

USER GUIDE

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Jump-In™ GripTite™ HEK293 Retargeting Kit

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

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Product Information

Kit Contents and Storage

Kit contents and storage

The Jump-In™ GripTite™ HEK293 Retargeting Kit contains the following components. The contents of each kit component are described below.

| 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) | 200 µL at 0.5 µg/µL in TE buffer, pH 8.0* | -20°C |
| pJTI™ R4 DEST CMV pA (destination vector) | 200 µL at 0.5 µg/µL in TE buffer, pH 8.0 | -20°C |
| Product Information Sheet | each | N/A |

*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 downloading at:

www.cdc.gov/od/ohs/biosfty/bmb15/bmb15toc.htm.

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

Product use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Continued on next page

Kit Contents and Storage, continued

Purpose of this manual

This manual 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 manual

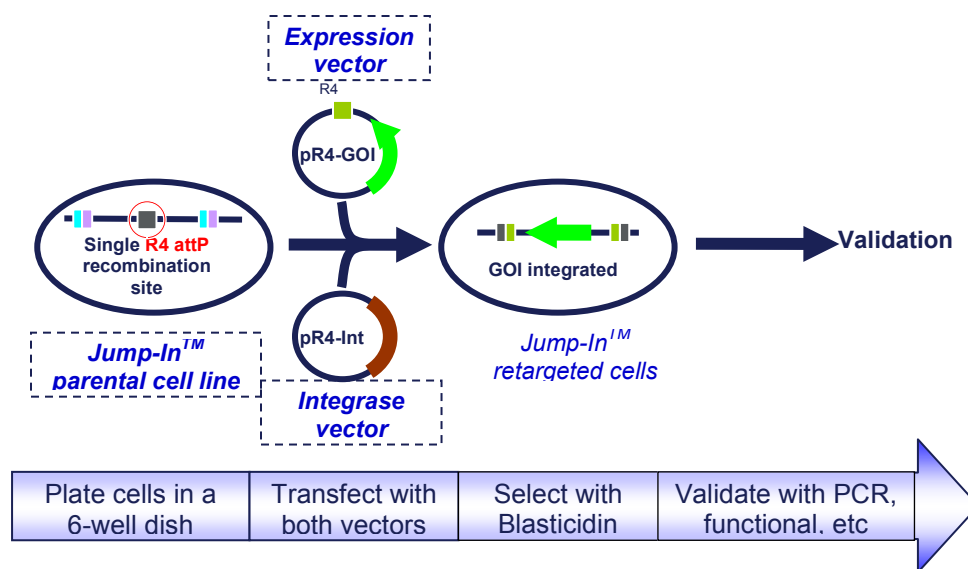
This manual **does not** provide detailed instructions for generating entry clones, but presents guidelines and suggestions to help you obtain the best results when constructing an entry clone. For more information on the Gateway® Technology and detailed instructions on generating an entry clone, refer to the Gateway® Technology with Clonase® II manual (Cat. nos. 12535-029 and 12535-037) and the manual for the specific donor vector you are using. These manuals are available on our website (www.lifetechnologies.com) or by contacting Technical Support (see page 43).

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.lifetechnologies.com) and the published literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001).

Continued on next page

Description of the System, continued

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 32–35. For instructions on how to propagate the Jump-In™ vectors, see page 36.

- 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.

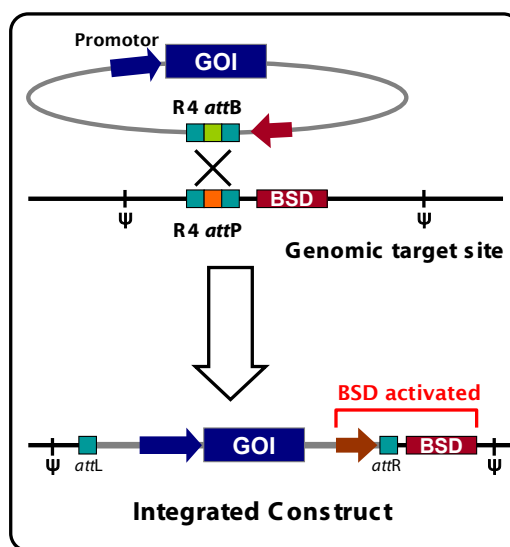
Note: To recombine your gene of interest into the pJTI™ R4 DEST CMV pA vector, you will need an entry clone containing the gene of interest (see page 15 for more information).

- 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, below). 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 *attP* target site. This integration event also positions the constitutive human EF1 α 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

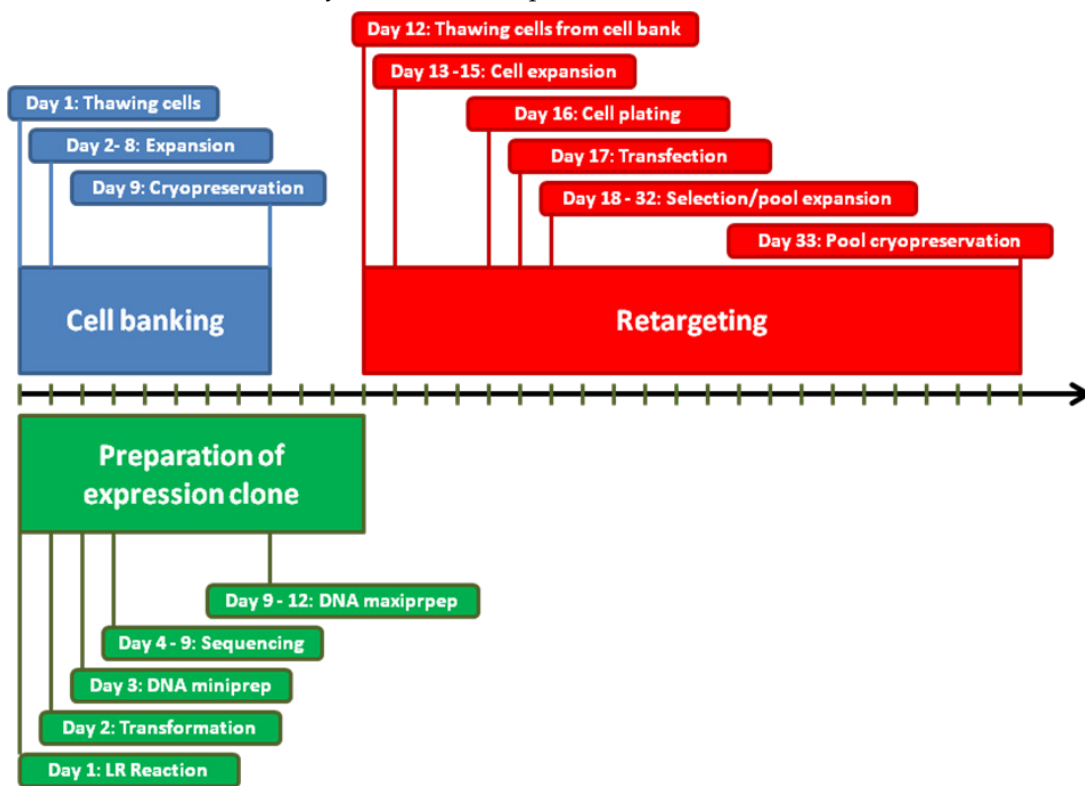
Workflow

The table below 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 | 11 |
| 2 | Create an entry clone by cloning your gene of interest into a Gateway® entry vector | 16 |
| 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) | 17 |
| 4 | Cotransfect your retargeting construct and the integrase vector into the Jump-In™ GripTite™ HEK293 cells | 21 |
| 5 | Select for retargeted Jump-In™ GripTite™ HEK293 cells in Selection Medium containing Blasticidin | 25 |
| 6 | Confirm retargeting of the Jump-In™ GripTite™ HEK293 cells by PCR | 27 |
| 7 | Characterize the retargeted clones | 29 |

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.



Materials Required, But Not Provided

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 | 10569-010 |
| Fetal Bovine Serum, Dialyzed (US) (FBS) | 100 mL | 26400-036 |
| MEM Non-Essential Amino Acids Solution 10 mM (100X), liquid (NEAA) | 100 mL | 11140-050 |
| HEPES Buffer Solution (1 M) | 100 mL | 15630-080 |
| Penicillin-Streptomycin, liquid | 100 mL | 15140-122 |
| Geneticin® Selective Antibiotic, liquid | 100 mL | 10131-027 |
| Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid | 1000 mL | 14190-136 |
| Trypsin, 0.05% (1X) with EDTA 4Na, liquid | 100 mL | 25300-054 |
| Recovery™ Cell Culture Freezing Medium, liquid | 50 mL | 12648-010 |
| Countess® Automated Cell Counter | 1 each | C10227 |
| Trypan Blue Stain | 100 mL | 10250-061 |

Jump-In™ vectors

The table below 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 table below 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 | 31985-062 |
| Lipofectamine® LTX and PLUS™ Reagent* | 1 mL | 15338-100 |
| Hygromycin B | 20 mL | 10687-010 |
| Blasticidin S HCl Selection Antibiotic, (liquid) | 20 mL | A11139-02 |
| Ampicillin, Sodium Salt | 200 mg | 11593-027 |
| PrestoBlue™ Cell Viability Reagent | 25 mL | A13261 |

Continued on next page

Materials Required, But Not Provided, continued

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.

| 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.

| Product | Quantity | Cat. no. |
|--|--------------|----------|
| One Shot® ccdB Survival™ 2 T1 ^R 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 |

PCR analysis

The table below 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 MgSO₄ (50 mM).

Culturing the Jump-In™ GripTite™ HEK293 Platform Cell Line

Guidelines for Culturing Jump-In™ GripTite™ HEK293 Cells

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 manual.

General guidelines for culturing Jump-In™ GripTite™ HEK293 cells

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 to 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 (see page 6 for ordering information).
 - 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), making Jump-In™ GripTite™ HEK293 cells resistant to Hygromycin B. In addition the Jump-In™ GripTite™ HEK293 cell lines also contains the Neomycin resistance gene (Neo^R). 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 table below 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 6 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 Life Technologies.

† 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 (see page 37).

Continued on next page

Jump-In™ GripTite™ HEK293 Media, continued

Volumes required for preparing

Jump-In™ GripTite™ HEK293 media

The table below provides an example for the volumes required to prepare the media as outlined in the media composition table on page 9.

Note that the volumes provided in the table below is accurate only for Life Technologies products listed on page 6.

| 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 Life Technologies.

† 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 (see page 37).

Thawing Jump-In™ GripTite™ HEK293 cells

- Materials required**
- Jump-In™ GripTite™ HEK293 cells (store frozen cells in liquid nitrogen until ready to use)
 - Thawing Medium (see page 9), pre-warmed to 37°C
 - Growth Medium (see page 9), 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
-

**Thawing
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% CO₂ 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 to 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% CO₂.
 9. At first passage, switch to Growth Medium.
Note: See page 13 for an image of the Jump-In™ GripTite™ HEK293 cells.
-

Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells

- Materials required**
- Growth Medium (see page 9), pre-warmed to 37°C
 - Dulbecco's Phosphate Buffered Saline (D-PBS) (1X) (see page 6 for ordering information)
 - Trypsin, 0.05% (1X) with EDTA (see page 6 for ordering information)
 - 37°C water bath
 - 37°C incubator with a humidified atmosphere of 5% CO₂
 - Disposable, sterile 15-mL conical tubes
 - T-75 cell culture flask or 100-mm cell culture dish
 - Hemocytometer or cell counter (e.g., Countess® Automated Cell Counter)
 - Trypan blue
-

Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells

Propagating and maintaining Jump-In™ GripTite™ HEK293 cells require passaging or feeding of cells at least twice a week and maintaining the cells between 5% and 95% confluency. To passage the Jump-In™ GripTite™ HEK293 cells:

1. Pre-warm fresh Growth Medium in a culture vessel in the 37°C incubator.
2. Aspirate the medium from growing 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 coat the cells evenly. Cells usually detach after ~2 to 10 minutes of exposure to 0.05% Trypsin/EDTA.
4. Add an equal volume of Growth Medium to inactivate the 0.05% Trypsin/EDTA.
5. Verify under a microscope that cells have detached and clumps have completely dispersed.
6. 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® Automated Cell Counter. We recommend determining cell health frequently to ensure optimal performance in retargeting experiments.
7. Centrifuge the cells at 200 × g for 5 minutes and resuspend then in Growth Medium.
8. 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.

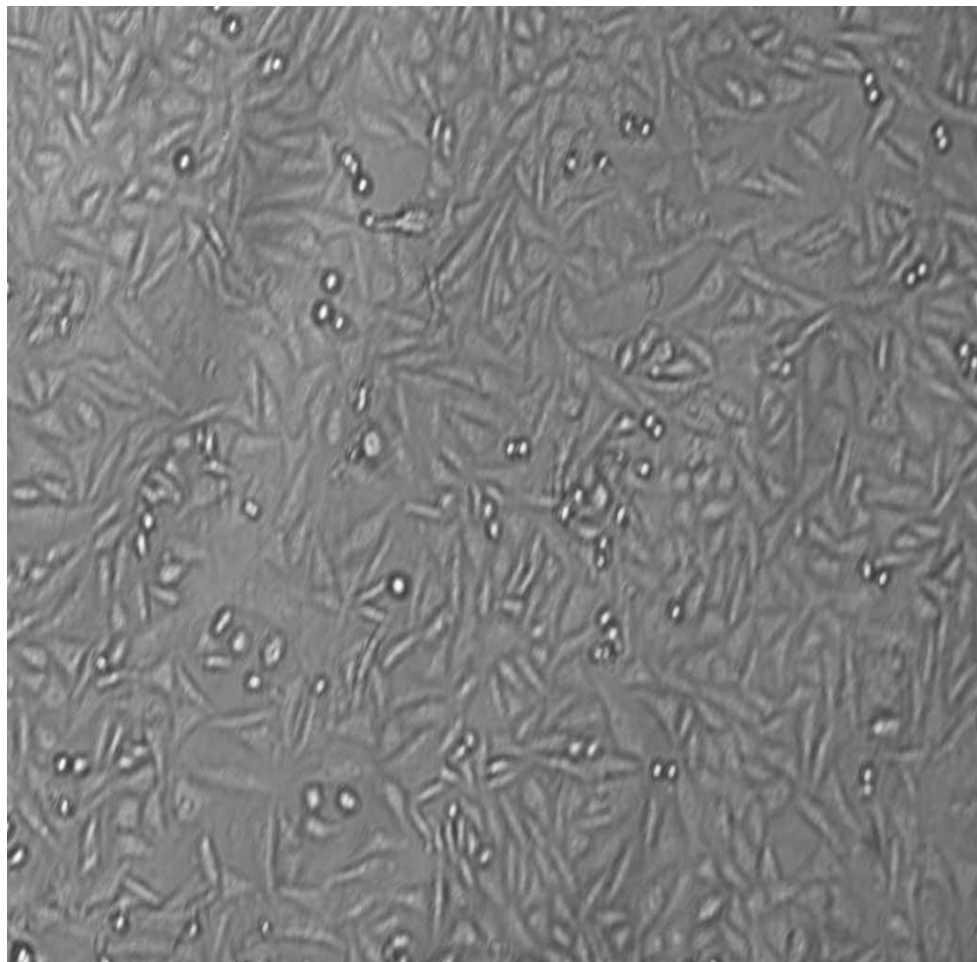
Important: Do **not** allow the cells to remain confluent for extended periods of time.

Continued on next page

Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells, continued

Morphology of
Jump-In™ GripTite™
HEK293 cells

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



Freezing Jump-In™ GripTite™ HEK293 cells

Materials required

- Plates with Jump-In™ GripTite™ HEK293 cells
 - Dulbecco's Phosphate Buffered Saline (D-PBS) (1X) (see page 6 for ordering information)
 - Growth Medium (see page 9)
 - Trypsin, 0.05% (1X) with EDTA (see page 6 for ordering information)
 - Recovery™ Cell Culture Freezing Medium (see page 6 for ordering information)
 - Disposable, sterile 15-mL conical tubes
 - Sterile freezing vials for liquid nitrogen storage
-

Freezing Jump-In™ GripTite™ HEK293 cells

1. Harvest the cells by trypsinization as described in Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells, page 12.
 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 may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen.
-



IMPORTANT! Before starting experiments, we strongly recommend that you first expand and bank at least 10 to 20 vials of frozen Jump-In™ GripTite™ HEK293 cells in Recovery™ Cell Culture Freezing Medium as described above.

Constructing 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:

- the generation of a Gateway® entry clone containing your gene of interest
- LR recombination reaction between the entry clone and the pJTI™ R4 DEST CMV pA 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.

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 *attL* sequences, which are then used to recombine with *attR* 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 *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. 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 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 (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)NNATGG

Creating 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, you will:

1. Perform an LR recombination reaction using the *attL*-containing entry clone and the *attR*-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 (*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 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 *recA*, *endA* *E. coli* strain including DH5α™, TOP10, or equivalent for transformation (see page 7 for ordering information).

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.

Continued on next page

Creating an Expression Clone, continued

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. 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.

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

Continued on next page

Creating an Expression Clone, continued

LR recombination reaction protocol

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 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 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*.

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 V5 epitope, 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) or contact Technical Support (page 43).

| 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' |

Retargeting 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 on:

- 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:** We strongly recommend that you establish a high-efficiency transfection protocol prior to performing retargeting experiments. Jump-In™ GripTite™ HEK293 cells can be transfected with high efficiency using a variety of different methods, including lipid-based transfection and electroporation.
 - **Cells:** Cells at 80–90% confluency are ideal for transfection. A higher confluency often results in a higher proportion of dead cells in culture. We recommend that you visually evaluate the cells prior to transfection. Alternatively, you can assess cell viability using the Countess® Automated Cell Counter or the Trypan Blue exclusion method.
 - **Selection conditions:** We recommend using Blasticidin from Life Technologies at a concentration of 10 µg/mL for selecting retargeted cells (for ordering information, see page 6). 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. Determine the Blasticiding toxicity using the Blasticidin Toxicity Assay with PrestoBlue™ Cell Viability Reagent (described on page 37), which has been optimized for Jump-In™ GripTite™ HEK293 cells. This assay also applies to most other selection antibiotics and cell types, but some modifications may be required in the protocol.
 - **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.
-

Transfecting 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. 15338-100) 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) will provide the best retargeting efficiency in the majority of all cases. Experimental evidence suggests that a plasmid ratio (retargeting vector to integrase vector) of between 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. We strongly recommend that you optimize the transfection procedure prior to any retargeting experiments.
- The following section outlines the design and execution of a typical transfection experiment for the retargeting of Jump-In™ GripTite™ HEK293 cells. The transfection is performed in a 6-well plate using Lipofectamine® LTX as the 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 prior to conducting 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 any 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 6 for ordering information for pJTI™ R4 EXP CMV EmGFP pA)

Continued on next page

Transfecting Jump-In™ GripTite™ HEK293 cells, continued

- 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 (see page 6 for ordering information)
 - 6-well plate
 - Sterile 15-mL conical tube
 - Trypsin, 0.05% (1X) with EDTA (see page 6 for ordering information)
 - D-PBS without Ca²⁺ or Mg²⁺ (see page 6 for ordering information)
 - Lipofectamine® LTX and PLUS™ Reagent (see page 6 for ordering information)
 - Growth Medium (see page 9 for recipe)
 - Transfection Medium (see page 9 for recipe)
 - Opti-MEM® I Reduced Serum Medium (see page 6 for ordering information)
 - Countess® Automated Cell Counter or Trypan Blue Stain for exclusion counting (see page 6 for ordering information)
-

Preparing 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 (see page 9) and place the plate in a 37°C incubator with a humidified atmosphere of 5% CO₂ 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 to 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® 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 × 10⁶ 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% CO₂.
-

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Transfecting Jump-In™ GripTite™ HEK293 cells, continued

Transfection protocol

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% confluency 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% CO₂.

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 as shown below.

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, and 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% CO₂ for at least 16 to 24 hours before adding the selection antibiotics or passaging the cells.



For more general information about transfection, visit our transfection web portal on our website at www.lifetechnologies.com. For more information about lipid-mediated transfection using the Lipofectamine® LTX Reagent and tips for optimization, refer to the product insert provided with the Lipofectamine® LTX and PLUS™ Reagents (Cat. nos. A12621 and 15338-100), which is also available for downloading at www.lifetechnologies.com.

Selecting Retargeted Jump-In™ GripTite™ HEK293 cells

Guidelines for selection

- After transfection, incubate the cells for at least 16 to 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 Life Technologies at a concentration of 10 µg/mL for selecting 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 (see page 37).
- 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.

Selection protocol

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

1. Passage the cells to a larger culture vessel (T-75 flask) in Transfection Medium (see page 9) and allow the cells to attach for several hours.
2. Pre-warm the Selection Medium (see page 9) 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 to 4 weeks.
5. After 2 to 4 week of culture in Selection Medium, negative control cell populations should be eliminated. The positive control (Sample C: pJTTM 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.

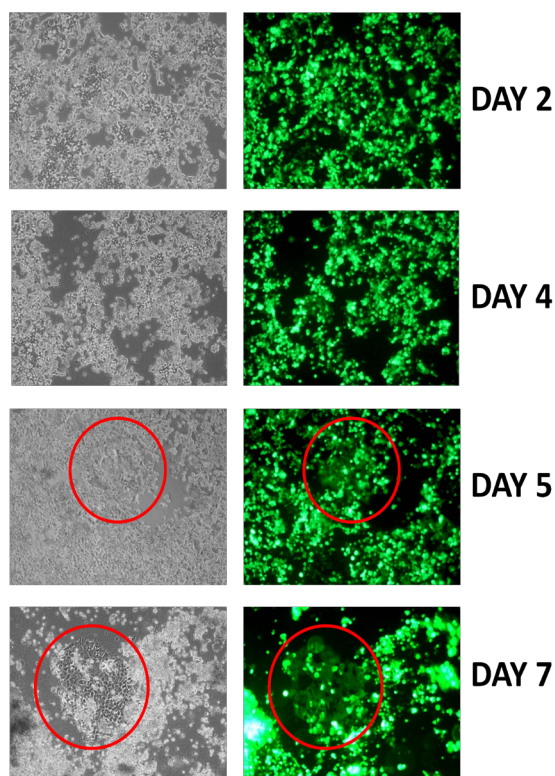
Figure 5 (next page) shows an example for a selection timecourse. The emerging retargeted Jump-In™ GripTite™ HEK293 colony is marked by a red circle.

Continued on next page

Selecting Retargeted Jump-In™ GripTite™ HEK293 cells, continued

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.



Isolating retargeted clones (optional)

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 43).

Propagating 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 **Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells**, page 12.

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

Confirming 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 EF1 α promoter upstream of the *bla* gene, resulting in Blasticidin resistance of successfully retargeted clones. PCR amplification of the EF1 α promoter–Blasticidin resistance gene junction in successfully retargeted clones using the recommended primers (see below for primer sequences) 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 may 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 (see page 41).

Materials required

- Retargeted Jump-In™ GripTite™ HEK293 cells
- Phosphate Buffered Saline (PBS)
- CellsDirect Resuspension and Lysis Buffers (see page 7 for ordering information)
- PCR primers (see below for primer sequences)
- AccuPrime™ Taq DNA Polymerase High Fidelity (see page 7 for ordering information)
- 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, Life Technologies offers a custom primer synthesis service. For more information, refer to our website (www.lifetechnologies.com) or contact Technical Support (page 43).

| Target | Primer | Sequence | Expected size |
|------------------------------------|------------------------|---------------------------------------|---------------|
| EF1 α – <i>bla</i> junction | EF1 α (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' | |

Continued on next page

Confirming Retargeting Events, continued

Preparing 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 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.

PCR protocol

1. Set up a PCR with the primers and conditions listed below, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page 27 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™ <i>Taq</i> DNA Polymerase High Fidelity (5 U/µL) | 1 µL |
| Cell lysate (from step 6, above) | 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.

Southern blot analysis (optional)

PCR is usually sufficient to confirm the presence of the retargeted sequences in Jump-In™ GripTite™ HEK293 cells after transfection. However, you may 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. 10503-027) to isolate the genomic DNA from the platform cell line (see page 7 for ordering information).

Characterizing 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.

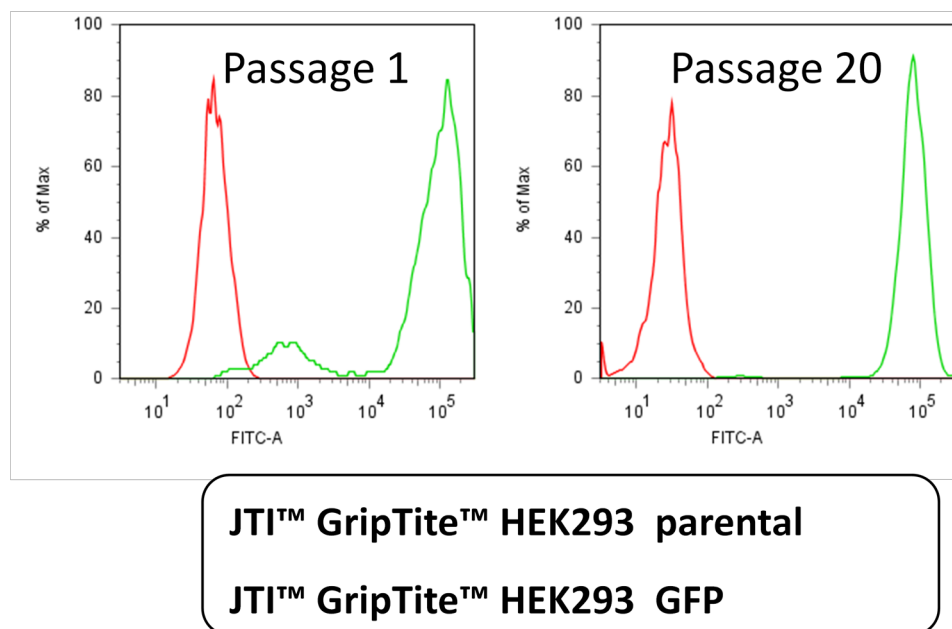
We strongly recommend that you include one sample with the pJTI™ R4 EXP CMV EmGFP pA vector as positive control in each retargeting experiment.

We also recommend that following characterization of your retargeted cell line you expand and bank at least 10 vials of retargeted Jump-In™ GripTite™ HEK293 cells in Recovery™ Cell Culture Freezing Medium as described on page 14.

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.



Troubleshooting

Culturing Jump-In™ GripTite™ HEK293 cells

The table below 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 9). |
| 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. | <ul style="list-style-type: none"> Use $10 \mu\text{g}/\text{mL}$ 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 (see page 37). |

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Troubleshooting, continued

Retargeting Jump-In™ GripTite™ HEK293 cells

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

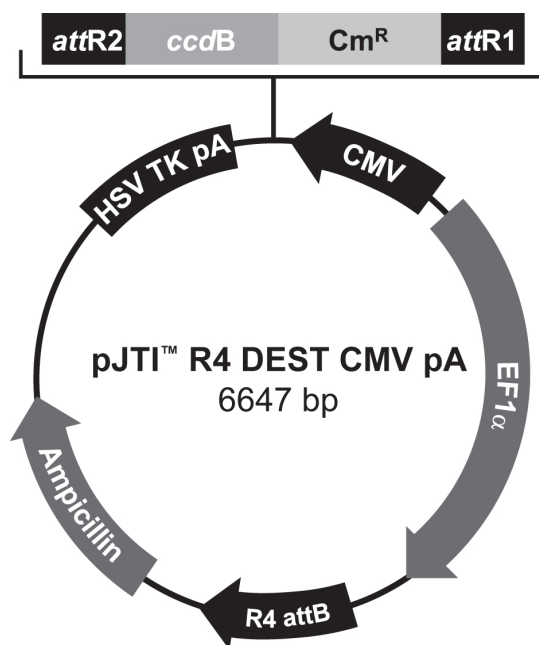
| Symptom | Cause | Solution |
|--------------------------------------|---|--|
| Low survival rate after transfection | Poor DNA quality | The quality of the retargeting construct DNA strongly influences the results of transfection experiments. Use endotoxin-free DNA for all transfections. Make sure 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 confluency often results in a higher proportion of dead cells in culture. Avoid excessive cell densities of high confluency. |
| | 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 manual |
| | Cells are damaged during harvesting and subsequent handling prior to transfection | <ul style="list-style-type: none"> • 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% confluency 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 | <ul style="list-style-type: none"> • Test cultures for Mycoplasma contamination. • Start a new culture from a fresh stock. |

Appendix

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 *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 Jump-In™ GripTite™ HEK293 genome, and the human EF1 α promoter for constitutive expression of Blasticidin resistance upon successful integration. The complete sequence of pJTI™ R4 DEST CMV pA is available from www.lifetechnologies.com or by contacting Technical Support (see page 43).



Features of pJTI™ R4 DEST CMV pA

6647 nucleotides

R4 *attB*: 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)

EF1 α promoter: bases 5418–6596

*(c): complementary strand

Continued on next page

pJTI™ R4 DEST CMV pA, continued

Features of pJTI™ R4 DEST CMV pA

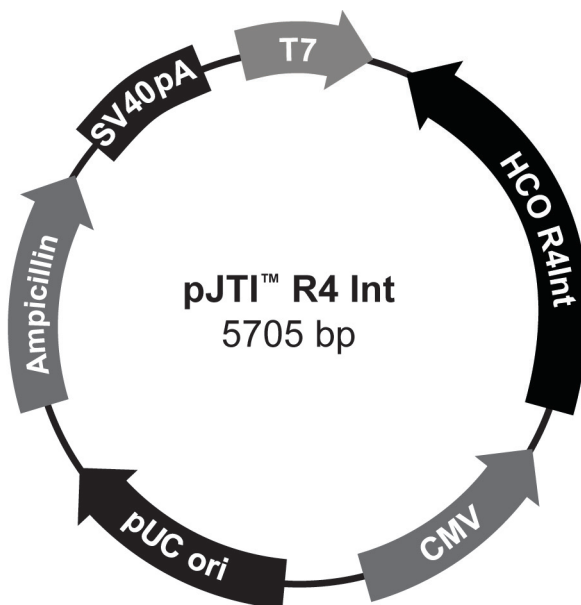
The pJTI™ R4 DEST CMV pA vector contains the following elements. All features have been functionally tested.

| Feature | Benefit |
|-------------------------------------|--|
| CMV promoter | Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987) |
| <i>attR1</i> and <i>attR2</i> 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 <i>et al.</i> , 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) |
| EF1 α 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 <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 gene | Allows the selection and propagation of the vector in <i>E. coli</i> |
| <i>ccdB</i> gene | Located between the two <i>attR</i> sites, allows for negative selection of the vector following the LR recombination reaction (Bernard & Couturier, 1992; Bernard <i>et al.</i> , 1993) |
| Chloramphenicol resistance gene | Located between the two <i>attR</i> 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.lifetechnologies.com or by contacting Technical Support (see page 43).



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

Continued on next page

pJTI™ R4 Int, continued

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 <i>et al.</i> , 2009) |
| CMV promoter | Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987) |
| pUC origin | Allows high-copy replication and maintenance in <i>E. coli</i> |
| Ampicillin resistance gene | Allows the selection and propagation of the vector in <i>E. coli</i> |
| SV40 polyadenylation signal | Allows transcript termination and polyadenylation of mRNA |

Propagating Jump-In™ Vectors

Propagating pJTI™ R4 DEST CMV pA

To propagate and maintain the pJTI™ R4 DEST CMV pA vector, we recommend using 10 ng of the vector to transform One Shot® *ccdB* Survival™ 2 T1^R Competent Cells, an *E. coli* strain resistant to *ccdB* effects that can support the propagation of plasmids containing the *ccdB* gene (see page 7 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 *ccdB* effects.

Propagating pJTI™ R4 Int

To propagate and maintain the pJTI™ R4 Int vector, we recommend using 10 ng of the vector to transform a *recA*, *endA* *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.

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).

Handling Blasticidin

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

Preparing and storing stock solutions

- Blasticidin is soluble in water and acetic acid.
 - Prepare a working stock solution of 5 to 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.
-

Assessing Blasticidin toxicity

We recommend using Blasticidin from Life Technologies at a concentration of 10 $\mu\text{g}/\text{mL}$ for selecting retargeted cells (for ordering information, see page 45). 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. Determine the Blasticiding toxicity using the Blasticidin Toxicity Assay with PrestoBlue™ Cell Viability Reagent (described on the next page), 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 9)
 - Blasticidin
 - 96-well cell culture plate
 - PrestoBlue™ Cell Viability reagent (see page 6 for ordering information)
-

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Blasticidin, continued

Blasticidin toxicity assay with PrestoBlue™ Cell Viability Reagent

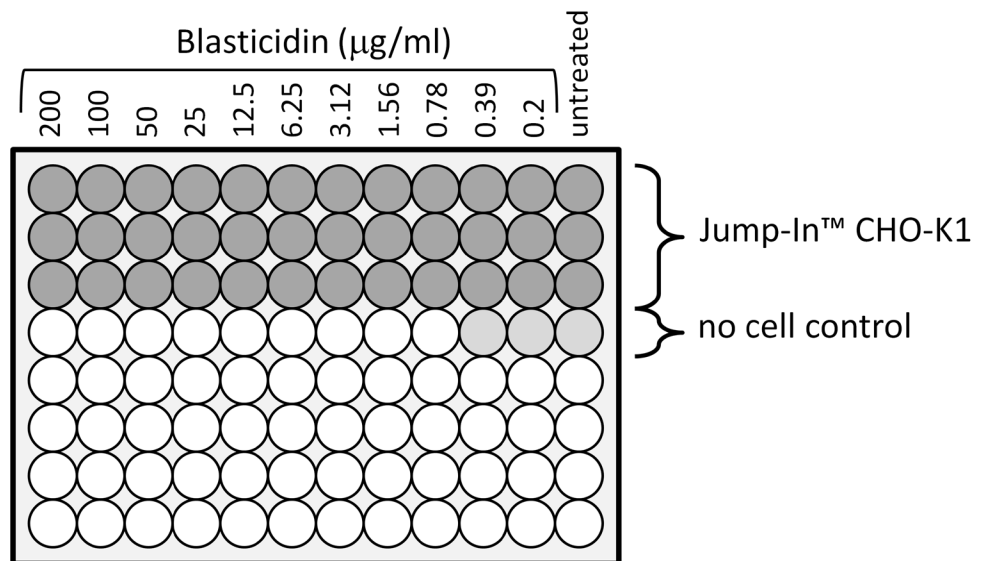
1. Harvest the Jump-In™ GripTite™ HEK293 cells by trypsinization as outlined in Propagating and Maintaining Jump-In™ GripTite™ HEK293 cells on page 12.
2. Prepare a cell suspension at a concentration of 500 cells/mL in Growth Medium.
3. Plate 100 µL of the cell suspension in three rows (3 × 12 wells) of a 96-well plate and incubate in a 37°C incubator with a humidified atmosphere of 5% CO₂ for 18 to 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 Blasticidin 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 µL of growth 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% CO₂.
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 (refer to www.lifetechnologies.com for more information on PrestoBlue™ Cell Viability Reagent). Prepare enough PrestoBlue™ reagent for 40 wells (36 wells for Blasticidin serial dilution and 3 wells for no cell control wells).
13. Remove the medium by aspiration and add 100 µL 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; for more information, refer to the instructions provided with the PrestoBlue™ Cell Viability Reagent, which are also available on our website at www.lifetechnologies.com).
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 the next page.

Continued on next page

Blasticidin, continued

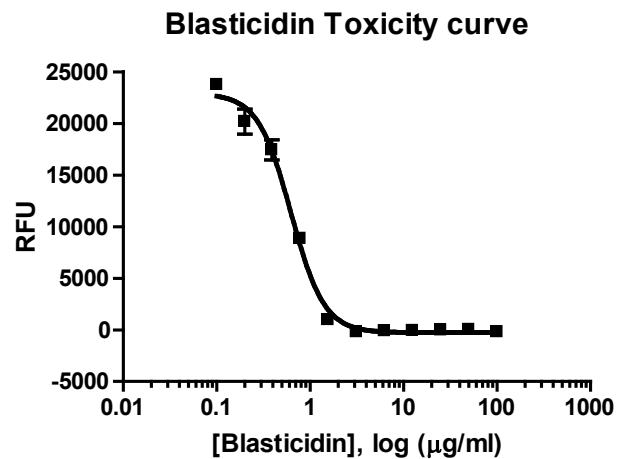
Blasticidin serial dilution

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 Life Technologies (see page 6 for ordering information)

Handling 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
-

Preparing and storing stock solutions

Hygromycin B is available from Life Technologies 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.

Optional: Confirming 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 7 for ordering information)
- PCR primers (see below for primer sequences)
- AccuPrime™ Taq DNA Polymerase High Fidelity (see page 7 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, Life Technologies offers a custom primer synthesis service. For more information, refer to our website (www.lifetechnologies.com) or contact Technical Support (page 43).

| 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' | |

Preparing 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.

Continued on next page

Optional: Confirming the R4 Site of Integration, continued

PCR protocol

1. Set up a PCR with the primers and conditions listed below, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see previous page 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™ <i>Taq</i> DNA Polymerase High Fidelity (5 U/µL) | 1 µL |
| Cell lysate (from step 6, above) | 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.

Technical Support

Obtaining support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

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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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