

Jump-In™ T-REx™ CHO-K1 Retargeting Kit

For targeted integration of your gene of interest in Jump-In™ T-REx™ CHO-K1 cells

Catalog Number A15007

Publication Number MAN0007277

Revision A.0



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Revision history: Pub. No. MAN0005450

Revision	Date	Description
A.0	21 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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Product information

Contents and storage

Kit contents and storage

The Jump-In™ T-REx™ CHO-K1 Retargeting Kit contains the following components.

Component	Amount/Composition	Storage
Jump-In™ T-REx™ CHO-K1 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-TO 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™ T-REx™ CHO-K1 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/bmb15/bmb15toc.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™ T-REx™ CHO-K1 platform cell line, and offers instructions and guidelines for:

- Maintaining the Jump-In™ T-REx™ CHO-K1 culture
- Using Gateway™ technology and the pJTI™ R4 DEST CMV-TO pA destination vector to generate a retargeting expression construct of your gene of interest
- Retargeting the Jump-In™ T-REx™ CHO-K1 platform cell line with the retargeting expression construct, and subsequent selection and expansion of retargeted cells
- Characterization and quality control of retargeted Jump-In™ T-REx™ CHO-K1 cells

Not covered by this user guide

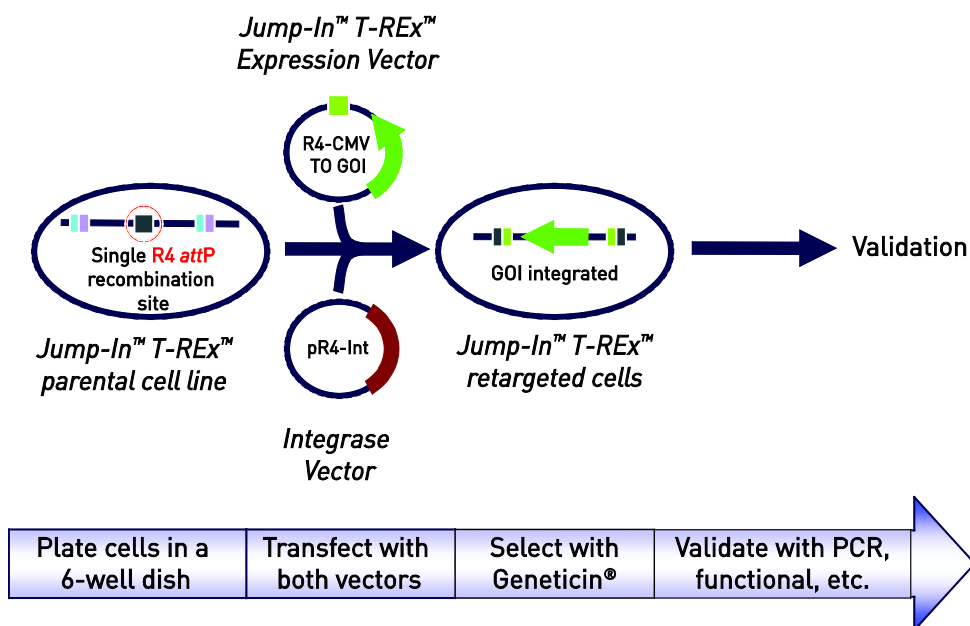
This user guide **does not** provide detailed instructions for generating entry clones, but presents guidelines and suggestions to help you obtain the best results when constructing an entry clone. For more information on the Gateway™ Technology and detailed instructions on generating an entry clone, 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 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™ T-REx™ CHO-K1 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™ T-REx™ parental cell line with your gene of interest (GOI)



Jump-In™ T-REx™ System

The Jump-In™ T-REx™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn10-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen *et al.*, 1983). We recommend the use of doxycycline in place of tetracycline for induction in the Jump-In™ T-REx™ System. Doxycycline is a water soluble form of tetracycline and works identically to induce of your gene of interest. Regulation in the Jump-In™ T-REx™ System is based on the binding of doxycycline to the Tet repressor and the derepression of the CMV promoter containing two tetracycline operator 2 (TetO₂) sites, allowing expression of the gene of interest (Yao *et al.*, 1998). After retargeting your inducible expression plasmid into Jump-In™ T-REx™ cells, expression of your gene of interest is inhibited until doxycycline is added to the growth media.

Jump-In™ T-REx™ cells

The Jump-In™ T-REx™ CHO-K1 cells supplied with this kit were generated by genomic integration of an R4 acceptor site and a “promoterless” Geneticin™ antibiotic resistance gene (Neo) 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. Clones with a single R4 integration site were validated for retargeting by transfection with the pJTI™ R4 EXP CMV-TO EmGFP pA (Cat. No. A15005) and JTI™ R4 Int vectors (included in this kit), followed by selection with Geneticin™ selective antibiotic for 2–3 weeks. Following induction with Doxycycline, retargeting efficiency was determined by analysis of green fluorescent protein expression using flow cytometry.

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

Jump-In™ T-REx™ vectors included in the kit

The following Jump-In™ vectors are included in the Jump-In™ T-REx™ CHO-K1 Retargeting Kit. For a map and features of each vector, and instructions for their propagation, see pages 39–42.

- pJTI™ R4 DEST CMV-TO 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 inducible CMV-TO promoter.

Note: To recombine your gene of interest into the pJTI™ R4 DEST CMV-TO 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 gene of interest into the parental target site when the Jump-In™ T-REx™ CHO-K1 parental cell line is cotransfected with both vectors for retargeting.

Jump-In™ T-REx™ Vectors not included in the kit

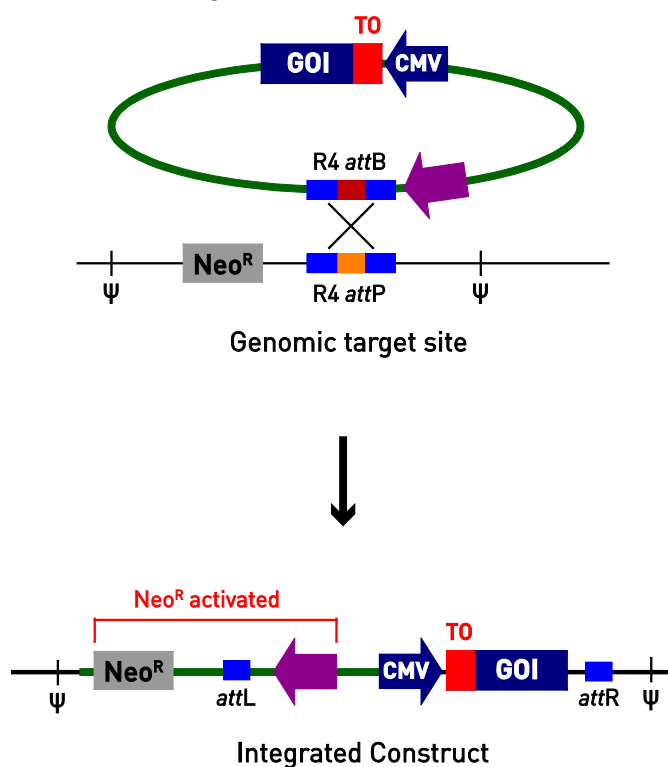
The table below lists the ordering information for additional Jump-In™ retargeting vectors that can be used with the Jump-In™ T-REx™ CHO-K1 cell line, including the GFP expression vector (positive control) and the multiple cloning site vector for restriction enzyme cloning of your gene of interest.

Product	Quantity	Cat. No.
pJTI™ R4 EXP CMV-TO EmGFP pA	100 µg	A15005
pJTI™ R4 CMV-TO MCS pA	100 µg	A15004

Jump-In™ targeted integration

The Jump-In™ T-REx™ CHO-K1 parental 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-TO pA vector using the Gateway™ Cloning Technology or the pJTI™ R4 CMV-TO MCS pA vector using restriction enzyme cloning) 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 parental cell line at the R4 *attP* target site. This integration event also positions the constitutive human EF1 α promoter upstream of the “promoterless” Geneticin™ antibiotic resistance gene, thus allowing the selection of successfully “retargeted” transformants using Geneticin™ antibiotic.

Figure 2 Targeted integration of the gene of interest (GOI) and a promoter for Geneticin™ (Neo) resistance gene.



Experiment Outline

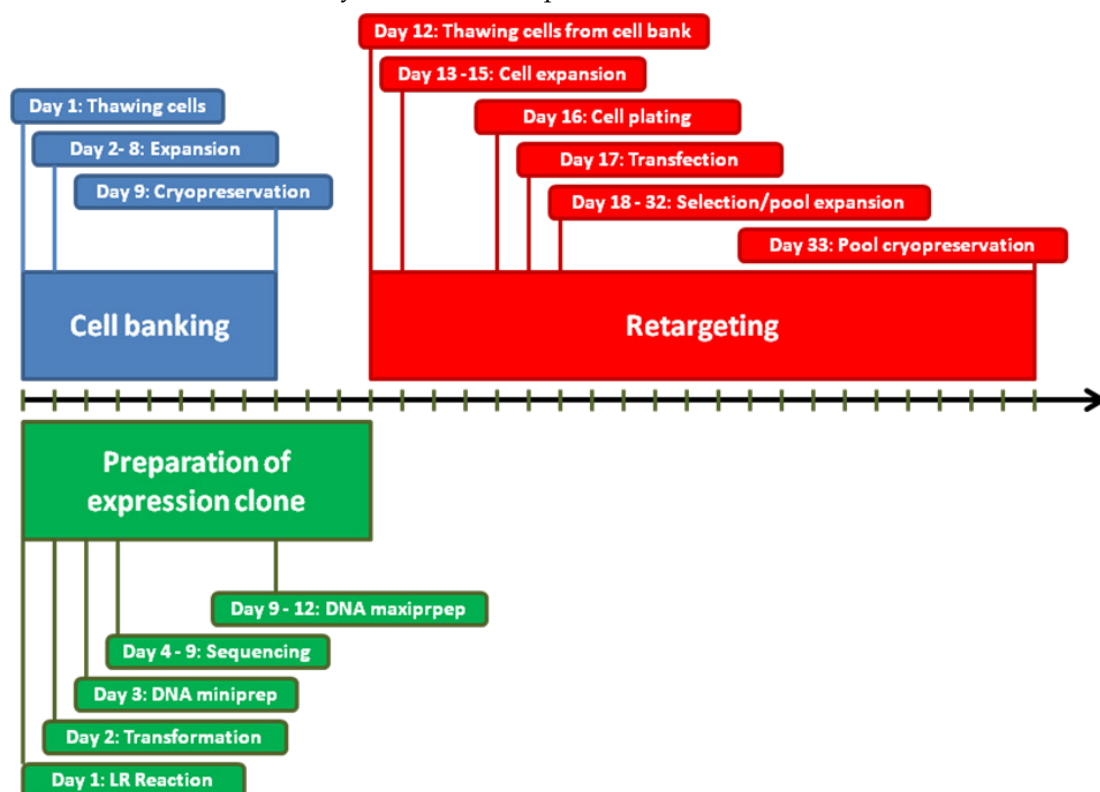
Workflow

The table below describes the major steps required for retargeting the Jump-In™ T-REx™ CHO-K1 cell line.

Step	Action	Page
1	Thaw and expand the Jump-In™ T-REx™ CHO-K1 cells	14
2	Create an entry clone by cloning your gene of interest into a Gateway™ entry vector	18
3	Generate a retargeting construct by performing an LR recombination reaction between the entry clone and pJTI™ R4 DEST CMV-TO pA vector (i.e., the destination vector)	19
4	Cotransfect your retargeting construct and the integrase vector into the Jump-In™ T-REx™ CHO-K1 cells	22
5	Select for retargeted Jump-In™ T-REx™ CHO-K1 cells in Selection medium containing Geneticin™	26
6	Confirm retargeting of the Jump-In™ T-REx™ CHO-K1 cells by PCR	28
7	Characterize the retargeted clones	30

Experiment timeline

Figure 3 Experiment timeline for retargeting the Jump-In™ T-REx™ CHO-K1 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™ T-REx™ CHO-K1 culture

We recommend the following accessory products for culturing, passaging, and maintaining the Jump-In™ T-REx™ CHO-K1 platform 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
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	AMQAF1000
Trypan Blue Stain	100 mL	10250061

Tetracycline – reduced serum

Note that many lots of fetal bovine serum (FBS) contain tetracycline, because FBS is generally isolated from cows that have been fed a diet containing tetracycline. If you culture your cells in medium containing FBS that is not reduced in tetracycline, you may observe low basal expression of your gene of interest in the absence of tetracycline.

We have cultured our mammalian cells in medium containing FBS that may not be reduced in tetracycline, and have observed undetectable-to-very low basal expression of GFP from the positive control vector in the absence of additional tetracycline. If your gene of interest produces a toxic protein, you may wish to culture your cells in tetracycline-reduced FBS. For more information, consult the supplier of your serum.

Transfection and selection

The table below lists ordering information for the products we recommend for transfection and selection of the Jump-In™ T-REx™ CHO-K1 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
Geneticin™ Selective Antibiotic (G418 Sulfate), 50 mg/mL Solution	20 mL	10131 035
Blasticidin S HCl Selection Antibiotic, (liquid)	10 × 1 mL	A1113903
Ampicillin, Sodium Salt	200 mg	11593027
PrestoBlue™ Cell Viability Reagent	25 mL	A13261

Induction and Expression

The table below lists ordering information for the products we recommend for induction and expression in the Jump-In™ T-Rex™ CHO-K1 platform cell line.

Product	Quantity	Cat. No.
Doxycycline*	1 g	MP Biomedicals, 19504401
* Prepare a 1 mg/mL solution in water and store in aliquots at –20°C.		

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 <i>E. coli</i>	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 <i>E. coli</i>	20 × 50 µL	C862003
One Shot™ TOP10 Chemically Competent <i>E. coli</i>	10 × 50 µL	C404010

PCR analysis

The following table lists ordering information about the products we recommend for PCR analysis of retargeted Jump-In™ CHO-K1 cells.

Product	Quantity	Cat. No.
CellsDirect Resuspension and Lysis Buffers*	1 kit	11739010
AccuPrime™ Taq DNA Polymerase High Fidelity**	200 reactions	12346086
DNAzol™ Reagent	100 mL	10503027
* 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™ T-REx™ CHO-K1 platform cell line

Guidelines for Jump-In™ T-REx™ CHO-K1 cell culture

Overview

The Jump-In™ T-REx™ CHO-K1 platform cell line, derived from the Chinese Hamster Ovary cell line (CHO-K1, 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™ T-REx™ CHO-K1 platform cells.

IMPORTANT! For optimal retargeting results, it is very important that you strictly follow the guidelines for culturing the Jump-In™ T-REx™ CHO-K1 cells provided in this user guide.

General guidelines for Jump-In™ T-REx™ CHO-K1 culture

Follow these general guidelines to grow and maintain the Jump-In™ T-REx™ CHO-K1 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™ T-REx™ CHO-K1 cells when they are near confluence.
- When thawing or sub-culturing cells, use pre-warmed medium for cell transfer.
- After initial thawing and passage, Jump-In™ T-REx™ CHO-K1 cells usually double in about 24 hours.
- Use 10 mL/L of antibiotic containing penicillin and streptomycin if required.
- Jump-In™ T-REx™ CHO-K1 cells can be adapted to suspension culture using CD OptiCHO™ Medium (Cat. No. 12681) following the recommended protocol.



IMPORTANT! The integration vector used in creating the Jump-In™ T-REx™ CHO-K1 platform cell line contains a hygromycin resistance gene (Hyg^R), making Jump-In™ T-REx™ CHO-K1 cells resistant to Hygromycin B. The Tet repressor is under the control of Blastidicin. If you want to integrate more genes stably into this cell line, do **not** use Hygromycin B or Blastidicin for selection for those genes.

Jump-In™ T-REx™ CHO-K1 media

Jump-In™ T-REx™ CHO-K1 media compositions

The table below lists the compositions of the various media required for culturing and retargeting Jump-In™ T-REx™ CHO-K1 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 9 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%	—	—
Hygromycin B†	—	200 µg/mL	—	—	—
Blasticidin	—	10 µg/mL	—	—	10 µg/mL
Geneticin™‡	—	—	—	—	5 mg/mL
<p>* Do not substitute.</p> <p>** Available together as Penicillin-Streptomycin, liquid from Thermo Fisher Scientific.</p> <p>† We recommend adding fresh selection antibiotics when the medium is added to the cells.</p> <p>‡ Because the concentration of Geneticin™ antibiotic required for effective selection of retargeted cells might vary depending on the source or the lot of Geneticin™, we recommend that you establish the sensitivity of Jump-In™ T-REx™ CHO-K1 cells to the Geneticin™ lot used for selection before conducting retargeting experiments (see page 33).</p>					



IMPORTANT! The CHO-K1 cell line requires proline for growth. Therefore, the use of MEM Non-Essential Amino Acids Solution as a media supplement is critical for the cultivation of CHO-K1 cells.

**Volumes required
for preparing
Jump-In™ T-REx™
CHO-K1 media**

The table below provides an example for the volumes required to prepare the media as outlined in the media composition table on page 12.

Note that the volumes provided in the table below is accurate only for Thermo Fisher Scientific products listed on pages 9–10.

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 **
Hygromycin B†	—	to 200 µg/mL	—	—
Blasticidin	—	10 µg/mL	—	10 µg/mL
Geneticin™‡	—	—	—	5 mg/mL
<p>* Do not substitute.</p> <p>** Available together as Penicillin-Streptomycin, liquid from Thermo Fisher Scientific.</p> <p>† We recommend adding fresh selection antibiotics when the medium is added to the cells.</p> <p>‡ Because the concentration of Geneticin™ antibiotic required for effective selection of retargeted cells might vary depending on the source or the lot of Geneticin™, we recommend that you establish the sensitivity of Jump-In™ T-REx™ CHO-K1 cells to the Geneticin™ lot used for selection before conducting retargeting experiments (page 33).</p>				

Thaw Jump-In™ T-REx™ CHO-K1 cells

- Materials required**
- Jump-In™ T-REx™ CHO-K1 cells (store frozen cells in liquid nitrogen until ready to use)
 - Thawing medium (page 12), pre-warmed to 37°C
 - Growth medium (page 12), 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™ T-REx™ CHO-K1 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 1–2 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.



IMPORTANT! Before starting experiments, we strongly recommend that you first expand and bank at least 10–20 vials of frozen Jump-In™ T-REx™ CHO-K1 cells in Recovery™ Cell Culture Freezing Medium as described on page 16.

For optimal retargeting efficiency, do not use the Jump-In™ T-REx™ CHO-K1 cells after 10 passages due to their increased resistance to Geneticin™ selection at high passage numbers.

Propagate and maintain Jump-In™ T-REx™ CHO-K1 cells

- Materials required**
- Growth medium (page 12), 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% 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™ II Automated Cell Counter)
 - Trypan blue

Propagate and maintain Jump-In™ T-REx™ CHO-K1 cells

Propagating and maintaining Jump-In™ T-REx™ CHO-K1 cells require passaging or feeding of cells at least twice a week and maintaining the cells between 5% and 95% confluence. To passage the Jump-In™ T-REx™ CHO-K1 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 ~2–10 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™ II 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 then resuspend in Growth medium.
8. Seed a 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 reach confluence.

Cryopreserve Jump-In™ T-REx™ CHO-K1 cells

- Materials required**
- Plates with Jump-In™ T-REx™ CHO-K1 cells
 - Dulbecco's Phosphate Buffered Saline (D-PBS) (1X)
 - Growth medium (page 12)
 - 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™
T-REx™ CHO-K1
cells**

1. Harvest the cells by trypsinization as described in “Propagate and maintain Jump-In™ T-REx™ CHO-K1 cells”, page 15.
 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.

Construct the retargeting expression vector

Before starting

Experiment overview

After you have expanded the Jump-In™ T-REx™ CHO-K1 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-TO 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 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-TO 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-TO 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.

Thermo Fisher Scientific offers a number of entry vectors, including the pENTR/D-TOPO™, that you can use to generate your 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 how to construct 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 are of good quality (see “Design PCR primers”). 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 overnight at 16°C 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™ technology. 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 *attB* 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)NNATGG

Create an expression clone

Overview

After you generate an entry clone, you will create your expression clone by performing the LR recombination reaction to transfer your gene of interest into the pJTI™ R4 DEST CMV-TO 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-TO pA vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
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, perform an LR recombination reaction between the entry clone and pJTI™ R4 DEST CMV-TO pA, then transform the reaction mixture into a suitable *E. coli* host to select for an expression clone.
- Include 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. Always add the LR clonase enzyme 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 can use any *recA*, *endA* *E. coli* strain including DH5α™, TOP10, or equivalent for transformation.

IMPORTANT! 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.

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.

Note: You can perform the LR recombination reaction using the 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 manual.

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. An LR recombination reaction between the pENTR™-gus plasmid and a destination vector (i.e., pJTI™ R4 DEST CMV-TO pA) generates an expression clone containing the gene encoding β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991).

Materials needed

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

Follow this procedure to perform the LR reaction between your entry clone and the pJTI™ R4 DEST CMV-TO 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	Pos. control
Entry clone (50–150 ng/reaction)	1 to 7 μL	—
pJTI™ R4 DEST CMV-TO 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 $\geq 1 \times 10^8$ 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*.

Confirm 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 are ampicillin-resistant and chloramphenicol-sensitive, while the transformants containing a plasmid with a mutated *ccdB* gene are 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 39).

Primer	Sequence
CMV promoter (forward)	5' -CGC AAA TGG GCG GTA GGC GTG-3'
V5 epitope (reverse)	5' -ACC GAG GAG AGG GTT AGG GAT-3'

Retarget the Jump-In™ T-REx™ CHO-K1 platform cell line

Before starting

Overview

The Jump-In™ T-REx™ CHO-K1 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-TO pA or the pJTI™ R4 CMV-TO MCS pA retargeting vector allows the efficient integration and expression of a gene of interest into the R4 target site on the Jump-In™ T-REx™ CHO-K1 genome. This section provides instructions for:

- Preparing the Jump-In™ T-REx™ CHO-K1 cells and the vector DNA for transfection
- Co-transfecting the Jump-In™ T-REx™ CHO-K1 cells with the pJTI™ R4 Int (integrase vector) and the retargeting construct (your expression vector based on pJTI™ R4 DEST CMV-TO pA or pJTI™ R4 CMV-TO MCS pA, or the GFP control vector pJTI™ R4 EXP CMV-TO EmGFP pA)
- Selecting, expanding, and characterizing the retargeted clones

General guidelines for retargeting experiments

Successful retargeting of Jump-In™ T-REx™ CHO-K1 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 Jump-In™ T-REx™ CHO-K1 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. We recommend that you visually evaluate the cells prior to transfection.

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 Geneticin™ from Thermo Fisher Scientific at a concentration of 5 mg/mL.

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

Determine the Geneticin™ toxicity using the Geneticin™ Toxicity Assay with PrestoBlue™ Cell Viability Reagent (described on page 34), which has been optimized for Jump-In™ T-REx™ CHO-K1 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™ T-REx™ CHO-K1 cells

Guidelines for transfection

The Jump-In™ T-REx™ CHO-K1 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™ T-REx™ CHO-K1 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-TO pA or pJTI™ R4 CMV-TO MCS 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™ T-REx™ CHO-K1 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 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 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-TO EmGFP pA positive control vector + pJTI™ R4 Int (integrase vector) (see page 6 for pJTI™ R4 EXP CMV-TO EmGFP pA ordering information)

Materials required

- Jump-In™ T-REx™ CHO-K1 cells
- Retargeting expression construct derived from pJTI™ R4 DEST CMV-TO pA or pJTI™ R4 CMV-TO MCS pA (Cat. No. A15004)
- pJTI™ R4 Int plasmid encoding the R4 Integrase
- pJTI™ R4 EXP CMV-TO EmGFP pA positive control vector
- 6-well plate
- Sterile 15-mL conical tube
- Trypsin, 0.05% (1X) with EDTA
- D-PBS without Ca²⁺ or Mg²⁺
- Lipofectamine™ LTX and PLUS™ Reagent
- Growth medium (page 12)
- Transfection medium (page 12)
- Opti-MEM™ I Reduced Serum Medium
- Countess™ II Automated Cell Counter or Trypan Blue Stain for exclusion counting

**Prepare Jump-In™
T-REx™ CHO-K1
cells for
transfection**

One day before transfection, prepare Jump-In™ T-REx™ CHO-K1 cells as follows:

1. Fill each well of a 6-well culture plate with 3.5 mL pre-warmed Growth medium (page 12) 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 ~2–10 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 transfer 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 × 10⁶ cells/mL.
8. Seed the Jump-In™ T-REx™ CHO-K1 cells into a 6-well culture plate so that they become ~80% confluent 24 hours later (~500,000 cells/well) in a volume of 4 mL Transfection medium (i.e., Growth medium without antibiotics; see page 12).
9. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO₂.

**Transfect Jump-In™
T-REx™ CHO-K1
cells**

Before starting

1. On the day of transfection, remove the spent medium from the 6-well plate containing the Jump-In™ T-REx™ CHO-K1 cells at ~80% confluence and replace it with 2 mL/well of pre-warmed Transfection medium (page 12).
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.

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-TO 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, then incubate the tubes for 5 minutes at room temperature.

Prepare transfection complexes

5. Mix the Lipofectamine™ LTX Reagent gently, then 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, then incubate the tubes for 30 minutes at room temperature.

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™ T-REx™ CHO-K1 cells in the 6-well plate.
8. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours before changing the medium, 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™ T-REx™ CHO-K1 cells

Guidelines for selection

- After transfection, incubate the cells for 24 hours before changing the medium, adding the selection antibiotics, or passaging the cells.
- To successfully select for your “retargeted” Jump-In™ T-REx™ CHO-K1 cells, you need to use the minimum concentration of Geneticin™ required to kill your untransfected Jump-In™ T-REx™ CHO-K1 cells.

We recommend using Geneticin™ from Thermo Fisher Scientific at a concentration of 5 mg/mL for selecting retargeted cells. However, if you prefer to use Geneticin™ from another source, we strongly recommend determining the effective toxic dose of each new Geneticin™ lot using a cytotoxicity assay before performing retargeting experiments (see 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 Geneticin™.

Selection protocol

16–24 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 (page 12) and allow the cells to attach for several hours.
2. Pre-warm the Selection medium (page 12) to 37°C.
Note: Selection medium does not contain Hygromycin B, but includes 5 mg/mL of Geneticin™ and 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 2–4 week of culture in Selection medium, negative control cell populations should be eliminated. The positive control (Sample C: pJTI™ R4 EXP CMV-TO EmGFP pA control vector) should contain >85% GFP positive cells after inducing cells for 24 hours by adding 1 µg/mL Doxycycline to the Growth medium. Varying numbers of cell colonies should emerge after 2 weeks in all retargeted wells.
6. After selection is complete, the expression of the gene of interest will be repressed until the addition of 1 µg/mL Doxycycline to the Growth medium for at least 16 hours.

***Optional: Isolate
retargeted clones***

Because the retargeting of the transgene reaches >95% of the cell population in most cases, isolation of a single retargeted clone 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 39).

**Propagating
retargeted clones**

Use pre-warmed Selection medium (**omit** Hygromycin B, but **include** Geneticin™ at 5 mg/mL and Blasticidin at 10 µg/mL), and follow the same propagation methods as outlined in “Propagate and maintain Jump-In™ T-REx™ CHO-K1 cells”, page 15.

Note: We recommend that you freeze a number of vials after the retargeted Jump-In™ T-REx™ CHO-K1 pool has been established.

Confirm retargeting events

Overview Upon retargeting the Jump-In™ T-REx™ CHO-K1 platform line, 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™ T-REx™ CHO-K1 genome introduces the human EF1 α promoter upstream of the *Neo* gene, resulting in Geneticin™ resistance of successfully retargeted clones. PCR amplification of the EF1 α promoter–Geneticin™ resistance gene junction in successfully retargeted clones using the recommended primers (for primer sequences, see “PCR primers”, page 28) results in a 528 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™ T-REx™ CHO-K1 genome in an optional PCR (page 37).

Materials required

- Retargeted Jump-In™ T-REx™ CHO-K1 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™ T-REx™ CHO-K1 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 39).

Target	Primer	Sequence	Expected size
EF1 α – <i>Neo</i> junction	EF1 α (forward)	5'–GCC TCA GAC AGT GGT TCA AAG TTT–3'	528 bp
	Geneticin™ (reverse)	5'–TCA GAG CAG CCG ATT GTC TG–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'	

Preparing retargeted clones for PCR

1. Pellet 10,000 to 30,000 retargeted Jump-In™ T-REx™ CHO-K1 cells by centrifugation.
2. Wash the cells by resuspending them in 500 μ L of PBS.
3. Centrifuge the cells to pellet and remove the PBS.
4. Resuspend the cell pellet in a mixture of 20 μ L of CellsDirect Resuspension Buffer and 2 μ L 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 μ L 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, 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.

Optional: Southern blot analysis

PCR is usually sufficient to confirm the presence of the retargeted sequences in Jump-In™ T-REx™ CHO-K1 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 the retargeted clones

Overview

While PCR and Southern blot analysis are usually sufficient to confirm the successful retargeting of the Jump-In™ T-REx™ CHO-K1 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.

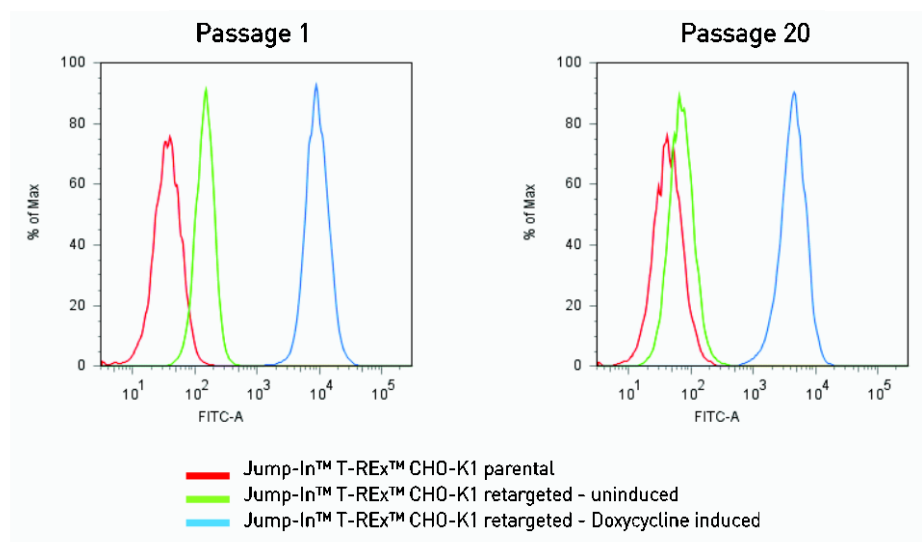
Include one sample with the pJTI™ R4 EXP CMV-TO EmGFP pA vector as positive control in each retargeting experiment. To analyze expression of the GFP control, cells must be induced by adding 1 µg/mL Doxycycline to the media. The best expression is observed 24 hours after induction.

Following characterization of your retargeted cell line, expand and bank at least 10 vials of retargeted Jump-In™ T-REx™ CHO-K1 cells in Recovery™ Cell Culture Freezing Medium (Cat. No. 12648010) as described on page 16.

FACS characterization

The following images and flow cytometry histograms provide an example of the sustained expression of a transgene (EmGFP) over long-term cultures of retargeted Jump-In™ T-REx™ CHO-K1 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 4 Jump-In™ T-REx™ CHO-K1 platform cell line was retargeted using a GFP cassette driven by a constitutive CMV-TO promoter. The retargeted cells were analyzed for EmGFP expression using flow cytometry. Retargeted cells were analyzed at passage 1 and 20 (after retargeting) before and after induction with 1 µg/mL Doxycycline.



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™ T-REx™ CHO-K1 cells.
Cells grow slowly	Thawing medium not correct	Use the specified Thawing medium (page 12).
	Growth medium not correct	Use correct Growth medium. CHO cells require the addition of non-essential amino acid supplement.
	Cells too old	Use healthy Jump-In™ T-REx™ CHO-K1 cells under passage 30. Do not overgrow.
No growth after transfection	Mycoplasma contamination	Discard the cells, media, and reagents, and use early stock of cells with fresh media and reagents.
	Incorrect amount of Geneticin™ is used for selection.	<ul style="list-style-type: none"> Use 5 mg/mL of Geneticin™ 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 Geneticin™. Because the concentration of Geneticin™ required for effective selection of retargeted cells might vary depending on the source or the lot of Geneticin™, we recommend that you establish the sensitivity of Jump-In™ T-REx™ CHO-K1 cells to the Geneticin™ lot used for selection before conducting retargeting experiments (page 33).

Retargeting Jump-In™ CHO-K1 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 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% 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	<ul style="list-style-type: none"> • Test cultures for Mycoplasma contamination. • Start a new culture from a fresh stock.

Appendix B: Support protocols

Geneticin™ selective antibiotic

Description	Geneticin™ selective antibiotic (also known as G-418 sulfate) blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial gene (APH), derived from Tn5, results in detoxification of Geneticin™ (Southern & Berg, 1982).
How to handle Geneticin™	Geneticin™ selective antibiotic is harmful and may cause sensitization by skin contact. It is irritating to eyes and skin. In case of contact with eyes, rinse eyes immediately with plenty of water and seek medical advice. Avoid contact with skin and eyes. Wear suitable protective clothing and gloves when handling Geneticin™ selective antibiotic and Geneticin™-containing solutions.
Prepare and store stock solutions	Follow the instructions provided with Geneticin™ selective antibiotic to prepare your working stock solution. Geneticin™ selective antibiotic in powder form should be stored at room temperature and at 4°C as a solution. The stability of Geneticin™ selective antibiotic is guaranteed for two years, if stored properly.
Assess Geneticin™ toxicity	<p>We recommend using Geneticin™ selective antibiotic from Thermo Fisher Scientific at a concentration of 5 mg/mL for selecting retargeted cells.</p> <p>However if you prefer to use Geneticin™ antibiotic from another source, we recommend determining the effective toxic dose of each new Geneticin™ selective antibiotic lot using a cytotoxicity assay before performing retargeting experiments.</p> <p>Determine the Geneticin™ antibiotic toxicity using the Geneticin™ Toxicity Assay with PrestoBlue™ Cell Viability Reagent (see page 34), which has been optimized for Jump-In™ T-REx™ CHO-K1 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.</p>
Materials required	<ul style="list-style-type: none">• 10-cm culture dish with Jump-In™ T-REx™ CHO-K1 cells• Growth medium (see page 12)• Geneticin™ selective antibiotic• 96-well cell culture plate• PrestoBlue™ Cell Viability reagent

**Perform Geneticin™
toxicity assay with
PrestoBlue™ Cell
Viability Reagent**

1. Harvest the Jump-In™ T-Rex™ CHO-K1 cells by trypsinization as outlined in “Propagate and maintain Jump-In™ T-Rex™ CHO-K1 Cells” on page 15.
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 18–24 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.

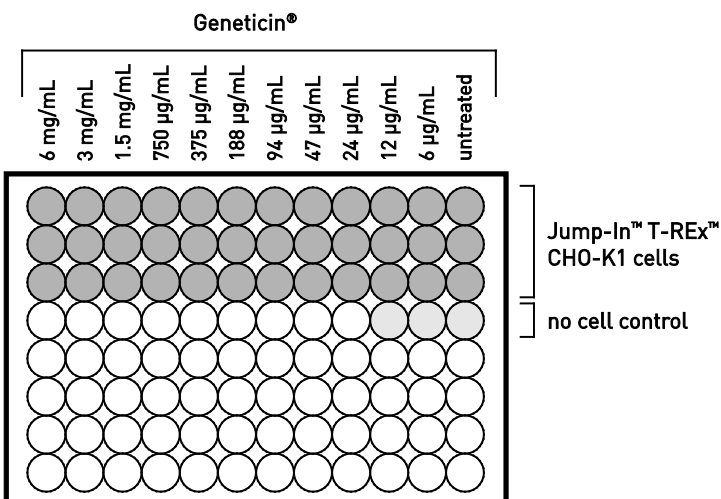
4. Prepare 1 mL of a 6 mg/mL Geneticin™ antibiotic stock solution in Growth medium in a 1.5-mL microcentrifuge tube. This is “Tube 1” of the serial dilution.
5. Prepare 10 additional 1.5-mL microcentrifuge tubes (numbered 2 to 11) containing 0.5 mL of Growth medium.
6. Prepare a 11-point two-fold serial dilution by transferring 0.5 mL of the Geneticin™ stock solution from tube 1 to tube 2 (mix by pipetting), then 0.5 mL from tube 2 to tube 3, and so on, until you have reached tube 11.
7. Add 100 µL of each dilution to one column of the 96-well plate containing the cells (e.g., 6 mg/mL of Geneticin™ in Column 1, 3 mg/mL Geneticin™ in Column 2, 1.5 mg/mL Geneticin™ in Column 3, etc.). Add 100 µL of Growth Medium to Column 12 as untreated control (see diagram page 35).
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 Geneticin™ serial dilution as described in steps 4–7.
11. Repeat media exchange cycle (steps 8 through 10) three times (for 8–10 days).
12. Prepare PrestoBlue™ reagent by diluting it 1:10 in Growth medium. Prepare enough PrestoBlue™ reagent for 40 wells (36 wells for Geneticin™ 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 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).
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.

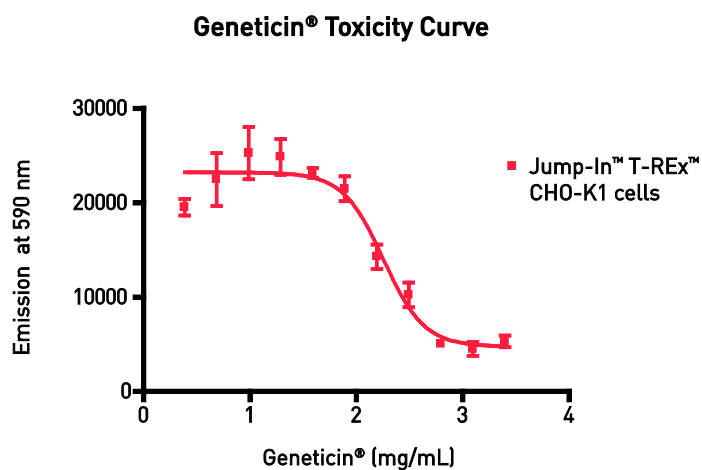
Geneticin™ serial
dilution

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



Geneticin™ toxicity curve

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



Hygromycin B

Description	The integration vector used in creating the Jump-In™ T-REx™ CHO-K1 platform cell line contains the hygromycin resistance gene (Hyg ^R) (Gritz & Davies, 1983; Palmer <i>et al.</i> , 1987), making Jump-In™ T-REx™ CHO-K1 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.
How to handle Hygromycin B	<ul style="list-style-type: none">• 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.

Doxycycline

Description	Doxycycline is used to induce gene expression in Jump-In™ T-REx™ cell lines. Doxycycline is a water soluble substitute for tetracycline.
How to handle Doxycycline	<ul style="list-style-type: none">• Doxycycline is light sensitive. Store stock solution at 4°C protected from exposure to light.• Doxycycline is harmful. Do not ingest solutions containing the drug.• Wear gloves, a laboratory coat, and safety glasses or goggles when handling doxycycline and doxycycline containing solutions.
Prepare and store stock solutions	Doxycycline is available from MP Biomedicals as a 1 g powder stock. Make a 1 mg/mL doxycycline stock solution in water, aliquot and store at –20°C. Doxycycline stock solution is stable at –20°C for 3 to 4 months.

Optional: Confirm the R4 site of integration

Overview The Jump-In™ T-REx™ CHO-K1 platform line was created by the PhiC31-mediated site-specific integration of the R4 retargeting sequences into the genome of CHO K1 cells. Follow the guidelines below for the optional assay to confirm the presence of R4 retargeting site in the Jump-In™ T-REx™ CHO-K1 genome.

Materials required

- Retargeted Jump-In™ T-REx™ CHO-K1 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 the site-specific integration of the R4 retargeting sequences into the genome of CHO-K1 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 39).

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™ T-REx™ CHO-K1 cells using the CellsDirect Resuspension and Lysis Buffers as described below.

1. Pellet 10,000 to 30,000 retargeted Jump-In™ T-REx™ CHO-K1 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 37 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.

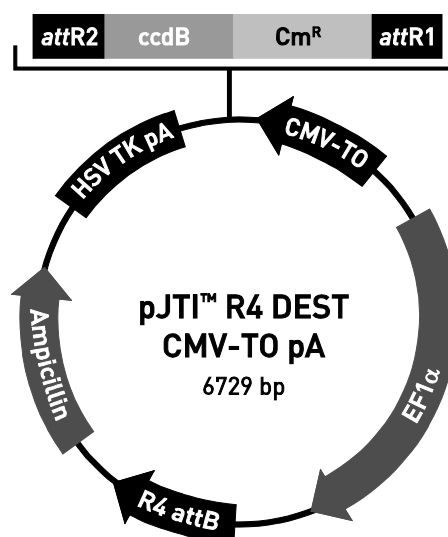
Appendix C: Vectors

pJTI™ R4 DEST CMV-TO pA

Map of pJTI™ R4 DEST CMV-TO pA

The pJTI™ R4 DEST CMV-TO pA vector (6,729 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™ T-REx™ CHO-K1 genome, and the human EF1 α promoter for constitutive expression of the Geneticin™ resistance gene upon successful integration.

The complete sequence of pJTI™ R4 DEST CMV-TO pA is available for download at www.thermofisher.com or by contacting Technical Support (page 39).



Features of pJTI™ R4 DEST CMV-TO pA Vector 6729 nucleotides

R4 *attB*: bases 28–322
Ampicillin resistance gene: bases 474–1331
HSV TK pA: bases 2532–2803 (c)*
attR2 recombination site: bases 2908–3032
ccdB gene: bases 3073–3378 (c)
Chloramphenicol resistance gene: bases 3720–4379 (c)
attR1 recombination site: bases 4488–4612 (c)
2X TetO₂: bases 4754–4793 (c)
CMV promoter: bases 4794–5380 (c)
EF1 α promoter: bases 5500–6678

*(c): complementary strand

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

Feature	Benefit
CMV-TO 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) The CMV promoter contains two tetracycline operator 2 (TetO ₂) sites allowing inducible expression of the gene of interest (Yao <i>et al.</i> , 1998)
<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)
EF1α promoter	Drives the high level expression of the Geneticin™ 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

Propagate pJTI™ R4 DEST CMV-TO pA To propagate and maintain the pJTI™ R4 DEST CMV-TO pA vector, use 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. 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.

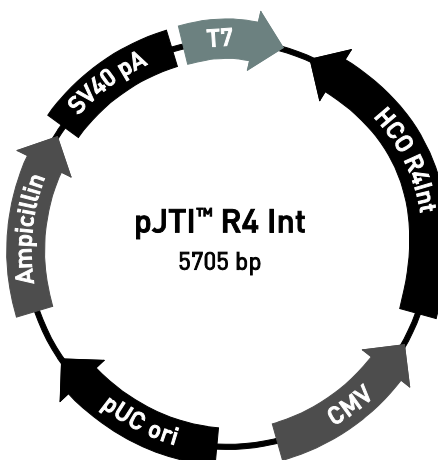
Note: Do not use general *E. coli* cloning strains (such as TOP10 or DH5α™) for propagation and maintenance of pJTI™ R4 DEST because these strains are sensitive to *ccdB* effects.

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™ T-REx™ CHO-K1 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 for download at www.thermofisher.com or by contacting Technical Support (page 39).



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 Geneticin™ resistance gene to the <i>attP</i> 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

Propagate pJTI™ R4 Int

To propagate and maintain the pJTI™ R4 Int vector, use 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.

Documentation and support

Obtaining support

Technical Support	<p>For the latest services and support information for all locations, go to www.thermofisher.com.</p> <p>At the website, you can:</p> <ul style="list-style-type: none">• 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)	<p>Safety Data Sheets (SDSs) are available at www.thermofisher.com/sds.</p> <p>IMPORTANT! For the SDSs of chemicals not distributed by Thermo Fisher Scientific contact the chemical manufacturer.</p>
Limited Product Warranty	<p>Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.</p>

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 <i>attL1</i> and <i>attL2</i> 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 <i>attB1</i> and <i>attB2</i> 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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