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## Contents and Storage

Component	Part no.	Amount	Storage
pJTI™ R4 Dest CMV-N-EmGFP pA vector	A14141	200 µL at 0.5 µg/µL in TE buffer, pH 8.0	-20°C

## Overview

The pJTI™ R4 Dest CMV-N-EmGFP pA vector (7,373 base pairs) is designed specifically to be used in a Gateway® LR recombination reaction to create a retargeting expression vector containing your gene of interest (GOI) with an N-terminal Emerald Green Fluorescent Protein (EmGFP) under the control of the CMV promoter. When co-transfected with the integrase vector (pJTI™ R4 Int vector) included in the Jump-In™ parental kits into a Jump-In™ parental cell line containing the genomic R4 site, the GOI-EmGFP fusion will be expressed and successfully integrated cells will fluoresce green.

### *Purpose of this User Guide*

This user guide provides a map and features of the pJTI™ R4 Dest CMV-N-EmGFP pA vector, a brief overview of how to propagate the vector, and information about constructing the retargeting expression vector containing your GOI-EmGFP fusion using Gateway® technology.

This manual **does not** provide detailed instructions for culturing the Jump-In™ parental cells, retargeting the Jump-In™ parental cells, or characterization and quality control of retargeted Jump-In™ cells. For more information, refer to the detailed manual for a Jump-In™ parental cell line kit manual on our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or by contacting Technical Support.

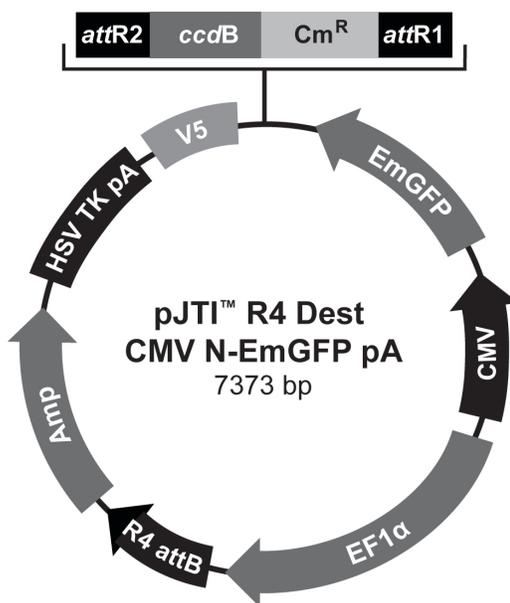
## Technical Support

For assistance, contact our technical support team at [drugdiscoverytech@lifetech.com](mailto:drugdiscoverytech@lifetech.com) or 760-603-7200 (enter 3 for “know your party’s extension”, then enter 40266).

## pJTI™ R4 Dest CMV-N-EmGFP pA Vector

### Map of pJTI™ R4 Dest CMV N-EmGFP pA

The pJTI™ R4 Dest CMV-N-EmGFP pA vector (7373 base pairs) can be used to create a Gateway® expression vector for the expression of Emerald Green Fluorescent Protein (EmGFP) fused to the N-terminus of your gene of interest after retargeting into the genomic R4 site of a Jump-In™ parental cell line. This vector contains the  $\lambda$  Integrase *attR1* and *attR2* sites for the transfer of a DNA element of interest from a Gateway® entry clone to generate the retargeting expression clone, the R4 *attB* site for site-specific integration of the DNA elements into the genome of a Jump-In™ parental cell line, and the human EF1 $\alpha$  promoter for constitutive expression of Blasticidin resistance upon successful integration. The complete sequence of the pJTI™ R4 Dest CMV-N-EmGFP pA vector is available from our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or by contacting Technical Support.



### pJTI™ R4 Dest CMV N-EmGFP pA 7373 nucleotides

R4 *attB*: bases 28–322

Ampicillin resistance gene (*Amp<sup>R</sup>*): bases 474–1331

HSV TK polyA: bases 2532–2803

V5 epitope: 2830–2871

*attR2* recombination site: bases 2879–3003

*ccdB* gene: bases 3044–3349 (c)\*

Chloramphenicol resistance gene (*Cm<sup>R</sup>*): bases 3691–4350 (c)

*attR1* recombination site: bases 4459–4583 (c)

EmGFP: bases 4599–5315 (c)

CMV promoter: bases 5439–6026 (c)

EF1 $\alpha$  promoter: bases 6144–7322

\*(c): complementary strand

## Features of pJTI™ R4 Dest CMV N-EmGFP pA

The pJTI™ R4 Dest CMV-N-EmGFP pA vector contains the following elements. All features have been functionally tested.

Feature	Benefit
CMV promoter	Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
R4 <i>attB</i> site	Allows R4 integrase-mediated integration into the complementary genomic R4 <i>attP</i> site in the parental Jump-In™ cell line
Ampicillin resistance	Allows the selection and propagation of the vector in <i>E. coli</i>
HSV TK polyA signal	The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
EF1 $\alpha$ promoter	Drives the high level expression of the Blasticidin resistance gene following the retargeting into the genomic R4 site of the parental Jump-In™ cell line
<i>attR1</i> and <i>attR2</i> sites	Two recombination sites for cloning of the gene of interest from an entry clone
<i>ccdB</i>	The <i>ccdB</i> gene located between the two <i>attR</i> sites for negative selection
Chloramphenicol resistance	Chloramphenicol resistance gene located between the two <i>attR</i> sites for counterselection
EmGFP	cDNA for Green Fluorescent Protein (Emerald) allows detection of retargeted cells by fluorescence microscopy or flow cytometry

## Propagating pJTI™ R4 Dest CMV N-EmGFP pA

To propagate and maintain the pJTI™ R4 Dest CMV N-EmGFP pA vector, we recommend using 10 ng of the vector to transform One Shot® *ccdB* Survival™ 2 T1<sup>R</sup> Competent Cells (Cat. no. A10460), an *E. coli* strain resistant to *ccdB* effects that can support the propagation of plasmids containing the *ccdB* gene. Select transformants on LB plates containing 50–100  $\mu$ g/mL ampicillin. Be sure to prepare a glycerol stock of a transformant containing plasmid for long-term storage.

**Note:** Do not use general *E. coli* cloning strains (such as TOP10 or DH5 $\alpha$ ™) for propagation and maintenance of pJTI™ R4 Dest CMV-N-EmGFP pA because these strains are sensitive to *ccdB* effects.

## Constructing the Retargeting Expression Construct

### Experiment Outline

The construction of the retargeting expression vector is a multi-step process that includes the following:

Step	Action
1	Create an entry clone by cloning your gene of interest into a Gateway® entry vector
2	Generate a retargeting expression clone by performing an LR recombination reaction between the entry clone and the pJTI™ R4 Dest CMV-N-EmGFP pA vector (i.e., the destination vector)

For generating the retargeting construct using Gateway® Technology, follow the protocol as outlined in this section. **Note that this section does not provide detailed instructions for generating entry clones, but presents guidelines and suggestions to help you obtain the best results when constructing an entry clone.** For more information on the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual (Cat. nos. 12535-029 and 12535-037) available at [www.lifetechnologies.com](http://www.lifetechnologies.com).

### Materials Required but Not Provided

#### Gateway® cloning

The table below lists ordering information for the products required to perform the Gateway® cloning reaction necessary to create a retargeting expression vector. For more information, refer to [www.lifetechnologies.com](http://www.lifetechnologies.com) or contact Technical Support.

Product	Quantity	Cat. no.
Gateway® LR Clonase® II enzyme mix*	20 reactions	11791-020
TE buffer, pH 8.0	500 mL	AM9849
One Shot® TOP10 Chemically Competent <i>E. coli</i>	20 reactions	C4040-03
Purelink® HQ Mini Plasmid DNA Purification Kit	100 preps	K2100-01

\* The Gateway® LR Clonase® II enzyme mix also includes the Proteinase K Solution (2 µg/µL) and the pENTR™-gus Positive Control vector (50 ng/µL).

#### Competent cells

The table below lists ordering information about the competent *E. coli* cells that you can use to propagate vectors. For more information, refer to [www.lifetechnologies.com](http://www.lifetechnologies.com) or contact Technical Support.

Product	Quantity	Cat. no.
One Shot® ccdB Survival™ 2 T1 <sup>R</sup> Competent Cells	10 reactions	A10460
One Shot® Mach1™ Phage-Resistant Chemically Competent <i>E. coli</i>	20 × 50 µL	C8620-03
One Shot® TOP10 Chemically Competent <i>E. coli</i>	10 × 50 µL	C4040-10

For Technical Support for this or other Drug Discovery Products, dial 760-603-7200, option 3, extension 40266.

## Gateway® entry vectors

To recombine your gene of interest into the pJTI™ R4 DEST CMV N-EmGFP pA destination vector, you will need an entry clone containing your gene of interest. An entry clone contains your gene of interest flanked by *attL* sequences, which are then used to recombine with *attR* sequences on the pJTI™ R4 DEST CMV N-EmGFP pA destination vector to create your desired expression clone.

To create entry clones containing your gene of interest, you may:

- Clone a PCR product or a restriction enzyme fragment into an entry (pENTR™) vector  
or
- Generate a PCR product containing *attB* sites and use this *attB*-PCR product in a BP recombination reaction with a donor (pDONR™) vector.

Many entry vectors, including pENTR/D-TOPO®, are available from Life Technologies to facilitate the generation of entry clones. A selection guide for choosing the most appropriate Gateway® entry vector for your application is available at [www.invitrogen.com/Gateway](http://www.invitrogen.com/Gateway). For detailed information on constructing an entry clone, refer to the manual for the specific entry vector you are using.

## Creating an Entry Clone Using the pDONR™ Vector Method

### Guidelines for creating an entry clone

- Ensure that the primers used for PCR amplification of your gene of interest (see below) are of good quality. Since these primers are generally ~45 bases in length, the possibility of mutations is greater. Mutations in the PCR primers may in turn lead to inefficient recombination with pDONR vectors.
- If possible, avoid using a plasmid containing the kanamycin resistance gene as the template for PCR, because most entry vectors contain the kanamycin resistance gene for selection in *E. coli*.
- If your gene of interest is longer than ~3 kb, incubate the BP reaction at 16°C overnight instead of 1 hour at room temperature.
- Sequence the entry clone with appropriate primers to ensure that the *att* sites do not have any mutations.

### Designing PCR primers

The design of the PCR primers to amplify your gene of interest is critical for recombinational cloning using Gateway®. Consider the following when designing your PCR primers:

- Sequences required to facilitate Gateway® cloning
- Sequence required for efficient expression of the native protein (i.e., Kozak consensus sequence), if necessary
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal fusion tag

For more detailed information on primer design, refer to section “Designing *attB* PCR Primers” in the Gateway® Technology with Clonase™ II manual (part no. 25-0749).

### Kozak consensus sequence

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation. An example of a Kozak consensus sequence is provided below. **Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus.** The ATG initiation codon is shown underlined.

(G/A)NNATGG

## Creating an Expression Clone

After you generate an entry clone, you will create your expression clone by performing the LR recombination reaction to transfer the gene of interest into the pJTI™ R4 Dest CMV-N-EmGFP pA vector. To ensure the best results, we recommend that you read this section before starting your experiments. To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *attL*-containing entry clone and the *attR*-containing pJTI™ R4 Dest CMV-N-EmGFP pA vector.
2. Transform the reaction mixture into a suitable *E. coli* host (see next page for more information on suitable *E. coli* hosts).
3. Select for expression clones on LB agar plates containing 100 µg/mL ampicillin.

### Guidelines for creating an expression clone

An LR recombination reaction occurs between two specific attachment sites (*attL* and *attR*) on the entry clone and the destination vector, allowing the recombination of fragments into the destination vector. Keep the following points in mind when generating your retargeting expression clone:

- After you have obtained an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and the pJTI™ R4 Dest CMV-N-EmGFP pA vector, and transform the reaction mixture into a suitable *E. coli* host to select for an expression clone.
- We recommend including a negative control (no LR Clonase® II) in your experiment to help you evaluate your results.
- While it may be tempting to use a “master mix” when setting up multiple LR reactions, this does not give the best results. LR clonase enzyme should always be added at the end. Add the DNA first, briefly centrifuge the tubes, and then add the enzyme to the liquid phase at the bottom.

### *E. coli* host

You may use any *recA*, *endA* *E. coli* strain including DH5α™, TOP10, or equivalent for transformation.

**Note:** Do not transform the LR reaction mixture into *E. coli* strains that contain the F' episome (e.g., TOP10F'). These strains contain the *ccdA* gene and will prevent negative selection with the *ccdB* gene.

### Positive control

The pENTR™-gus plasmid is provided with the LR Clonase® II Enzyme Mix for use as a positive control for recombination and expression. Using the pENTR™-gus entry clone in an LR recombination reaction with a destination vector (i.e., pJTI™ R4 Dest CMV-N-EmGFP pA vector) will allow you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*).

### LR Clonase® II enzyme mix

The Gateway® LR Clonase® II enzyme mix (Cat. no. 11791-020) catalyzes the LR recombination reaction. This enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase® Reaction Buffer previously supplied as separate components into an optimized single-tube format for easier setup of the LR recombination reaction. Use the protocol provided below 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 (Cat. no. 11791-019), if desired. Because reaction conditions differ, use the protocol provided with the LR Clonase® enzyme mix. Do **not** use the protocol for LR Clonase® II enzyme mix provided in this manual.

## LR Recombination Reaction

### Materials needed

- Purified plasmid DNA of your entry clone (50–150 ng/μL in TE, pH 8.0)
- pJTI™ R4 Dest CMV-N-EmGFP pA 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)
- Proteinase K Solution (2 μg/μL; supplied with LR Clonase® II enzyme mix; thaw and keep on ice until use)
- pENTR™-gus Positive Control (50 ng/μL; supplied with LR Clonase® II enzyme mix; use as a control for the LR reaction)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates containing 100 μg/mL ampicillin

### LR recombination reaction protocol

Follow this procedure to perform the LR reaction between your entry clone and the pJTI™ R4 Dest CMV-N-EmGFP pA destination vector. To include a negative control, set up a second sample reaction, but omit the LR Clonase® II enzyme mix.

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

Component	Sample	Positive control
Entry clone (50–150 ng/reaction)	1 to 7 μL	—
pJTI™ R4 Dest CMV-N-EmGFP pA vector (150 ng/μL)	1 μL	1 μL
pENTR™-gus Positive Control (50 ng/mL)	—	1 μL
TE buffer, pH 8.0	to 8 μL	to 8 μL

2. Remove the LR Clonase® II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Mix the LR Clonase® II enzyme mix by vortexing it briefly twice (2 seconds each time).
4. To each sample above, add 2 μL of LR Clonase® II enzyme mix. Mix well by pipetting up and down. Return the LR Clonase™ II enzyme mix to –20°C immediately after use.
5. Incubate the reaction mixtures at 25°C for 1 hour.

**Note:** Extending the incubation time to 18 hours typically yields more colonies.

6. Add 1 μL of the Proteinase K Solution to each reaction mixture and incubate for 10 minutes at 37°C.

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

7. Transform 1 μL of the LR recombination reaction into a suitable *E. coli* host following the manufacturer's instructions and select for expression clones.

### What you should see

If you have used *E. coli* cells with a transformation efficiency of  $\geq 1 \times 10^8$  cfu/μg, and transformed and plated the entire reaction, the LR reaction should give > 5,000 colonies. Note that the colony number can also depend on insert size and toxicity. Larger inserts often result in fewer colonies. Incubation and culture growth at 30°C may also be necessary if the insert is abnormally large or toxic to *E. coli*.

## Confirming the Expression Clone

### False positives

The *ccdB* gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL of chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

### Sequencing the expression clone

To confirm that your gene of interest is in frame with the C-terminal GFP, you may sequence your expression construct, if desired. We suggest using the following primer sequences.

For your convenience, Life Technologies offers a custom primer synthesis service. For more information, refer to our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or contact Technical Support.

Primer	Sequence
T7 promoter (forward)	5'-TAA TAC GAC TCA CTA TAG GG-3'
V5 epitope (reverse)	5'-ACC GAG GAG AGG GTT AGG GAT-3'

# Purchaser Notification

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## Gateway® Clone Distribution Policy

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

## *Gateway® Clone Distribution Policy*

### Introduction

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

### Gateway® Entry Clones

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

### Gateway® Expression Clones

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

### Additional Terms and Conditions

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

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