Jump-In™ GripTite™ HEK293 Retargeting Kit

For targeted integration of your gene of interest in Jump-In™ GripTite™ HEK293 cells

Catalog Number A14150

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Revision A.0

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Revision	Date	Description
A.0	19 June 2017	Updated the concentration of expression vectors and rebranded the user guide.
1.0	12 October 2011	Basis for the current revision.

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Contents

Product information

Contents and storage

Kit contents and storage

The Jump-In™ GripTite™ HEK293 Retargeting Kit (Cat. No. A14150) contains the following components.

Component	Amount/Composition	Storage
Jump-In™ GripTite™ HEK293 Cells	2 vials (~3 × 10^6 cells/vial) in Freezing medium*	Liquid nitrogen
pJTI™ R4 Int (integrase vector)	100 µg at 1.5 µg/µL in TE buffer, pH 8.0*	–20°C
pJTI™ R4 DEST CMV pA (destination vector)	100 µg at 1.5 µg/µL in TE buffer, pH 8.0	–20°C

*Recovery™ Cell Culture Freezing medium
**TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

CAUTION! Handle the Jump-In™ GripTite™ HEK293 cells as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. For more information on BL-1 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories,* 5th ed., published by the Centers for Disease Control, which is available for download at **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Safety Data Sheet (SDS) before handling.

Purpose of this user guide

This user guide provides an overview of the Jump-In™ GripTite™ HEK293 platform cell line, and offers instructions and guidelines for:

- Maintaining the Jump-In™ GripTite™ HEK293 culture
- Using Gateway™ technology and the pJTI R4 DEST CMV pA destination vector to generate an expression construct of your gene of interest for retargeting the Jump-In™ GripTite™ HEK293 platform cell line
- Retargeting the Jump-In™ GripTite™ HEK293 platform cell line with the retargeting expression construct, and subsequent selection and expansion of retargeted cells
- Characterization and quality control of retargeted Jump-In™ GripTite™ HEK293 cells

Not covered by this user guide

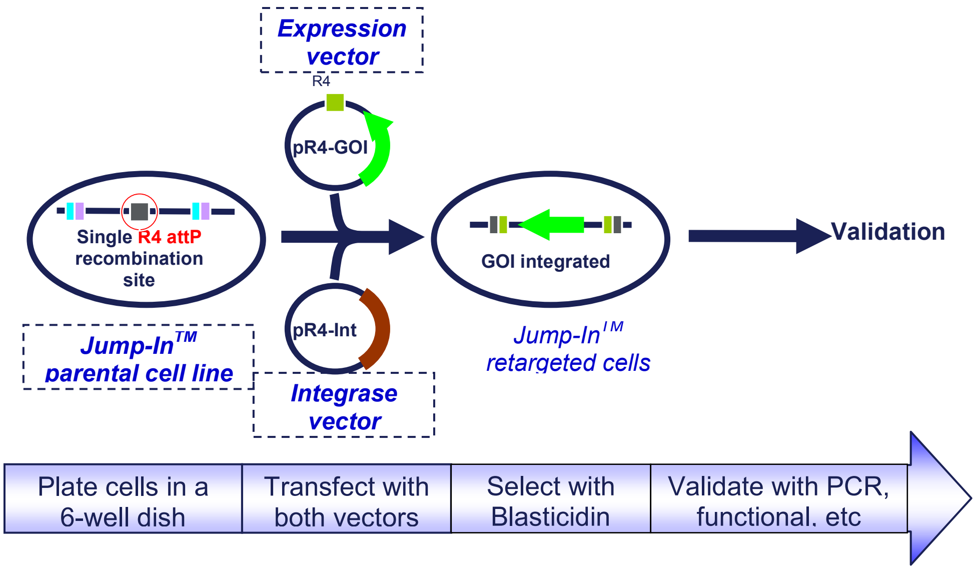
This user guide **does not** provide detailed instructions for generating entry clones, but presents guidelines and suggestions to help you obtain the best results when constructing an entry clone. For more information on the Gateway™ Technology and detailed instructions on generating an entry clone, refer to the Gateway™ Technology with Clonase™ II user guide (Cat. Nos. 12535029 and 12535037) and the user guide for the specific donor vector you are using. These user guides are available for download at **www.thermofisher.com** or by contacting Technical Support (page 44)

Description of the system

Overview of Jump-In™ technology

Jump-In™ cell engineering is a novel gene targeting technology based on R4 integrase-mediated site-specific homologous recombination. This technology allows the targeted integration of genetic material into a specific pre-engineered site on a platform cell line (such as the Jump-In™ GripTite™ HEK293 cell line) and reduces by design the effort required for the generation of stable cell lines compared to standard methods. Isogenic expression from a defined genomic locus provides the ideal solution for comparative analysis of gene families, isotypes, or orthologs.

Figure 1 Retargeting a Jump-In™ parental cell line with your gene of interest (GOI)



Jump-In™ GripTite™ HEK293 cell line

The Jump-In™ GripTite™ HEK293 cells supplied with this kit were generated by genomic integration of an R4 acceptor site and a "promoterless" Blasticidin resistance gene (BSD) using the Jump-In™ Cell Engineering Technology. Positive clones containing stable genomic integrations were selected based on their newly acquired Hygromycin resistance, picked by single-cell sorting, and then tested to determine the number of R4 sites present in the host cell genome by Southern blot and copy number analysis. Clones with a single R4 integration site were validated for retargeting by transfection with the pJTI™ R4 EXP CMV EmGFP pA (Cat. No. A14146) and JTI™ R4 Int vectors (included in this kit), followed by antibiotic selection with Blasticidin for 2 to 3 weeks. Retargeting efficiency was determined by analysis of green fluorescent protein expression using flow cytometry.

For more information on the Jump-In™ Cell Engineering Technology, refer to our website (**www.thermofisher.com)** and the published literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001).

Jump-In™ vectors included in the kit

The following Jump-In™ vectors are included in the Jump-In™ GripTite™ HEK293 Retargeting Kit. For a map and features of each vector, see pages 40–43. For instructions on how to propagate the Jump-In™ vectors, see page 43.

- pJTI™ R4 DEST CMV pA vector (i.e., the "retargeting construct" when containing the gene of interest) is designed specifically to be used in a Gateway™ LR recombination reaction to create your retargeting expression clone containing your gene of interest under the control of the CMV promoter.

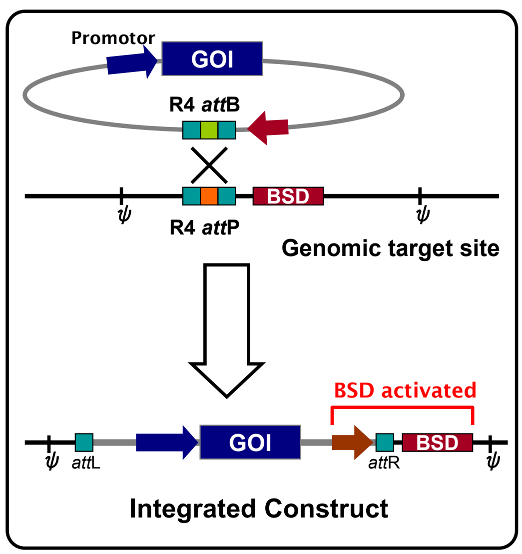
  Note: To recombine your gene of interest into the pJTI™ R4 DEST CMV pA vector, you will need an entry clone containing the gene of interest (see page 17 for more information).

- pJTI™ R4 Int vector allows the expression of the R4 Integrase, which facilitates the site-specific integration of your multiple DNA elements into the platform target site when the Jump-In™ GripTite™ HEK293 platform cell line is cotransfected with both vectors for retargeting.

Jump-In™ targeted integration

The Jump-In™ GripTite™ HEK293 platform cell line, generated using the Jump-In™ cell engineering technology, can be retargeted by cotransfection with a retargeting expression construct (generated from pJTI™ R4 DEST CMV pA using the Gateway™ cloning technology) and the pJTI™ R4 Int vector expressing the R4 Integrase (Figure 2). During retargeting, the genetic elements of interest carried by the retargeting expression construct are site-specifically integrated into the genome of the platform cell line at the R4 *att*P target site. This integration event also positions the constitutive human EF1α promoter upstream of the "promoterless" Blasticidin resistance gene, thus allowing the selection of successfully "retargeted" transformants using Blasticidin.

Figure 2 Targeted integration of the gene of interest (GOI) and a promoter for Blasticidin (BSD).



Experiment outline

Workflow

The following table describes the major steps required for retargeting the Jump-In™ GripTite™ HEK293 cell line.

Step	Action	Page
1	Thaw and expand the Jump-In™ GripTite™ HEK293 cells	13
2	Create an entry clone by cloning your gene of interest into a Gateway™ entry vector	18
3	Generate a retargeting expression construct by performing an LR recombination reaction between the entry clone and pJTI™ R4 DEST CMV pA (i.e., the destination vector)	19
4	Cotransfect your retargeting construct and the integrase vector into the Jump-In™ GripTite™ HEK293 cells	22
5	Select for retargeted Jump-In™ GripTite™ HEK293 cells in Selection medium containing Blasticidin	26
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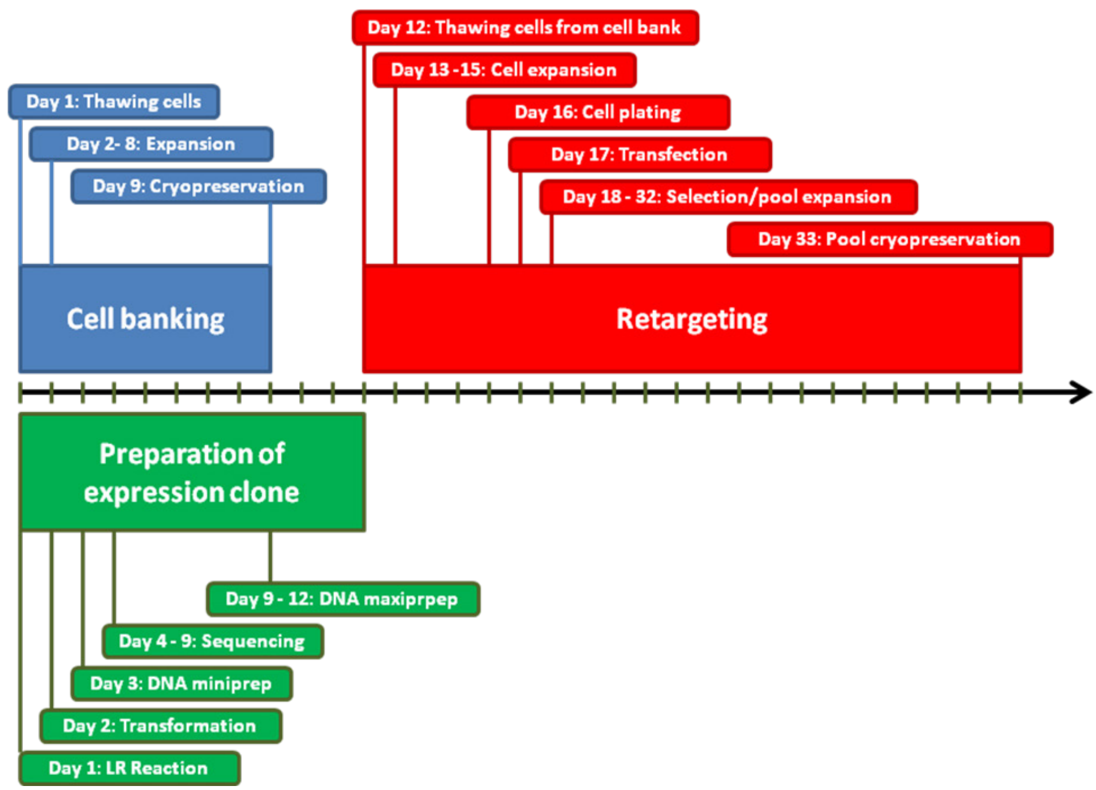
Experiment timeline

Figure 3 Experiment timeline for retargeting the Jump-In™ GripTite™ HEK293 cell line. These timeline are provided as a guideline for experimental planning; actual timelines can vary based on the experimental conditions.



Required materials not supplied

Jump-In™ GripTite™ HEK293 culture

We recommend the following accessory products for culturing, passaging, and maintaining the Jump-In™ GripTite™ HEK293 platform cell line.

Product	Amount	Cat. No.
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose, GlutaMAX™, pyruvate)	500 mL	10569010
Fetal Bovine Serum, Dialyzed (US) (FBS)	100 mL	26400036
MEM Non-Essential Amino Acids Solution 10 mM (100X), liquid (NEAA)	100 mL	11140050
HEPES Buffer Solution (1 M)	100 mL	15630080
Penicillin-Streptomycin, liquid	100 mL	15140122
Geneticin™ Selective Antibiotic, liquid	100 mL	10131027
Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	1000 mL	14190136
Trypsin, 0.05% (1X) with EDTA 4Na, liquid	100 mL	25300054
Recovery™ Cell Culture Freezing medium, liquid	50 mL	12648010
Countess™ II Automated Cell Counter	1 each	C10227
Trypan Blue Stain	100 mL	10250061

Jump-In™ vectors

The following table lists ordering information for additional Jump-In™ retargeting vectors that can be used with the Jump-In™ GripTite™ HEK293 cell line including the GFP expression vector (positive control) and the N-terminal GFP-fusion expression destination vector.

Product	Quantity	Cat. No.
pJTI R4 EXP CMV EmGFP pA	100 µg	A14146
pJTI R4 Dest CMV N-EmGFP pA	100 µg	A14141

Transfection and selection

The following table lists ordering information for the products we recommend for transfection and selection of the Jump-In™ GripTite™ HEK293 platform cell line.

Product	Quantity	Cat. No.
Opti-MEM™ I Reduced Serum Medium (1X), liquid	100 mL	31985062
Lipofectamine™ LTX and PLUS™ Reagent*	1 mL	15338100
Hygromycin B	20 mL	10687010
Blasticidin S HCl Selection Antibiotic, (liquid)	20 mL	A1113902
Ampicillin, Sodium Salt	200 mg	11593027
PrestoBlue™ Cell Viability Reagent	25 mL	A13261

Gateway™ cloning

The following table lists ordering information for the products required to perform the Gateway™ cloning reaction necessary to create a retargeting expression vector.

Product	Quantity	Cat. No.
Gateway™ LR Clonase™ II enzyme mix*	20 reactions	11791020
TE buffer, pH 8.0	500 mL	AM9849
One Shot™ TOP10 Chemically Competent *E. coli*	20 reactions	C404003
Purelink™ HQ Mini Plasmid DNA Purification Kit	100 preps	K210001
* 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 following table lists ordering information about the competent *E. coli* cells that you can use to propagate vectors.

Product	Quantity	Cat. No.
One Shot™ ccdB Survival™ 2 $T1^R$ Competent Cells	10 reactions	A10460
One Shot™ Mach1™ Phage-Resistant Chemically Competent *E. coli*	20 × 50 µL	C8620-03
One Shot™ TOP10 Chemically Competent *E. coli*	10 × 50 µL	C4040-10

PCR analysis

The following table lists ordering information about the products we recommend for PCR analysis of retargeted Jump-In™ GripTite™ HEK293 cells.

Product	Quantity	Cat. No.
CellsDirect Resuspension and Lysis Buffers*	1 kit	11739-010
AccuPrime™ *Taq* DNA Polymerase High Fidelity**	200 reactions	12346-086
DNAzol™ Reagent	100 mL	10503-027
* The product includes 10 mL resuspension buffer and 1 mL lysis buffer. ** The product includes the AccuPrime™ *Taq* DNA Polymerase High Fidelity enzyme (5 U/µL), 10X AccuPrime™ PCR Buffer I, 10X AccuPrime™ PCR Buffer II, and $MgSO_4$ (50 mM).		

Culture the Jump-In™ GripTite™ HEK293 platform cell line

Guidelines for Jump-In™ GripTite™ HEK293 cell culture

Introduction

The Jump-In™ GripTite™ HEK293 platform cell line, derived from the GripTite™ 293 MSR cell line (GripTite™ HEK293, ATCC CCL-61), is engineered to contain a single chromosomal target for the R4 Integrase. This allows for efficient and site-specific insertion of an expression cassette from a retargeting construct generated using Gateway™ cloning technology.

This section provides instructions and guidelines for thawing, propagating, and freezing the Jump-In™ GripTite™ HEK293 platform cells.

IMPORTANT! For optimal retargeting results, it is very important that you strictly follow the guidelines for culturing the Jump-In™ GripTite™ HEK293 cells provided in this user guide.

General guidelines for Jump-In™ GripTite™ HEK293 cell culture

Follow these general guidelines to grow and maintain the Jump-In™ GripTite™ HEK293 platform cell line:

- All solutions and equipment that may contact cells must be sterile. Always use proper sterile technique and work in a laminar flow hood.
- We strongly recommend generating at least 10–20 vials of frozen parental cell stocks before starting your retargeting experiment.
- For general maintenance of the cell line, pass Jump-In™ GripTite™ HEK293 cells when they are near confluence.
- When thawing or sub-culturing cells, use pre-warmed medium for cell transfer.
- Use 10 mL/L of antibiotic containing penicillin and streptomycin, if required
- After initial thawing and passage, Jump-In™ GripTite™ HEK293 cells usually double in about 24 hours.

⚠ **IMPORTANT!** The integration vector used in creating the Jump-In™ GripTite™ HEK293 platform cell line contains a hygromycin resistance gene (Hyg^R) and a Neomycin resistance gene (Neo^R), making the Jump-In™ GripTite™ HEK293 cells resistant to Hygromycin B and Geneticin™. If you want to integrate more genes stably into this cell line, do **not** use Hygromycin B or Geneticin™ for selection for those genes.

Jump-In™ GripTite™ HEK293 media

Jump-In™ GripTite™ HEK293 media compositions

The following table lists the compositions of the various media required for culturing and retargeting Jump-In™ GripTite™ HEK293 cells. Unless otherwise stated, we recommend that you equilibrate all media and solutions to 37°C for optimal performance before adding to cells. For any media substitution you make, we strongly recommend that you validate cell viability and performance first. See page 8 for ordering information

Component	Thawing medium	Growth medium	Freezing medium	Transfection medium	Selection medium
D-MEM with GlutaMAX™-I (high glucose)	90%	90%	—	90%	90%
FBS, Dialyzed*	10%	10%	—	10%	10%
MEM Non-Essential Amino Acids Solution	0.1 mM	0.1 mM	—	0.1 mM	0.1 mM
HEPES Buffer (pH 7.3)	25 mM	25 mM	—	25 mM	25 mM
Penicillin (antibiotic)**	100 U/mL	100 U/mL	—	—	100 U/mL
Streptomycin (antibiotic)**	100 µg/mL	100 µg/mL	—	—	100 µg/mL
Recovery™ Cell Culture Freezing medium	—	—	100%	—	—
Geneticin™†	—	600 µg/mL	—	—	600 µg/mL
Hygromycin B†	—	200 µg/mL	—	—	—
Blasticidin†‡	—	—	—	—	10 µg/mL

* Do **not** substitute.

** Available together as Penicillin-Streptomycin, liquid from Thermo Fisher Scientific.

† We recommend adding fresh selection antibiotics when the medium is added to the cells.

‡ Because the concentration of Blasticidin required for effective selection of retargeted cells might vary depending on the source or the lot of Blasticidin, we recommend that you establish the sensitivity of Jump-In™ GripTite™ HEK293 cells to the Blasticidin lot used for selection before conducting retargeting experiments (page 33).

Volumes required to prepare Jump-In™ GripTite™ HEK293 media

The following table provides an example for the volumes required to prepare the media as outlined in the media composition table on page 11.

Note that the volumes provided in the following table are accurate only for Thermo Fisher Scientific products listed on page 8.

Component	Thawing medium	Growth medium	Transfection medium	Selection medium
D-MEM with GlutaMAX™-I (high glucose)	500 mL	500 mL	500 mL	500 mL
FBS, Dialyzed*	50 mL	50 mL	50 mL	50 mL
MEM Non-Essential Amino Acids Solution	5 mL	5 mL	5 mL	5 mL
HEPES Buffer (pH 7.3)	5 mL	5 mL	5 mL	5 mL
Penicillin (antibiotic)**	5 mL	5 mL	—	5 mL
Streptomycin (antibiotic)**	See **	See **	—	See **
Recovery™ Cell Culture Freezing medium	—	—	—	—
Geneticin™†	—	to 600 µg/mL	—	to 600 µg/mL
Hygromycin B†	—	to 200 µg/mL	—	—
Blasticidin†‡	—	—	—	See ‡

* Do not substitute.

** Available together as Penicillin-Streptomycin, liquid from Thermo Fisher Scientific.

† We recommend adding fresh selection antibiotics when the medium is added to the cells.

‡ Because the concentration of Blasticidin required for effective selection of retargeted cells might vary depending on the source or the lot of Blasticidin, we recommend that you establish the sensitivity of Jump-In™ GripTite™ HEK293 cells to the Blasticidin lot used for selection before conducting retargeting experiments (page 33).

Thaw Jump-In™ GripTite™ HEK293 cells

Materials required

- Jump-In™ GripTite™ HEK293 cells (store frozen cells in liquid nitrogen until ready to use)
- Thawing medium (page 11), pre-warmed to 37°C
- Growth medium (page 11), pre-warmed to 37°C
- Disposable, sterile 15-mL conical tubes
- 37°C water bath
- T-75 cell culture flask or 100-mm cell culture dish

Thaw Jump-In™ GripTite™ HEK293 cells

1. Place 20 mL of Thawing medium into a T-75 flask.
2. Place the flask in a 37°C incubator with a humidified atmosphere of 5% CO_2 for 15 minutes to allow the medium to equilibrate to the proper pH and temperature.
3. Remove the vial of cells from liquid nitrogen and place it in a 37°C water bath for 1–2 minutes to thaw the cells rapidly with gentle agitation. Do **not** submerge the vial in the water.
4. Decontaminate the vial by wiping it with 70% ethanol before opening it in a Class II biological safety cabinet.
5. Transfer the vial contents slowly into 10 mL of Thawing medium in a sterile 15-mL conical tube.
6. Centrifuge the cells at 200 × *g* for 5 minutes.
7. Aspirate the supernatant and resuspend the cell pellet in 1 mL of fresh Thawing medium.
8. Transfer contents of the tube to the T-75 tissue culture flask containing pre-equilibrated Thawing medium and place the flask in the 37°C incubator with a humidified atmosphere of 5% CO_2.
9. At first passage, switch to Growth medium.

   Note: See Figure 4 for an image of the Jump-In™ GripTite™ HEK293 cells (page 15).

Propagate and maintain Jump-In™ GripTite™ HEK293 cells

Materials required

- Growth medium (page 11), pre-warmed to 37°C
- Dulbecco's Phosphate Buffered Saline (D-PBS) (1X)
- Trypsin, 0.05% (1X) with EDTA
- 37°C water bath
- 37°C incubator with a humidified atmosphere of 5% CO_2
- Disposable, sterile 15-mL conical tubes
- T-75 cell culture flask or 100-mm cell culture dish
- Hemocytometer or cell counter (e.g., Countess™ II Automated Cell Counter)
- Trypan blue

Propagate and maintain Jump-In™ GripTite™ HEK293 cells

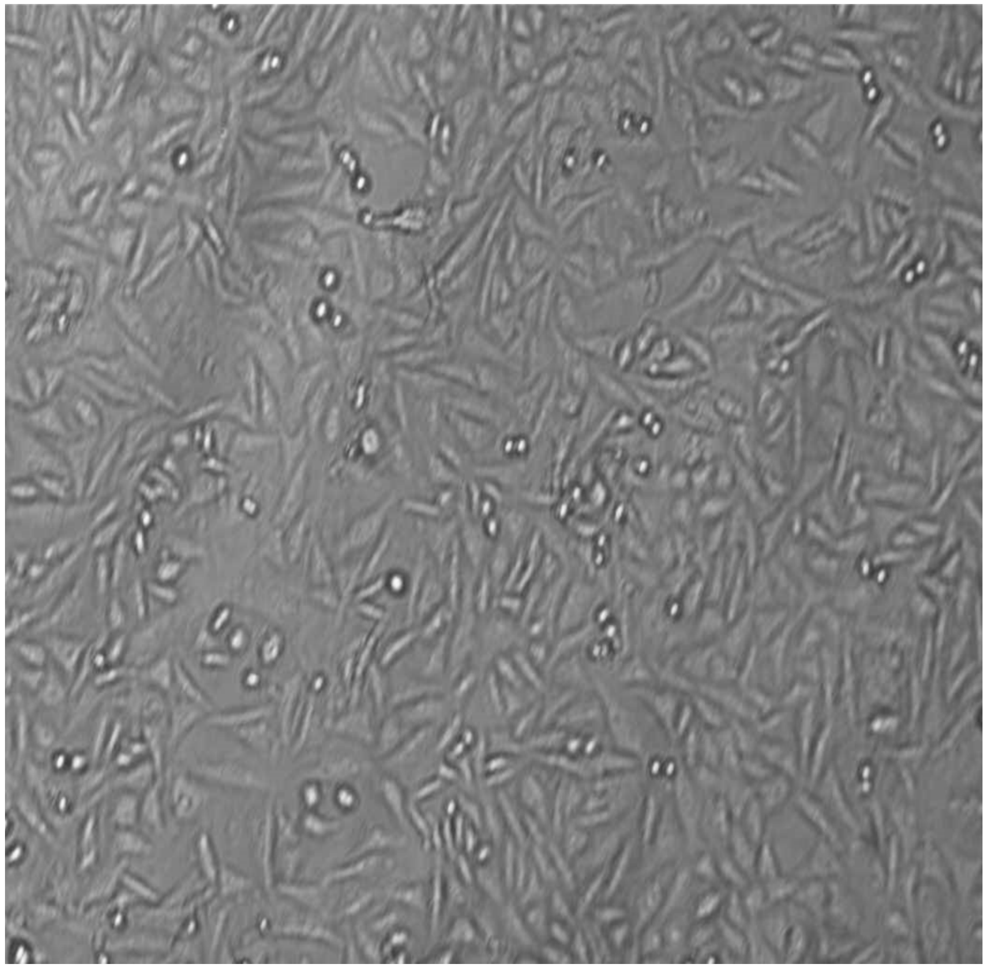
IMPORTANT! Passage or feed Jump-In™ GripTite™ HEK293 cells at least twice a week and maintain the culture between 5% and 95% confluence.Pre-warm fresh Growth medium in a culture vessel in the 37°C incubator.

1. Aspirate the medium from growing cells and rinse the cells once with PBS.
2. Add 0.05% Trypsin/EDTA to the cells (3 mL for a 100-mm dish or 5 mL for a T-75 flask), and swirl the culture vessel to coat the cells evenly. Cells usually detach after ~2–10 minutes of exposure to 0.05% Trypsin/EDTA.
3. Add an equal volume of Growth medium to inactivate the 0.05% Trypsin/EDTA.
4. Verify under a microscope that cells have detached and clumps have completely dispersed.
5. Determine the viable cell number using a hemocytometer or a cell counter. Cell number and viability can be quickly and conveniently determined using the Countess™ II Automated Cell Counter. We recommend determining cell health frequently to ensure optimal performance in retargeting experiments.
6. Centrifuge the cells at 200 × *g* for 5 minutes and resuspend then in Growth medium.
7. Seed fresh culture vessel containing pre-warmed Growth medium at the appropriate cell density. We recommend a split ratio of 1:3 to 1:10.

   IMPORTANT! Do **not** allow the cells to remain confluent for extended periods of time.

Morphology of Jump-In™ GripTite™ HEK293 cells

Figure 4 Typical morphology of proliferating Jump-In™ GripTite™ HEK293 cells.



Cryopreserve Jump-In™ GripTite™ HEK293 cells

Materials required

- Plates with Jump-In™ GripTite™ HEK293 cells
- Dulbecco's Phosphate Buffered Saline (D-PBS) (1X)
- Growth medium (page 11)
- Trypsin, 0.05% (1X) with EDTA
- Recovery™ Cell Culture Freezing medium
- Disposable, sterile 15-mL conical tubes
- Sterile freezing vials for liquid nitrogen storage

Freeze Jump-In™ GripTite™ HEK293 cells

1. Harvest the cells by trypsinization as described in "Propagate and maintain Jump-In™ GripTite™ HEK293 cells", page 14.
2. After detachment and trypsin inactivation, determine the viable cell count.
3. Centrifuge the cells at $200 \times g$ for 5 minutes, and resuspend them in Recovery™ Cell Culture Freezing medium at 4°C to a density of 3×10^6 cells/mL.
4. Dispense 1 mL aliquots of cells into cryogenic vials.
5. Place the cryogenic vials in an insulated container for slow cooling and store overnight at –80°C.
6. The next day transfer the cell to liquid nitrogen for long-term storage.

   Note: You can check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen.

IMPORTANT! Before starting experiments, expand and bank at least 10 to 20 vials of frozen Jump-In™ GripTite™ HEK293 cells in Recovery™ Cell Culture Freezing medium.

Construct the retargeting expression vector

Before starting

Experiment overview

After you have expanded the Jump-In™ GripTite™ HEK293 platform cell line and prepared frozen master stocks, you need to generate the retargeting expression vector containing your gene of interest. The construction of the retargeting expression vector is a multi-step process that requires:

1. the generation of a Gateway™ entry clone containing your gene of interest (page 18)
2. LR recombination reaction between the entry clone and the pJTI™ R4 DEST CMV pA destination vector (page 19)

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 user guide (Cat. Nos. 12535029 and 12535037) available for download at **www.thermofisher.com**.

Gateway™ entry vectors

To recombine your gene of interest into the pJTI™ R4 DEST CMV pA destination vector, you will need an entry clone containing your gene of interest. An entry clone contains your gene of interest flanked by *att*L sequences, which are then used to recombine with *att*R sequences on the pJTI™ R4 DEST CMV 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 *att*B sites and use this *att*B-PCR product in a BP recombination reaction with a donor (pDONR™) vector.

Many entry vectors, including pENTR/D-TOPO™, are available from Thermo Fisher Scientific 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.thermofisher.com/Gateway**. For detailed information on constructing an entry clone, refer to the user guide for the specific entry vector you are using.

Create an entry clone (pDONR™ vector method)

Guidelines for creating an entry clone

- Ensure that the primers used for PCR amplification of your gene of interest (see "Design PCR primers") 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 mutations.

Design 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 detailed information on primer design, refer to the section "Designing *att*B PCR Primers" in the "Gateway™ Technology with Clonase™ II" user guide (Cat. Nos. 12535029 and 12535037), available for download at **www.thermofisher.com**.

Kozak consensus sequence

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

(G/A)NN<u>ATG</u>**G**

Create an expression clone

Introduction

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 pA vector. To ensure the best results, we recommend that you read this section before starting your experiments.

To generate an expression clone:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pJTI™ R4 DEST CMV pA vector.
2. Transform the reaction mixture into a suitable *E. coli* host (see below).
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 (*att*L and *att*R) 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 can perform an LR recombination reaction between the entry clone and pJTI™ R4 DEST CMV pA, and transform the reaction mixture into a suitable *E. coli* host (see below) 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 *rec*A, *end*A *E.coli* strain including DH5α™, TOP10, or equivalent for transformation (see page 9 for ordering information).

IMPORTANT! Do not transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (e.g., TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B 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 pA) will allow you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991).

LR Clonase™ II enzyme mix

The Gateway™ LR Clonase™ II enzyme mix (Cat. No. 11791020) 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 can perform the LR recombination reaction using LR Clonase™ enzyme mix (Cat. No. 11791019), 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 user guide.

Materials needed

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE, pH 8.0)
- pJTI™ R4 DEST CMV 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

Perform LR recombination reaction

Follow this procedure to perform the LR reaction between your entry clone and the pJTI™ R4 DEST CMV 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 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 can 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 ≥ 1 × 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 the insert size and toxicity of the gene of interest. 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*.

Confirm the expression clone

False positives

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

Sequence the expression clone

To confirm that your gene of interest is in frame with the C-terminal V5 epitope, you may sequence your expression construct, if desired. We suggest using the following primer sequences.

For your convenience, Thermo Fisher Scientific offers a custom primer synthesis service. For more information, go to **www.thermofisher.com** or contact Technical Support (page 44).

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′

Retarget the Jump-In™ GripTite™ HEK293 platform cell line

Before starting

Introduction

The Jump-In™ GripTite™ HEK293 platform line is designed for site-specific retargeting mediated by the R4 Integrase, and shows sustained expression and appropriate regulation of the transgenes over long-term culture. The pJTI™ R4 DEST CMV pA retargeting vector allows the efficient integration and expression of a gene of interest into the R4 target site on the Jump-In™ GripTite™ HEK293 genome. This section provides instructions and guidelines for:

- Preparing the Jump-In™ GripTite™ HEK293 cells and the vector DNA for transfection
- Co-transfecting the Jump-In™ GripTite™ HEK293 cells with the pJTI™ R4 Int (integrase vector) and the retargeting construct (your expression vector based on pJTI™ R4 DEST CMV pA or the GFP control vector pJTI™ R4 EXP CMV EmGFP pA, Cat. No. A14146)
- Selecting, expanding, and characterizing the retargeted clones

General guidelines for retargeting experiments

Successful retargeting of Jump-In™ GripTite™ HEK293 platform cell line is directly dependent on a number of factors. It is extremely important to optimize experimental conditions for the following parameters:

- **Transfection efficiency**: Establish a high-efficiency transfection protocol before performing retargeting experiments. You can transfect the Jump-In™ GripTite™ HEK293 cells with high efficiency using a variety of different methods, including lipid-based transfection and electroporation.
- **Cells:** Cells at 80–90% confluence are ideal for transfection. A higher confluence often results in a higher proportion of dead cells in culture.

  Before transfection, visually evaluate the cells and assess cell viability using the Countess™ II Automated Cell Counter or the Trypan Blue exclusion method.
- **Selection conditions:** To select retargeted cells, use Blasticidin from Thermo Fisher Scientific at a concentration of 10 µg/mL.

  If you prefer to use Blasticidin from another source, we strongly recommend determining the effective toxic dose of each new Blasticidin lot using a cytotoxicity assay before performing retargeting experiments.

  Determine the Blasticiding toxicity using the Blasticidin Toxicity Assay with PrestoBlue™ Cell Viability Reagent (page 34), which has been optimized for Jump-In™ GripTite™ HEK293 cells. This assay also applies to most other selection antibiotics, but some modifications may be required for use with other selection antibiotics and cell types.
- **DNA:** The quality and the concentration of DNA used play a central role for the efficiency of transfection. It is crucial that the DNA is free of endotoxins. If using large quantities of DNA, we recommend commercially prepared plasmid DNA. For smaller quantities, use a commercial kit that delivers pure DNA that is free of endotoxins. Do **not** precipitate DNA with ethanol to concentrate because it reduces efficiency and viability due to the salt contamination.

Transfect Jump-In™ GripTite™ HEK293 cells

Guidelines for transfection

The Jump-In™ GripTite™ HEK293 platform cell line was validated for retargeting using lipid-based transfection with Lipofectamine™ LTX and Plus™ Reagent (Cat. No. 15338100) as described in this section. When co-transfecting Jump-In™ GripTite™ HEK293 cells with the integrase vector and the retargeting construct, consider the following factors:

- Typically a plasmid ratio of 1:1 for the retargeting expression construct (derived from pJTI™ R4 DEST CMV pA) to pJTI™ R4 Int (integrase vector) provides the best retargeting efficiency. Experimental evidence suggests that a plasmid ratio (retargeting vector to integrase vector) of 1:5 to 5:1 will yield a similar number of retargeted colonies. Plasmid ratios outside this range will likely result in a reduced retargeting efficiency.
- Retargeting efficiency is directly dependent on overall transfection efficiency. Optimize the transfection procedure before performing any retargeting experiments.
- The following section outlines the design and execution of a typical transfection experiment to retarget of Jump-In™ GripTite™ HEK293 cells. The transfection is performed in a 6-well plate using the Lipofectamine™ LTX transfection reagent. This format has proven reliable and convenient. We strongly recommend that you validate and optimize any alternative transfection format for the retargeting procedure before conducting any experiments.

Suggested controls

We strongly recommend that you include all the suggested negative and positive controls in your transfection experiments. The controls provide a good indicator for the principle success of the retargeting reaction and are crucial for troubleshooting.

- **Negative control 1**: mock (transfection reaction in the absence of plasmid DNA)
- **Negative control 2:** Retargeting expression vector only
- **Positive control:** pJTI™ R4 EXP CMV EmGFP pA positive control vector + pJTI™ R4 Int (integrase vector) (see page 8 for ordering information for pJTI™ R4 EXP CMV EmGFP pA)

Materials required

- Jump-In™ GripTite™ HEK293 cells
- Retargeting expression construct derived from pJTI™ R4 DEST CMV pA
- pJTI™ R4 Int plasmid encoding the R4 Integrase
- pJTI™ R4 EXP CMV EmGFP pA positive control vector
- 6-well plate
- Sterile 15-mL conical tube
- Trypsin, 0.05% (1X) with EDTA
- D-PBS without Ca^{2+} or Mg^{2+}
- Lipofectamine™ LTX and PLUS™ Reagent
- Growth medium (page 11)
- Transfection medium (page 11)
- Opti-MEM™ I Reduced Serum Medium
- Countess™ II Automated Cell Counter or Trypan Blue Stain for exclusion counting

Prepare Jump-In™ GripTite™ HEK293 cells for transfection

One the day before transfection, prepare Jump-In™ GripTite™ HEK293 cells as follows:

1. Fill each well of a 6-well culture plate with 3.5 mL pre-warmed Growth medium (page 11) and place the plate in a 37°C incubator with a humidified atmosphere of 5% CO_2 until use.
2. Aspirate the spent Growth medium from the cells and rinse the cells once with PBS.
3. Add 0.05% Trypsin/EDTA to the cells (3 mL for a 100-mm dish or 5 mL for a T-75 flask) and swirl the culture vessel to evenly coat the cells. Cells usually detach after ~2–10 minutes of exposure to 0.05% Trypsin/EDTA.
4. Add an equal volume of Growth medium to the culture vessel to inactivate the 0.05% Trypsin/EDTA, and transfed the cells to a sterile 15-mL conical tube.
5. Verify under a microscope that the cells have detached and cell clumps have completely dispersed.
6. Determine the cell number using the Countess™ II Automated Cell Counter or a hemocytometer.
7. Centrifuge the cells at 200 × *g* for 5 minutes and resuspend the cell pellet in Growth medium at a concentration of 1×10^6 cells/mL.
8. Seed the Jump-In™ GripTite™ HEK293 cells into a 6-well culture plate so that they become ~80% confluent 24 hours later (~750,000 cells/well) in a volume of 4 mL Growth medium
9. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

Transfect Jump-In™ GripTite™ HEK293 cells

Before starting

1. On the day of transfection, remove the Growth medium from the 6-well plate containing the Jump-In™ GripTite™ HEK293 cells at ~80% confluence and replace it with 2 mL of pre-warmed Transfection medium in each well.
2. Place the 6-well plate in a 37°C incubator with a humidified atmosphere of 5% CO_2.

Dilute DNA and PLUS™ Reagent

3. In four 1.5-mL microcentrifuge tubes labeled A–D, dilute the vectors used for transfection in 0.5 mL of Opti-MEM™ I Reduced Serum Medium.

   Note: Each tube should contain 2.5 µg of total DNA in 0.5 mL of Opti-MEM™ I medium except "negative control 1" (i.e., mock transfection), which should not have any DNA.

Component	A (Neg. control 1)	B (Neg. control 2)	C (Pos. control)	D (Retargeting)
Opti-MEM™ I Medium	0.5 mL	0.5 mL	0.5 mL	0.5 mL
pJTI™ R4 Int vector	—	—	1.25 µg	1.25 µg
pJTI™ R4 EXP CMV EmGFP pA	—	—	1.25 µg	—
Retargeting construct	—	2.5 µg	—	1.25 µg

4. Mix the PLUS™ Reagent, then add 2.5 µL into each tube from step 3. Mix well by pipetting or vortexing, and incubate the tubes at room temperature for 5 minutes.

Prepare transfection complexes

5. Mix the Lipofectamine™ LTX Reagent gently, and add 6.25 µL into each Opti-MEM™ I +DNA + PLUS™ Reagent mixture from step 4 (i.e., tubes A–D).
6. Mix the contents of each tube gently by pipetting up and down 3 times, and incubate the tubes at room temperature for 30 minutes.

Add transfection complexes to cells

7. Add the Opti-MEM™ I + DNA + PLUS™ Reagent + Lipofectamine™ LTX mixtures (i.e., transfection complexes) from step 5 to the Jump-In™ GripTite™ HEK293 cells in the 6-well plate.
8. Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 for at least 16–24 hours before adding the selection antibiotics or passaging the cells.

Note: For more information about transfection, visit our transfection web portal at **www.thermofisher.com/transfection**.

For more information about lipid-mediated transfection with the Lipofectamine™ LTX Reagent and tips for optimization, refer to the user guide for Lipofectamine™ LTX and PLUS™ Reagents (Cat. Nos. A12621 and 15338100), which is available for download at **www.thermofisher.com**.

Select retargeted Jump-In™ GripTite™ HEK293 cells

Guidelines for selection

- After transfection, incubate the cells for at least 16–24 hours before adding the selection antibiotics or passaging the cells.
- To successfully select for your "retargeted" Jump-In™ GripTite™ HEK293 cells, you need to use the minimum concentration of Blasticidin required to kill your untransfected Jump-In™ GripTite™ HEK293 cells.

  We recommend using Blasticidin from Thermo Fisher Scientific at a concentration of 10 µg/mL to select the retargeted cells. However, if you prefer to use Blasticidin from another source, we strongly recommend determining the effective toxic dose of each new Blasticidin lot using a cytotoxicity assay before performing retargeting experiments (page 33).
- Most likely, you will need to move the cells to a larger culture vessel (100-mm dish or T-75 flask) to avoid overcrowding the transfected cell population, which can result in increased resistance to antibiotic treatment.
- If you choose to transfer the transfected cells to a larger culture vessel, split the cells such that they are no more than 25% confluent, because antibiotic selection works best with actively dividing cells. After splitting the cells, allow them to attach to the culture vessel for several hours **before** adding Blasticidin.

Select retargeted Jump-In™ GripTite™ HEK293 cells

16–24 hours after transfection (or when the cells have sufficiently recovered and the colonies have become well-defined), proceed with selection.

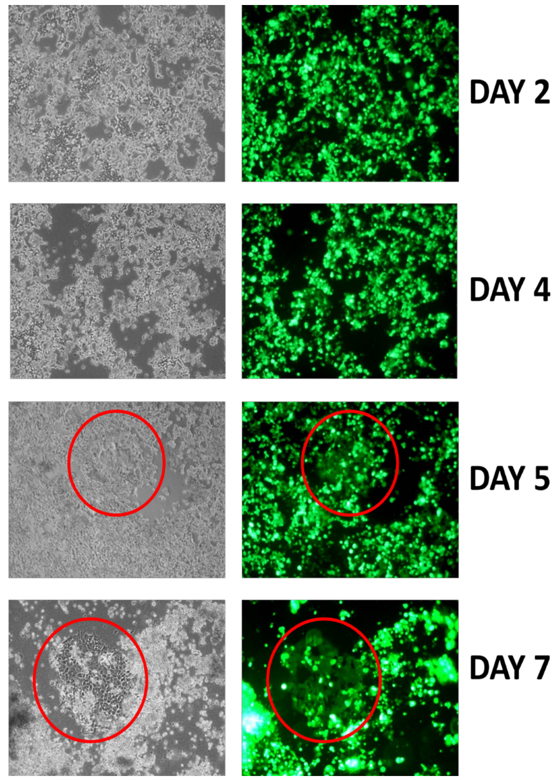
1. Passage the cells to a larger culture vessel (T-75 flask) in Transfection medium (see page 11) and allow the cells to attach for several hours.
2. Pre-warm the Selection medium (see page 11) to 37°C.

   Note: Selection medium does not contain Hygromycin B, but includes 10 µg/mL of Blasticidin.
3. Remove the Transfection medium from the attached cells and replace it with pre-warmed Selection medium.
4. Change the Selection medium at least twice a week for the next 2–4 weeks.
5. After 2–4 week of culture in Selection medium, negative control cell populations should be eliminated. The positive control (Sample C: pJTI™ R4 EXP CMV EmGFP pA control vector) should contain >85% GFP positive cells. Varying numbers of cell colonies should emerge after 2 weeks in all retargeted wells.

   Figure 5 (page 27) shows an example for a selection timecourse. The emerging retargeted Jump-In™ GripTite™ HEK293 colony is marked by a red circle.

Example of selection timecourse

Figure 5 Jump-In™ GripTite™ HEK293 cells were imaged following transfection with pJTI™ R4 Exp CMV-GFP-pA. Brightfield and fluorescence microscopy (GFP) images (10X objective) were taken at the indicated day of antibiotic selection with blasticidin. The red circles indicate colonies of blasticidin resistant retargeted Jump-In™ GripTite™ HEK293 cells.



Optional: Isolate the retargeted clones

Because the penetration of the transgene reaches >95% of the cell population in most cases, isolation of the retargeted clones is usually not required. If desired, you can obtain individual clones from the retargeted pool by picking individual colonies or from single cells using established cell isolation methods, including limiting dilution or cell sorting. Typically only a few clones need to be isolated and analyzed due to the high percentage of retargeted cells that express the transgene. For more information, contact Technical Support (page 44).

Propagate the retargeted clones

Use pre-warmed Selection medium (**omit** Hygromycin B, but **include** Blasticidin at 10 µg/mL and Geneticin™ at 600 µg/mL), and follow the same propagation methods as outlined in "Propagate and maintain Jump-In™ GripTite™ HEK293 cells", page 14.

Note: We recommend that you freeze a number of vials after the retargeted Jump-In™ GripTite™ HEK293 cells pool has been established.

Confirm retargeting events

Introduction

Upon retargeting the Jump-In™ GripTite™ HEK293 platform line, follow the guidelines below to use PCR to screen for successful retargeting events with genomic DNA isolated from the retargeted pool or individual clones. To eliminate the high background observed with only the primary PCR, use of nested PCR with primary and secondary reactions is required.

PCR targets

Successful retargeting of the Jump-In™ GripTite™ HEK293 genome introduces the human EF1α promoter upstream of the *bla* gene, resulting in Blasticidin resistance of successfully retargeted clones. PCR amplification of the EF1α promoter–Blasticidin resistance gene junction in successfully retargeted clones using the recommended primers (for the primer sequences, see "PCR primers", page 28) results in a 608 bp product. The primer sequences for amplifying the Hygromycin resistance gene are given for use in a positive control reaction. You can use the primers for the target site to confirm the presence of the retargeting sequences in the Jump-In™ GripTite™ HEK293 genome in an optional PCR (page 38).

Materials required

- Retargeted Jump-In™ GripTite™ HEK293 cells
- Phosphate Buffered Saline (PBS)
- CellsDirect Resuspension and Lysis Buffers
- PCR primers (see below for primer sequences)
- AccuPrime™ *Taq* DNA Polymerase High Fidelity
- Thermocycler
- Water bath or heat block at 75°C

PCR primers

To confirm that the Jump-In™ GripTite™ HEK293 cells platform cell line has been successfully retargeted, use the PCR primers listed below.

For your convenience, Thermo Fisher Scientific offers a custom primer synthesis service. For more information, go to **www.thermofisher.com** or contact Technical Support (page 44).

Target	Primer	Sequence	Expected size
EF1α–*bla* junction	EF1α (forward)	5′-GCC TCA GAC AGT GGT TCA AAG TTT-3′	608 bp
	Blasticidin (reverse)	5′-GAT CGC GAC GAT ACA AGT CA-3′	
Hygromycin resistance gene	Hygromycin (forward)	5′-ATG AAA AAG CCT GAA CTC ACC-3′	430 bp
	Hygromycin (reverse)	5′-ATT GAC CGA TTC CTT GCG-3′	

Prepare retargeted clones for PCR

1. Pellet 10,000 to 30,000 retargeted Jump-In™ GripTite™ HEK293 cells by centrifugation.
2. Wash the cells by resuspending them in 500 µL of PBS.
3. Centrifuge the cells to pellet and remove the PBS.
4. Resuspend the cell pellet in a mixture of 20 µL of CellsDirect Resuspension Buffer and 2 µL of Lysis Solution.
5. Incubate the cell suspension for 10 minutes at 75°C.
6. Centrifuge for 1 minute to pellet cell debris. You will need 3 µL of the cell lysate to set up your PCR.

Perform PCR

1. Set up a PCR with the primers and conditions listed below, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page 28 for PCR primers).

   Note: To eliminate the high background observed with only the primary PCR, use of nested PCR with primary and secondary reactions is required.

Component	Volume
10X AccuPrime™ PCR Buffer II	5 µL
Forward PCR primer (10 µM stock)	1 µL
Reverse PCR primer (10 µM stock)	1 µL
AccuPrime™ *Taq* DNA Polymerase High Fidelity (5 U/µL)	1 µL
Cell lysate (from step 6, page 28)	3 µL
Sterile, distilled water	39 µL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial denaturation	2 minutes	94°C	1X
Denaturation	30 seconds	94°C	25–35X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 5–10 µL from the reaction and analyze by agarose gel electrophoresis.

Optional: Southern blot analysis

PCR is usually sufficient to confirm the presence of the retargeted sequences in Jump-In™ GripTite™ HEK293 cells after transfection. However, you can also perform a Southern blot analysis as an additional check to screen for a single copy number by using a radiolabeled probe from the expression vector used to retarget the cells. We recommend using the DNAzol™ Reagent (Cat. No. 10503027) to isolate the genomic DNA from the platform cell line.

Characterize retargeted clones

Introduction

While PCR and Southern blot analysis are usually sufficient to confirm the successful retargeting of the Jump-In™ GripTite™ HEK293 platform cell line, we recommend that you perform quality control assays on retargeted clones to confirm expression and function of the retargeted transgene using the appropriate methodology.

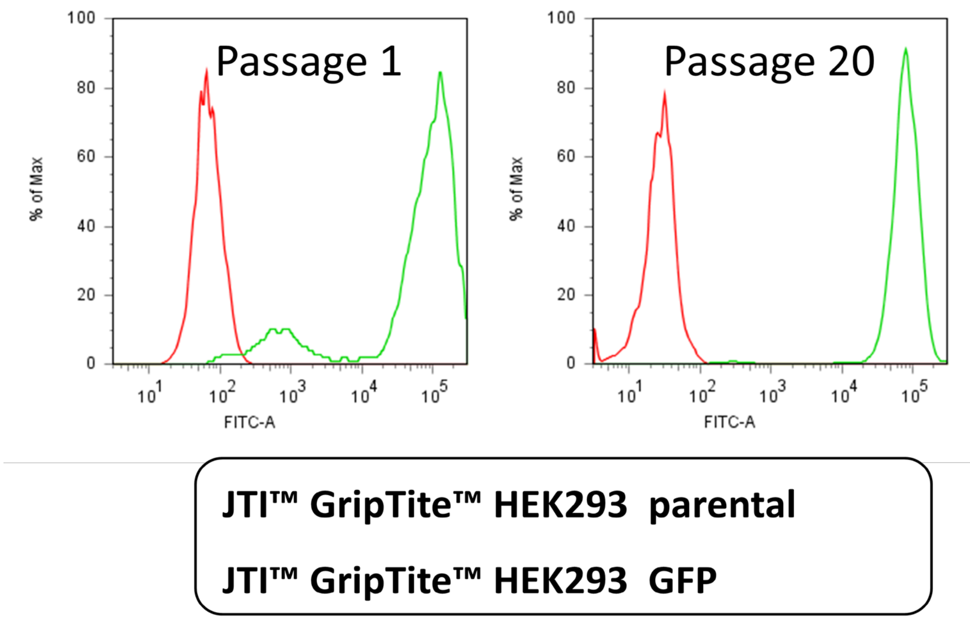
Include one sample with the pJTI™ R4 EXP CMV EmGFP pA vector as positive control in each retargeting experiment.

Following characterization of your retargeted cell line, expand and bank at least 10 vials of retargeted Jump-In™ GripTite™ HEK293 cells in Recovery™ Cell Culture Freezing medium as described on page 16.

FACS characterization

The images and flow cytometry histograms below provide an example of the sustained expression of a transgene (EmGFP) over long-term cultures of retargeted Jump-In™ GripTite™ HEK293 cells. For more information on the long-term expression and function of various transgenes in different Jump-In™ cell backgrounds, refer to (Lieu *et al.*, 2009).

Figure 6 Jump-In™ GripTite™ HEK293 platform cell line was retargeted using a GFP cassette driven by a constitutive CMV promoter. The retargeted cells were analyzed at passage 1 and 20 (after retargeting) for EmGFP expression using flow cytometry.



Appendix A: Troubleshooting

Cell culture

The following table lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Symptom	Cause	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep the cells in liquid nitrogen until thawing. Do not store the cells for extended periods at –80°C.
	Homemade stock not viable	Freeze the cells at a density of 2×10^6 to 3×10^6 viable cells/mL.
		Use low-passage cells to prepare your own stocks.
		Follow the freezing procedure for your type of cell culture exactly. Slow freezing and fast thawing are crucial.
		At the time of thawing, thaw quickly and do not expose vial to air but quickly change from nitrogen tank to 37°C water bath.
		Obtain new Jump-In™ GripTite™ HEK293 cells.
	Thawing medium not correct	Use the specified Thawing medium (see page 11).
Cells grow slowly	Cells too old	Use healthy Jump-In™ GripTite™ HEK293 cells under passage 30. Do not overgrow.
	Mycoplasma contamination	Discard the cells, media, and reagents, and use early stock of cells with fresh media and reagents.
No growth after transfection	Incorrect amount of Blasticidin is used for selection.	• Use 10 µg/mL of Blasticidin for selection. • It is normal to observe a large number of dead cells in the culture right after passaging to a larger culture vessel and after the addition of Blasticidin. • Because the concentration of Blasticidin required for effective selection of retargeted cells might vary depending on the source or the lot of Blasticidin, we recommend that you establish the sensitivity of Jump-In™ GripTite™ HEK293 cells to the Blasticidin lot used for selection before conducting retargeting experiments (page 33).

Retargeting Jump-In™ GripTite™ HEK293 cells

The following table lists some potential problems and solutions that help you troubleshoot your problems during transfection for retargeting.

Symptom	Cause	Solution
Low survival rate after transfection	Poor DNA quality	The quality of the retargeting construct DNA strongly influences the results of transfection experiments. Use endotoxin-free DNA for all transfections. Make sure that the A_{260}:A_{280} ratio of the DNA is between 1.8 and 2.0. Do not use phenol:chloroform extraction, or ethanol precipitation.
	Cells are cultured in suboptimal conditions	Cells that are 80–90% confluent are ideal for transfection. A higher confluence often results in a higher proportion of dead cells in culture. Avoid excessive cell densities of high confluence.
	Suboptimal transfection conditions	Certain transfection methods can cause cell death. We recommend optimizing transfection conditions, especially if you choose a protocol different from the protocol suggested in this user guide
	Cells are damaged during harvesting and subsequent handling prior to transfection	• Avoid damaging cells conditions during harvesting. • Centrifuge cells at lower speeds (150–200 × *g*). • Avoid overexposure to trypsin. • Pipette the cells gently.
Low transfection efficiencies	Poor optimization of transfection parameters	Optimize transfection parameters following manufacturers' recommendations.
	Amount of DNA too low	Use the correct amount of DNA for the transfection method of choice as described on page 24.
	Cell density too low or too high	Too low or too high cell densities could drastically reduce the transfection efficiency. Adjust the cell density to 80–90% confluence on the day of transfection.
	Poor DNA quality	Use endotoxin-free DNA for all transfections. Make sure that the A_{260}:A_{280} ratio of the DNA is between 1.8 and 2.0. Do not use phenol:chloroform extraction, or ethanol precipitation.
	Cells are contaminated with Mycoplasma	• Test cultures for Mycoplasma contamination. • Start a new culture from a fresh stock.

Appendix B: Support protocols

Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells. Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

How to handle Blasticidin

Always wear gloves, mask, goggles, and a laboratory coat when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Prepare and store stock solutions

- Blasticidin is soluble in water and acetic acid.
- Prepare a working stock solution of 5–10 mg/mL Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use and freeze at –20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1 week at 4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should not exceed 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Assess Blasticidin toxicity

We recommend using Blasticidin from Thermo Fisher Scientific at a concentration of 10 µg/mL for selecting retargeted cells.

However if you prefer to use Blasticidin from another source, we recommend that you determine the effective toxic dose of each new Blasticidin lot using a cytotoxicity assay before performing retargeting experiments.

Determine the Blasticiding toxicity using the Blasticidin Toxicity Assay with PrestoBlue™ Cell Viability Reagent (page 34), which has been optimized for Jump-In™ GripTite™ HEK293 cells. This assay also applies to most other selection antibiotics, but some modifications may be required for use with other selection antibiotics and cell types.

Materials required

- 10-cm culture dish with Jump-In™ GripTite™ HEK293 cells
- Growth medium (see page 11)
- Blasticidin
- 96-well cell culture plate
- PrestoBlue™ Cell Viability reagent

Perform Blasticidin toxicity assay with PrestoBlue™ Cell Viability Reagent

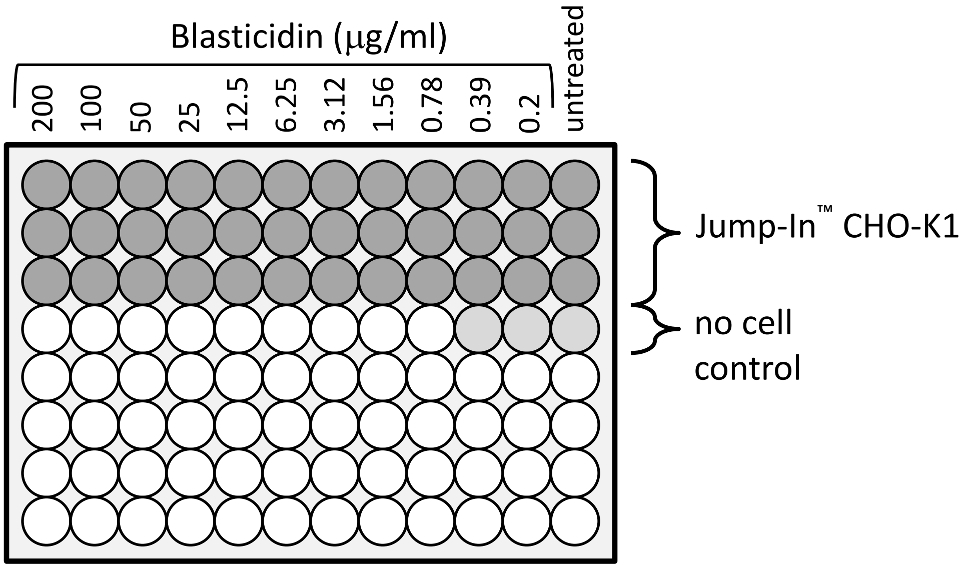
1. Harvest the Jump-In™ GripTite™ HEK293 cells by trypsinization as outlined in "Propagate and maintain Jump-In™ GripTite™ HEK293 cells" on page 14.
2. Prepare a cell suspension at a concentration of 500 cells/mL in Growth medium.
3. Plate 100 µL of the cell suspension in three rows (3 × 12 wells) of a 96-well plate and incubate in a 37°C incubator with a humidified atmosphere of 5% CO_2 for 18–24 hours.

   Note: It is important to start with a relatively small number cells because the experiment requires 7–8 days of continuous cultivation. Using more cells will require a replating step.
4. Prepare 1 mL of a 200 µg/mL Blastcidin stock solution in Growth medium in a 1.5-mL microcentrifuge tube. This is "Tube 1" of the serial dilution.
5. Prepare 10 additional 1.5-mL microcentrifuge tubes (numbered 2 to 11) containing 0.5 mL Growth medium.
6. Prepare a 11-point two-fold serial dilution by transferring 0.5 mL of the Blasticidin stock solution from tube 1 to tube 2 (mix by pipetting), followed by transferring 0.5 mL from tube 2 to tube 3 and so on until you reached tube 11.
7. Add 100 µL of each dilution to one column of the 96-well plate containing the cells (e.g., 200 µg/mL of Blasticidin in Column 1, 100 µg/mL Blasticidin in Column 2, 50 µg/mL Blasticidin in Column 3, etc.). Add 100 mL of growh medium to column 12 as untreated control (see diagram on the next page).
8. Incubate the cells for 48–72 hours in a 37°C incubator with a humidified atmosphere of 5% CO_2.
9. After the incubation, remove the medium by aspiration and add 100 µL of fresh Growth medium.
10. Prepare and add the Blasticidin serial dilution as described in step 4–7.
11. Repeat media exchange cycle (steps 8 through 10) three times (for 8–10 days total).
12. Prepare PrestoBlue™ reagent by diluting it 1:10 in Growth medium 1:10. Prepare enough PrestoBlue™ reagent for 40 wells (36 wells for Blasticidin serial dilution and 3 wells for no cell control wells).

    Note: For more information on the PrestoBlue™ Cell Viability Reagent, go to **www.thermofisher.com**.
13. Remove the medium by aspiration and add 100 mL of PrestoBlue™ reagent
14. Incubate the cells for at least 10 minutes at 37°C. Longer incubation time increases the sensitivity of detection.
15. Read the fluorescence or absorbance. Fluorescence is more sensitive than absorbance and is the preferred detection method (excitation 540–570 nm, emission 580–610 nm).
16. Calculate and plot the results (the no-cell control values can be used to determine background fluorescence). Higher values correlate with higher metabolic activity. A sample plot is shown on page 35.

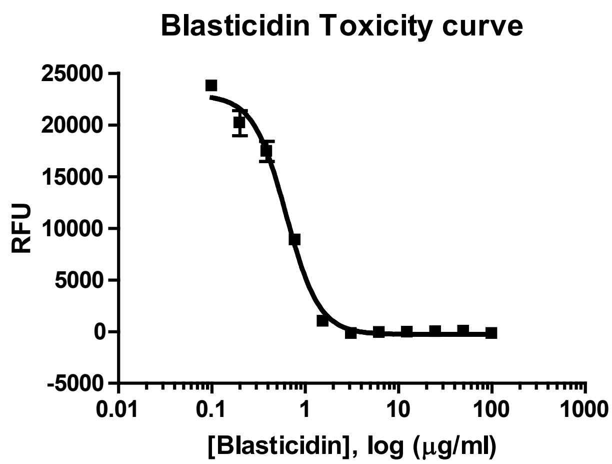
Blasticidin serial dilution

The diagram below shows the Blasticidin serial dilution performed in a 96-well plate.



Blasticidin toxicity curve

The graph below illustrates an example of a Blasticidin toxicity curve. (RFU = relative fluorescence units; low RFU indicates low viability).



Hygromycin B

Description

The integration vector used in creating the Jump-In™ GripTite™ HEK293 platform cell line contains the hygromycin resistance gene (Hyg^R) (Gritz & Davies, 1983; Palmer *et al.*, 1987), making Jump-In™ GripTite™ HEK293 cells resistant to Hygromycin B. When added to cultured mammalian cells, Hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin B liquid is available separately from Thermo Fisher Scientific (see page 8 for ordering information)

How to handle Hygromycin B

- Hygromycin B is light sensitive. Store the liquid stock solution at 4°C protected from exposure to light.
- Hygromycin B is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Hygromycin B and Hygromycin B-containing solutions

Prepare and store stock solutions

Hygromycin B is available from Thermo Fisher Scientific as a 50 mg/mL stock solution in autoclaved, deionized water, and is filter-sterilized. The solution is brown in color. The stability of Hygromycin B is guaranteed for six months, if stored at 4°C. Medium containing Hygromycin B is stable for up to six weeks.

Propagate the Jump-In™ Vectors

Propagate the pJTI™ R4 DEST CMV pA vector

To propagate and maintain the pJTI™ R4 DEST CMV pA vector, we recommend using 10 ng of the vector to transform One Shot™ *ccd*B Survival™ 2 T1^R Competent Cells, an *E. coli* strain resistant to *ccd*B effects that can support the propagation of plasmids containing the *ccd*B gene (see page 9 for ordering information). Select transformants on LB plates containing 50–100 µg/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 DH5α™) for propagation and maintenance of pJTI™ R4 DEST because these strains are sensitive to *ccd*B effects.

Propagate the pJTI™ R4 Int vector

To propagate and maintain the pJTI™ R4 Int vector, we recommend using 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain such as TOP10F′, DH5α™-T1^R, TOP10, or equivalent. Select transformants on LB plates containing 50–100 µg/mL ampicillin. Be sure to prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Optional: Confirm the R4 Site of Integration

Introduction

The Jump-In™ GripTite™ HEK293 platform cell line was created by the PhiC31-mediated site-specific integration of the R4 retargeting sequences into the genome of GripTite™ HEK293 cells. Follow the guidelines below for the optional assay to confirm the presence of R4 retargeting site in the Jump-In™ GripTite™ HEK293 genome.

Materials required

- Retargeted Jump-In™ GripTite™ HEK293 cells
- Phosphate Buffered Saline (PBS)
- CellsDirect Resuspension and Lysis Buffers (see page 9 for ordering information)
- PCR primers (see below for primer sequences)
- AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page 9 for ordering information)
- Thermocycler
- Water bath or heat block at 75°C

PCR primers

To confirm the site-specific integration of the R4 retargeting sequences into the genome of GripTite™ HEK293 cells, use the PCR primers listed below.

For your convenience, Thermo Fisher Scientific offers a custom primer synthesis service. For more information, go to **www.thermofisher.com** or contact Technical Support (page 44).

Target	Primer	Sequence	Expected size
Hygromycin resistance gene	Hygromycin (forward)	`5′-ATG AAA AAG CCT GAA CTC ACC-3′`	430 bp
	Hygromycin (reverse)	`5′-ATT GAC CGA TTC CTT GCG-3′`	

Prepare genomic DNA for PCR

Prepare genomic DNA from Jump-In™ GripTite™ HEK293 cells using the CellsDirect Resuspension and Lysis Buffers as described below.

1. Pellet 10,000 to 30,000 retargeted Jump-In™ GripTite™ HEK293 cells by centrifugation.
2. Wash the cells by resuspending them in 500 µL of PBS.
3. Centrifuge the cells to pellet and remove the PBS.
4. Resuspend the cell pellet in a mixture of 20 µL of CellsDirect Resuspension Buffer and 2 µL of Lysis Solution.
5. Incubate the cell suspension at 75°C for 10 minutes.
6. Centrifuge for 1 minute to pellet cell debris. You will need 3 µL of the cell lysate to set up your PCR.

Perform PCR

1. Set up a PCR with the primers and conditions listed below, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page 38 for PCR primers).

   Note: To eliminate the high background observed with only the primary PCR, use of nested PCR with primary and secondary reactions is required.

Component	Volume
10X AccuPrime™ PCR Buffer II	5 µL
Forward PCR primer (10 µM stock)	1 µL
Reverse PCR primer (10 µM stock)	1 µL
AccuPrime™ *Taq* DNA Polymerase High Fidelity (5 U/µL)	1 µL
Cell lysate (from step 6, page 38)	3 µL
Sterile, distilled water	39 µL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial denaturation	2 minutes	94°C	1X
Denaturation	30 seconds	94°C	40X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 5–10 µL from the reaction and analyze by agarose gel electrophoresis.

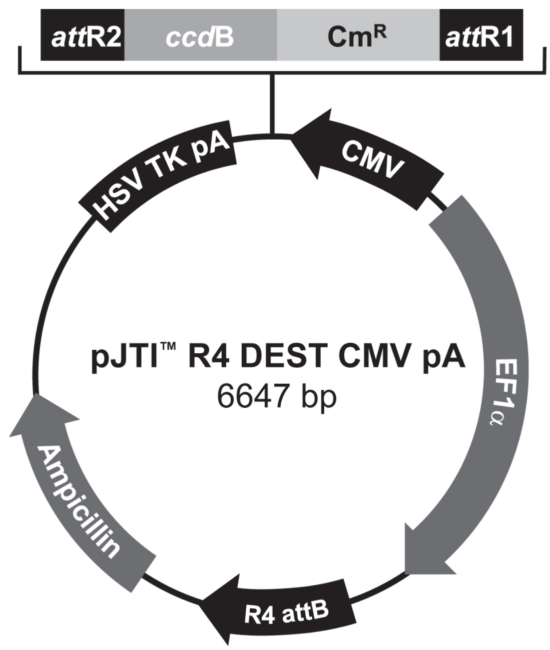
Appendix C: Vectors

pJTI™ R4 DEST CMV pA

Map of pJTI™ R4 DEST CMV pA

The pJTI™ R4 DEST CMV pA vector (6,647 base pairs) contains the λ Integrase *att*R1 and *att*R2 sites for the transfer of a DNA element of interest from a Gateway™ entry clone to generate the retargeting expression clone, the R4 *att*B site for site-specific integration of the DNA elements into the Jump-In™ GripTite™ HEK293 genome, and the human EF1α promoter for constitutive expression of Blasticidin resistance upon successful integration.

The complete sequence of pJTI™ R4 DEST CMV pA is available from **www.thermofisher.com** or by contacting Technical Support (page 44).



Features of pJTI™ R4 DEST CMV pA
6647 nucleotides

R4 *aat*B: bases 28–322
Ampicillin resistance gene: bases 474–1331
HSV TK pA: bases
attR2 recombination site: bases 2879–3003
ccdB gene: bases 3044–3349 (c)*
Chloramphenicol resistance gene: bases 3670–4350 (c)
attR1 recombination site: bases 4459–4583
CMV promoter: bases 4711–5289 (c)
EF1α promoter: bases 5418–6596

*(c): complementary strand

Features of pJTI™ R4 DEST CMV pA

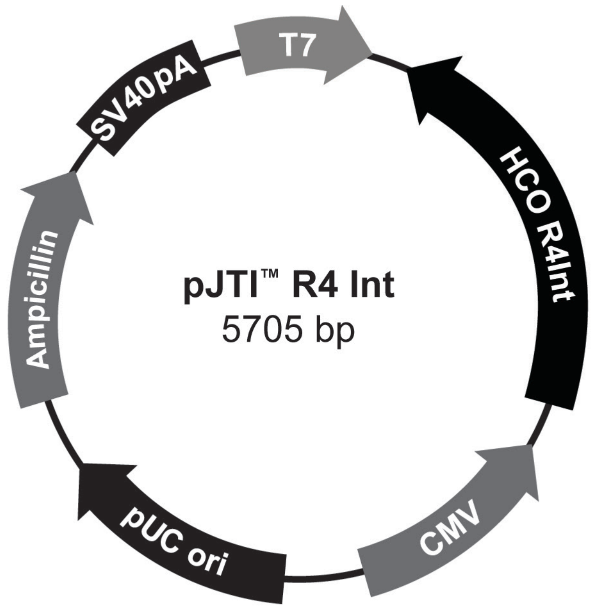
The pJTI™ R4 DEST CMV pA vector contains the following elements. All features have been functionally tested.

Feature	Benefit
CMV promoter	Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
*att*R1 and *att*R2 sites	Allows recombinational cloning from a Gateway™ entry construct (Landy, 1989)
V5 epitope	Allows the detection of the recombinant fusion protein using anti-V5 antibodies (Southern *et al.*, 1991)
HSV TK polyA signal	The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript (Cole & Stacy, 1985)
EF1α 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
R4 *att*B site	Allows R4 integrase-mediated integration into the complementary genomic R4 *att*P site in the parental Jump-In™ cell line
Ampicillin resistance gene	Allows the selection and propagation of the vector in *E. coli*
*ccd*B gene	Located between the two *att*R sites, allows for negative selection of the vector following the LR recombination reaction (Bernard & Couturier, 1992; Bernard *et al.*, 1993)
Chloramphenicol resistance gene	Located between the two *att*R sites, allows for counterselection of the vector

pJTI™ R4 Int

Map of pJTI™ R4 Int

The pJTI™ R4 Int vector (5,705 bp) contains the gene for R4 Integrase from the *Streptomyces* PhiC31 phage. The R4 Integrase allows the site-specific integration of DNA elements into the Jump-In™ GripTite™ HEK293 genome from the pJTI™ R4 DEST retargeting expression construct upon cotransfection of the platform line with both vectors. The complete sequence of pJTI™ R4 Int is available from **www.thermofisher.com** or by contacting Technical Support (page 44).



Features of pJTI™ R4 Int
5705 nucleotides

T7 promoter: bases 1–20
HCO R4Int: bases 43–1452 (c)*
CMV promoter: bases 1590–2113 (c)
pUC origin: bases 2598–3271
Ampicillin resistance gene: bases 3458–4318 (c)
SV40 polyA site: bases 5254–5616 (c)

*(c): complementary strand

Features of pJTI™ R4 Int

The pJTI™ R4 Int vector contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows in vitro transcription in the sense orientation and sequencing through the insert
HCO R4Int	Encodes the R4 Integrase that facilitates the site-specific integration of the gene of interest (contained in the retargeting expression construct) and the promoter for the Blasticidin resistance gene to the attP site in an existing Jump-In™ platform cell line (Lieu *et al.*, 2009)
CMV promoter	Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
pUC origin	Allows high-copy replication and maintenance in *E. coli*
Ampicillin resistance gene	Allows the selection and propagation of the vector in *E. coli*
SV40 polyadenylation signal	Allows transcript termination and polyadenylation of mRNA

Documentation and Support

Obtaining support

Technical Support

For the latest services and support information for all locations, go to **www.thermofisher.com**.

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

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.thermofisher.com/sds**.

IMPORTANT! For the SDSs of chemicals not distributed by Thermo Fisher Scientific contact the chemical manufacturer.

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Gateway™ clone distribution policy

Introduction

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

Gateway™ entry clones

Thermo Fisher Scientific Corporation 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. Thermo Fisher Scientific Corporation agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Thermo Fisher Scientific Corporation.

Gateway™ expression clones

Thermo Fisher Scientific Corporation 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. Thermo Fisher Scientific Corporation agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Thermo Fisher Scientific Corporation. Organizations other than academia and government may also distribute such Gateway™ expression clones for a nominal fee ($10 per clone) payable to Thermo Fisher Scientific 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 Thermo Fisher Scientific 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 Thermo Fisher Scientific 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 Thermo Fisher Scientific Corporation's licensing department at 760-603-7200.

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