invitrogen™

Jump-In™ TI™ Gateway® Targeted Integration System

MultiSite Gateway®-adapted Vector System for Generation of Isogenic Stable Mammalian Cell Lines

Catalog nos. A10895, A10896, and A10897

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User Manual

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Kit Contents and Storage

Introduction

This manual provides guidelines and instructions for generating isogenic stable mammalian cell lines, and is supplied with the products listed below.

Product	Cat. no.
Jump-In™ TI™ Gateway® System	A10895
Jump-In™ TI™ Gateway® Vector Kit	A10896
Jump-In™ TI™ Platform Kit	A10897

System Components

Each product contains the following components. For a detailed description of the contents of each component, see vi–viii.

Component	Cat. no.		
	A10895	A10896	A10897
Jump-In™ TI™ Platform Kit	√		√
Jump-In™ TI™ Gateway® Vector Kit	√	√	
MultiSite Gateway® Pro Plus Kit	√		
Jump-In™ TI™ Gateway® System Manual	√	√	√

Shipping/Storage

The Jump-In™ TI™ Gateway® System and all its components are shipped on dry ice. Upon receipt, store each component as detailed below. All reagents are guaranteed for a minimum of six months if stored properly.

Item	Shipping	Storage
Vectors	Dry ice	**–20°C**
LR Clonase™ II Plus Enzyme Mix	Dry ice	**–20°C (6 months)** **–80°C (long term)**
BP Clonase™ II Enzyme Mix	Dry ice	**–20°C (6 months)** **–80°C (long term)**
One Shot® Mach1™ Chemically Competent *E. coli*	Dry ice	**–80°C**

Important

Jump-In™ TI™ (**T**argeted **I**ntegration) Gateway® System Kit is designed to help you genetically engineer stable isogenic cell lines that express multiple genetic elements of interest using the Jump-In™ and MultiSite Gateway® Technologies. **Although the kits have been designed to help you construct your cell engineering vectors in the simplest, most direct fashion, as well as to perform transfection and selection procedures to generate your recombinant cell line expressing your gene(s) of interest in the most efficient way, the use of these products is geared towards users who are familiar with the concepts of the Gateway® Technology, site-specific recombination, and culturing mammalian and stem cells.** If you are unfamiliar with these technologies, we recommend that you acquire a working knowledge of the Gateway® Technology, and mammalian and stem cell culture.

Continued on next page

Kit Contents and Storage, continued

Kit Components

The Jump-In™ TI™ Gateway® System contains the following components. The contents of each kit component are described below.

Jump-In™ TI™ Platform Kit

The Jump-In™ TI™ Platform Kit supplied with the Jump-In™ TI™ Gateway® System is also available individually (Cat. no. A10897). It contains the vectors used for platform cell line generation. **Store the vectors at –20°C.**

Vector	Composition	Amount
pJTI™/Bsd	20 µl of vector at 500 ng/µl in TE buffer, pH 8.0	10 µg
pJTI™/Neo	20 µl of vector at 500 ng/µl in TE buffer, pH 8.0	10 µg
pJTI™/Zeo	20 µl of vector at 500 ng/µl in TE buffer, pH 8.0	10 µg
pJTI™ PhiC31 Int	20 µl of vector at 500 ng/µl in TE buffer, pH 8.0	10 µg

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Jump-In™ TI™ Gateway® Vector Kit

The Jump-In™ TI™ Gateway® Vector Kit supplied with the Jump-In™ TI™ Gateway® System is also available individually (Cat no. A10896). It contains the vectors for retargeting the platform cell line. **Store the vectors at –20°C.**

Vector	Composition	Amount
pJTI™ R4 DEST	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	6 µg
pJTI™ R4 Int	20 µl of vector at 500 ng/µl in TE buffer, pH 8.0	10 µg

Continued on next page

Kit Contents and Storage, continued

MultiSite Gateway® Pro Plus Vector Module

The following vectors and primers are supplied with the MultiSite Gateway® Pro Plus Vector Module for creating the entry and expression clones in a multi fragment recombination reaction. **Store the contents of the vector module at –20°C**.

Vector	Composition	Amount
pDONR™ 221 P1-P5r	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pDONR™ 221 P5-P2	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pDONR™ 221 P1-P4	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pDONR™ 221 P4r-P3r	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pDONR™ 221 P3-P2	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pDONR™ 221 P5-P4	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ L1-pLac-lacZalpha-R5	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ L5-pLac-Spect-L2	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ L1-pLac-lacZalpha-L4	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ R4-pLac-Spect-R3	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ L3-pLac-Tet-L2	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
pENTR™ L5-LacI-L4	60 µl of vector at 100 ng/µl in TE Buffer, pH 8.0	6 µg
M13 (–20) Forward primer	20 µl of primer at 100 ng/µl in TE Buffer, pH 8.0	2 µg
M13 Reverse primer	20 µl of primer at 100 ng/µl in TE Buffer, pH 8.0	2 µg
pDONR™ 221	Lyophilized in TE Buffer, pH 8.0	6 µg

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Kit Contents and Storage, continued

LR Clonase™ II Plus Enzyme Mix

The following reagents are supplied with LR Clonase™ II Plus enzyme mix. Store **at –20°C for up to 6 months. For long-term storage, store at –80°C.**

Item	Composition	Amount
LR Clonase™ II Plus Enzyme Mix	Proprietary	40 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl

BP Clonase™ II Enzyme Mix

The following reagents are supplied with BP Clonase™ II enzyme mix. **Store at –20°C for up to 6 months. For long-term storage, store at –80°C.**

Item	Composition	Amount
BP Clonase™ II Enzyme Mix	Proprietary	40 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl
30% PEG/Mg solution	30% PEG 8000/30 mM $MgCl_2$	1 ml
pEXP7-tet	50 ng/µl in TE Buffer, pH 8.0	20 µl

One Shot® Mach1™ T1^R Chemically Competent *E. coli*

The following reagents are included with the One Shot® Mach1™ T1^R Chemically Competent *E. coli.* **Store the competent cells at –80°C.**

Reagent	Composition	Amount
Mach1™ T1^R chemically competent cells	–	21 × 50 µl
S.O.C. Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Genotype of Mach1™ T1^R

F$^-$ ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R($r_K^- m_K^+$) Δ*rec*A1398 *end*A1 *ton*A

Accessory Products

Introduction

The products listed in this section may be used with the Jump-In™ TI™ Gateway® System. For accessory products that may be used with the MultiSite Gateway® Pro Plus Vector Module, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit. For more information, refer to our website at www.invitrogen.com or contact Technical Support (see page 42).

Media and Buffers for Cell Culture

We recommend the following media and buffers for culturing, passaging, and maintaining your mammalian and stem cell cultures. For more information on these and other cell culture products available from Invitrogen, refer to our website at www.invitrogen.com or contact Technical Support (see page 42).

Product	Amount	Cat. no.
Dulbecco's Modified Eagle Medium (D-MEM)	500 ml	11965-092
Dulbecco's Modified Eagle Medium (D-MEM) high glucose with L-glutamine and sodium pyruvate	500 ml	11995-065
D-MEM/F-12 containing GlutaMAX™ (1X), liquid	500 ml	10565-018
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985-062 31985-070
OptiPRO™ SFM (1X)	1000 ml	12309-019
CD CHO Medium	1000 ml	10743-029
CD 293 Medium	1000 ml	11913-019
293 SFM II	1000 ml	11686-029
CD DG44 Medium	1000 ml	12610-010
StemPro® hESC SFM Complete Medium (contains StemPro® supplement, D-MEM/F-12 with GlutaMAX™, 25% BSA, FGF basic, and 2-mercaptoethanol)	1 kit	A1000701
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid (Ca- and Mg-free)	500 ml 1000 ml 10 × 500 ml	14190-144 14190-136 14190-250
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid (contains Ca and Mg)	500 ml 10 × 500 ml	14040-133 14040-182
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml 1000 ml	10010-023 10010-031

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Accessory Products, continued

Serum and Supplements for Cell Culture

We recommend the following accessory products for culturing, passaging, and maintaining your mammalian cell and embryonic stem cell cultures. For more information on these and other cell culture products available from Invitrogen, refer to www.invitrogen.com or contact Technical Support (see page 42).

Product	Amount	Cat. no.
GlutaMAX™-I Supplement	100 ml	35050-061
200 mM L-Glutamine	100 ml	25030-081
MEM Non-Essential Amino Acids Solution 10 mM (100X)	100 ml	11140-050
HT Supplement	50 ml	11067-030
bFGF (FGF Basic, Human Recombinant)	50 µg	PHG0026
Fetal Bovine Serum, Certified	500 ml	16000-044
Fetal Bovine Serum, Qualified	500 ml	26140-079
Fetal Bovine Serum, ES Cell-Qualified (US)	500 ml	16141-079
Pluronic F-68, 10% (100X)	100 ml	24040-032
Knockout™ Serum Replacement (KSR)	500 ml	10828-028
Bovine Albumin Fraction V Solution (7.5%)	100 ml	15260-037
BSA, 10% Ultrapure Molecular Biology Grade	1000 ml	P2458
2-Mercaptoethanol	50 ml	21985-023

Fetal Bovine Serum, ES Cell-Qualified

Invitrogen also provides ES Cell-Qualified Fetal Bovine Serum originating from countries other than the US. These can be more appropriate for your situation, and may be used to maintain your stem cell culture. For more information, refer to www.invitrogen.com.

Mitomycin C Treated MEFs

Mitomycin C treated, Hygromycin resistant primary MEFs are available from Millipore (Cat. no. PMEF-H) or ATCC (SCRC-1045.2). Hygromycin resistant primary MEF that are **not** Mitomycin treated are also available separately from Millipore (Cat. no. PMEF-HL) or ATCC (Cat. no. SCRC-1045). One vial of cells (~5×10^6–6×10^6 cells/vial) can be used to plate ten 60-mm dishes. MEFs which are not mitotically arrested must be treated with Mitomycin C before use. Mitomycin C is available separately from Sigma, St. Louis (Cat no. M4287).

Porcine Skin Gelatin

Porcine Skin Gelatin can be obtained from Sigma, St. Louis (Cat no. G1890).

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Accessory Products, continued

Additional Products

For more information on the following accessory products, refer to our website at www.invitrogen.com or contact Technical Support (see page 42).

Product	Amount	Cat. no.
Trypsin-EDTA (0.05% Trypsin, EDTA•4Na) (1X), liquid	100 ml 20 × 100 ml	25300-054 25300-120
Versene-EDTA (0.05% Versene, EDTA•4Na) (1X), liquid	100 ml	15040-066
TrypLE™ Express Dissociation Enzyme without Phenol Red	100 ml 20 × 100 ml	12604-013 12604-039
Antibiotic-Antimycotic (100X), liquid	100 ml	15240-062
Penicillin-Streptomycin	100 ml	15070-063
Lipofectamine™ 2000 Transfection Reagent	1.5 ml 15 ml	11668-019 11668-500
Geltrex™	5 ml	12760-021
Geltrex™, hESC qualified	1 ml	A10480-01
Collagenase Type IV	1 g	17104-019
StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit	100 reactions	23191-050
StemPro® EZPassage™ Disposable Stem Cell Passaging Tool	10 tools (disposable)	23181-010
Anti-Clumping Agent	20 ml	01-0057AE
LIVE/DEAD® Cell Vitality Assay Kit	1000 assays	L34951
Trypan Blue Stain	100 ml	10250-061
ProLong® Gold Antifade Reagent	10 ml	P36930
ProLong® Gold Antifade Reagent with DAPI	10 ml	P36931
CellsDirect Resuspension and Lysis Buffers	1 kit	11739-010
AccuPrime™ *Taq* DNA Polymerase High Fidelity	1000 reactions	12346-094
DNAzol® Reagent	100 ml	10503-027
HEPES Buffer Solution (1M)	20 ml 100 ml 20 × 100 ml	15603-106 15630-080 15630-130
Quant-iT™ dsDNA Assay Kit (0.2–100 ng)	1 kit	Q-33120
UltraPure™ Glycogen	100 µl	10814-010
UltraPure™ Salmon Sperm DNA Solution (10 mg/ml)	5 × 1 ml	15632-011
UltraPure™ 20X SSC	1000 ml	15557-044
UltraPure™ 10% SDS Solution	4 × 100 ml	15553-027
Water, distilled	500 ml	15230-162

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Accessory Products, continued

Selection Agents

The table below lists ordering information for the selection agents required for use with the Jump-In™ TI™ Gateway® System Kits.

Product	Amount	Cat. no.
Hygromycin B	20 ml	10687-010
Blasticidin S HCl	50 mg	R210-01
Geneticin®, powder	1 g 5 g 25 g	11811-023 11811-031 11811-098
Geneticin®, liquid	20 ml 100 ml	10131-035 10131-027
Zeocin™	1 g 5 g	R250-01 R250-05

MultiSite Gateway® Pro Kits

Invitrogen offers several MultiSite Gateway® Pro kits for rapid construction of expression clones containing your choice of up to four separate DNA elements, which allow the opportunity to perform pathway reconstitution, multiple gene expression and regulation, and protein interaction studies. All MultiSite Gateway® Pro kits are compatible with the pJTI™ vectors included in the Jump-In™ TI™ Gateway® System kits. Each kit supplies enough reagents for 20 recombination reactions.

Product	Cat. no.
MultiSite Gateway® Pro 2.0 Kit for 2-fragment recombination	12537-102
MultiSite Gateway® Pro 3.0 Kit for 3-fragment recombination	12537-103
MultiSite Gateway® Pro 4.0 Kit for 4-fragment recombination	12537-104
MultiSite Gateway® Pro Plus Kit for 2-, 3- or 4-fragment recombination	12537-100

Competent Cells

The table below lists ordering information for competent *E. coli* cells that can be used to propagate your vectors.

Product	Amount	Cat. no.
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 reactions	A10460
One Shot® Mach1™ T1^R Chemically Competent Cells	20 × 50 µl	C8620-03
One Shot® TOP10 Chemically Competent Cells	10 × 50 µl	C4040-10
E-Shot™ DH10B™-T1^R Electrocompetent Cells	20 × 25 µl	C5100-03

Introduction

Overview

Introduction

The Jump-In™ TI™ (**T**argeted **I**ntegration) Gateway® System combines Invitrogen's MultiSite Gateway® Pro cloning and Jump-In™ cell engineering technologies for efficient generation of isogenic mammalian cell lines by enabling irreversible insertion of multiple genetic elements (such as promoter-reporter pairs) at specific locations in the mammalian genome.

For a detailed explanation of the technology behind the Jump-In™ TI™ Gateway® System, see **Jump-In™ TI™ Gateway® Cell Engineering Technology** on the next page.

Components of the Jump-In™ TI™ Gateway® System

The Jump-In™ TI™ Gateway® System consists of the following components:

- The Jump-In™ TI™ Platform Kit for the generation of a stable platform cell line that can later be retargeted using an expression construct containing your genetic elements of interest. The Jump-In™ TI™ Platform Kit consists of three platform vectors, pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo, expressing the blasticidin, neomycin, and zeocin resistance markers, respectively, and the pJTI™ PhiC31 Int vector that expresses the PhiC31 Integrase. For a map and features of each vector, see pages 30–33.
- The MultiSite Gateway® Pro Plus Vector Module for simultaneous cloning of up to four DNA fragments to generate a retargeting construct. Based on the Gateway® Technology (Hartley *et al.*, 2000; Sasaki *et al.*, 2005; Sasaki *et al.*, 2004) the MultiSite Gateway® uses site-specific recombinational cloning to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation.
- The Jump-In™ TI™ Gateway® Vector Kit for retargeting of platform cell lines. The Jump-In™ TI™ Gateway® Vector Kit consists of the pJTI™ R4 DEST vector (*i.e.*, the "retargeting construct" when containing your DNA elements of interest) and the pJTI™ R4 Int vector expressing the R4 Integrase. For a map and features of each vector, see pages 34–35. For the recombination region of the pJTI™ R4 DEST, see page 17.

Note

In addition to the complete Jump-In™ TI™ Gateway® System (Cat. no. A10895) containing all the components listed above, Invitrogen also offers the individual component kits as stand alone products (Jump-In™ TI™ Gateway® Vector Kit, Cat. no. A10896, and Jump-In™ TI™ Platform Kit, Cat. no. A10897). For more information on the Jump-In™ TI™ Gateway® System and its component kits, visit our website at www.invitrogen.com or contact Technical Support (page 42).

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Overview, continued

Jump-In™ TI™ Gateway® Targeted Integration Technology

The Jump-In™ TI™ (**T**argeted **I**ntegration) technology uses PhiC31 integrase-mediated recombination to stably integrate DNA sequences of choice at specific genomic locations called pseudo-*att*P sites in mammalian cells. Unlike the better-known recombinases such as Cre and Flp, PhiC31 integrase catalyzes recombination between two non-identical sites. Further, the lack of a corresponding excisionase enzyme makes the integration events catalyzed by PhiC31 unidirectional and virtually irreversible. The Jump-In™ TI™ Platform Kit, as part of the Jump-In™ TI™ Gateway® System, places a target in the chromosomal DNA for a second site-specific integration event mediated by the R4 Integrase (*i.e.*, "retargeting").

The first step in targeted integration is the creation of the R4 platform line. This is accomplished by the PhiC31 integrase-mediated, site-specific insertion of the R4 integrase target sequences (*i.e.*, *att*P) along with the Hygromycin resistance gene from a pJTI™ platform vector. The pJTI™ platform vector also contains the sequences for resistance against a second selection agent (blasticidin, neomycin, or zeocin resistance genes in pJTI™/Bsd, pJTI™/Neo, or pJTI™/Zeo, respectively), but lacks the promoter to express from this resistance gene. Transformants containing the desired R4 "*att*P retargeting sequences" and the promoterless selection marker are selected using Hygromycin B and expanded for the "retargeting event." Step 1 on the next page schematically depicts platform line creation.

The second step in targeted integration is the retargeting event mediated by the R4 integrase expressed from the pJTI™ R4 Int vector. At this step, the genetic elements of interest carried by the retargeting expression construct (generated from pJTI R4 DEST using the MultiSite Gateway® Pro Plus Vector Module, see pages 14–18) are site-specifically integrated into the platform line genome at the R4 *att*P target site (introduced into the cell line at the first step). This integration event also positions the constitutive human EF1α promoter upstream of the blasticidin, neomycin, or zeocin resistance gene (*i.e.*, "promoterless" selection marker), thus allowing the selection of successfully "retargeted" transformants using the appropriate selection agent. Step 2 on the next page depicts retargeting of the platform line.

For more information on PhiC31 and R4 Integrases, and their uses in targeted integration refer to our website at www.invitrogen.com and published literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001).

In addition to the Jump-In™ TI™ Gateway® System, which enables the rapid creation of isogenic stable cell lines, Invitrogen also offers the Jump-In™ Fast Gateway® System (Cat. no. A10893) which facilitates the generation of a polyclonal pool of mammalian cells that over-expresses your protein of interest. In the Jump-In™ Fast Gateway® System, your gene of interest is directly inserted into the genome in a single recombination step mediated by the PhiC31 integrase.

For more information on Jump-In™ TI™ Gateway® and Jump-In™ Fast Gateway® Systems, visit our website at www.invitrogen.com or contact Technical Support (page 42).

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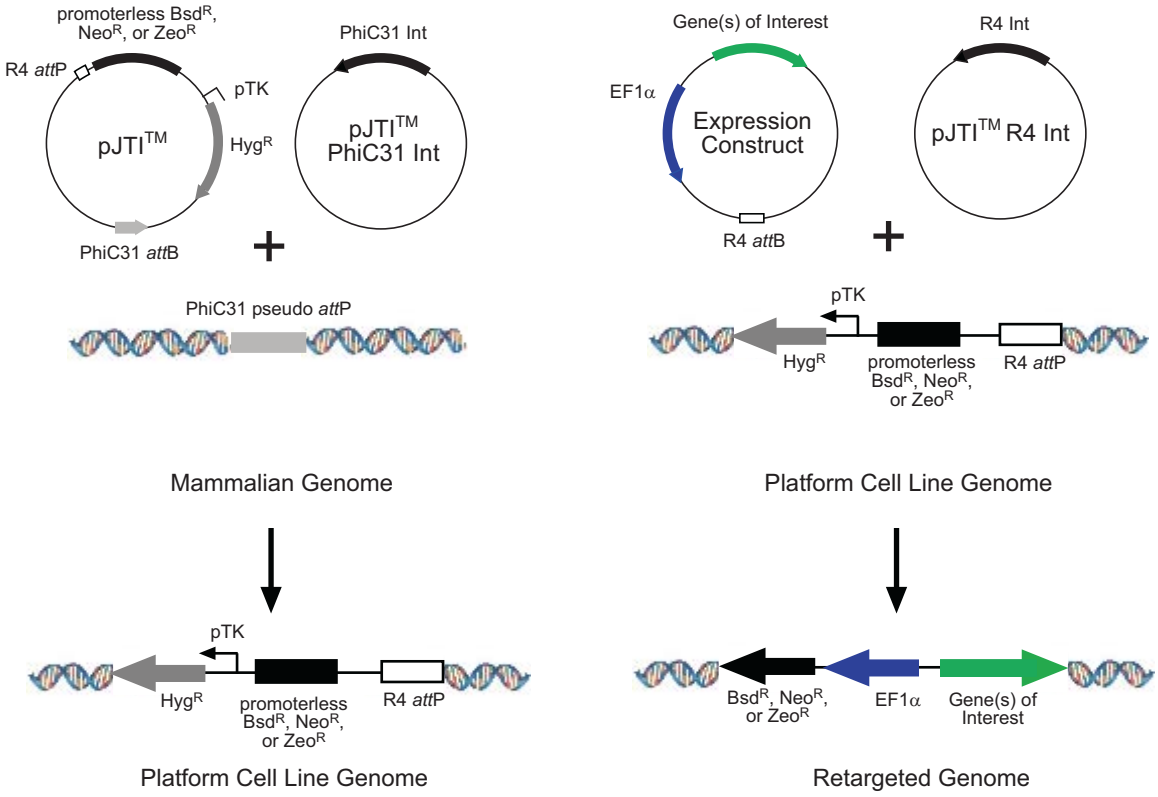
Overview, continued

Jump-In™ TI™ Gateway® System Workflow

The schematic below depicts the major steps of the targeted integration reaction using the Jump-In™ TI™ Gateway® System.



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Overview, continued

Purpose of This Manual

This manual provides an overview of the Jump-In™ TI™ Gateway® System, and offers instructions and guidelines for:

- Generating, selecting, and expanding your platform cell line using the Jump-In™ TI™ Platform Kit
- Creating your "retargeting expression construct" using the MultiSite Gateway® Pro Plus Vector Module module and the Jump-In™ TI™ Gateway® Vector Kit
- Retargeting your platform line with your retargeting expression construct using the Jump-In™ TI™ Gateway® Vector Kit, and the subsequent selection and expansion of your retargeted cell line
- Characterization and quality control of your cell line after targeted integration events (*i.e.*, platform line creation and retargeting)

This manual **does not** provide detailed protocols for maintaining your mammalian cell culture as each cell line behaves differently under different laboratory conditions. However, you will find general instructions on maintaining your cells before and after the retargeting events, and suggestions and tips on cell culture to ensure successful targeted integration experiments.

For more information about the MultiSite Gateway® Technology, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit. For more information on targeted integration, see published literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001). For more information on culturing mammalian cell lines and human stem cells, refer to www.invitrogen.com or contact Technical Support (see page 42).

Important

Jump-In™ TI™ Gateway® Kit is designed to help you genetically engineer stable isogenic mammalian cell lines that express multiple genetic elements of interest using the Jump-In™ Targeted Integration and MultiSite Gateway® Technologies. **Although the kits have been designed to help you construct your cell engineering vectors in the simplest, most direct fashion, as well as to perform transfection and selection procedures to generate your recombinant cell line expressing your gene(s) of interest in the most efficient way, the use of these products is geared towards users who are familiar with the concepts of the Gateway® Technology, site-specific recombination, and culturing mammalian and stem cells.** If you are unfamiliar with these technologies, we recommend that you acquire a working knowledge of the Gateway® Technology and methods for maintaining mammalian cell cultures and stem cells.

Methods

General Information

Introduction

This section provides instructions and guidelines for creating and retargeting your platform cell line using the Jump-In™ TI™ Gateway® System, as well as the subsequent selection and expansion. It also includes general information on maintaining your mammalian or stem cell culture before and after each transformation. **However, we recommend that you to tailor your cell culture protocols to the specific needs and requirements of your particular cell line, as these vary considerably between different cell lines and under different laboratory conditions.**

This manual **does not** provide instructions for generating the retargeting construct using MultiSite Gateway® Technology. For instructions on designing and creating the retargeting construct, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit.

For more information on the MultiSite Gateway® Technology and general cell culture maintenance, visit our website at www.invitrogen.com or contact Technical Support (see page 42).

Propagating Jump-In™ TI™ Gateway® System Vectors

To propagate and maintain the pJTI™ R4 DEST vector, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (see page xii) from Invitrogen. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

To propagate and maintain the pJTI™ R4 Int, pJTI™ PhiC31 Int, pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo vectors, we recommend using 10 ng of each vector to separately transform a *rec*A, *end*A *E. coli* strain like 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.

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

For information on propagating and maintaining the pDONR vectors included in the MultiSite Gateway® Pro Plus Vector Module, refer top the MultiSite Gateway® Pro manual supplied with Jump-In™ TI™ Gateway® System. The MultiSite Gateway® Pro manual is also available online at www.invitrogen.com or by contacting Technical Support (see page 42).

Important

Preparation of Plasmid DNA: For targeted integration experiments, it is essential that the plasmid DNA used for transfection is of very high quality. Typically, best results have been obtained using plasmid DNA that has **very low levels of endotoxins**. If using large quantities of DNA, we recommend that the plasmid DNA is commercially prepared. If smaller quantities are required, use a commercial kit that delivers **pure DNA that is free of endotoxins**. Follow the manufacturer's recommended protocol for DNA preparation.

Continued on next page

General Information, continued

When working with mammalian cells, including stem cells, handle 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*, 4th ed., published by the Centers for Disease Control, or see the following web site: www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

General Cell Handling

For established cell lines (*e.g.*, HeLa, COS-1) consult original references or the supplier of your cell line for detailed instructions on maintaining your cells and the optimal method of transfection. Pay particular attention to the exact medium requirements, when to passage the cells, and at what dilution to split the cells. The guidelines below are general instructions that pertain to many cell lines; for best results, **we recommend that you follow the protocols of your cell line exactly**.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper aseptic technique and work in a laminar flow hood.
- Before starting experiments, be sure to have your cells established (at least 5 passages) and also have at least 10–20 vials of frozen stocks on hand. We recommend using early-passage cells for your experiments.
- For general maintenance of cell culture, passage your cells when they are near confluence (>80–90% confluent). Avoid overgrowing cells before passaging.
- Use Trypan Blue exclusion or the LIVE/DEAD® Cell Vitality Assay (Cat. no. L34951) to determine cell viability. Log phase cultures should be >90% viable.
- When thawing or subculturing, transfer your cells into pre-warmed medium.
- 10 µl/ml of antibiotic-antimycotic containing penicillin, streptomycin, and amphotericin B may be used if required (see page xi for ordering information).
- Cells should be at the appropriate confluence (usually 70–90% confluency in a 60-mm dish) and at greater than 90% viability prior to transfection.
- If you are using stem cells in your experiments, you must maintain your culture on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells or in an appropriate medium conditioned on a MEF feeder layer (MEF-CM) for at least two weeks, and as a feeder-free culture on MEF-CM for at least one passage prior to transfection. Make sure to start preparing the feeder layer two days before culturing your stem cells.
- It is crucial to allow your cells to recover for at least one day after transfection before you start selection with the appropriate agent.

Important

If you are using stem cells, it is very important to strictly follow the guidelines for culturing your stem cells to keep them undifferentiated.

Generating the R4 Platform Cell Line

Introduction

The first step in targeted integration is the generation of the R4 platform line, which is accomplished by cotransfecting the pJTI™ PhiC31 Int vector (expressing the PhiC31 integrase) and one of the platform vectors (pJTI™/Bsd, pJTI™/Neo, or pJTI™/Zeo, depending on your choice of selection agent for retargeting) into your mammalian or stem cells. Since the platform vector also contains the Hygromycin resistance gene driven by the thymidine kinase promoter, transformants with the desired retargeting sequences are selected in media containing Hygromycin B. This section provides instructions and guidelines for generating the R4 platform line.

For a map and features of the pJTI™ PhiC31 Int vector and of the each platform vector (pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo), see pages 30–33. **The vector sequences of pJTI™ PhiC31 Int, pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo are available on our website at www.invitrogen.com or by contacting Technical Support (see page 42).**

Important

You will select stable transformants containing the R4 retargeting sequences by their resistance to Hygromycin B. You **will not** use blasticidin, Geneticin® (G-418, a neomycin analog), or Zeocin™ to select for your "platform line" as the genes that confer resistance to these agents are promoterless and cannot be expressed at this stage. You will use blasticidin, Geneticin®, or Zeocin™ resistance to select for successfully "retargeted" clones after the second integration step, which will position a constitutive human EF1α promoter upstream of the appropriate resistance gene (see the schematic on page 3).

Hygromycin B

All pJTI™ platform vectors contain the *E. coli* hygromycin resistance gene (*HPH*) (Gritz & Davies, 1983) for selection of transfectants with the antibiotic, Hygromycin B (Palmer *et al.*, 1987). When added to cultured mammalian cells, Hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin B is available separately from Invitrogen (see page xii for ordering information).

CAUTION

- 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 Hygromycin B

Follow the instructions provided with Hygromycin B to prepare your working stock solution. The stability of Hygromycin B is guaranteed for six months, if stored at 4°C **in the dark**. Medium containing Hygromycin B is stable for up to six weeks.

Continued on next page

Generating the R4 Platform Cell Line, continued

Determining the Hygromycin B Sensitivity

To successfully generate an R4 platform cell line containing the R4 *att*P retargeting sequence, you need to determine the minimum concentration of Hygromycin B required to kill your untransfected cells. Typically, concentrations ranging from
10 to 400 µg/ml of Hygromycin B are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to determine the minimum concentration necessary for your cell line of choice.

1. Plate or split a confluent plate so that the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Hygromycin B (0, 10, 50, 100, 200, 400, 600 µg/ml).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Hygromycin B that kills the cells within 1–2 weeks after the addition of Hygromycin B.

Method of Transfection

For established cell lines, consult original references or the supplier of your cell line for optimal method of transfection. Methods of transfection include lipid-mediated transfection (Felgner *et al.*, 1989; Felgner & Ringold, 1989), calcium phosphate precipitation (Chen & Okayama, 1987; Wigler *et al.*, 1977), and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988).

We have achieved satisfactory results with two nonviral gene delivery methods, lipid-mediated transfection using Lipofectamine™ 2000 (see page xi for ordering information), and electroporation or microporation. Both methods do not seem to affect the growth characterisitics of the cells; however, certain variant stem cell lines are refractory to transfection by Lipofectamine™ 2000. Note that if you use calcium phosphate or lipid-mediated transfection methods, the amount of total DNA required for transfection is typically higher than for electroporation.

We have obtained the **best results** using **high-efficiency transfection methods such as microporation or electroporation,** and we recommend that you use these methods as well.

Continued on next page

Transfection Considerations

The following factors are important for successful transfection:

- **Cells**: Cells that are 80–90% confluent are ideal for transfection. A higher confluency often results in a higher proportion of dead cells in culture. Carry out a live/dead assay using either FACS (LIVE/DEAD® Cell Vitality Assay Kit, see page xi for ordering information) or Trypan Blue exclusion counting. For more information on how to distinguish metabolically active cells from cells that are dead or injured using the LIVE/DEAD® Cell Vitality Assay Kit, refer to **Assessing Cell Vitality** on page 36 in the **Appendix**.
- **Quality of 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 using 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.
- **Amount of DNA**: We generally use 10 µg **total** plasmid DNA per 1×10^6–8×10^6 cells per transfection, but the amount of plasmid DNA may vary depending on the nature of the cell line, the transfection efficiency of your cells, and the method of transfection used. When transfecting your mammalian cell line of choice, we recommend that you try a range of plasmid DNA concentrations to optimize transfection conditions for your cell line.

Important

If you are transforming stem cells, you must maintain your culture on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells or in an appropriate medium conditioned on a MEF feeder layer (MEF-CM) for at least two weeks, and as a feeder-free culture on MEF-CM for at least one passage prior to transfection. Make sure to start preparing the feeder layer two days before culturing your stem cells.

Transfection Procedure

You may use any of the recommended procedures to co-tranfect pJTI™ PhiC31 Int and pJTI™/Bsd, pJTI™/Neo, or pJTI™/Zeo into your cell line of choice. Follow the manufacturer's recommendations for transfection. Be sure to follow the guidelines outlined below:

- Remember to include negative controls where either the PhiC31 integrase vector or the platform vector is omitted.
- Plate the transformed cells in 60-mm culture dishes containing the appropriate medium and allow the cells to recover **without selection** for at least 24 hours, if you have used lipid-mediated transfection, or 48–72 hours, if you have used electroporation or microporation.
- Wash the cells and provide with fresh medium every day.
- Each colony recovers at a different rate. Monitor morphology and size of the colonies.
- When your targeted cells have recovered from transfection and the colonies are well-defined, proceed to **Selecting Stable Integrants**, next page.

Continued on the next page

Generating the R4 Platform Cell Line, continued

Selecting Stable Integrants

After your cells have sufficiently recovered from transfection, proceed with Hygromycin B selection as described below. Use the medium appropriate for your cell line.

1. 48 to 72 hours after transfection, transfer your cells into 100-mm dishes containing fresh medium. Split cells such that they are no more than 25% confluent as the selection antibiotics work best at actively dividing cells.
2. Incubate the cells at 37°C for 2–3 hours until they have attached sufficiently to the culture dish.
3. Remove the medium and add fresh medium containing the appropriate amount of Hygromycin B (see page 8).
4. Feed the cells with selective medium every 2–3 days until foci can be identified. Depending on the cell line, colonies will start appearing as early as day 5 of drug selection. Mark the colonies and observe them for an additional period of time (total of 12–21 days under selection).
5. Manually pick single, well-defined colonies and expand using the appropriate medium under selection for further analysis.

Screening R4 Platform Cell Line Clones

Introduction

The PhiC31 integrase catalyzes recombination between two nonidentical sites and lacks a corresponding excisionase enzyme, thus making the integration event unidirectional, and ensuring that the constructs integrated into the genome do not act subtsrates for the reverse reaction. Therefore, the Hygromycin B resistance conferred to your cell line by the integration of the platform vector and the subsequent selection in selective medium virtually guarantees that your clones contain the R4 retargeting sequence. However, you may still screen your expanded clones by Southern blot analysis to ascertain that only a single integration event has taken place, and by PCR analysis for the presence of R4 retargeting sequences.

Southern Blot Analysis

You can use Southern blot analysis to determine the number of integrations in each of your Hygromycin B-resistant clones. When performing Southern blot analysis, you should consider the following factors:

- **Probe:** We recommend that you use a fragment of the Hygromycin resistance gene (~1 kb) as the probe to screen your samples. You may amplify the Hygromycin expression cassette from one of the pJTI™ platform vectors using the appropriate primers. To label the probe, we generally use a standard random priming kit (*e.g.*, Ambion, DECAprime II™ Kit, Cat. no. 1455). Other random priming kits are suitable.
- **Genomic DNA:** We recommend using the DNAzol® Reagent (see page xi) to isolate the genomic DNA from the Hygromycin B-resistant clones.
- **Restriction digest:** When choosing a restriction enzyme to digest the genomic DNA, we recommend choosing an enzyme that cuts at a single known site outside of the Hygromycin resistance gene in the pJTI™ platform vector used (such as *Bam*H I or *Hin*d III). Hybridization of the Hygromycin probe to the digested DNA should then allow you to detect a single band containing the Hygromycin resistance gene from pJTI™ platform vector if only one integration event has occurred.
- **Protocol:** You may use any Southern blotting protocol of your choice. Refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) for detailed protocols.

What You Should See

If you digest genomic DNA from your transfectants with an appropriate restriction enzyme that cuts at a single known site outsite the Hygromycin resistance gene, and use a Hygromycin resistance gene fragment as a probe in your Southern analysis, you should be able to easily distinguish between single and multiple integration events.

- DNA from single integrants should contain only one hybridizing band corresponding to a single copy of the integrated pJTI™ platform vector.
- DNA from multiple integrants should contain more than one hybridizing band. If the pJTI™ platform vector integrates into multiple chromosomal locations, the bands may be of varying sizes.

Continued on next page

Screening R4 Platform Cell Line Clones, continued

PCR Analysis

When performing PCR analysis on the genomic DNA isolated from your R4 platform line clones, you should consider the following factors:

- We recommend using nested PCR with primary and secondary reactions to eliminate the high background observed with only primary PCR.
- You should design your primers for the R4 retargeting sequence from the R4 *att*P site to the appropriate resistance marker (Bsd, Neo, or Zeo, depending on the platform vector used). You may use the Hygromycin resistance gene from the plasmid DNA as a positive control.
- For a map and a description of the features of each platform vector (pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo) and of the pJTI™ PhiC31 Int vector, see pages 30–33.
- The vector sequences of pJTI™/Bsd, pJTI™/Neo, pJTI™/Zeo, and pJTI™ PhiC31 Int vectors are available on our website at www.invitrogen.com or by contacting Technical Support (see page 42).
- We recommend a high fidelity thermostable DNA polymerase such as the AccuPrime™ *Taq* DNA Polymerase for the nested PCR (see page xi for ordering information).
- Be sure to include a final extension step (7 minutes at 72°C) in your PCR.
- Follow the protocol below to prepare genomic DNA from crude cell lysates for your PCR.

  Note: Other genomic DNA isolation methods are also suitable.

Preparation of Genomic DNA for PCR

1. Pellet a total of 10,000 to 30,000 cells.
2. Wash the cells with 500 µl PBS.
3. Centrifuge cells to pellet and remove PBS.
4. Resuspend the cell pellet in a mixture of 20 µl of Resuspension Buffer and 2 µl of Lysis Solution (CellsDirect Resuspension and Lysis Buffers, see page xi).
5. Incubate the cell suspension at 75°C for 10 minutes.
6. Centrifuge for 1 minute to pellet cell debris.
7. Use 3 µl of the cell lysate to set up your PCR.

What You Should See

Successful integration of the pJTI™ platform vector into the genome of your cell line will result in a PCR product representing the amplified DNA sequence between the R4 *att*P site and the respective selection marker (Bsd, Zeo or Neo).

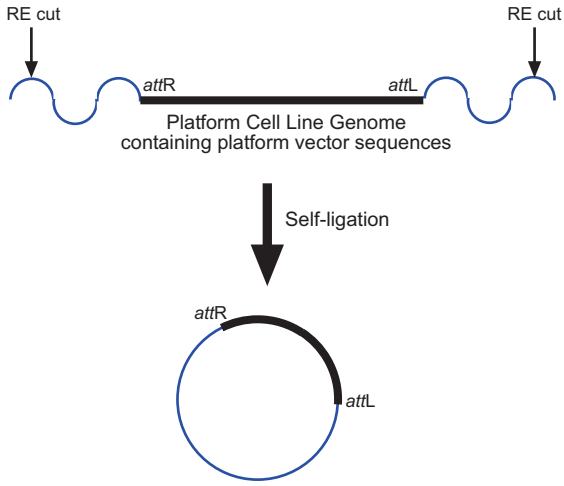
Freezing R4 Platform Cells

We highly recommend that you freeze and bank at least 10–20 vials of your R4 platform cells once you have expanded the cell line and confirmed that a single integration event has occurred. For instructions on cryopreserving your R4 platform cell line see page 37, **Freezing Mammalian Cells**, in the **Appendix.**

Determining Site of Integration

Introduction

To determine the site of integration in the genome, you can perform a plasmid rescue assay and map the site of integration by comparing the recovered sequences to the genomic sequences of your cell line. The figure below schematically depicts the plasmid rescue assay, where the thin lines represent the genomic DNA from your cell line prior to targeting, and the bold lines represent the integrated pJTI™ platform vector sequences (adapted from Chalberg *et al.*, 2006).



Plasmid Rescue Assay

1. Isolate genomic DNA from individual Hygromycin B-resistant clones grown to confluency using your preferred method.
2. Digest the genomic DNA with a restriction enzyme that does not cut within the pJTI™ platform vector you have used. Stop the restriction digest by heat inactivation. If the restriction enzyme cannot be heat-inactivated, perform a phenol:chloroform extraction of the genomic DNA and ethanol precipitate.
3. Incubate the restriction fragments with T4 DNA ligase overnight at 16°C under dilute conditions that favor self-ligation.
4. Extract the DNA from the ligation mixture with phenol:chloroform, ethanol precipitate the DNA, and resuspend in water.
5. Electroporate a fraction (25%) of the ligated DNA into DH10B™-T1^R electrocompetent *E. coli* (see page xii for ordering information) using the recommended conditions for the electroporator.
6. Plate electroporated cells on LB-agar plates containing 100 µg/ml ampicillin.
7. Isolate the plasmid DNA from resulting colonies, and sequence with the following primer to the PhiC31 *att*B site :

   5’-TCC CGT GCT CAC CGT GAC CAC-3’
8. Determine the genomic integration site by matching the sequence read to the database at BLAT (www.genome.ucsc.edu/cgi-bin/hgBlat).

Constructing the Retargeting Expression Vector

Introduction

Once you have established your R4 platform cell line and confirmed that a single integration event has occurred, you may proceed to retargeting your platform line by cotransfecting with your "retargeting expression construct" and the pJTI™ R4 Int vector to generate a stable, isogenic cell line expressing your genetic elements of interest. This section provides suggestions and helpful hints for generating the retargeting expression construct.

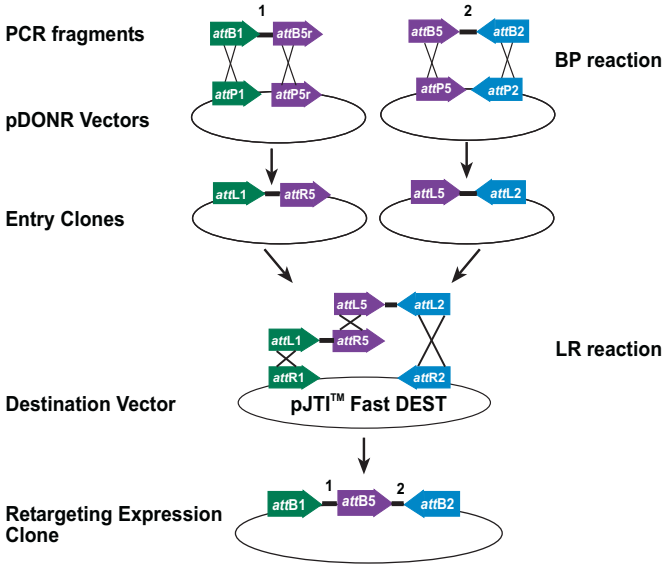
Important

For generating the retargeting construct using MultiSite Gateway® Technology, follow the protocol as outlined in the MultiSite Gateway® Pro manual (25-0942) supplied with the kit. **This section does not provide instructions for generating the retargeting construct, but provides additional comments and suggestions to help you obtain the best results in multi-fragment vector construction.** Note that the successful assembly of more than 3 fragments is dependent on many variables, and following the suggestions below will help maximize the chances of getting the right clone.

For more information on the MultiSite Gateway® Technology, visit our website at www.invitrogen.com or contact Technical Support (see page 42).

MultiSite Gateway® Pro 2-Fragment Recombination

Two PCR products flanked by specific *att*B or *att*Br sites and two MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate two entry clones. The two entry clones and the pJTI™ Fast DEST destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression construct containing two DNA elements. Refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit for detailed instructions.



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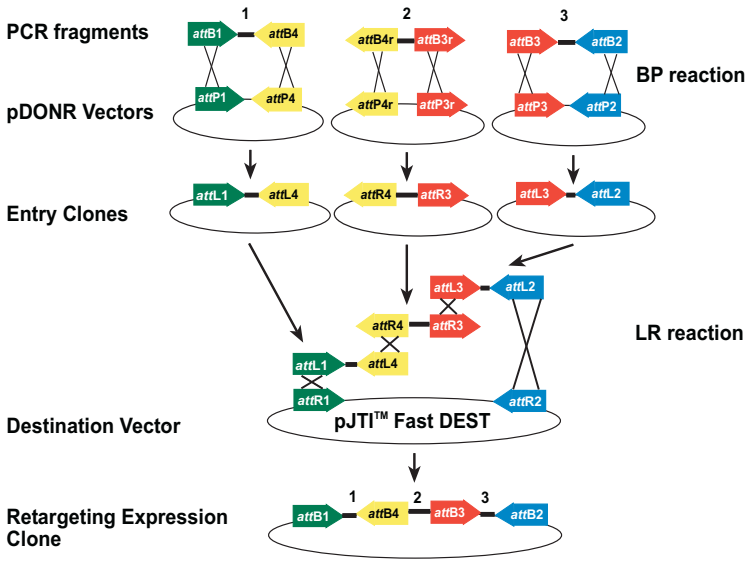
Constructing the Retargeting Expression Vector, continued

MultiSite Gateway® Pro 3-Fragment Recombination

Three PCR products flanked by specific *att*B or *att*Br sites and three MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate three entry clones. The three entry clones and the pJTI™ Fast DEST destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression construct containing three DNA elements. Refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit for detailed instructions.



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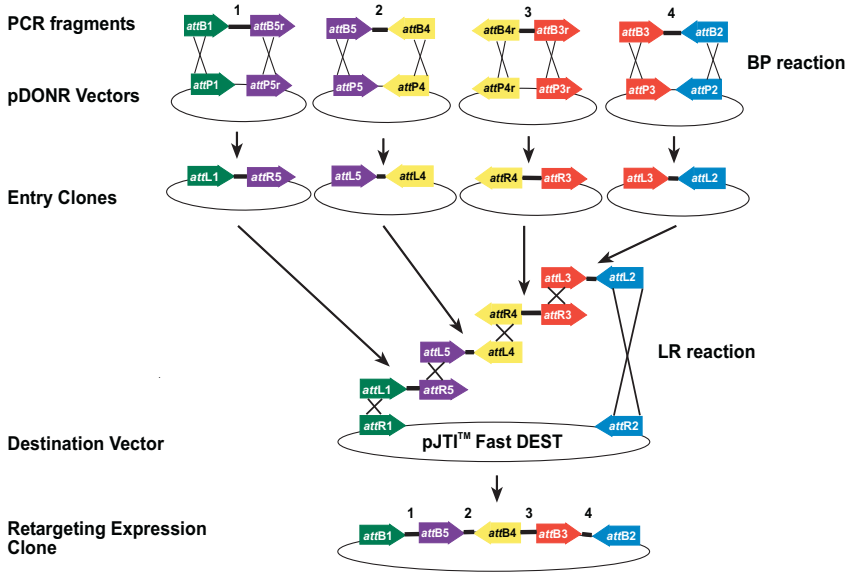
Constructing the Retargeting Expression Vector, continued

MultiSite Gateway® Pro 4-Fragment Recombination

Four PCR products flanked by specific *att*B or *att*Br sites and four MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate two entry clones. The four entry clones and the pJTI™ Fast DEST destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression construct containing four DNA elements. Refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit for detailed instructions.



MultiSite Gateway® Pro Donor Vectors

The MultiSite Gateway® Pro donor vectors are used to clone *att*B- or *att*Br-flanked PCR products to generate entry clones, and contain similar elements as other Gateway® donor vectors. However, because different *att*B sites will flank your PCR products, different donor vectors are required to facilitate generation of entry clones, which are later used in creating your retargeting expression construct. The table below lists the specific donor vectors required to assemble a retargeting expression construct containing one, two, three, or four DNA elements of interest.

For a map and a description of the features of each MultiSite Gateway® Pro donor vector, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit.

Note: pDONR™ 221 is provided as a positive control for the BP recombination reaction, and should not be used to generate multi-fragment entry clones.

Number of Fragments	Donor Vectors Required
1	pDONR201 or pDONR221
2	pDONR221 P1P5r and pDONR221 P5P2
3	pDONR221 P1P4, pDONR221 P4rP3r, and pDONR221 P3P2
4	pDONR221 P1P5r, pDONR221 P5P4, pDONR221 P4rP3r, and pDONR221 P3P2

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Constructing the Retargeting Expression Vector, continued

pJTI™ R4 DEST Destination Vector

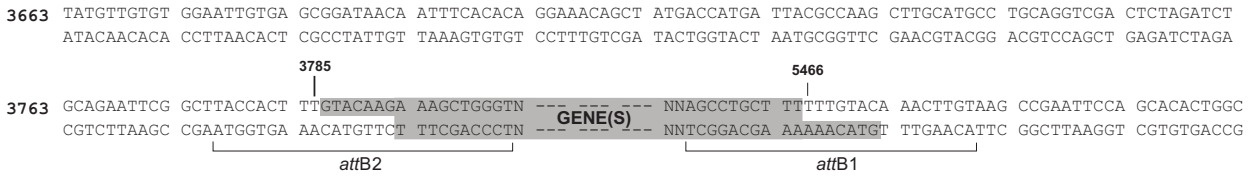
The pJTI™ R4 DEST vector is specifically designed to be used in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression clone to site-specifically integrate your multiple DNA elements into the genome of your R4 platform cell line. The pJTI™ R4 DEST vector contains the constitutive human EF1α promoter, which when integrated upstream of the promoterless resistance gene by the R4 Integrase, results in Blasticin, Geneticin®, or Zeocin™ resistance of the successfully retargeted clones. For a map and features of pJTI™ R4 DEST, see page 34.

Recombination Region of pJTI™ R4 DEST

The recombination region of the retargeting expression clone resulting from pJTI™ R4 DEST × pDONR entry clone is shown below.

Shaded regions correspond to those DNA sequences recombinationally transferred from the entry clone into pJTI™ R4 DEST vector. Non-shaded regions are derived from the pJTI™ R4 DEST vector.

The vector sequence of pJTI™ R4 DEST is available on our website at www.invitrogen.com or by contacting Technical Support (see page 42).



Important

Preparing Plasmid DNA: For targeted integration experiments, it is essential that the plasmid DNA used for transfection is of very high quality. Typically, best results have been obtained using plasmid DNA that has **very low levels of endotoxins**. If using large quantities of DNA, we recommend that the plasmid DNA is commercially prepared. If smaller quantities are required, use a commercial kit that delivers **pure DNA that is free of endotoxins**. Follow the manufacturer's recommended protocol for DNA preparation.

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Constructing the Retargeting Expression Vector, continued

Generating Entry Clones

- Ensure that primers used for PCR amplification 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 the pDONR vectors.
- If possible, avoid using a plasmid containing the kanamycin resistance gene as the template for PCR.
- If the fragment of interest is longer than ~3 kb, incubate the BP reaction at 16°C overnight instead of 1 hour at room temperature.
- When picking colonies for analysis, replica plate them on kanamycin and the drug resistance of the PCR template to reduce the background from template that is inadvertently purified. The colonies should only grow on kanamycin.

Generating Retargeting Expression Clones

- Produce clean DNA preparations of the entry clones to use in the LR reaction. DNA from "minipreps" will suffice for the assembly of up to two fragments. For assembly of 3 or more fragments, "midiprep" or "maxiprep" amount and quality DNA is essential.
- Sequence the entry clones with appropriate primers to ensure that the *att* sites do not have mutations.
- Dilute the DNA to a convenient concentration for the reactions. Since the MultiSite Gateway® Pro manual recommends 20 femtomoles of the DEST vector and 10 femtomoles of each of the entry vectors per reaction, we recommend maintaining a working concentration of 20 fmoles/µl for the DEST vector and 10 fmoles/µl for each of the entry vectors to allow the addition of 1 µl of each vector to the recombination reaction. The vector aliquots should be stored at –20°C.
- 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.
- Longer incubation times are essential if you are assembling more than two fragments. Generally, overnight incubation at either room temperature or at 16°C should work.
- Performing multiple transformations is more efficient than performing one large transformation. For a 4-fragment assembly, it may be necessary to transform the complete reaction volume to get enough colonies for analysis. Five transformations of 2 µl each will yield more colonies than two transformations of 5 µl each.
- Replica plate the colonies obtained from transformations on ampicillin and kanamycin plates. True recombinant clones will only grow on ampicillin plates.

Establishing Sensitivity to Selection Agents

Introduction

After you cotransfect your retargeting expression construct and the pJTI™ R4 Int vector into your R4 platform cells to create your isogenic cell line, you will select stable transformants containing your genetic elements of interest by their resistance to Blasticidin, Geneticin® (G-418, a neomycin analog), or Zeocin™. Successful retargeting of your R4 platform cell line will position the constitutive human EF1α promoter upstream of the "promoterless resistance gene" and confer resistance to the appropriate selective agent depending on the pJTI™ platform vector used.

To succefully create your isogenic cell line by retargeting, you need to determine the minimum concentration of the selective agent required to kill your untransfected mammalian R4 platform cells. This section provides instructions for establishing the sensitivity of your platform cell line to each of the selection agents.

Blasticidin

The pJTI™/Bsd platform vector contains the Blasticidin S deaminase gene for the selection of transfectants with the antibiotic Blasticidin. The deaminase converts Blasticidin S to a nontoxic deaminohydroxy derivative (Izumi et al., 1991). Blasticidin S HCl is available separately from Invitrogen (see page xii).

- Blasticidin S is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Blasticidin S and Blasticidin S-containing solutions.
- Always weigh Blasticidin S and prepare solutions in a hood.

Preparing and Storing Blasticidin S

Follow the instructions provided with Blasticidin S to prepare your working stock solution. Aliquot in small volumes suitable for one time use and store at 4ºC (short-term) or at –20ºC (long-term). Do not subject stock solutions to freeze/thaw cycles and do not store in a frost-free freezer. Aqueous stock solutions are stable for 1–2 weeks at 4ºC and 6–8 weeks at –20ºC. Medium containing Blasticidin may be stored at 4ºC for up to 2 weeks.

Determining Blasticidin S Sensitivity

The Blasticidin concentration required for selection in mammalian cells varies depending on the cell line used. Use 2–10 µg/ml Blasticidin for selection in mammalian cells. We recommend performing a kill curve as described below to determine the appropriate Blasticidin concentration to use for selecting resistant cells.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin (*e.g.*, 0, 2, 4, 6, 8, 10 µg/ml Blasticidin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10–14 days after addition of the antibiotic.

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Establishing Sensitivity to Selection Agents, continued

Geneticin® (G-418)

The pJTI™/Neo platform vector contains the neomycin resistance gene which confers resistance to the antibiotic Geneticin® (also known as G-418 sulfate). Geneticin® is available separately from Invitrogen (see page xii for ordering information).

- Geneticin® is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Geneticin® and Geneticin®-containing solutions.

Preparing and Storing Geneticin®

Follow the instructions provided with Geneticin® to prepare your working stock solution. Geneticin® in powder form should be stored at room temperature and at 4°C as a solution. The stability of Geneticin® is guaranteed for six months, if stored properly.

Determining Geneticin® Sensitivity

The amount of Geneticin® required to be present in culture media to select for resistant cells varies with a number of factors including cell type. We recommend that you re-evaluate the optimal concentration whenever experimental conditions are altered (including use of Geneticin® from a different lot. Note that Geneticin® in powder form has only 75% of the potency of Geneticin® available in liquid form.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Geneticin® (0, 50, 100, 250, 500, 750, and 1000 µg/ml Geneticin®).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Geneticin® that kills the cells within 1–2 weeks after addition of Geneticin®.

Continued on next page

Establishing Sensitivity to Selection Agents, continued

Zeocin™

The pJTI™/Zeo platform vector contains the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), the product of which is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™ (Calmels *et al.*, 1991; Drocourt *et al.*, 1990).

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989). Zeocin™ is available separately from Invitrogen (see page xii for ordering information)

- Zeocin™ is light sensitive. Store Zeocin™, plates, and medium containing Zeocin™ **in the dark**.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Preparing and Storing Zeocin™

Follow the instructions provided with Zeocin™ to prepare your working stock solution. For your convenience, the drug is prepared in autoclaved, deionized water and is available in 1.25 ml aliquots at a concentration of 100 mg/ml. Store Zeocin™ at –20°C **in the dark**, and thaw on ice before use. The stability of Zeocin™ is guaranteed for six months, if stored properly.

Determining Zeocin™ Sensitivity

To successfully retarget your platform cell line containing the promoterless zeocin resistance gene, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected R4 platform cell line. Typically, concentrations ranging from 50 to 1000 µg/ml Zeocin™ are sufficient to kill most untransfected mammalian cell lines, with the average being 100 to 400 µg/ml. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so that the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (0, 50, 100, 250, 500, 750, and 1000 µg/ml Zeocin™).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Zeocin™ that kills the cells within 1–2 weeks after addition of Zeocin™.

Continued on next page

Establishing Sensitivity to Selection Agents, continued

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from other antibiotics including Hygromycin B, Geneticin® (G-418), and blasticidin. **Cells exposed to fatal concentrations of Zeocin™ do not round up and detach from the plate.** Sensitive cells may exhibit the following morphological changes upon exposure to Zeocin™:

- Vast increase in size, similar to the effects of cytomegalovirus infecting permissive cells
- Abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and Golgi apparatus, or other scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)

Eventually, these "cells" will completely break down and only "strings" of protein remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™.

Important

We have observed that stem cells display a considerably elevated sensitivity to selective antibiotics. If you are retargeting platform cell lines generated from stem cells, we recommend that you use a 10-fold lower range of concentrations for each of the selective agents when determining the sensitivity of your untransfected platform cell line to Blasticidin, Geneticin®, and Zeocin™.

Retargeting the R4 Platform Cell Line

Introduction

The second step in targeted integration experiments is the retargeting event mediated by the R4 integrase (expressed from pJTI™ R4 Int vector) where the genetic elements of interest are site-specifically integrated into the platform line genome when the retargeting expression construct (created using the MultiSite Gateway® Pro module, see the preceding pages) is targeted to the R4 *att*P sequences. This integration event also positions the constitutive human EF1α promoter upstream of the blasticidin, neomycin, or zeocin resistance gene (*i.e.*, "promoterless" selection marker), thus allowing the selection of transformants that are successfully "retargeted" using the appropriate selection agent. For a map and features of the pJTI™ R4 Int vector, see page 35. **The vector sequence of pJTI™ R4 Int vector is available on our website at www.invitrogen.com or by contacting Technical Support (see page 42).**

This section provides instructions and guidelines for:

- Cotransfecting your retargeting expression construct and the pJTI™ R4 Int vector into your R4 platform cell line
- Selecting, expanding, and characterizing your retargeted clones

Method of Transfection

Consult the original references or the supplier of your cell line for optimal method of transfection. Methods of transfection include lipid-mediated transfection (Felgner *et al.*, 1989; Felgner & Ringold, 1989), calcium phosphate precipitation (Chen & Okayama, 1987; Wigler *et al.*, 1977), and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988).

We have obtained the best results for retargeting using **high-efficiency transfection methods** such as microporation or electroporation.

Transfection Considerations

The following factors are important for successful transfection:

- **Cells**: Cells that are 80–90% confluent are ideal for transfection. A higher confluency often results in a higher proportion of dead cells in culture. Carry out a live/dead assay using either FACS (LIVE/DEAD® Cell Vitality Assay Kit, see page xi for ordering information) or Trypan Blue exclusion counting. For more information on using the LIVE/DEAD® Cell Vitality Assay Kit, refer to **Assessing Cell Vitality** on page 36.
- **Quality of 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 using 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.
- **Amount of DNA**: We generally use 10 µg **total** plasmid DNA per 2×10^6–8×10^6 cells per transfection, but the amount of plasmid DNA may vary depending on the nature of the cell line, the transfection efficiency of your cells, and the method of transfection used. When transfecting your mammalian cell line of choice, we recommend that you try a range of plasmid DNA concentrations to optimize transfection conditions for your cell line.

Continued on next page

Retargeting the R4 Platform Cell Line, continued

Important

If you are transforming stem cells, you must maintain your culture on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells or in an appropriate medium conditioned on a MEF feeder layer (MEF-CM) for at least two weeks, and as a feeder-free culture on MEF-CM for at least one passage prior to transfection. Make sure to start preparing the feeder layer two days before culturing your stem cells.

Transfection Procedure

Use a **high-efficiency transfection methods** such as electroporation or microporation to co-tranfect pJTI™ R4 Int vector and the "retargeting expression construct" (generated using the MultiSite Gateway® Pro module) into your R4 platform cells. Follow the instructions provided by the manufacturer of the microporation or electroporation apparatus for best results. Be sure to follow the guidelines outlined below:

- Passage your platform cell line at least once **without** Hygromycin B selection prior to transfection.
- Remember to include negative controls where either the R4 integrase vector or the retargeting expression construct is omitted.
- Plate the transformed cells in 60-mm culture dishes containing the appropriate medium and allow the cells to recover **without selection** until the colonies become well-defined.
- Wash the cells and provide with fresh medium every day.
- Each colony recovers at a different rate. Monitor morphology and size of the colonies.
- When your targeted cells have recovered from transfection and the colonies are well-defined (usually 5 days post-microporation or 2 days post-electroporation), proceed to **Selecting Retargeted Clones**, next page.

Continued on the next page

Important

To succefully select for your isogenic "retargeted" cell line, you need to use the minimum concentration of the appropriate selective agent required to kill your untransfected mammalian R4 platform cell line. See pages 19–22 for more information on detemining the sensitivity of your untransfected platform cell line to the selection agents.

Selecting Retargeted Clones

After your cells have sufficiently recovered from transfection, proceed with selection as described below. Use the selection agent appropriate for the pJTI™ platform vector you have used to create your R4 platform cell line, and incubate your cells in the suitable medium.

1. 48 to 72 hours after transfection (or when the cells have sufficiently recovered and the colonies have become well-defined), transfer the cells into 100-mm dishes containing fresh medium. Split cells such that they are no more than 25% confluent as the selection antibiotics work best at actively dividing cells.
2. Incubate the cells at 37°C for 2–3 hours until they have attached sufficiently to the culture dish.
3. Remove the medium and add fresh medium containing the appropriate selection agent at the proper concentration (see **Establishing Sensitivity to Selection Agents**, pages 19–22). If you have retargeted stem cells growing on MEF feeders, you should also start Hygromycin B selection to prevent overgrowth of your colonies by MEFs.
4. Feed the cells with selective medium every 2–3 days until foci can be identified. Depending on the cell line, colonies will start appearing as early as day 5 of drug selection. Mark the colonies and observe them for an additional period of time (total of 12–21 days under selection).
5. Manually pick single, well-defined colonies and expand using the appropriate medium under selection for further analysis.

Note

We recommend that you continue with the Blasticidin, Geneticin®, or Zeocin™-based selective pressure even after your retargeted clones have been selected and expanded for downstream experiments. Continuous selective pressure ensures that expression from your gene(s) of interest is maintained.

Screening Retargeted Clones

Introduction

Upon retargeting your R4 platform line, follow the guidelines below to PCR screen for successful retargeting events using genomic DNA isolated from individual clones. Use of nested PCR with primary and secondary reactions is required to eliminate the high background observed with only the primary PCR.

Preparing Genomic DNA for PCR

1. Pellet 10,000 to 30,000 cells total.
2. Wash the cells with 500 µl PBS.
3. Centrifuge cells to pellet and remove PBS.
4. Resuspend cell pellet in a mixture of 20 µl of Resuspension Buffer and 2 µl of Lysis Solution (CellsDirect Resuspension and Lysis Buffers, see page xi).
5. Incubate the cell suspension at 75°C for 10 minutes.
6. Centrifuge for 1 minute to pellet cell debris.
7. Use 3 µl of the cell lysate to set up your PCR.

PCR Analysis

When performing PCR analysis on the genomic DNA isolated from your retargeted clones, you should consider the following factors:

- We recommend using nested PCR with primary and secondary reactions to eliminate the high background observed with only primary PCR.
- Successful retargeting of your platform line genome introduces the human EF1α promoter upstream of the resistance gene to the selection marker, resulting in blasticidin, Geneticin®, or Zeocin™ resistance of successfully retargeted clones depending on the platform vector used during platform line creation. You should design your primers from the EF1α promoter to the appropriate resistance marker. You may use plasmid DNA or the Hygromycin resistance gene as a positive control.
- You may also design PCR primers specific to your gene(s) of interest in the retargeting construct to check for the presence of successful integrations.
- For a map and a description of the features of each platform vector (pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo) and of the pJTI™ PhiC31 Int vector, see pages 30–33. **The vector sequences of pJTI™/Bsd, pJTI™/Neo, and pJTI™/Zeo vectors are available on our website at www.invitrogen.com or by contacting Technical Support (see page 42).**
- We recommend a high fidelity thermostable DNA polymerase such as the AccuPrime™ *Taq* DNA Polymerase (see page xi) for the nested PCR.
- Be sure to include a final extension step (7 minutes at 72°C) in your PCR.

Southern Blot Analysis (optional)

PCR is usually sufficient to confirm the presence of the retargeted sequences in your cell line after transfection. However, you may also perform a Southern blot analysis as an additional check to screen for a single copy number. Use the Southern blot protocol of your choice with a radiolabeled probe from the expression vector used to retarget the cells. We recommend using the DNAzol® Reagent (see page xi) to isolate genomic DNA from the platform cell line.

Troubleshooting

Introduction

The following tables list some potential problems and possible solutions to help you troubleshoot your targeted integration experiments. For troubleshooting any potential problems that might arise when generating your retargeting expression construct, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit.

Culturing Cells

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

Problem	Cause	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Home-made stock not viable	Freeze cells at a density of 2×10^6–3×10^6 viable cells/ml.
		Use low-passage cells to make your own stocks.
		Follow the freezing procedure for your type of cell culture exactly. Slow freezing and fast thawing are crucial. Add the cold freezing medium in a dropwise manner (slowly), swirling the tube after each drop. At the time of thawing, thaw quickly and do not expose vial to the air but quickly change from nitrogen tank to 37°C water bath.
		Obtain new cells.
	Thawing medium not correct	Use specified medium.
	Cells too diluted	Generally, we recommend thawing one vial in a 35-mm dish. If you need to concentrate cells, spin down the culture for 4 minutes at 200 × *g* at room temperature and dilute the cells at higher density.
	MEFs sub-optimal and do not support recovery of your stem cells (if using stem cells thawed on MEF feeders)	Purchase or make a new batch of mitotically inactivated MEFs (see page 36).
MEFs overgrow plate	MEFs not inactivated	Inactivate mitosis in MEFs as described on pages 34–41, or purchase inactivated MEFs (see page x).

Continued on next page

Troubleshooting, continued

Culturing Cells

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

Problem	Cause	Solution
Cells grow slowly	Growth medium not correct	Use correct growth medium.
	bFGF inactive	bFGF is not stable when frequently warmed and cooled. Add bFGF to medium just before use, or store medium with bFGF in aliquots at –20°C.
	Cells too old	Use healthy cells under passage 30; do not overgrow.
	Cells too diluted	Spin down cells for 4 minutes 200 × *g* at room temperature; aspirate media and dilute cells at higher density.
	Clump size is to small and differentiated	Be gentle at time of passage so the clumps of cells don't get too small.
	Mycoplasma contamination	Discard cells, media and reagents, and use early stock of cells with fresh media and reagents.
Cells differentiated (if using stem cells)	Cells not thawed and established on correct medium	Thaw and culture a fresh vial of stem cells. Make sure to thaw into the correct medium as recommended by the supplier.
	Suboptimal quality of feeder layer (if cells are maintained on feeder layers)	Check the concentration of feeder cells used. Purchase (see page x) or make (see page 34) new batch of mitotically inactivated MEFs, if necessary. Use Hygromycin resistant MEFs after platform creation.
	Culture conditions not correct	Thaw and culture fresh vial of stem cells. Follow thawing instructions and subculture/maintenance procedures exactly.
	Cells overexposed to collagenase	Stem cells are very sensitive to collagenase overexposure. Avoid exposing cells to collagenase for more than 3 minutes. Do **not** use lower concentrations of collagenase and treat for longer periods.
	Cells passaged too early	Passaging stem cells too early causes poor plating and differentiation. Grow to cells to near-confluence, *i.e.*, a day or two longer than when the colonies are just touching.
No growth after transfection	Incorrect amount of selection agent is used.	Determine the minimum concentration of the selection agent required to kill untransfected cells as described on page 8 and pages 19–22, and use this amount for selection

Continued on next page

Troubleshooting, continued

Transfecting Cells

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

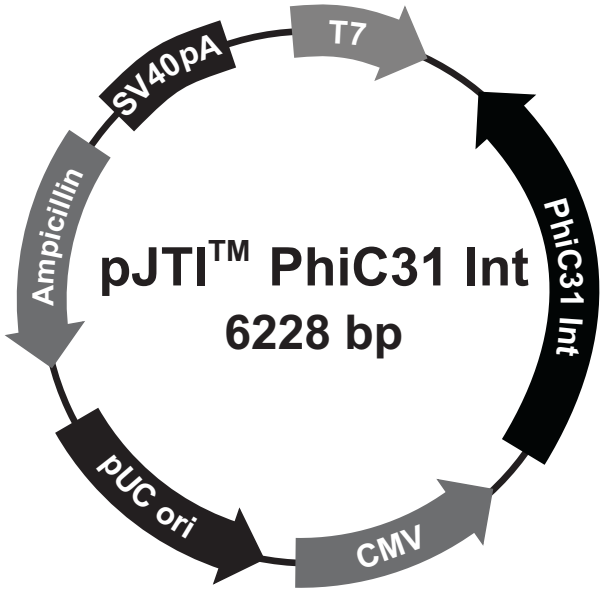
Problem	Cause	Solution
Low survival rate after transfection	Poor DNA quality	The quality of the plasmid DNA strongly influences the results of transfection experiments. Use **endotoxin-free DNA** for all transfections. Make sure that the A260:A280 ratio of the DNA is between 1.8 and 2.0. **Do not** use 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.
	Cells are harvested from selective plates prior to transfection for retargeting	You must passage your platform cell lines at least once **without drug selection** prior to transfection. Stem cell platform lines must be passaged at least once as a feeder-free culture on MEF-CM and Geltrex™ **without drug** selection prior to transfection.
	Cells are damaged during harvesting and subsequent handling prior to transfection	Avoid damaging cells conditions during harvesting. Centrifuge cells at lower speeds (150–200 × *g*). Avoid overexposure to TrypLE™, trypsin, accutase, or other dissociation reagents. Pipette cells gently.
	Cells remained too long in electroporation cuvette or the Gold-Tip.	Immediately after electroporation/microporation, transfer cells into pre-warmed medium at 37°C to prevent damage.
	Multiple use of Gold-Tip (if MT-100 MicroPorator is used for transfection)	Maximum recommend use Gold-Tip is between 1 and 3 times, because the electric pulses that are applied drastically reduce its quality and impair its physical integrity.
Low transfection efficiencies	Poor optimization of transfection parameters	Optimize transfection parameters following electroporator/microporator manufacturers' recommendations.
	Amount of DNA too low	Use the correct amount of DNA for the transfection method of choice following recommended conditions.
	Cell density too low or too high	Too low or too high cell densities could drastically reduce the transfection efficiency. Use 1×10^6 cells per microporation, or 0.6×10^7–1.0×10^7 cells per electroporation.
	Poor DNA quality	Use endotoxin-free DNA for all transfections. Make sure that the A260:A280 ratio of the DNA is between 1.8 and 2.0. **Do not** use phenol:chloroform extraction, or ethanol precipitation.
	Cells are contaminated with Mycoplasma	Test cultures for Mycoplasma contamination. Start a new culture from a fresh stock.

Appendix

pJTI™ PhiC31 Int

Map of pJTI™ PhiC31 Int

The pJTI™ PhiC31 Int vector (6228 bp) contains the *Streptomyces* phage PhiC31 integrase gene under the control of the Cytomegalovirus immediate-early promoter (CMV). The PhiC31 integrase mediates the site specific integration at pseudo-*att*P sites. In the Jump-In™ TI™ Gateway® System, it is used for site-specifically integrating R4 *att*P retargeting sequences in the genome of the mammalian cell line of choice to create the platform cell line. **The vector sequence of pJTI™ PhiC31 Int is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



Features of pJTI™ PhiC31 Int
6228 nucleotides

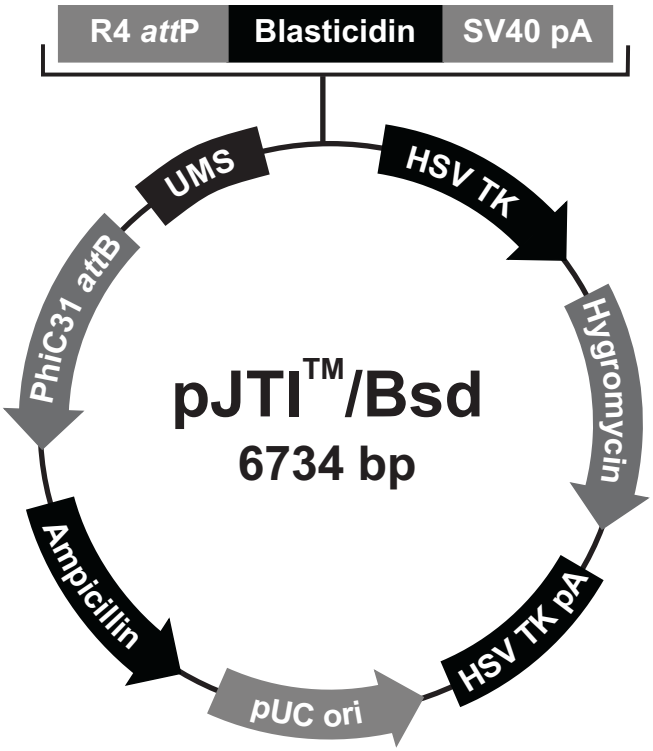
T7 promoter: bases 1-20
PhiC31Int: bases 83-1924 (c)*
CMV promoter: bases 2113-2636 (c)
pUC origin: bases 3121-3794 (c)
Ampicillin resistance gene (ORF): bases 3981-4841 (c)
SV40 polyA site: bases 5777-6139 (c)

*(c): complementary strand

pJTI™/Bsd

Map of pJTI™/Bsd

The pJTI™/Bsd vector (6734 bp) contains the PhiC31 *att*B site for PhiC31 integrase-mediated integration into the genome of cell line of choice and the Hygromycin resistance gene under the control of Herpes simplex virus-thymidine kinase promoter for subsequent selection. It also contains the R4 *att*P site for R4 integrase-mediated retargeting and the promoterless Blasticidin resistance gene for the selection of retargeted clones. **The vector sequence of pJTI™/Bsd is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



Features of pJTI™/Bsd
6734 nucleotides

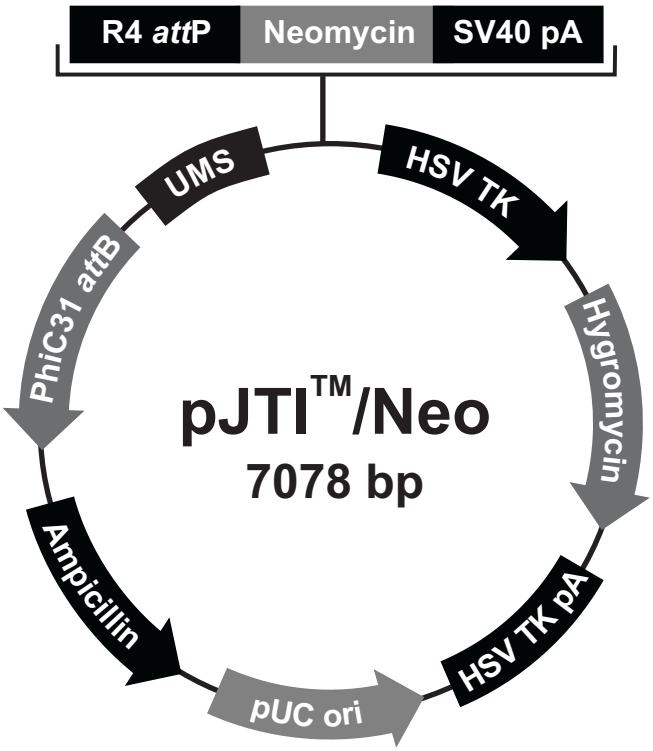
HSV TK: bases 1-249
Hygromycin resistance gene: 262-1296
HSV TK pA: bases 1300-1790
pUC origin: bases 1810-2483 (c)*
Ampicillin resistance gene: bases 2631-3488 (c)
PhiC31 *att*B integration site: bases 3629-3907 (c)
UMS (terminator): bases 4437-5467
R4 *att*P recombination site: bases 5512-5575
Blasticidin resistance gene: bases 5649-6047
SV40 pA: bases 6205-6286

*(c): complementary strand

pJTI™/Neo

Map of pJTI™/Neo

The pJTI™/Neo vector (7078 bp) contains the PhiC31 *att*B site for PhiC31 integrase-mediated integration into the genome of cell line of choice and the Hygromycin resistance gene under the control of Herpes simplex virus-thymidine kinase promoter for subsequent selection. It also contains the R4 *att*P site for R4 integrase-mediated retargeting and the promoterless neomycin resistance gene for the selection of retargeted clones with Geneticin®. **The vector sequence of pJTI™/Neo is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



Features of pJTI™/Neo
7078 nucleotides

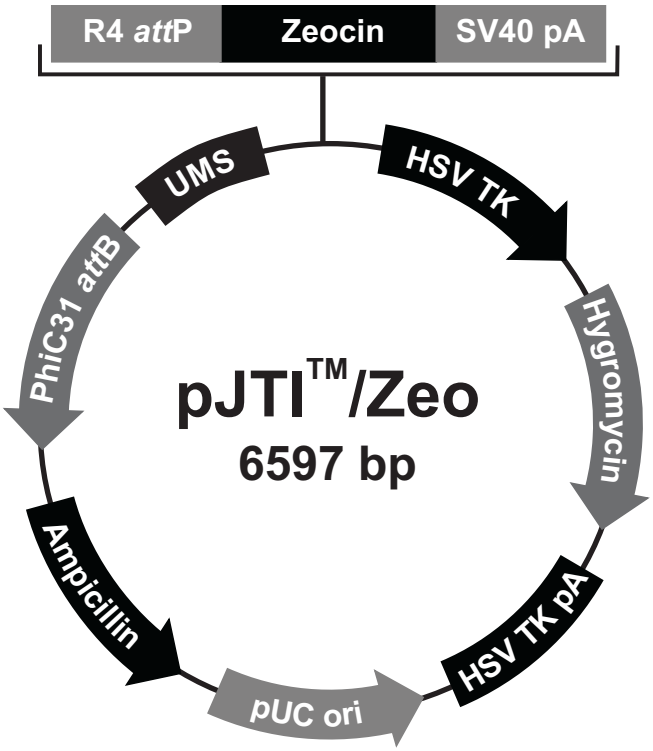
HSV TK: bases 1-249
Hygromycin resistance gene: 262-1296
HSV TK pA: bases 1300-1790
pUC origin: bases 1810-2483 (c)*
Ampicillin resistance gene: bases 2631-3488 (c)
PhiC31 *att*B integration site: bases 3629-3907 (c)
UMS (terminator): bases 4437-5467
R4 *att*P recombination site: bases 5512-5575
Neomycin resistance gene: bases 5582-6376
SV40 pA: bases 6550-6680

*(c): complementary strand

pJTI™/Zeo

Map of pJTI™/Zeo

The pJTI™/Zeo vector (6597 bp) contains the PhiC31 *att*B site for PhiC31 integrase-mediated integration into the genome of cell line of choice generating the platform cell line and the Hygromycin resistance gene under the control of Herpes simplex virus-thymidine kinase promoter for selection. It also contains the R4 *att*P site for R4 integrase mediated retargeting and the promoterless Zeocin™ resistance gene for the selection of retargeted clones. **The vector sequence of pJTI™/Zeo is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



Features of pJTI™/Zeo
6597 nucleotides

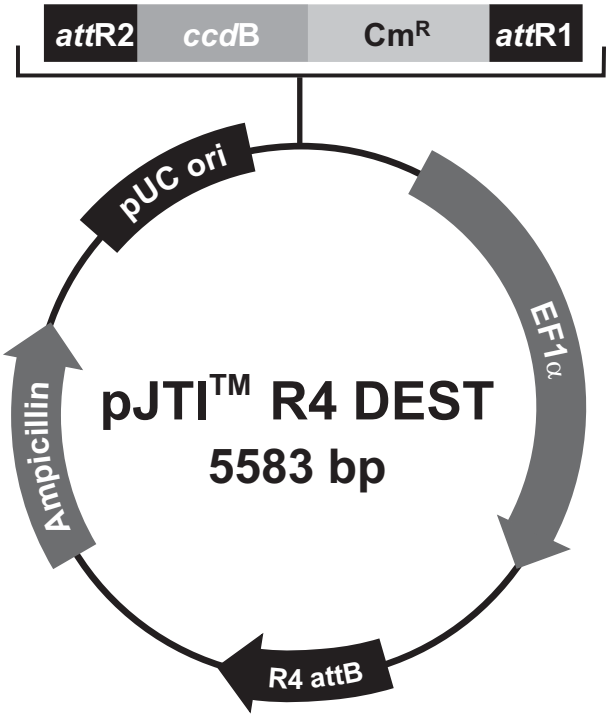
HSV TK: bases 1-249
Hygromycin resistance gene: 262-1296
HSV TK pA: bases 1300-1790
pUC origin: bases 1810-2483 (c)*
Ampicillin resistance gene: bases 2631-3488 (c)
PhiC31 *att*B integration site: bases 3629-3907 (c)
UMS (terminator): bases 4437-5467
R4 *att*P recombination site: bases 5512-5575
Zeocin resistance gene: bases 5659-6030
SV40 pA: bases 6047-6128

*(c): complementary strand

pJTI™ R4 DEST

Map of pJTI™ R4 DEST

The pJTI™ R4 DEST vector (5583 bp) contains the λ Integrase *att*R1 and *att*R2 sites for the MultiSite Gateway® transfer of DNA elements of interest from pDONR entry clones to generate the retargeting expression clone, the R4 *att*B site for site-specific integration of the DNA elements into the R4 platform cell line genome, and the human EF1α promoter for constitutive expression of resistance to the appropriate selection marker upon successful integration (Blasticidin, Geneticin®, or Zeocin™, depending on the platform vector used). **The vector sequence of pJTI™ R4 DEST is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



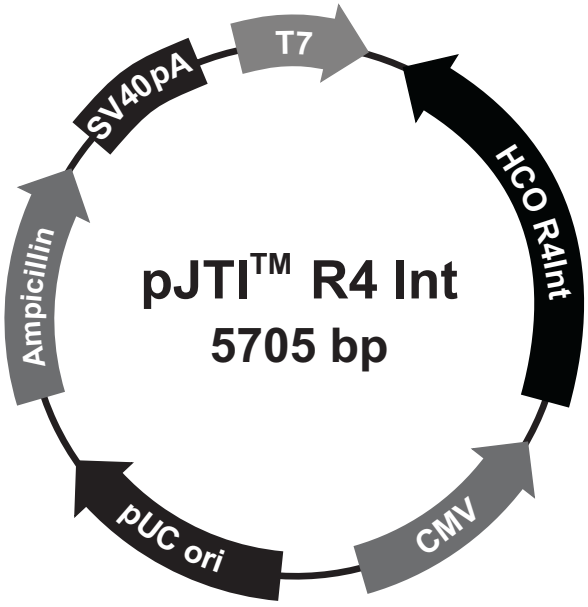
Features of pJTI™ R4 DEST
5583 nucleotides

EF1α: bases 66-1244
R4 *att*B: bases 1323-1617
Ampicillin resistance gene (ORF): bases 1761-2621
pUC origin: bases 2766-3439
*att*R2 recombination site: bases 3842-3966
*ccd*B gene: bases 4007-4312 (complementary strand)
Chloramphenicol resistance gene: bases 4632-5312 (complementary strand)
*att*R1 recombination site: bases 5421-5545 (complementary strand)

pJTI™ R4 Int

Map of pJTI™ R4 Int

The pJTI™ R4 Int vector (5705 bp) contains the gene for R4 Integrase from the *Steptomyces* PhiC31 phage. The R4 Integrase allows the site-specific integration of DNA elements into the genome of the platform cell line from the pJTI™ R4 DEST retargeting expression construct upon cotransfection of the platform line with both vectors. **The vector sequence of pJTI™ R4 Int is available from www.invitrogen.com or by contacting Technical Support (see page 42).**



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 (ORF): bases 3458-4318 (c)
SV40 polyA site: bases 5254-5616 (c)

*(c): complementary strand

Assessing Cell Vitality

Introduction

We recommend using the LIVE/DEAD® Cell Vitality Assay Kit, available separately from Invitrogen, to assess the vitality of your cells by flow cytometry. For more information on how to distinguishes metabolically active cells from cells that are dead or injured, refer to the manual provided with the LIVE/DEAD® Cell Vitality Assay Kit (Cat. no. L34951). For ordering information, see page xi.

LIVE/DEAD® Cell Vitality Assay

The assay has been optimized using Jurkat cells. Some modifications may be required for use with other cell types. A negative control for necrosis should be prepared by incubating cells with 2 mM hydrogen peroxide for 4 hours at 37°C. Untreated cells should be used as a positive control for C_{12}-resazurin staining.

1. **Prepare a 1 mM stock solution of C12-resazurin.** Dissolve the contents of the vial of C_{12}-resazurin (Component A) in 100 µL of DMSO (Component C). It may be necessary to agitate the solution in an ultrasonic water bath to fully dissolve the C_{12}-resazurin. The C_{12}-resazurin stock solution should be stable for 3 months if stored at ≤ –20°C, protected from light. Prepare a fresh 50 µM working solution of C_{12}-resazurin by diluting 1 µL of the 1 mM C_{12}-resazurin stock solution in 19 µL of DMSO.
2. **Prepare a 1 µM working solution of SYTOX Green stain.** For example, dilute 5 µL of the 10 µM SYTOX Green stain stock solution (Component B) in 45 µL of DMSO (Component C). The unused portion of this working solution may be stored at ≤ –20°C for up to 1 month.
3. **Prepare a 1X phosphate-buffered saline (PBS) solution.** For example, for about 20 assays, add 2 ml of 10X PBS (Component D) to 18 ml of deionized water (dH2O). Pass the 1X PBS through a 0.2 micron filter before use.
4. **Harvest the cells and dilute as necessary to about 1×10^6 cells/ml using the 1X PBS.** The cells may be washed with 1X PBS if desired.
5. **Add the dyes to the cell suspension.** Add 1 µL of the 50 µM C_{12}-resazurin working solution (prepared in step 1) and 1 µL of the 1 µM SYTOX Green stain working solution (prepared in step 2) to each 100 µL of cell suspension (final concentrations of 500 nM C_{12}-resazurin and 10 nM SYTOX Green dye).

   Note: If the fluorescence intensity of the SYTOX Green dye is too low, the final dye concentration can be increased up to 50 nM.
6. **Incubate the cells at 37°C in an atmosphere of 5% CO_2 for 15 minutes.**
7. **Dilute the cell suspension.** After the incubation period, add 400 µL of the 1X PBS, mix gently, and keep the samples on ice.
8. **Analyze the cell sample.** As soon as possible, analyze the stained cells by flow cytometry, exciting at 488 nm and measuring the fluorescence emission at 530 nm and 575 nm. The population should separate into two groups: live cells with a low level of green and a high level of orange fluorescence and necrotic cells with a high level of green fluorescence and a low level of orange fluorescence. Confirm the flow cytometry results by viewing the cells with a fluorescence microscope, using filters appropriate for fluorescein (FITC) and tetramethylrhodamine (TRITC).

Freezing Mammalian Cells

Introduction

We highly recommend that you freeze and bank at least 10–20 vials of cells at each stage of genetic manipulation. The cryopreserved cells will supply you with a low passage culture for future genetic manipulations and will ensure that you avoid loss by contamination and minimize genetic changes resulting from continuous culture. Cryopreservation will also help prevent aging and transformation if you are using a finite cell line. The following freezing protocols have been adapted from Freshney, 1987.

Freezing Medium

There are several common media used to freeze cells. For serum-containing medium, the constituents may be as follows:

- complete medium containing 10% DMSO (dimethylsulfoxide), or
- 50% cell-conditioned medium with 50% fresh medium with 10% DMSO

If you prefer to cryopreserve your cells in serum-free media, you should include a protein source to protect the cells from the stress of the freeze-thaw process. A serum-free medium generally has low or no protein, but you can still use it as a base for a cryopreservative medium in the following formulations:

- 50% cell-conditioned serum free medium and 50% fresh serum-free medium containing 7.5% DMSO
- fresh serum-free medium containing 7.5% DMSO and 10% cell culture grade BSA

Freezing Protocol for Suspension Cultures

1. Count the number of viable cells to be cryopreserved. Cells should be in log phase.
2. Centrifuge the cells at ~200–400 × *g* for 5 minutes to pellet.
3. Using a pipette, remove the supernatant down to the smallest volume without disturbing the cells.
4. Resuspend cells in freezing medium to a concentration of 1×10^7–5×10^7 cells/ml for serum containing medium, or 0.5×10^7–1×10^7 cells/ml for serum-free medium. Aliquot into cryogenic storage vials.
5. Place vials on wet ice or in a 4°C refrigerator, and start the freezing procedure within 5 minutes.
6. Freeze the cells slowly by decreasing the temperature at 1°C per minute. This can be done by programmable coolers or by placing the vials in an insulated box placed in a –70°C to –90°C freezer, then transferring to liquid nitrogen storage.

Continued on next page

Freezing Mammalian Cells, continued

Freezing Protocol for Adherent Cultures

1. Detach cells from the substrate with the appropriate dissociation agent. Detach as gently as possible to minimize damage to the cells.
2. Resuspend the detached cells in a complete growth medium and establish the viable cell count.
3. Centrifuge at ~200 × *g* for 5 minutes to pellet the cells.
4. Using a pipette, withdraw the supernatant down to the smallest volume without disturbing the cells.
5. Resuspend cells in freezing medium to a concentration of 0.5×10^7–1×10^7 cells/ml.
6. Aliquot into cryogenic storage vials. Place vials on wet ice or in a 4°C refrigerator, and start the freezing procedure within 5 minutes.
7. Freeze the cells slowly by decreasing the temperature at 1°C per minute. This can be done by programmable coolers or by placing the vials in an insulated box placed in a –70°C to –90°C freezer, then transferring to liquid nitrogen storage.

Thawing Mammalian Cells

Introduction

Cryopreserved cells are fragile and require gentle handling. Thaw cells quickly and plate directly into complete growth medium. If cells are particularly sensitive to cryopreservation, centrifuge the cells to remove the cryopreservative (DMSO or glycerol) and then plate into growth medium. We recommend the following procedures adapted from Freshney, 1987, for thawing cryopreserved cells.

Centrifugation Method

1. Remove the cells from storage and thaw quickly in a 37°C water bath.
2. Place 1 or 2 ml of frozen cells in ~25 ml of complete growth medium. Mix very gently.
3. Centrifuge cells at ~80 × *g* for 2–3 minutes, and discard the supernatant.
4. Gently resuspend the cells in complete growth medium and perform a viable cell count.
5. Plate the cells at ≥ 3×10^5 cells/ml.

Direct Plating Method

1. Remove the cells from storage and thaw quickly in a 37°C water bath.
2. Plate the cells directly, using 10–20 ml of complete growth medium per 1 ml of frozen cells. Cell inoculum should be at least 3×10^5 cells/ml.
3. Incubate cells for 12–24 hours, and replace the medium with fresh complete growth medium to remove the cryopreservative.

Generating Mitomycin C Treated MEFs

Introduction

If you are using stem cells in your targeted integration experiments, you must maintain your culture on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells or in an appropriate medium conditioned on a MEF feeder layer (MEF-CM) for at least two weeks, and as a feeder-free culture on MEF-CM for at least one passage prior to transfection. This section provides instructions for generating Mitomycin C-treated, mitotically inactivated MEFs.

Mitomycin C is highly toxic. Read and understand the **MSDS** and handle accordingly.

Preparing Gelatin-Coated Plates

Prepare 0.1% (w/v) porcine skin gelatin (Sigma Cat no. G1890) in sterile, distilled water, and sterilize by filtration using a 0.2 micron filter. Store up to 1 year at 4°C. Coat plates for 20–60 minutes at room temperature with 0.1% gelatin in distilled water.

Preparing Mitomycin C

Prepare 10 µg/ml Mitomycin C in MEF medium (see below); filter sterilize and store at –20°C in the dark until use. Mitomycin C can also be kept at 4°C in the dark for up to 2 weeks. Mitomycin C is available separately from Sigma, St. Louis (Cat no. M4287).

Note: Used Mitomycin C **must** be neutralized by addition of 15ml bleach (Clorox) per 500 ml Mitomycin C solution. Swirl to mix, incubate for 15 minutes, and discard.

MEF Medium

To prepare 500 ml of MEF medium, mix the following reagents (see pages ix–x for ordering information):

Component	Volume	Final Concentration
D-MEM	445 ml	1X
FBS	50 ml	10%
NEAA (10 mM)	5 ml	0.1 mM
2-Mercaptoethanol, 1,000X (55 mM)	500 µl	55 µM

Filter through a 0.22 micron filtration unit to sterilize. Pre-heat the medium to 37°C before use.

Obtaining MEFs

Hygromycin resistant primary MEFs that are **not** Mitomycin C treated are available separately from Millipore (Cat. no. PMEF-HL) or ATCC (Cat. no. SCRC-1045). One vial of cells (~5×10^6–6×10^6 cells/vial) can be used to plate ten 60-mm dishes. MEFs which are not mitotically arrested must be treated with Mitomycin C before use.

Continued on next page

Generating Mitomycin C Treated MEFs, continued

Mitomycin C Inactivation

Use the procedure below to generate mitotically inactivated MEFs in T175 culture flasks. Make sure that the MEFs to be treated with Mitomycin C are 90–95% confluent in T175 flasks 3 days after the initial thawing. Observe each flask individually under the microscope to ensure cell growth and culture sterility.

1. Culture MEFs in MEF medium (see page 40 for recipe).
2. In a biosafety cabinet, aspirate the medium from T175 flasks and add 16 ml of Mitomycin C solution (10 µg/ml).
3. Incubate MEFs treated with 10 µg/ml Mitomycin C in the flasks for 2–3 hours at 37°C, 5% CO_2. Work in sets of no more than six flasks at a time.
4. After 2–3 hours of incubation, aspirate off the Mitomycin C solution and neutralize the waste with bleach (see above).
5. Wash cells five times with Dulbecco's Phosphate-Buffered Saline (D-PBS) containing Mg^{2+} and Ca^{2+} (see page ix for ordering information).
6. Aspirate D-PBS and wash cells with 20 ml D-PBS that is Mg^{2+} and Ca^{2+}-free (see page ix ordering information).
7. Add 3 ml of 0.05% Trypsin-EDTA solution per flask to trypsinize cells (see page xi for ordering information). At room temperature, monitor the degree of cell detachment, while gently rocking and tapping the flask.
   Note: MEFs are trypsin sensitive. 1–2 minutes of incubation is sufficient to detach cells. **Do not** overexpose
8. When cells are sufficiently detached from the flask, add 5 ml of MEF medium to each flask, rock to disperse and pool cell suspensions from 1–6 flasks into 2 × 50-ml conical tubes.
9. Add 15 ml of MEF medium to the first flask to rinse out the cells. Rinse the subsequent flask using the same 15 ml MEF medium, and pool with cell suspension. Discard the flasks.
10. Adjust the volume in each tube to 50 ml with MEF medium and centrifuge cells at 200 × *g* for 4 minutes at room temperature.
11. Resuspend cell pellets with MEF medium and pool into one 50-ml tube, using a maximum of 12 × T175 flasks of cells per 50-ml tube.
12. Centrifuge cells at 200 × *g* for 4 minutes at room temperature.
13. Resuspend the cell pellet in 40 ml of MEF medium, using a 10-ml serological pipette and ensuring that the cells are resuspended fully. Adjust the volume to 50 ml with MEF medium.
14. Centrifuge cells at 200 × *g* for 4 minutes at room temperature. At this stage, the cells will have been washed a total of 9 times: 6 times before trypsin, once at trypsinization, and twice post-trypsinization.
15. Resuspend the cell pellet in 10 ml of MEF medium and then bring to a final volume of 40 ml with MEF medium, mixing vigorously before counting cells with trypan blue. Mixing is critical to get an accurate cell count.
16. Plate MEFs at a density of 3×10^4 cells/cm^2 of culture surface area in MEF medium with 2.5 ml per well of a gelatin-coated 6-well dish.
17. Freeze the cells for later use, or use within 2 to 5 days after plating for hESC cell culture. The medium should be changed every other day if they are not used immediately.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
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Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

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