USER GUIDE

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Jump-In™ Fast Gateway® Targeted Integration System

MultiSite Gateway®-adapted vector system for rapid generation of well-expressing mammalian cell lines

Catalog numbers A10893 and A10894

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For Research Use Only. Not for use in diagnostic procedures.

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Contents

Kit Contents and Storage

Introduction

This manual provides guidelines and instructions for efficiently generating stable mammalian cell lines that overexpress your protein(s) of interest using the Jump-In™ Fast Gateway® technology. It is supplied with the products listed below.

Product	Cat. no.
Jump-In™ Fast Gateway® System	A10893
Jump-In™ Fast Gateway® Core Kit	A10894

System Components

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

Component	Cat. no.	
	A10893	A10894
Jump-In™ Fast Gateway® Core Kit	√	√
MultiSite Gateway® Pro Plus Kit	√	
Jump-In™ Fast Gateway® System Manual	√	√

Shipping/Storage

The Jump-In™ Fast 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™ Fast Gateway® System Kit is designed to help you genetically engineer well-expressing stable mammalian cell lines that contain 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 lines 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 cell lines and stem cells.** If you are unfamiliar with these technologies, we recommend that you acquire a working knowledge of the Gateway® Technology and mammalian cell culture.

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Kit Contents and Storage, continued

Kit Components

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

Jump-In™ Fast Gateway® Core Kit

Jump-In™ Fast Gateway® Core Kit supplied with the Jump-In™ Fast Gateway® System is also available as an individual kit (Cat no. A10894). It contains the vectors for targeting your mammalian cell line. **Store the vectors at –20°C.**

Vector	Composition	Amount
pJTI™ Fast DEST	40 µL of vector at 150 ng/µL in TE buffer, pH 8.0	6 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

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 2-, 3- or 4-fragment recombination reactions. Each module supplies enough reagents for 20 recombination reactions. **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

Product Use

For research use only. Not for use in diagnostic procedures.

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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™ Fast 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.lifetechnologies.com** or contact Technical Support (see page 30).

Media and Buffers for Cell Culture

We recommend the following media and buffers 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 Life Technologies, refer to our website at **www.lifetechnologies.com** or contact Technical Support (see page 30).

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 and stem cell cultures. For more information on these and other cell culture products available from Life Technologies, refer to **www.lifetechnologies.com** or contact Technical Support (see page 30).

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

Life Technologies 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.lifetechnologies.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.lifetechnologies.com** or contact Technical Support (see page 30).

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 used with the Jump-In™ Fast Gateway® System.

Product	Amount	Cat. no.
Hygromycin B	20 mL	10687-010
Ampicillin, Sodium Salt	200 mg	11593-027

MultiSite Gateway® Pro Kits

Life Technologies 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 Jump-In™ Fast 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™ Fast Gateway® System combines Life Technologies' MultiSite Gateway® Pro cloning and Jump-In™ cell engineering technologies for efficiently generating well-expressing mammalian cell lines. The system enables irreversible integration of multiple genetic elements (such as promoter-reporter pairs) at specific locations in the mammalian genome of choice.

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

Components of the Jump-In™ Fast Gateway® System

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

- The Jump-In™ Fast Gateway® Core Kit containing the vectors for targeting your mammalian cell line of interest. The Jump-In™ Fast Gateway® Core Kit consists of the pJTI™ Fast DEST vector to deliver your genetic elements of interest into your mammalian cell line of choice, and the pJTI™ PhiC31 Int vector expressing the PhiC31 Integrase to mediate the stable integration of your "targeting expression construct" (i.e., pJTI™ Fast DEST vector) into the genome of your cell line. For a map and features of each vector, see pages 22–23. For the recombination region of the pJTI™ Fast DEST, see page 9.
- The MultiSite Gateway® Pro Plus Vector Module for simultaneous cloning of up to four DNA fragments to generate your targeting expression clone. 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 into your pJTI™ Fast DEST vector.

Note

In addition to the complete Jump-In™ Fast Gateway® System (Cat. no. A10893) containing all the components listed above, Life Technologies offers the targeting vectors pJTI™ Fast DEST and pJTI™ PhiC31 Int as a separate kit (Jump-In™ Fast Gateway® Core Kit, Cat. no. A10894) which can be used with any of the Life Technologies MultiSite Gateway® Pro Kits (see page x for ordering information) to generate expression clones containing up to five individual DNA elements.

For more information on the Jump-In™ Fast Gateway® System and other available MultiSite Gateway® Pro Kits, visit our website at **www.lifetechnologies.com** or contact Technical Support (see page 30).

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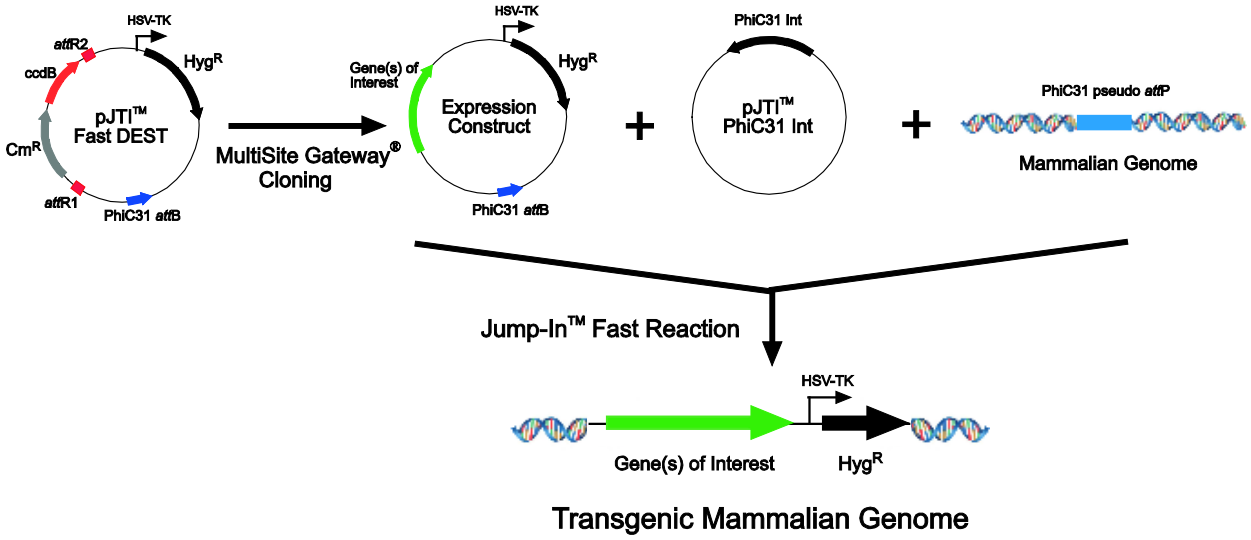
Overview, continued

Jump-In™ Fast Gateway® Targeted Integration Technology

The Jump-In™ targeted integration 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. In the Jump-In™ Fast Gateway® System, the integrated DNA sequences include your genetic elements of interest (such as promoter-reporter pairs) from the targeting expression construct that is generated using the pJTI™ Fast DEST vector and the MultiSite Gateway® Pro Plus Vector Module. Since the pJTI™ Fast DEST vector also encodes the Hygromycin resistance gene, transformants containing stably integrated sequences are selected using Hygromycin B and expanded for downstream applications. The figure below schematically depicts the workflow for generating your well-expressing mammalian cell line using the Jump-In™ Fast Gateway® targeted integration technology.

For more information on the PhiC31 Integrase, and its use in targeted integration refer to our website at **www.lifetechnologies.com** and published the literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001).



Other Jump-In™ Systems

In addition to the Jump-In™ Fast Gateway® System, which enables the rapid creation of well-expressing mammalian cell lines, Life Technologies also offers the Jump-In™ TI™ Gateway® Targeted Integration System (Cat. no. A10895) which facilitates the generation of **isogenic** stable cell lines expressing your genetic element(s) of interest, and allows you to eliminate chromosomal positioning effects from your experiments. For more information on Jump-In™ TI™ Gateway® System, visit our website at **www.lifetechnologies.com** or contact Technical Support (see page 30).

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Overview, continued

Purpose of This Manual

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

- Creating your "targeting expression construct" using the MultiSite Gateway® Pro Plus Vector module and the pJTI™ Fast DEST vector
- Targeting your mammalian cell line by co-transfecting with your targeting expression construct and the pJTI™ PhiC31 Int vector
- Selecting and expanding of your targeted cell line that expresses your genetic elements of interest
- Characterization of your cell line after targeted integration

In addition to our website at **www.lifetechnologies.com**, you may also refer to recent published literature (Thyagarajan *et al.*, 2008; Thyagarajan *et al.*, 2001) for more information on PhiC31-mediated recombination.

Not Covered by This Manual

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 on culturing mammalian cell lines and human stem cells, refer to **www.lifetechnologies.com** or contact Technical Support (page 30).

This manual **does not** provide detailed instructions for generating your targeting expression construct. Detailed protocols for creating your expression construct using the MultiSite Gateway® technology are included in the MultiSite Gateway® Pro manual (25-0942) supplied with the kit.

Important

The Jump-In™ Fast Gateway® Kit is designed to help you genetically engineer stable mammalian cell lines that overexpress multiple genetic elements of interest. **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 cell lines 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 engineering your mammalian cell line to express your genetic elements of interest using the Jump-In™ Fast Gateway® System, as well as the subsequent selection and expansion of the best expressing clones.

Although this section also includes general information on maintaining your mammalian or stem cell culture before and after the targeted integration reaction, **we emphasize that you should 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.lifetechnologies.com** or contact Technical Support (see page 30).

Propagating Jump-In™ Fast Gateway® System Vectors

To propagate and maintain the pJTI™ Fast DEST vector, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells from Life Technologies (see page x). 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™ PhiC31 Int vector, we recommend using 10 ng of the vector to 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™ Fast 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 to the MultiSite Gateway® Pro manual supplied with Jump-In™ Fast Gateway® System. The MultiSite Gateway® Pro manual is also available online at **www.lifetechnologies.com** or by contacting Technical Support (see page 30).

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.

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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, 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 particular 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 (see page ix) 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 ix 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.

Assembling the Targeting Expression Construct

Introduction

The first step in engineering your mammalian cell line to stably express your genetic elements of interest is the cloning of these genetic elements into the pJTI™ Fast DEST vector to generate your targeting expression construct using the MultiSite Gateway® Pro Plus Module. This section provides suggestions and helpful hints for generating the targeting expression construct.

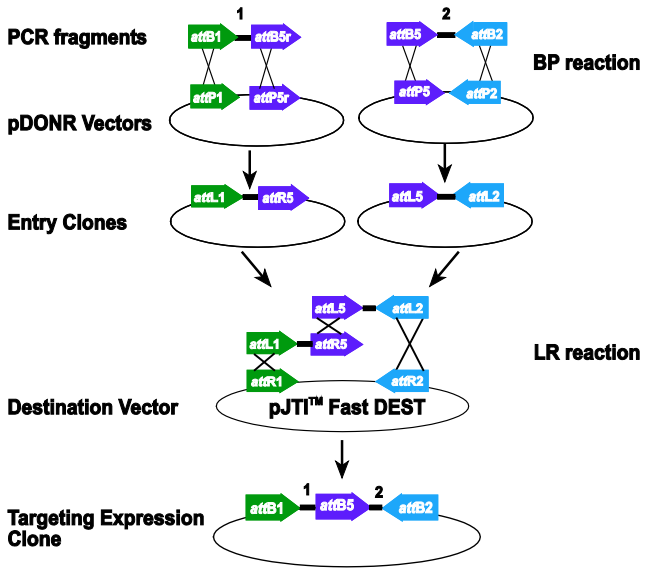
Important

For generating the targeting 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 targeting 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.lifetechnologies.com** or contact Technical Support (see page 30).

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 targeting 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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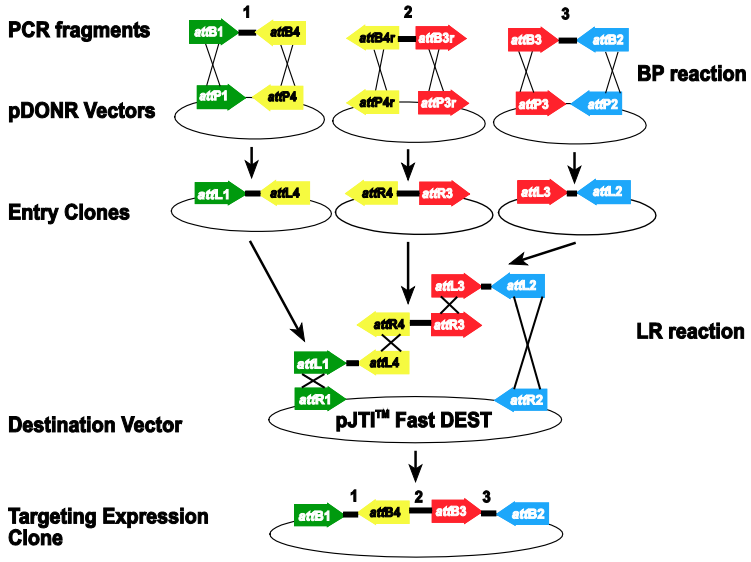
Assembling the Targeting Expression Construct, continued

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 targeting 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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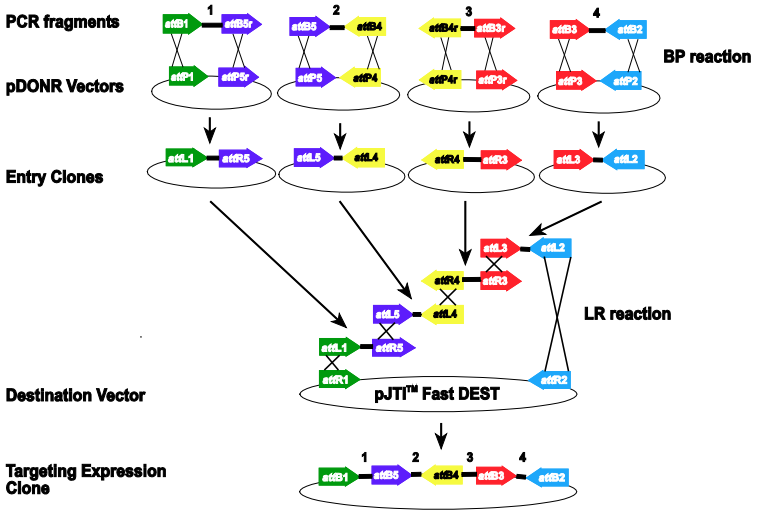
Assembling the Targeting Expression Construct, continued

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 targeting 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 the entry clones, which are later used in creating your targeting expression construct. The table below lists the specific donor vectors required to assemble a targeting 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	pDONR™201 or pDONR™221
2	pDONR™221 P1P5r and pDONR™221 P5P2
3	pDONR™221 P1P4, pDONR™221 P4rP3r, and pDONR™221 P3P2
4	pDONR™221 P1P5r, pDONR™221 P5P4, pDONR™221 P4rP3r, and pDONR™221 P3P2

Continued on next page

Assembling the Targeting Expression Construct, continued

pJTI™ Fast DEST Destination Vector

The pJTI™ Fast DEST vector is specifically designed to be used in a MultiSite Gateway® Pro LR recombination reaction to create your targeting expression construct. The pJTI™ Fast DEST vector contains Hygromycin resistance gene under the control of the thymidine kinase promoter, which when integrated into your cell line results in Hygromycin B resistance of the successfully retargeted clones. For a map and features the pJTI™ Fast DEST, see page 23.

Recombination Region of pJTI™ Fast DEST

The recombination region of the targeting expression construct resulting from pJTI™ Fast DEST × pDONR entry clone is shown below.

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

The vector sequence of pJTI™ Fast DEST is available on our website at www.lifetechnologies.com or by contacting Technical Support (see page 30).

```
4324 GGGTAACGCC AGGGTTTTCC CAGTCACGAC GTTGTAAAAC GACGGCCAGT GAATTCGAGC TCGGTACCCG GGGATCCTCT AGAGTCGACT AGTAACGGCC
     CCCATTGCGG TCCCAAAAGG GTCAGTGCTG CAACATTTTG CTGCCGGTCA CTTAAGCTCG AGCCATGGGC CCCTAGGAGA TCTCAGCTGA TCATTGCCGG

                                    4454                                                    6135
                                     |                                                       |
4424 GCCAGTGTGC TGGAATTCGG CTTACAAGTT TGTACAAAAA AGCTGAAC  GENE(S)  TTCAGCTTTC TTGTACAAAG TGGTAAGCCG AATTCTGC
     CGGTCACACG ACCTTAAGCC GAATGTTCAA ACATGTTTTT TCGACTTG          AAGACGAAAG AACATGTTTC ACCATTCGGC TTAAGACG
```

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.

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.

Continued on next page

Assembling the Targeting Expression Construct, continued

Assembling the Targeting Expression Clone

- 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.

Generating Transgenic Cell Line by Targeted Integration

Introduction

The Jump-In™ Fast Gateway® System is used for stably integrating your genetic elements of interest into the genome of your mammalian cell line of choice. The irreversible integration is mediated by the *Streptomyces* phage PhiC31 Integrase (expressed from pJTI™ PhiC31 Int vector) which catalyzes the recombination between your targeting expression construct (containing the native PhiC31 *att*B site) and the pseudo-*att*P sites in the genome of your cell line. Since your targeting expression construct also contains the Hygromycin resistance gene driven by the thymidine kinase promoter, you will select for stable transformants using Hygromycin B after co-transfecting your cells with your targeting expression construct and the pJTI™ PhiC31 Int vector. This section provides instructions and guidelines for generating your transgenic mammalian cell line to express your gene(s) of interest.

For a map and features of the pJTI™ PhiC31 Int, see page 22. **The vector sequence of pJTI™ PhiC31 Int is available on our website at www.lifetechnologies.com or by contacting Technical Support (see page 30).**

Hygromycin B

The pJTI™ Fast DEST vector (thus the targeting expression construct after the MultiSite Gateway® recombination reaction) contains 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 Life Technologies (see page x for ordering information).

- 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 Transgenic Cell Line by Targeted Integration, continued

Determining the Hygromycin B Sensitivity

To successfully target your mammalian cell line, 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 ensure that you 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 ix for ordering information), and electroporation or microporation. Both methods do not seem to affect the growth characteristics 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

Generating Transgenic Cell Line by Targeted Integration, continued

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 ix 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 24 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 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 (i.e., targeting expression construct **and** pJTI™ PhiC31 Int vector) per 1×10^6 to 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. To increase the probability of getting single-copy integration of the targeting expression construct, we recommended that you use the amount of vector that results in less than five drug-resistant colonies in the **absence** of integrase. Using that amount in the presence of integrase should result in >15 colonies.

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.

Continued on next page

Generating Transgenic Cell Line by Targeted Integration, continued

Transfection Procedure

You may use any of the recommended procedures to co-transfect pJTI™ PhiC31 Int and your targeting expression construct 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 targeting 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** (at least 24 hours for lipid-mediated transfection, 48–72 hours for 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**, below.

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. 48–72 hours after transfection or when your cells have sufficiently recovered, 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 12).
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 Transgenic Cell Line Clones

Introduction

The PhiC31 integrase catalyzes recombination between two non-identical 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 as substrates for the reverse reaction (Chalberg *et al.*, 2006). Therefore, the Hygromycin B resistance conferred to your cell line by the integration of your targeting expression construct and the subsequent selection in selective medium virtually guarantees that your clones contain your gene(s) of interest. 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 to confirm the presence of your genetic elements of interest.

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 the pJTI™ Fast DEST vector 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 also suitable.
- **Genomic DNA:** We recommend using the DNAzol® Reagent to isolate the genomic DNA from the Hygromycin B-resistant clones (see page ix for ordering information).
- **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™ Fast DEST vector (such *Hin*d III or *Spe* I). Hybridization of the Hygromycin probe to the digested DNA should then allow you to detect a single band containing the Hygromycin resistance gene from the pJTI™ Fast DEST vector.
- **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 outside 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 targeting expression construct.
- DNA from multiple integrants should contain more than one hybridizing band. If the targeting expression construct integrates into multiple chromosomal locations, the bands may be of varying sizes.

Continued on next page

Screening Transgenic Cell Line Clones, continued

PCR Analysis

When performing PCR analysis on the genomic DNA isolated from your transgenic cell 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.
- Design your primers for your gene(s) of interest to confirm their presence in the genome of Hygromycin-resistant clones.
- You may design your positive control primers for the Hygromycin resistance gene. You may use both the genomic DNA from your clones and the plasmid DNA from your targeting expression construct as positive controls.
- For a map and features of the pJTI™ Fast DEST vector, see page 23. The vector sequence of pJTI™ Fast DEST is available on our website at **www.lifetechnologies.com** or by contacting Technical Support (see page 30).
- We recommend a high fidelity thermostable DNA polymerase such as the AccuPrime™ *Taq* DNA Polymerase for the nested PCR (see page ix 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 ix for ordering information).
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 your targeting expression construct into the genome of your cell line will provide the target for the PCR analysis. When run on an agarose gel, you should see a band of appropriate size representing the sequences from your gene of interest.

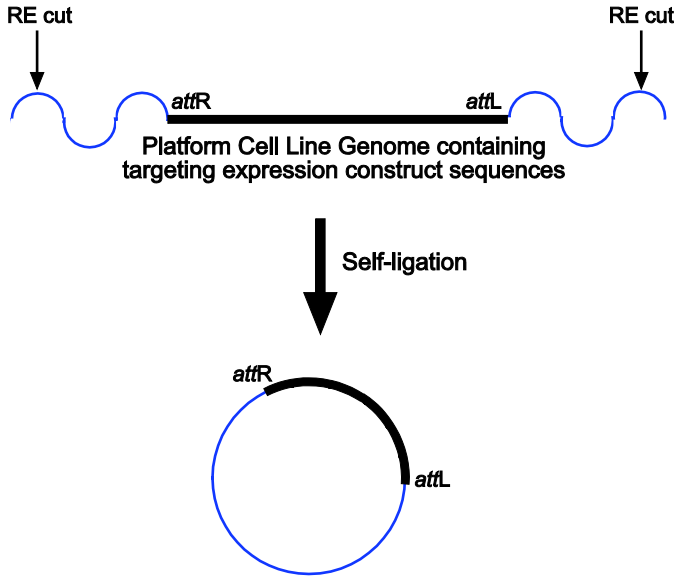
Freezing Your Transgenic Mammalian Cells

We highly recommend that you freeze and bank at least 10–20 vials of your transgenic mammalian cells once you have expanded the cells and confirmed that a single integration event has occurred. For instructions on cryopreserving mammalian cells, see page 25, **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 targeting expression construct (i.e., pJTI™ Fast DEST) sequences (adapted from Chalberg *et al.*, 2006).



Plasmid Rescue

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 targeting expression construct 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 x 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).

Clonal Selection

Chromosomal Positioning Effects

The pseudo-*att*P sites targeted by the PhiC31 integrase have partial sequence identity with the native *att*P sites, and have been found to be present in the intronic regions of genes and transcriptionally active areas (Chalberg *et al.*, 2006). Since these pseudo-*att*P sites are typically in open chromatin regions, there is usually less interference with transgene expression, which allows the use of different promoters to differentially regulate transgene expression (Thyagarajan *et al.*, 2008). However, although it is unlikely, the possibility of interference with the expression of transgenes in your cell line due to chromosomal positional effects still exists amongst the Hygromycin B-resistant clones.

Clonal Selection or Polyclonal Pools?

Depending on your downstream applications, you may decide to simply pool all the drug resistant foci for expression of your protein of interest, or assess the expression levels of individual clones and choose the highest- or lowest-expressing ones. An extremely useful feature of the Jump-In™ Fast Gateway® System is the small number of chromosomal pseudo-*att*P hotspots for PhiC31-mediated integration which considerably reduces the number of clones you will have to screen for the clone that best suits your experimental needs.

Clonal Selection by Limiting Dilution

To obtain a clonal cell line (*i.e.*, derived from a single colony), you may dilute your pool of stably transfected cells at 1–2 cells per well in a 24-, 48-, or 96-well plate and culture under selection in the appropriate medium for your cell line. After scaling-up your cultures derived from single-colonies, you may check for expression of your transgenes by SDS-PAGE, protein-specific ELISA, or a bioactivity assay of choice.

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 targeting 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 2–3 × 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 24).
MEFs overgrow plate	MEFs not inactivated	Inactivate mitosis in MEFs as described on pages 23–29, or purchase inactivated MEFs (see page viii).

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 viii) or make (see page 23) 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 Hygromycin B required to kill untransfected cells as described on page 12 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.

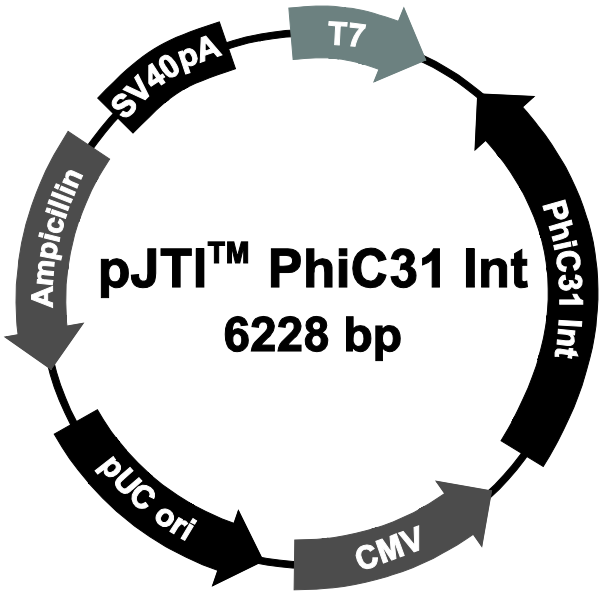
Problem	Cause	Solution
Low survival rate after transfection	Poor DNA quality	The quality of the plasmid DNA strongly influences the results of transfection experiments. Use **endotoxin-free DNA** for all transfections. Make sure that the A260:A280 ratio of the DNA is between 1.8 and 2.0. **Do not** use 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 $0.6–1.0 \times 10^7$ 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™ Fast Gateway® System, it is used for site-specifically integrating your gene(s) of interest in the genome of the mammalian cell line of choice. **The vector sequence of pJTI™ PhiC31 Int is available from www.lifetechnologies.com or by contacting Technical Support (see page 30).**



Features of pJTI™ PhiC31 Int
6228 nucleotides

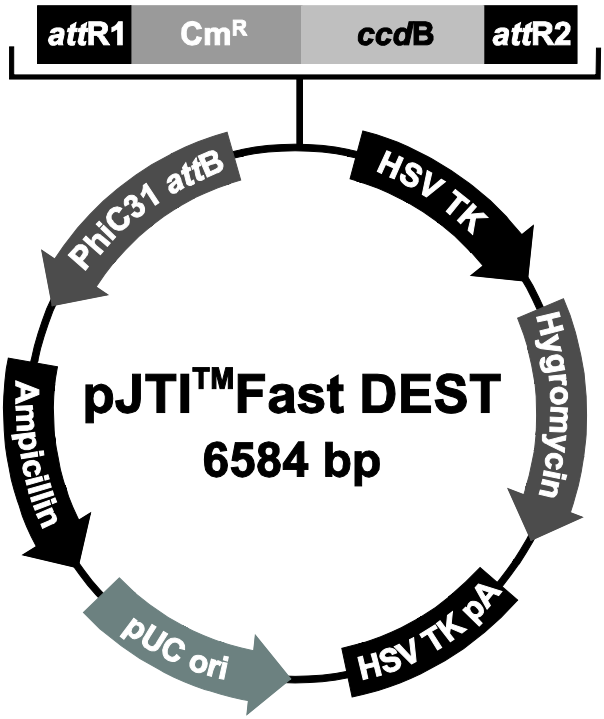
T7 promoter: bases 1-20
PhiC31Int: bases 83-1924 (c)*
CMV promoter: bases 2113-2636 (c)
pUC origin: bases 3121-3794 (c)
Ampicillin resistance gene (ORF): bases 3981-4841 (c)
SV40 polyA site: bases 5777-6139 (c)

*(c): complementary strand

pJTI™ Fast DEST

Map of pJTI™ Fast DEST

The pJTI™ Fast DEST vector (6584 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 targeting expression construct, the PhiC31 *att*B site for the PhiC31-mediated integration into genome of your mammalian cell line, and the Hygromycin resistance gene for the selection of your transgenic cell line clones. **The vector sequence of pJTI™ Fast DEST is available from www.lifetechnologies.com or by contacting Technical Support (see page 30).**



Features of pJTI™Fast DEST
6584 nucleotides

HSV TK: bases 1-249
Hygromycin resistance gene: bases 262-1296
HSV TK pA: bases 1300-1790
pUC origin: bases 1810-2483 (c)*
Ampicillin resistance gene: bases 2631-3488 (c)
PhiC *att*B integration site: bases 3629-3907
OriP: bases 5027-7002 (c)
*att*R1 recombination site: bases 4447-4571
Chloramphenicol resistance gene: bases 4680-5360
*ccd*B gene: bases 5680-5985
*att*R2 recombination site: bases 6026-6150 (c)

*(c): complementary strand

Assessing Cell Vitality

Introduction

We recommend using the LIVE/DEAD® Cell Vitality Assay Kit, available separately from Life Technologies, 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 (see page ix for ordering information).

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, ensure that you will avoid loss by contamination, and will 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 1–5 × 10^7 cells/mL for serum containing medium, or 0.5–1 × 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 0.5–1 × 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 $\geq 3 \times 10^5$ 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 15 mL 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 vii–viiifor 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 (~5–6 × 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 28 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 vii 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 vii ordering information).
7. Add 3 mL of 0.05% Trypsin-EDTA solution per flask to trypsinize cells (see page ix 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 2–5 days after plating for hESC cell culture. The medium should be changed every other day if they are not used immediately.

Technical Support

Obtaining Support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Purchaser Notification

Introduction

Use of the StemPro® TARGET™ hESC - BG01v Kit is covered under the licenses detailed below.

Information for European Customers

StemPro® TARGET™ hESC - BG01v cells (variant hESC BG01V) are genetically modified and carry a chromosomal target site for R4 Integrase and a Hygromycin Resistance gene. The paternal human stem cells were derived March 2001 from a supernumerary IVF embryo that would have otherwise been discarded, and was obtained with informed consent. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Life Technologies understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

Life Technologies also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

Additional Terms and Conditions

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

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Notes

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