select for the proper number of clones containing full-length fusion protein by kanamycin selection.

One Shot® TOP10 Electrocomp *E. coli* cells

The SureFrame™ Allele Library Construction Kit includes One Shot® TOP10 Electrocomp *E. coli* cells. For transformation, use two 50 µl vials of competent cells for every allele library transfer; pool two separate transformation reactions and use 80 µl with 1 µl of BP recombination reaction; the remaining 20 µl can be used for controls if desired. Dispose of any unused thawed cells.

The One Shot® TOP10 Electrocomp *E. coli* kit includes the pUC19 plasmid, which you can use to test the efficiency of the competent cells, if desired. Transform 1 µl (10 pg) of pUC19 into 20 µl of competent cells; plate on LB agar plates containing 100 µg/ml ampicillin.

Materials Needed

You will need to provide the following items for transformation:

- 37°C shaking and **30°C** non-shaking incubator
- Three 10 cm diameter LB agar plates with 1 mM IPTG and the appropriate concentration of kanamycin determined in the titration experiment (see **Kanamycin Titration**, page 8). Kanamycin and IPTG are provided with the kit
- LB medium; sterile glycerol
- Ice bucket with ice
- Electroporator
- 0.1 cm cuvettes
- 15 ml snap-cap tubes (one for each transformation)
- Sterile 1.5 ml microcentrifuge tubes
- Two vials of One Shot® TOP10 Electrocomp *E. coli* cells
- One vial of S.O.B. Medium (supplied with the kit)
- If performing positive transformation control: pUC19 and one vial of S.O.C Medium (supplied with the kit); LB agar plates containing 100 µg/ml ampicillin

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Transforming Electrocomp Cells with Allele Library,
continued

Preparation

- Thaw one vial of S.O.B medium and bring to room temperature. **Do not** use the S.O.C. medium for the allele library transformation, as it interferes with the kanamycin selection. S.O.C. medium is used for the transformation control. Thaw pUC19 and a vial of S.O.C. medium if performing the transformation control.
- Thaw IPTG and the allele library BP recombination reaction if frozen (from page 25).
- Warm selective plates at 37°C for 30 minutes.
- Place cuvettes on ice.
- Thaw **on ice** 2 vials of One Shot® TOP10 Electrocomp cells.
- Thaw the pUC19 transformation control plasmid.
- Add 1 µl IPTG (1 M) to one vial containing 1 ml S.O.B. medium (final concentration 1 mM IPTG)

Electroporation Procedure

Important: To avoid arcing, use **only** One Shot® TOP10 Electrocomp cells for electroporation (supplied with the kit). Do not use chemically competent cells.

1. Set up your electroporator for bacterial transformation. Follow the manufacturer's instructions. Generally, the settings are: 1700 V; 200Ω; 25µF.
2. Add 1 µl of the BP recombination reaction to 80 µl of cells. Add 1 µl of pUC19 transformation control to a separate vial with 20 µl of competent cells. Mix all tubes gently with pipette tip. **Do not mix by pipetting up and down.**
3. Transfer the cells to the chilled electroporation cuvette on ice.
4. Electroporate the cells as per the manufacturer's recommended protocol.
5. Quickly add 920 µl room temperature S.O.B medium + 1 mM IPTG and mix gently. Do not use S.O.C. medium for the allele library, as it interferes with the kanamycin selection.

   Note: To recover the 20 µl transformation reaction for the **pUC19 transformation control**, use 230 µl S.O.C. medium. Do not add IPTG.
6. Transfer the solution to a 15 ml snap-cap tube.
7. Shake tubes for at least 1 hour at 37 °C to allow expression of the antibiotic resistance genes.
8. Add sterile glycerol to a final concentration of 20%, mix well.
9. Remove 50 µl for serial dilution; store the rest of the glycerol stock at -80°C for plating on kanamycin + IPTG LB plates later on.

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Transforming Electrocomp Cells with Allele Library,
continued

Titering Transformed Cells

1. Aliquot in five 1.5 ml microcentrifuge tubes 90 µl of LB medium per tube. Label the tubes 1 to 4 and "C".
2. Make 10-fold serial dilutions of the allele library transformation in tubes 1 to 4. Add 10 µl from the transformation reaction to the first tube, mix by inverting. Add 10 µl from the first tube to the second tube and mix by inverting. In the same manner, add 10 µl from the second to the third tube, and from the third to the fourth. Make sure to mix every time you make a new dilution.
3. Make a 10-fold dilution of the transformation control by adding 10 µl from the transformation reaction to the tube labeled "C", mix by inverting.
4. Spread 20 µl from dilutions 2 to 4 (allele library transformation) on a prewarmed LB plate containing the appropriate concentration of kanamycin. Spread 20 µl of 10-fold dilution "C" (transformation control) on a prewarmed LB ampicillin plate.
5. Incubate the plates 24-36 hrs at **30°C** (which results in better expression of the fusion proteins).
6. Count colonies on the control plate. Count colonies on the serial dilution plate that contains 50-200 colonies. Count at least one plate for every allele library transformation.

Calculating Transformation Efficiency

Calculate the transformation efficiency for pUC19 transformation control as transformants per 1 µg of plasmid DNA.

$$\frac{\text{\# of colonies}}{\text{10 pg transformed pUC19}} \times \frac{10^6 \text{ pg}}{\text{µg}} \times \frac{\text{940 µl transformation volume}}{\text{20 µl plated}} \times \frac{10}{\text{(fold dilution)}} = \frac{\text{\# transformants}}{\text{µg plasmid DNA}}$$

The expected transformation efficiency should be $\geq 1 \times 10^9$ transformants/µg pUC19

Calculating Titer

Calculate the titer for every allele library transformation as transformants per ml.

$$\frac{\text{\# of colonies counted}}{\text{20 µl plated}} \times \frac{10^3 \text{ µl}}{\text{ml}} \times \frac{\text{Dilution}}{\text{of counted plate}} = \frac{\text{\# transformants}}{\text{ml transformation reaction}}$$

As a reminder, the fold dilution in the serial dilution is as indicated below:

Tube	1	2	3	4
Fold Dilution	10	10^2	10^3	10^4

Isolating Allele Library DNA

Introduction

Once you have transformed One Shot® TOP10 Electrocomp *E. coli* cells with the allele library and determined the titer, you can plate out the required number of colonies of the allele library, and isolate DNA. This allele library DNA can then be used in an LR recombination reaction with a suitable destination vector to generate an expression allele library.

Library Representation

The generation of an allele library requires a minimum number of clones to be isolated for suitable library representation. This target number of clones/colonies will depend on the size of the ORF under study, with larger ORFs requiring a higher target number of clones. Errors generated by *Taq* polymerase are reported to occur in a biased manner (i.e. not all types of nucleotide changes occur at equal frequencies). As a result, the number of mutations per DNA sequence generated during PCR are not expected to follow the Poisson distribution (Matsumura & Ellington). In a effort to create general guidelines, we reason that for a 1kb ORF, which possesses ~333 codons, approximately 1,000-2,000 x 333, (or 333,000 to 666,000) clones should be sufficient to generate suitable library representation.

Kanamycin Plates Required

1. Based on the size of your ORF, determine the amount of clones required for suitable library representation as explained above.
2. Divide this number by 20,000-30,000, as you will aim to plate 20,000 to 30,000 clones on a single plate.

This is the number of 10 cm diameter LB agar plates containing the appropriate concentration of kanamycin you need to plate with the transformation reaction for plasmid isolation. Add three more plates to the experiment, these will be used to verify to make sure the titer of the glycerol stock. **Do not** forget to add 1 mM IPTG to each plate.

Materials Supplied by the User

You will need the following items:

- 37°C non-shaking incubator
- 10 cm diameter LB agar plates with 1 mM IPTG and the appropriate concentration kanamycin (determined in **Kanamycin Titration**, page 10)
- Ice bucket with ice
- LB medium
- 15 ml polypropylene tubes
- PureLink™ HiPure Plasmid Midiprep Kit, or other suitable kit or protocol to purify plasmid DNA

Continued on next page

Isolating Allele Library DNA, continued

Library DNA Isolation

1. Thaw the glycerol stock of the allele library transformation on ice (step 9, page 27).
2. Plate 20,000 to 30,000 colonies per selective plate, as calculated from the titer determined in the previous section (page 28).
3. Inoculate as many plates as needed to reach the target number of colonies needed, as determined above.
4. To make sure the glycerol stock has not lost viability, re-titer the stock:
   a. Aliquot 90 µl of LB medium each in four 1.5 ml microcentrifuge tubes. Label the tubes 1 to 4.
   b. Make 10-fold serial dilutions of the glycerol stock in tubes 1 to 4. Add 10 µl from the transformation reaction to the first tube, mix by inverting. Add 10 µl from the first tube to the second tube and mix by inverting. In the same manner, add 10 µl from the second to the third tube, and from the third to the fourth. Make sure to mix every time you make a new dilution.
   c. Spread 20 µl from dilutions 2 to 4 (allele library transformation) on a prewarmed LB plate containing the appropriate concentration of kanamycin.
5. Incubate all plates at **30°C** for 24-36 hours.
6. If the titer of the glycerol stock has not decreased significantly (i.e. there are still an estimated 20,000 – 30,000 clones per plate), proceed. Otherwise store the plates at 4°C and plate more cells accordingly or re-transform the recombination reaction.
7. Add 1 ml LB to each plate; scrape colonies using a sterile spreader and collect the suspension of 5 plates in a 15 ml polypropylene tube.
8. Spin down cells and perform a plasmid isolation using the PureLink™ HiPure Plasmid Midiprep Kit or equivalent according to the instructions provided with the kit. Use one midiprep reaction per five plates with a total of 100,000 to 150,000 colonies. If performing more than one Midiprep reaction, pool DNA when finished.

Generating Allele Library Expression Clones

Introduction

This section explains how to perform the LR recombination reaction to transfer your full-length allele library to a suitable destination vector to form Allele Library Expression Clones.

Substrates for the LR Recombination Reaction

In most cases, we recommend performing the LR recombination reaction using a:

- **Supercoiled** pENTR™-Express allele library (contains *att*L1 and *att*L2)
- **Supercoiled** destination vector (contains *att*R1 and *att*R2)

Note

Although the Gateway® Technology manual has previously recommended using a linearized destination vector and entry clone for more efficient LR recombination, further testing at Invitrogen has found that linearization of destination vectors and entry clones is generally **NOT** required to obtain optimal results for any downstream application.

LR Clonase™ II Enzyme Mix

The LR Clonase™ II enzyme mix is supplied with the SureFrame™ Allele Library Construction Kit 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. Use the protocol provided on the next page 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, but follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided on the next page.

Destination Vector

The SureFrame™ Allele Library Construction Kit does not contain a destination vector. If you are generating an allele library for use in the ProQuest™ Reverse Two-Hybrid System, use the destination vector pDEST™22 provided with the ProQuest™ Reverse Two-Hybrid System.

If you do not have a destination vector, visit the Gateway® portal at http://www.invitrogen.com/Gateway, or contact Technical Service (page 47) to choose a destination vector suitable for your downstream application.

Continued on next page

Generating Allele Library Expression Clones, continued

Materials Needed

You should have the following materials on hand before beginning:

Purified plasmid DNA of the allele library (50-250 ng/µl in TE, pH 8.0)

Destination Vector (150 ng/µl in TE, pH 8.0)

LR Clonase™ II enzyme mix (keep at -20°C until immediately before use)

TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)

Setting Up the LR Recombination Reaction

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

   Note: To include a negative control, set up a second sample reaction and omit the LR Clonase™ II enzyme mix (see Step 4).

	Amount	Sample	Negative Control
pENTR™-Express	250 ng	1.0-4.7 µl	1.0-4.7 µl
Destination vector (150 ng/µl)	500 ng	3.3 µl	3.3 µl
TE Buffer, pH 8.0		to 8 µl	to 10 µl

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

   Reminder: Return LR Clonase™ II enzyme mix to -20°C immediately after use.
5. Incubate reactions at 25°C for **20 hours**. This is longer than a standard LR recombination reaction to increase the recombination efficiency of the library.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform One Shot® TOP10 Electrocomp *E. coli* cells, next page.

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

Continued on next page

Generating Allele Library Expression Clones, continued

Transforming Competent Cells with Expression Clones

Transform One Shot® TOP10 Electrocomp *E. coli* cells with the allele library LR recombination reaction as described in **Transforming Electrocomp Cells with Allele Library**, page 26). Make sure to pay attention to the following points:

- Use the LR Recombination Reaction from the previous section (page 32).
- Use two 50 µl vials of One Shot® TOP10 Electrocomp *E. coli* cells for every allele library transfer; pool two separate transformation reactions and use 80 µl with 1 µl of transformation reaction; the remaining 20 µl can be used for controls.
- Prepare LB agar plates containing the proper antibiotic for your destination vector; for destination vector pDEST™22 provided with the ProQuest™ Reverse Two-Hybrid System, use 100 µg/ml ampicillin for selection.
- Use S.O.C. medium instead of S.O.B. medium for recovery after electroporation.
- Incubate plates at 37°C for 20-24 hours.
- Make sure you determine the titer for the transformation with the allele library LR recombination reaction as described

Note: Use of Electrocomp cells is required to get a significant number of transformants necessary for a suitable library representation.

Isolating DNA of Expression Clones

Isolate DNA of the allele library expression clones as described in **Isolating Allele Library DNA,** page 29. Make sure to pay attention to the following points:

- Use the glycerol stock generated above (**Transforming Competent Cells with Expression Clones**)
- Prepare LB agar plates containing the proper antibiotic for your destination vector; for destination vector pDEST™22 provided with the ProQuest™ Reverse Two-Hybrid System, use 100 µg/ml ampicillin.
- Aim for the same target number of colonies as for the allele library generation (see **Library Representation**, page 29).
- Incubate plates at 37°C for 20-24 hours.
- Scrape and isolate DNA as described before (page 29).

What to Do Next

Once you have obtained allele library DNA from the expression clones, you are ready to perform your downstream experiments. Some options for downstream applications are listed below:

- Reverse two-hybrid experiments to select for interaction defective alleles. Use the ProQuest™ Reverse Two-Hybrid System; see the ProQuest™ Two-Hybrid System manual for details to perform the screen.
- Experiments to screen for functionally deficient proteins
- *In vitro* interaction experiments

Troubleshooting

Introduction

Use the information in this section to troubleshoot allele library generation procedures.

Generation of PCR Products

Below problems for the generation of PCR products are identified and potential solution are given.

Problem	Reason	Solution
No PCR product	PCR suboptimal	Search for "pcr optimization" at our website (www.invitrogen.com) and read the tips, or refer to Molecular Biology handbooks, such as *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).
Low yield of *att*B-PCR product obtained after purification	*att*B-PCR product not diluted with TE	Dilute with 150 µl of 1X TE, pH 8.0 before adding the PEG/$MgCl_2$ solution.
	Centrifugation step too short or centrifugation speed too low	Increase time and speed of the centrifugation step to 30 minutes and 15,000 x g.
	Lost PEG pellet	When removing the tube from the microcentrifuge, keep track of the orientation of the outer edge of the tube where the pellet is located. When removing the supernatant from the tube, take care not to disturb the pellet.

Kanamycin Titration

Below problems for the kanamycin titration are identified and potential solution are given.

Problem	Reason	Solution
Few or no colonies obtained from the transformation control	Check electroporation settings	Generally, the electroporation settings are: 1700 V; 200Ω; 25µF.
	Was appropriate amount of DNA transformed	Transform 10 ng DNA
	Competent cells stored incorrectly	Store competent cells at -80°C.
	Transformation performed incorrectly	If you are using One Shot® TOP10 Electrocomp *E. coli* follow the protocol on page 26 to transform cells.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.

Continued on next page

Troubleshooting, continued

Kanamycin Titration, continued

Continued from previous page

Problem	Reason	Solution
Few or no colonies obtained from sample BP reaction on spectinomycin plates and the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 100 µg/ml spectinomycin
	Did not use the suggested BP Clonase™ II enzyme mix or BP Clonase™ II enzyme mix was inactive	Make sure to store the BP Clonase™ II enzyme mix at -20°C or -80°C. Do not freeze/thaw the BP Clonase™ II enzyme mix more than 10 times. Use the recommended amount of BP Clonase™ II enzyme mix (see page 25). Test another aliquot of the BP Clonase™ II enzyme mix.
	Not enough BP reaction transformed	Increase the amount of BP reaction transformed into One Shot® TOP10 Electrocomp *E. coli.*
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add the appropriate medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating.
	*att*B PCR primers contaminated with incomplete sequences	Use HPLC or PAGE-purified oligonucleotides to generate your *att*B-PCR product.
	*att*B-PCR product not purified sufficiently	Gel purify your *att*B-PCR product to remove *att*B primers and *att*B primer-dimers.
Few or no colonies obtained from sample BP reaction on kanamycin plates and colonies on spectinomycin plates	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing your determined kanamycin concentration
	Did you use One Shot® TOP10 Electrocomp *E. coli* cells	Use One Shot® TOP10 Electrocomp *E. coli* cells, supplied with the kit.
	Improper fusion protein expression	No IPTG added during recovery or to plates Incubation at 37°C instead of 30°C
	Upstream ORF is not compatible with downstream Kan^R expression	Use fragments of ORF
Too many colonies on the uninduced (–IPTG) plates, even at the highest kanamycin concentration	Switched plates, antibiotic	Make sure you use the right plates.
	IPTG contamination in media, plates	Make new plates.
	Cryptic promoter activity in the insert	Delete parts of the insert to pinpoint the cryptic promoter; use constructs without the cryptic promoter or mutate the insert to silence the cryptic promoter.

Continued on next page

Troubleshooting, continued

Allele Library Generation/ DNA Isolation

Below problems for the allele library generation / DNA isolation are identified and potential solution are given.

Problem	Reason	Solution
Fewer colonies than expected when selecting for full-length protein	Switched plates, antibiotic	Make sure you use the right plates.
	Titer calculated incorrectly	Recalculate the titer.
	Glycerol stock old	Retiter glycerol stock.
Poor DNA yield	Used too much cell mass per midiprep	Should only use cell mass from 5 plates per midiprep

Expression Library Generation/ DNA Isolation

Below problems for the expression library generation/ DNA isolation are identified and potential solution are given.

Problem	Reason	Solution
Low titer for expression library	LR recombination reaction set up incorrectly	Use the recommended amount of reagents (see page 32).
	Did not use the suggested LRClonase™ II enzyme mix or LR Clonase™ II enzyme mix was inactive	Make sure to store the LR Clonase™ II enzyme mix at -20°C or -80°C. Do not freeze/thaw the LR Clonase™ II enzyme mix more than 10 times. Use the recommended amount of LR Clonase™ II enzyme mix (see page 32). Test another aliquot of the LR Clonase™ II enzyme mix.
	Incubation time incorrect	Incubate LR recombination reaction for 20 hours.
	Not enough LR reaction transformed	Increase the amount of LR reaction transformed into One Shot® TOP10 Electrocomp *E. coli.*
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing the antibiotic for your destination vector.
Fewer colonies than expected after plating out for DNA isolation	Switched plates, antibiotic	Make sure you use the right plates.
	Titer calculated incorrectly	Recalculate the titer.
	Glycerol stock old	Retiter glycerol stock.
Poor DNA yield	Used too much cell mass per midiprep	Should only use cell mass from 5 plates per Midiprep.

Appendix

Gateway® Recombination Reactions

Introduction

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

Review the information in this section to briefly familiarize yourself with the Gateway® recombination reactions. For details, refer to the Gateway® Technology with Clonase™ II manual available from our web site at www.invitrogen.com or by contacting Technical Service (see page 47).

Recombination Reactions

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

BP Reaction

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

LR Reaction

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

Characteristics of Modified *att* Sites

The wild-type λ *att* recombination sites have been modified In the Gateway® System, thereby ensuring specificity of the recombination reactions to maintain orientation and reading frame. The modified *att* sites have the following characteristics:

Site	Length	Found in…
*att*B	25 bp	Expression vector Expression clone
*att*P	200 bp	Donor vector
*att*L	100 bp	Entry vector Entry clone
*att*R	125 bp	Destination vector

Continued on next page

Gateway® Recombination Reactions, Continued

Specificity of Modified *att* Sites

The modified *att* sites have the following specificity.

*att*B1 sites react only with *att*P1 sites

*att*B2 sites react only with *att*P2 sites

*att*L1 sites react only with *att*R1 sites

*att*L2 sites react only with *att*R2 sites

Vectors in ProQuest™

Each of the vectors supplied in the SureFrame™ Allele Library Construction Kit and ProQuest™ Reverse Two-Hybrid System is Gateway®-adapted, *i.e.* contains the appropriate *att* sites that allow site specific recombination to facilitate the transfer of heterologous DNA sequences between vectors.

Gateway® Vectors

There are four different types of Gateway®-adapted vectors available from Invitrogen to generate your desired entry and expression clones. Indicated also are the types of cloning vectors present in the SureFrame™ Allele Library Construction Kit and ProQuest™ Reverse Two-Hybrid System.

Gateway® Vector	Vector Characteristics	Cloning Vectors
Donor vector	Contains *att*P sites Used to clone *att*B-flanked PCR products and genes of interest to generate entry clones	pDONR™-Express
Entry vector	Contains *att*L sites Used to clone PCR products or restriction fragments that do not contain *att* sites to generate entry clones	--
Destination vector	Contains *att*R sites Recombines with the entry clone in an LR reaction to generate an expression clone Contains elements necessary to express the gene of interest in the appropriate system (*i.e. E. coli*, mammalian, yeast, insect)	pDEST™22[1] pDEST™32[1]
Expression vector	Contains *att*B sites Used to clone PCR products or restriction fragments that do not contain *att* sites to generate expression clones Contains elements necessary to express the gene of interest in the appropriate system (*i.e. E. coli*, mammalian, yeast, insect)	pEXP™-AD502[1]

[1]only in the ProQuest™ Reverse Two-Hybrid System

Continued on next page

Selection of Gateway® Vectors

To enable recombinational cloning and efficient selection of entry or expression clones, most Gateway® vectors contain two *att* sites flanking a cassette containing:

- The *ccd*B gene (see below) for negative selection (present in donor, destination, and supercoiled entry vectors)
- Chloramphenicol resistance gene (Cm^R) for counterselection (present in donor and destination vectors)

After a BP or LR recombination reaction, this cassette is replaced by the gene of interest to generate the entry clone and expression clone, respectively.

*ccd*B Gene

The presence of the *ccd*B gene allows negative selection of the donor and destination (and some entry) vectors in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard & Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* OmniMAX™ 2-$T1^R$, DH5α™, TOP10). When recombination occurs (*i.e.* between a destination vector and an entry clone or between a donor vector and an *att*B-PCR product), the *ccd*B gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Purifying Mutated *att*B-PCR Products

Introduction

After you have generated your *att*B-PCR product, we recommend purifying the PCR product to remove *att*B primers and any *att*B primer-dimers. Primers and primer-dimers can recombine efficiently with the donor vector in the BP reaction and may increase background after transformation into *E. coli*. A protocol is provided below to purify your PCR product.

Important

Standard PCR product purification protocols using phenol/chloroform extraction followed by sodium acetate and ethanol or isopropanol precipitation are not recommended for use in purifying *att*B-PCR products. These protocols generally have exclusion limits of less than 100 bp and do not efficiently remove large primer-dimer products.

Note

If your PCR template is a plasmid that contains the kanamycin resistance gene, we suggest treating your PCR reaction mixture with *Dpn* I before purifying the *att*B-PCR product. This treatment degrades the plasmid (*i.e. Dpn* I recognizes methylated GATC sites) and helps to reduce background in the BP recombination reaction associated with template contamination.

Materials Needed:

- 10X REact® 4 Buffer (Invitrogen, Catalog no. 16304-016)
- *Dpn* I (Invitrogen, Catalog no. 15242-019)

Protocol:

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer and ≥5 units of *Dpn* I.
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purifying *att*B-PCR Products**, next page.

Materials Needed

You should have the following materials on hand before beginning:

*att*B-PCR product (in a 50 µl volume)

TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

30% PEG 8000/30 mM $MgCl_2$ Solution

Agarose gel of the appropriate percentage to resolve your *att*B-PCR product

continued on next page

Purifying Mutated *att*B-PCR Products, continued

PEG Purification Protocol

Use the protocol below to purify *att*B-PCR products. Note that this procedure removes DNA less than 300 bp in size.

1. Add 150 µl of TE, pH 8.0 to a 50 µl amplification reaction containing your *att*B-PCR product.
2. Add 100 µl of 30% PEG 8000/30 mM $MgCl_2$. Vortex to mix thoroughly and centrifuge immediately at 10,000 x g for 15 minutes at room temperature.

   Note: In most cases, centrifugation at 10,000 x g for 15 minutes results in efficient recovery of PCR products. To increase the amount of PCR product recovered, the centrifugation time may be extended or the speed of centrifugation increased.
3. Carefully remove the supernatant. The pellet will be clear and nearly invisible.
4. Dissolve the pellet in 25 µl of TE, pH 8.0 (to concentration > 20 ng/µl).
5. Check the quality of the recovered *att*B-PCR product on an agarose gel. Estimate the quantity and concentration of DNA in your sample compared to the marker.
6. If the PCR product is suitably purified, proceed to **Creating Allele Library Using the BP Recombination Reaction**, page 25. If the PCR product is not suitably purified (*e.g. att*B primer-dimers are still detectable), see below.

Additional Purification

If you use the procedure above and your *att*B-PCR product is not suitably purified, or if your PCR product is less than 300 bp, you may gel purify your *att*B-PCR product. We recommend using the S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25).

Recipes

LB (Luria-Bertani) Medium and Plates

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

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

   For LB agar plates:
5. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
6. Autoclave on liquid cycle for 20 minutes at 15 psi.
7. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
8. Let harden, then invert and store at +4°C.

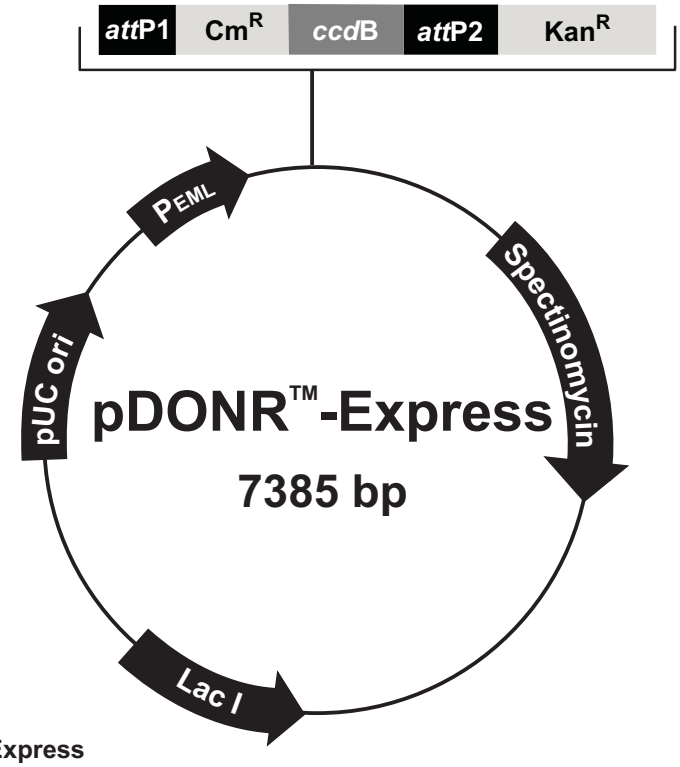
TE Buffer

10 mM Tris-HCl
1 mM EDTA
pH 7.5
Autoclave

Map of pDONR™-Express

Map of pDONR™-Express

The figure below shows the map of the pDONR™-Express vector. **The complete sequence of pDONR™-Express is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).**



Comments for pDONR™ Express
7385 nucleotides

EML promoter: bases 76-162
*att*P1 site: bases 163-391
*ccd*B gene: bases 2166-2462: bases 790-1095 (c)
Chloramphenicol resistance (Cm^R) gene: bases 1440-2099 (c)
*att*P2 site: bases 2346-2558
Kanamycin resistance gene: bases 2598-3392
Spectinomycin resistance gene: bases 3535-4545
Lac I ORF: bases 5379-6470
pUC origin: bases 6661-7334

(c) = complementary strand

Features of pDONR™-Express

Features of pDONR™-Express

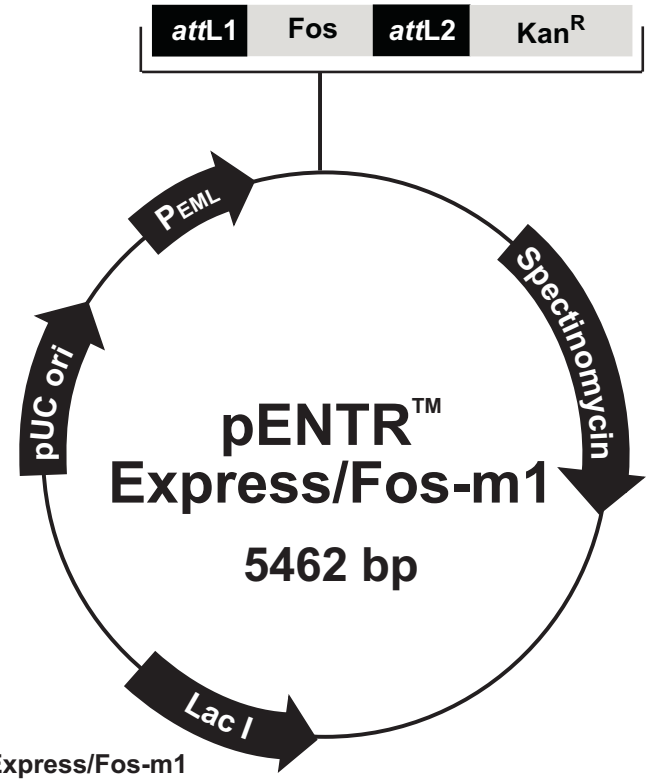
The table below shows the features of the pDONR™-Express vector.

Feature	Benefit
EML promoter	Permits high-level, IPTG inducible expression of the gene of interest fused to the kanamycin resistance gene. The EML promoter is an adapted EM7 promoter that has a lac operator engineered in it.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the expression construct with a Gateway® donor vector (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
Kanamycin resistance gene	Permits selection for the plasmid in *E. coli* if expressed in one contiguous frame (without stops) with the insert cloned in between the *att* sites.
Spectinomycin resistance gene	Permits selection for the plasmid in *E. coli*
lacI ORF	Encodes lac repressor which binds to the EML promoter to block basal transcription of the gene of interest
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

Map of pENTR™-Express/Fos-m1

pENTR™-Express/Fos-m1

The figure below shows the map of the pENTR™-Express/Fos-m1 clone. **The complete sequence of pENTR™-Express/Fos-m1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47)**



Comments for pENTR™ Express/Fos-m1
5462 nucleotides

EML promoter: bases 76-162
*att*L1 site: bases 163-391
Fos gene:282-530
*att*L2 site: bases 556-655
Kanamycin/Neomycin resistance gene: bases 678-1469
Spectinomycin resistance gene: bases 1612-2622
Lac I ORF: bases 3456-4547 (c)
pUC origin: bases 4738-5411

(c) = complementary strand

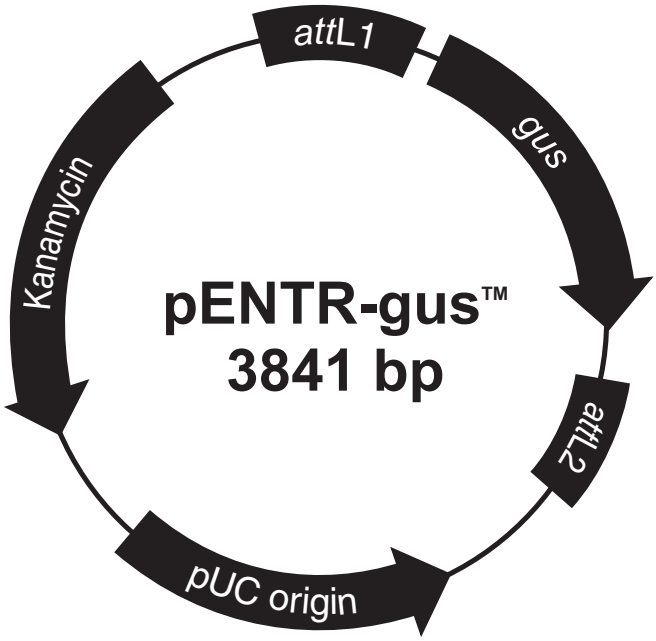
Map of pENTR™-gus

Description

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

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The complete sequence for pENTR™-gus is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).**



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

Technical Service

Web Resources

Visit the Invitrogen Web site at www.invitrogen.com for:

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

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Purchaser Notification

Introduction

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the SureFrame™ Allele Library Construction Kit.

Vectors

The structure of each vector is verified by restriction enzyme digestion; the purity assessed by measuring the OD_{260}/OD_{280} ratio. Additional functional qualification is also performed as below.

pDONR™-Express vector

pDONR™-Express vector is qualified in BP recombination reaction using the Gateway® BP Clonase™ II. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

pENTR™-Express vector

The Fos-m1 Kan^R fusion expression is tested using IPTG, kanamycin and spectinomycin.

Gateway® Clonase™ II Enzyme Mix

Gateway® BP and LR Clonase™ II Enzyme Mixes are functionally tested in a one hour recombination reaction followed by a transformation assay.

One Shot® TOP10 Electrocomp *E. coli*

Each lot of One Shot® TOP10 Electrocomp *E. coli* competent cells is tested for transformation efficiency using the pUC19 control plasmid included in the kit and following the procedure described in this manual. Test transformations are performed on 3 to 20 vials per lot, depending on batch size. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and incubated overnight. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA. In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and the absence of phage contamination.

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