USER GUIDE

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StemPro® TARGET™ hESC BG01v Kit

For Site-specific Retargeting in Human Embryonic Stem Cells Using MultiSite Gateway® Technology

Catalog number R7799-300

Revision date 10 February 2012
Publication Part number A10722

MAN0000707

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Contents

Kit Contents and Storage

Introduction

This manual is supplied with the StemPro® TARGET™ hESC BG01v Kit (Cat. no. R7799-300) and provides guidelines and instructions for maintaining and retargeting the StemPro® TARGET™ hESC - BG01v (**h**uman **e**mbryonic **s**tem **c**ell) platform line.

Shipping/Storage

The StemPro® TARGET™ hESC BG01v Kit is shipped on dry ice. Upon receipt, store each component as detailed below. All reagents are guaranteed for six months if stored properly.

Item	Shipping	Storage
StemPro® TARGET™ hESC - BG01v Cells	Dry ice	**Liquid Nitrogen**
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**

Handle the StemPro® TARGET™ hESC - BG01v cells as potentially biohazardous material under at least Biosafety Level 1 containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Material Safety Data Sheet before handling

Important

The StemPro® TARGET™ hESC BG01v Kits are designed to help you create a multiple-fragment retargeting clone using the MultiSite Gateway® Technology, and using this construct to insert multiple genetic elements into the chromosomal target site on the platform hESC line. **Although the kits have been designed to help you produce your retargeting construct in the simplest, most direct fashion, as well as to maintain the platform hESC line undifferentiated and to accomplish its retargeting in the most efficient way, the use of these products are geared towards users who are familiar with the concepts of the Gateway® Technology, site-specific recombination, and culturing hESC lines.** If you are unfamiliar with these technologies, we recommend that you acquire a working knowledge of the Gateway® Technology and methods for maintaining hESC cultures.

Intended Use

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

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Kit Contents and Storage, continued

Kit Components

The StemPro® TARGET™ hESC BG01v Kit contains the following components. The contents of each kit component are described below.

StemPro® TARGET™ hESC - BG01v Cells

StemPro® TARGET™ hESC - BG01v cells are shipped in a cryovial on dry ice. Upon receipt, promptly store the vial containing the cells in **liquid nitrogen**. For the composition of the Freezing medium, see page 12.

Component	Amount	Quantity
StemPro® TARGET™ hESC - BG01v Cells	~3 × 10^6 cells/ml in Freezing medium	1 ml

Jump-In™ Vector Kit

The Jump-In™ Vector Kit contains the necessary vectors for retargeting the platform hESC line. Upon receipt, store the vectors at –20°C.

Component	Composition	Amount
pJTI™ R4 Int	20 µl of vector at 0.5 µg/µl in TE buffer, pH 8.0*	10 µg
pJTI™ R4 DEST	40 µl of vector at 150 ng/µl in TE buffer, pH 8.0	6 µg

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

MultiSite Gateway® Pro 2.0 Vector Module

The following vectors and primers are supplied with the MultiSite Gateway® Pro 2.0 Vector Module for creating the entry and expression clones in a 2-fragment recombination reaction. Store the contents of the module at –20°C.

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
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
pDONR™ 221	Lyophilized 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

Note

For detailed information on the MultiSite Gateway® Pro 2.0 Vector Module and the additional products that may be used with it, refer to the MultiSite Gateway® Pro manual (25-0942) supplied with the kit.

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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 StemPro® TARGET™ hESC BG01v Kits. For accessory products that may be used with the MultiSite Gateway® Pro 2.0 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 45).

Products for hESC Culture

We recommend the following accessory products for culturing, passaging, and maintaining the StemPro® TARGET™ hESC - BG01v platform line. 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 45).

Product	Amount	Cat. no.
StemPro® hESC SFM Complete Medium (contains StemPro® supplement, D-MEM/F-12 with GlutaMAX™, 25% BSA, FGF basic, and 2-mercaptoethanol)	1 kit	A1000701
Collagenase Type IV	1 g	17104-019
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
GlutaMAX™-I Supplement	100 ml	35050-061
KnockOut™ Serum Replacement (KSR)	500 ml	10828-028
MEM Non-Essential Amino Acids Solution 10 mM (100X)	100 ml	11140-050
bFGF (FGF Basic, Human Recombinant)	50 µg	PHG0026
2-Mercaptoethanol	50-ml	21985-023
Fetal Bovine Serum, ES Cell-Qualified (US)	500 ml	16141-079
Bovine Albumin Fraction V Solution (7.5%)	100 ml	15260-037
BSA, 10% Ultrapure Molecular Biology Grade	1000 ml	P2458
Geltrex™	5 ml	12760-021
Geltrex™, hESC qualified	1 ml	A10480-01
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid	1000 ml 6 × 1000 ml	14190-136 14190-235
Antibiotic-Antimycotic (100X), liquid	100 ml	15240-062
StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit	100 reactions	23191-050
StemPro® EZPassage™ Disposable Stem Cell Passaging Tool	10 tools (disposable)	23181-010
Water, distilled	500 ml	15230-162

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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 grow StemPro® TARGET™ hESC - BG01v. 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 (~5–6 × 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).

Characterization and Quality Control of hESC

Following accessory products, available separately from Life Technologies, may be used in assays for characterization and quality control of StemPro® TARGET™ hESC - BG01v after retargeting. For more information, refer to our website at **www.lifetechnologies.com** or contact Technical Support (see page 45

Product	Amount	Cat. no.
LIVE/DEAD® Cell Vitality Assay Kit	1000 assays	L34951
Trypan Blue Stain	100 ml	10250-061
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml 1 L	10010-023 10010-031
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	1 L	15557-044
UltraPure™ 10% SDS Solution	4 × 100 ml	15553-027

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Transferring the Platform Line to Feeders

We recommend the following accessory products for transferring the StemPro® TARGET™ hESC - BG01v platform cell line to mouse feeders. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 45).

Product	Amount	Cat. no.
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid (Ca- and Mg-free)	500 ml 10 × 500 ml	14190-144 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 1 L	10010-023 10010-031
Fetal Bovine Serum, ES Cell-Qualified (US)	500 ml	16141-079
Trypsin-EDTA (0.05% Trypsin, EDTA•4Na) (1X), liquid	100 ml 20 × 100 ml	25300-054 25300-120

Retargeting the Platform Line

We recommend the following accessory products to prepare the StemPro® TARGET™ hESC - BG01v platform line for retargeting. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 45).

Product	Amount	Cat. no.
TrypLE™ Express Dissociation Enzyme without Phenol Red	100 ml 20 × 100 ml	12604-013 12604-039
OptiPRO™ SFM	1000 ml	12309-019

Selection Agents

The table below lists ordering information for the selection agents required for use with the StemPro® TARGET™ hESC BG01v Kits.

Product	Amount	Cat. no.
Hygromycin B	20 ml	10687-010
Zeocin™	1 g 5 g	R250-01 R250-05

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MultiSite Gateway® Pro Kits

Life Technologies offers a 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 StemPro® TARGET™ hESC BG01v kits. Each kit supplies enough reagents for 20 recombination reactions.

Product	Catalog 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

Introduction

Overview

Introduction

The StemPro® TARGET™ hESC BG01v Kit combines Life Technologies' MultiSite Gateway® Pro technology with the StemPro® TARGET™ hESC - BG01v platform cell line for efficient and specific insertion of multiple genetic elements such as promoter-reporter pairs into a chromosomal target site on the platform line. The kit includes:

- The MultiSite Gateway® Pro 2.0 Module for simultaneous cloning of two DNA fragments to generate a retargeting construct that contains the two DNA elements. Based on the Gateway® Technology (Hartley *et al.*, 2000; Sasaki *et al.*, 2005; Sasaki *et al.*, 2004) the MultiSite Gateway® Technology uses site-specific recombinational cloning to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation.
- The StemPro® TARGET™ hESC - BG01v cell line containing an "engineering platform" in the transcriptionally active and stable chromosomal locus 13q32 for site-specific "retargeting" of the cell line.
- pJTI™ R4 DEST vector (i.e., the "retargeting construct" when containing the DNA elements of interest) and pJTI™ R4 Int vector (expressing the R4 Integrase) for co-transfecting the StemPro® TARGET™ hESC - BG01v platform cell line for retargeting.

Jump-In™ Cell Engineering Technology

The StemPro® TARGET™ hESC - BG01v cells supplied with this kit were generated using Life Technologies' Jump-In™ cell engineering technology. The Jump-In™ technology uses phiC31 mediated recombination to stably integrate DNA sequences of choice at specific genomic locations in mammalian cells called PhiC31 psuedo sites. The StemPro® TARGET™ hESC - BG01v platform cell line was engineered to contain a chromosomal target for the R4 Integrase for efficient and site-specific insertion of multiple genetic elements using the pJTI™ R4 DEST and pJTI™ R4 Int vectors (see page 3).

The Jump-In™ TI™ Expression System, available separately from Life Technologies, enables rapid generation of isogenic stable cell lines that allow long –term and regulatable expression from your gene of interest.

The Jump-In™ Fast Expression System, also available separately from Life Technologies, facilitates the production of a polyclonal pool of mammalian cells that over-express your protein of interest.

For more information on Jump-In™ TI™ and Jump-In™ Fast Expression Systems, visit our website at **www.lifetechnologies.com** or contact Technical Support (page 45).

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Purpose of This Manual

This manual provides an overview of the StemPro® TARGET™ hESC - BG01v platform line, and offers instructions and guidelines for:

- Maintaining the StemPro® TARGET™ hESC - BG01v culture in StemPro® hESC SFM (serum- and feeder-free medium), as well as in MEF-CM (mouse embryonic fibroblast conditioned medium) and on MEF feeders.
- Characterization and quality control of StemPro® TARGET™ hESC - BG01v platform cell line after retargeting.
- Retargeting the StemPro® TARGET™ hESC - BG01v platform line with the retargeting construct, and subsequent selection and expansion of transformants.

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 the StemPro® TARGET™ hESC - BG01v platform line, see Liu *et al.*, 2008 and Thyagarajan *et al.*, 2008 (Liu *et al.*, 2008; Thyagarajan *et al.*, 2008). For more information on culturing hESC using StemPro® hESC SFM, refer to **www.lifetechnologies.com** or contact Technical Support (see page 45).

StemPro® TARGET™ hESC - BG01v Cells

Introduction

The StemPro® TARGET™ hESC - BG01v platform cell line is engineered to contain a chromosomal target for the R4 Integrase for efficient and site-specific insertion of multiple genetic elements (e.g., promoter-reporter pairs) from a retargeting construct that has been adapted for complex element assembly using MultiSite Gateway® Technology. Retargeted clones exhibit sustained expression and appropriate regulation of the transgenes over long-term undifferentiated culture, as well as upon their random differentiation and directed induction into various cell lineages. For more information on the StemPro® TARGET™ hESC - BG01v platform cell line, refer to Liu *et al*., 2008 and Thyagarajan *et al*., 2008.

Characteristics of StemPro® TARGET™ hESC - BG01v cells

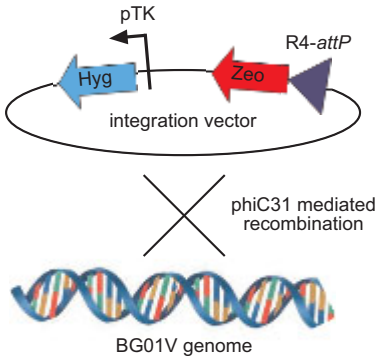
The StemPro® TARGET™ hESC - BG01v cells exhibit the following characteristics:

- Derived from the BG01V human embryonic stem cell line (ATCC No. SCRC-2002)
- 48, XY, +12, +17
- Prepared from low passage parental BG01V cells.
- Pluripotent: can differentiate to representatives of the three primary germ layers
- Contains R4 Integrase target site on chromosome 13q32 for site-specific retargeting
- Chromosomal target locus 13q32 remains transcriptionally active upon differentiation

Generation of StemPro® TARGET™ hESC - BG01v Platform Cell Line

We constructed an integration vector containing the R4 *attP* target sequence and the Hygromycin resistance gene, and used phiC31 mediated recombination to stably integrate the target site into the genome of BG01V cells. This placed the R4 *attP* site upstream of a selectable marker lacking a promoter (Zeocin™ resistance gene, *Sh ble*) on the 13q32 chromosomal locus. The Hygromycin resistant colonies were tested extensively to make sure that they contained a single copy of the target site, maintained parental BG01V karyotype, and retained hESC properties. Further examination of these clones revealed that they were pluripotent and were able to differentiate into representatives of all three primary germ layers. The resulting cell line was called StemPro® TARGET™ hESC - BG01v.

Note: Since the integration vector used contains a Hygromycin resistance gene (Hyg^R), StemPro® TARGET™ hESC - BG01v platform line is resistant to Hygromycin B. If you want to stably integrate more genes into this cell line, do not use Hygromycin B for selection.



Methods

General Information

Introduction

This section provides instructions and guidelines for maintaining and retargeting the StemPro® TARGET™ hESC - BG01v platform line, as well as characterization and quality control of the cell line after retargeting.

This section **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 hESC culture maintenance, visit our website at **www.lifetechnologies.com** or contact Technical Support (see page 45)

pJTI™ R4 DEST and pJTI™ R4 Int Vectors

The pJTI™ R4 DEST vector is designed specifically to be used in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression clone containing multiple DNA elements of your choice. The pJTI™ R4 Int vector allows the expression of the R4 Integrase, which facilitates the site specific integration of your multiple DNA elements into the platform target site when the StemPro® TARGET™ hESC - BG01v platform line is contransfected with both vectors. For a map and features of each vector, see pages 38–39. For the recombination region of the pJTI™ R4 DEST, see page 16.

Propagating pJTI™ R4 DEST and pJTI™ R4 Int 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 (Cat. no. A10460) from Life Technologies. 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 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™ 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 2.0 Module, refer top the MultiSite Gateway® Pro manual supplied with the StemPro® TARGET™ hESC BG01v kit. The MultiSite Gateway® Pro manual is also available online at **www.lifetechnologies.com** or by contacting Technical Support (see page 45).

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As with other human cell lines, when working with StemPro® TARGET™ hESC - BG01v cells, handle as potentially biohazardous material under at least Biosafety Level 1 containment.

General Cell Handling

Follow the general guidelines below to grow and maintain the StemPro® TARGET™ hESC - BG01v platform line:

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper sterile technique and work in a laminar flow hood.
- When subculturing StemPro® TARGET™ hESC - BG01v cells on mouse embryonic fibroblast feeder layers (MEF feeders), always use mitotically inactivated MEFs. Make sure to start preparing the feeder layer two days before culturing StemPro® TARGET™ hESC - BG01v cells.
- 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 (below 30 passages) for your experiments. Upon receipt of the cells from Life Technologies, grow and freeze multiple vials of the StemPro® TARGET™ hESC - BG01v cells to ensure that you have an adequate supply of early-passage cells.
- For general maintenance of the cell line, pass StemPro® TARGET™ hESC - BG01v cells when they are near confluence.
- When thawing or subculturing cells, transfer cells into pre-warmed medium.
- 10 ml/l of antibiotic-antimycotic containing penicillin, streptomycin, and amphotericin B may be used if required (see page vii for ordering information).

Important

It is very important to strictly follow the guidelines for culturing the StemPro® TARGET™ hESC - BG01v cells in this manual to keep them undifferentiated.

Thawing StemPro® TARGET™ hESC - BG01v Cells in StemPro® hESC SFM

Introduction

Follow the protocol below to thaw StemPro® TARGET™ hESC - BG01v cells to initiate cell culture in StemPro® hESC SFM. The StemPro® TARGET™ hESC - BG01v line is supplied in a vial containing 1 ml of cells at 3×10^6 cells/ml in freezing medium. For harvesting and freezing StemPro® TARGET™ hESC - BG01v cells in StemPro® hESC SFM, see below.

Materials Needed

- StemPro® TARGET™ hESC - BG01v cells (store frozen cells in liquid nitrogen until ready to use).
- StemPro® hESC SFM pre-warmed to 37°C (see page vii for ordering information).
- Disposable, sterile 15-ml conical tubes.
- 37°C water bath.
- Geltrex™-coated 35-mm dish (see page vii for ordering Geltrex™, and page 8 for instructions on preparing Geltrex™-coated dishes).

Important

The StemPro® TARGET™ hESC - BG01v cells supplied with this kit were harvested and banked after they were established on StemPro® hESC SFM; therefore, therefore they should be thawed into StemPro® hESC SFM. Although you may also thaw these cells on Mouse Embryonic Fibroblast-Conditioned Medium (MEF-CM) or MEF feeder layers, we recommend using StemPro® hESC SFM to minimize the stress from the thawing procedure. However, **if you have frozen hESCs harvested from MEF-CM or MEF feeder layers, you must thaw your cells into either MEF-CM or MEF feeder layers** to achieve high plating/survival of your hESC culture. See page 19 for thawing and establishing your hESC culture on MEF feeder layers.

Thawing Procedure

Store frozen cells in liquid nitrogen until ready to use. To thaw and establish StemPro® TARGET™ hESC - BG01v cells in StemPro® hESC SFM:

1. Remove the cryovial of cells from the liquid nitrogen and thaw quickly in a 37°C water bath (to prevent crystal formation).
2. When thawed, immediately transfer cells into 15-ml conical tube.
3. Add 1 ml of pre-warmed StemPro® hESC SFM to the 15-ml tube with thawed cells in a **dropwise** fashion**.**
   Note: Dropwise addition of pre-warmed StemPro® hESC SFM during the thawing procedure is crucial.
4. Centrifuge the 15-ml tube with cells at 1000 rpm (200 × g) for 2 minutes.
5. Repeat 2–3 times.
6. Aspirate medium, resuspend cells in warm StemPro® hESC SFM, and plate onto Geltrex™ coated dish (2 ml for a 35-mm dish).
7. Grow cells in a 37°C incubator with a humidified atmosphere of 5% CO_2. Change the medium everyday.

See page 11 for an image of the StemPro® TARGET™ hESC - BG01v platform line on the third day of incubation after thawing on StemPro® hESC SFM.

Human Embryonic Stem Cell Culture on StemPro® hESC SFM

Introduction

Traditional hESC culture methods require the use of mouse or human fibroblast feeder layers, which are labor-intensive and hard to scale, and the undefined conditions on feeder cultures make it difficult to maintain hESCs undifferentiated. StemPro® hESC SFM enables culture of hESCs in a Serum Free Medium (SFM) without feeder cells (see page vii for ordering information).
Go to **www.lifetechnologies.com/stempro/hesc** for an instructional video on how to use StemPro® hESC SFM

Important

Before starting experiments, we recommend that you first freeze 10–20 vials of the StemPro® TARGET™ hESC - BG01v cells in SFM Freezing Medium, as described in **Freezing StemPro® TARGET™ hESC - BG01v Cells Cultured on StemPro® hESC SFM** on page 12.

Important Points for hESC culture in StemPro® hESC SFM

To prevent differentiation and slow growth of StemPro® TARGET™ hESC - BG01v cells grown in StemPro® hESC SFM, follow these guidelines:

- **Starter culture:** This must be a high-quality culture, with a high density of cells, and primarily undifferentiated. The starter culture should be cells maintained on StemPro® hESC SFM, or on Geltrex™ in Mouse Embryonic Fibroblast-Conditioned Medium (MEF-CM) (see page vii for Geltrex™ ordering information). The cells should not be maintained on MEF feeders.
  Note: You may also maintain your starter culture on Matrigel® MEF-CM or on CELLstart™ without MEF-CM.
- **Passaging:** Passaging the cells is the most likely point of difficulty. It is critical to achieve high plating/survival of colony pieces. The pieces must be smaller than typical collagenase passaging on Geltrex™/MEF-CM. We recommend using the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool for reproducible and optimal passaging (see page vii for ordering information).
- Some **cell death** at passaging is normal; however, wide-scale cell death (i.e., <20% survival) indicates poor passaging.
- **Timing of passaging is a critical factor**. Do **not** passage the cells too early as they will plate poorly and differentiate. The cultures need to grow to near-confluence before they are harvested, i.e., a day or two after the colonies are just touching. This usually results in a harvest of 5–8 million cells per 60-mm culture dish.
- **Exposure to collagenase:** hESCs in StemPro® hESC SFM are very sensitive to overexposure to collagenase, which causes poor plating and differentiation. Do **not** expose cells to collagenase for longer than 3 minutes. Do **not** use lower concentrations of collagenase or treat for longer periods.
- **Density**: The cultures must be maintained at a high density (200+ colonies in a 60-mm dish). A lower cell density will cause the culture to deteriorate, slowing proliferation and causing differentiation. If this happens, allow the culture to proliferate to near confluence before splitting.
- hESCs grown in culture are always under pressure between **proliferation** and **differentiation**. The cultures should be fed every day; do not exhaust medium by not feeding. Scrape clearly differentiated areas out with a 21½-gauge needle.

Continued on next page

hESC Culture on StemPro® hESC SFM, continued

Physical Conditions for hESC Culture

Media: StemPro® hESC SFM (Cat. no. A10007-01) contains D-MEM/F-12 with GlutaMAX™, StemPro® hESC Supplement, and Bovine Serum Albumin 25% (BSA). Refer to the manual supplied with StemPro® hESC SFM for storage and handling information.

Incubator: 36 to 38°C, humidified atmosphere of 4 to 6 % CO_2 in air

Culture Conditions: Adherent; ensure proper gas exchange and minimize exposure to light

Recommended Culture Vessels: 35-mm or 60-mm dishes

Materials and Reagents Needed

You will need to have the following materials and reagents on hand before beginning (see page vii for ordering information):

- **bFGF (10 µg/ml)**: Prepare 10 µg/ml bFGF working solution in D-MEM/F-12 with 0.1% BSA, aliquot 80 µl per tube and store frozen at –20°C. Thaw at 37°C water bath immediately before use.
- **StemPro® hESC SFM complete medium**, pre-warmed to 37°C (see **Media Preparation**, next page).
- **Collagenase Type IV**: Dissolve 10 mg/ml collagenase in D-MEM/F-12, filter to sterilize and freeze in aliquots. Thaw immediately before use.
- **Geltrex™** Reduced Growth Factor Basement Membrane Matrix: Thaw the Geltrex™ bottle at 4°C overnight to prevent polymerization. Next day, dilute Geltrex™ 1:2 with D-MEM/F-12 at 4°C to make 100X stock solution, using an ice bucket to keep the bottles cold. Quickly prepare 0.5 ml aliquots in 50-ml conical tubes (pre-chilled on ice), and store at –20°C. Thaw at 4°C before use.
- Dulbecco's Phosphate Buffered Saline (D-PBS) (1X).
- D-MEM/F-12.
- 37°C water bath.
- Geltrex™-coated 35-mm culture plate (see page vii for ordering Geltrex™, and below for instructions on preparing Geltrex™-coated plates).

Coating Plates with Geltrex™

1. Thaw 1 tube of Geltrex™ (0.5 ml, aliquoted as above) slowly at 4°C and add 49.5 ml of cold D-MEM/F-12 (1:100 dilution). Mix gently.
2. Cover the whole surface of each culture plate with the Geltrex™ solution (1.5 ml for a 35-mm dish, 3 ml for 60-mm dish).
3. Seal each dish with parafilm to prevent drying, and incubate 1 hour at room temperature in a laminar flow hood.
4. You may store the Geltrex™-treated dish at 4°C for up to 1 month.
5. Before plating cells, tip the plate slightly and aspirate the Geltrex™ solution. Immediately plate cells in pre-equilibrated complete medium.

Continued on next page

Media Preparation

Follow the instructions below for preparing the various media required to maintain your hESC culture in StemPro® hESC SFM.

Storage and Handling of StemPro® hESC SFM Supplement

- StemPro® hESC SFM supplement is supplied as a frozen sample. Thaw supplement prior to use, re-freeze in desired volumes, and store them immediately at –20°C.
- **Avoid multiple freeze thaw cycles** of supplement.
- Thawed StemPro® hESC SFM Growth Supplement must be stored at 2–8°C (Stable up to 1 week).

BSA Wash Medium

Add BSA 25% (supplied with the StemPro® hESC SFM kit, Cat. no. A10007-01) at a final concentration of 0.1% to D-MEM/F-12 containing GlutaMAX™.

StemPro® hESC SFM Complete Medium

Thaw the StemPro® hESC SFM Supplement in 37 °C water bath (minimize dwell time), and prepare the StemPro® hESC SFM Complete Medium according to the table below.

Component	Final Concentration	For 500 ml	For 100 ml
D-MEM/F-12 with GlutaMAX™	1X	454 ml	90.8 ml
StemPro® hESC SFM Supplement (50X)	1X	10 ml	2 ml
BSA 25%	1.8%	36 ml	7.2 ml
bFGF (10 µg/ml)	8 ng/ml	400 µl	80 µl
2-Mercaptoethanol (55 mM)	0.1 mM	909 µl	182 µl

Important

StemPro® hESC SFM Complete Medium can be stored at 2–8°C **in dark** for up to 7 days. **Add 2-Mercaptoethanol daily during storage**, at volumes listed in the table above.

Collagenase Preparation

Prepare 1 mg/ml and 10 mg/ml aliquots of collagenase IV in D-MEM/F-12. Filter to sterilize and freeze at –20°C.
Note: 1 mg/ml aliquots of collagenase IV are used with hESCs maintained on MEF feeders, while the 10 mg/ml aliquots are used with hECSs cultured on StemPro® hESC SFM.

Continued on next page

Passaging Using Collagenase

1. Warm appropriate amount of 10 mg/ml Collagenase IV solution (~2000 U/ml), StemPro® hESC SFM Complete Medium, and BSA wash medium to 37°C in a water bath. Minimize dwell time.
2. Set up hESC plate on a dissecting microscope in a bio-safety cabinet or laminar flow hood to comfortably observe colonies.
3. Cut out and remove any overtly differentiated colonies with a 21½-gauge needle.
4. Aspirate the medium and gently add 1–2 ml of collagenase.
5. Leave for 3 minutes to loosen the cells in between colonies and to round up colony edges.
6. Gently tap the sides of the dish to dislodge cells.
7. Remove collagenase, rinse with D-PBS, and then add 3 ml of BSA wash medium.
8. Gently scrape dish using a sterile 1000 µL pipette tip.
9. Gently transfer clumps using a 5 ml pipette and place into a 15-ml tube.
10. Wash plate with 3 ml of BSA wash medium and add to tube.
11. Centrifuge cells at 200 × g for 2 minutes at room temperature.
12. Gently aspirate media and flick tube to loosen cells from the bottom.
13. Gently resuspend the cells in pre-equilibrated complete medium using a 1 ml or 5 ml serological pipette.
14. Remove a Geltrex™-coated plate from 2–8°C and tip slightly to aspirate the Geltrex™ solution. Immediately plate the cells. Do not allow the surface to dry out before plating.
15. Mix plates gently to evenly spread out the clumps and place the plate into an incubator set at 37°C with 5% CO_2 in air. See the next page for an image of the StemPro® TARGET™ hESC - BG01v platform line immediately after passaging using collagenase.
16. Gently change media the next day to remove excess cells and provide fresh nutrients, and every day thereafter.
17. Observe cells every day and passage by the above protocol whenever required (approximately every 5 to 7 days).

Important

If you are transferring your cells into StemPro® hESC SFM from MEF feeder layers or from MEF-conditioned medium (MEF-CM), prepare a frozen stock of the cells as a precaution. See page 23 for freezing cells cultured in MEF feeders.

Images of the StemPro® TARGET™ hESC - BG01v platform line

Below are representative images of the StemPro® TARGET™ hESC - BG01v platform line 3 days after thawing on StemPro® hESC SFM (Figure 1), and right after passaging using collagenase (Figure 2). The images are provided to display examples of typical StemPro® TARGET™ hESC - BG01v colony morphology.

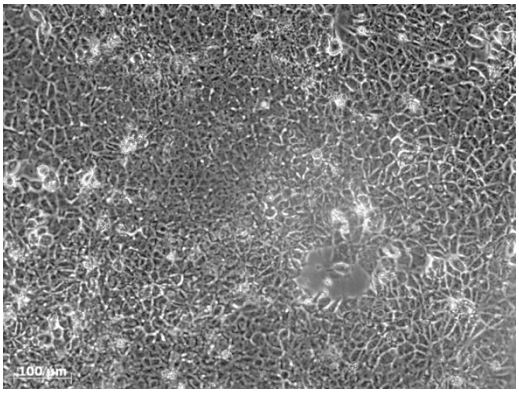


Figure 1. StemPro® TARGET™ hESC - BG01v cells 3 days after being thawed on StemPro® hESC SFM.

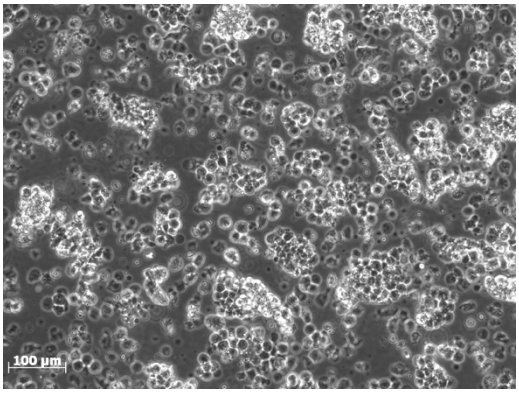


Figure 2. Clumps of StemPro® TARGET™ hESC - BG01v platform line immediately after passaging.

Freezing StemPro® TARGET™ hESC - BG01v Cells Cultured on StemPro® hESC SFM

Introduction

When freezing StemPro® TARGET™ hESC - BG01v cells that are cultured on StemPro® hESC SFM, we recommend the following:

- Freeze cells at a density of 2–3 × 10^6 viable cells/ml.
- For every 20 cm^2 of cells (one 60-mm dish), prepare 0.5 ml of SFM Freezing Medium 1 and 0.5 ml of SMF Freezing Medium 2 (see below).
- Bring StemPro® TARGET™ hESC - BG01v cells into freezing medium in two steps, as described in this section.

Guidelines for preparing freezing medium and freezing cells are provided in this section.

Materials Needed

You will need to have the following reagents on hand before beginning (see page vii for ordering information):

- Plates with StemPro® TARGET™ hESC - BG01v cells on StemPro® hESC SFM.
- BSA Wash Medium (see page 9).
- D-MEM/F-12 with GlutaMAX™.
- Collagenase Type IV working solution (10 mg/ml) in D-MEM/F-12 (see page 9).
- KnockOut™ Serum Replacement (KSR).
- DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood).
- Disposable, sterile 15-ml conical tubes.
- Sterile freezing vials.

Preparing SFM Freezing Medium

Prepare SFM Freezing Medium 1 and 2 immediately before use. Discard any unused medium.

SFM Freezing Medium 1: For every 1 ml, mix together the following in a separate sterile 15-ml tube:

D-MEM/F-12 with GlutaMAX™	0.5 ml
KnockOut™ Serum Replacement (KSR)	0.5 ml

SFM Freezing Medium 2: For every 1 ml, mix together the following in a separate sterile 15-ml tube:

DMEM/F12 with GlutaMAX™	0.8 ml
DMSO	0.2 ml

Keep SFM Freezing Medium 1 at room temperature, and place tube with SFM Freezing Medium 2 on ice.

Continued on next page

Freezing StemPro® TARGET™ hESC - BG01v Cells Cultured on StemPro® hESC SFM, continued

Freezing Protocol for hESCs Cultured on StemPro® hESC SFM

1. Aspirate serum-free culture medium from the cells and gently add 1–2 ml of 10 mg/ml collagenase solution. (Alternatively, use the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool to cut the cell colonies into pieces; follow the protocol provided with the tool and then proceed to step 4.)
2. Leave for 3 minutes to dislodge cell colonies from the substrate.
3. Remove collagenase and rinse with D-PBS.
4. Add 3 ml of BSA wash medium (see page 9).
5. Gently scrape the dish using a sterile 1000-µl pipette tip.
6. Gently transfer the cell clumps using a 5-ml pipette and place into a 15-ml tube.
7. Wash plate with 3 ml of wash medium and add to the tube.
8. Spin cells down for 2 minutes at 200 × g at room temperature.
9. Gently aspirate media and resuspend the StemPro® TARGET™ hESC - BG01v cells in SFM Freezing Medium 1 at room temperature, using 1 ml of freezing medium per cells from one 60-mm dish.
10. Add the same volume of cold SFM Freezing Medium 2 to cells in a **dropwise** manner, swirling the tube after each drop.
11. Resuspend the cells by gently pipetting 2–3 times. Aliquot 1 ml of the cell suspension to each freezing vial and store at –80°C overnight in isopropanol chamber.
12. Transfer frozen vials to liquid nitrogen tank for long-term storage.

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

Important

Before starting experiments, we recommend that you first expand and bank at least 10–20 vials of frozen StemPro® TARGET™ hESC - BG01v cells in SFM Freezing Medium, as described above.

StemPro® TARGET™ hESC - BG01v Culture in MEF-CM

StemPro® TARGET™ hESC - BG01v Culture in Feeder-free Conditions

You must maintain your StemPro® TARGET™ hESC - BG01v cells in MEF-conditioned medium (MEF-CM) as a feeder-free culture for at least one passage prior to transfection with your retargeting construct, as well as for the duration of selection and expansion after transfection. See page 20 for MEF and hESC media recipes.

Preparation of MEF-conditioned Medium

1. Plate mitotically inactivated MEF cells as described in the **Plating MEF Feeder Cell Layer**, page 20.
2. Change the MEF medium to hESC medium after 24 hours of incubation.
3. Collect the hESC medium, now MEF-conditioned medium, from culture dishes every 24 hours and supplement with bFGF to a final concentration of 4 ng/ml before using for hESCs.

Passage of StemPro® TARGET™ hESC - BG01v on Geltrex™ Coated Dishes

1. Prepare Geltrex™-coated dishes as described on page 8, steps 1–4.
2. Aspirate medium from cells and add 1ml collagenase IV per well of a 6-well plate.
3. Incubate for 5–20 minutes at 37°C. Incubation time will vary among different batches of collagenase, therefore you need to determine the appropriate incubation time by examining the colonies. Stop incubation when the edges of the colonies are starting to pull away from the plate.
4. Aspirate the collagenase and add 2 ml of MEF-CM into each well.
5. Gently scrape cells using a cell scraper or a 10 ml pipette to collect most of the cells on the well and transfer cells into a 15-ml tube.
6. Gently dissociate cells into small clusters (50–500 cells) by gentle pipetting. Do not take cells to a single cell suspension.
7. Remove the Geltrex™ from the Geltrex™-coated plates and wash once with D-MEM/F-12.
8. Seed the cells into each well of Geltrex-coated plates. The final volume of medium should be 2–3 ml per well.
9. Return the plate to the 37°C incubator. Make sure to obtain an even distribution of cells by gently shaking the plate left to right and back to front.
10. The day after seeding, undifferentiated cells should be visible as small colonies. Single cells in between the colonies will begin to differentiate. As the cells proliferate, the colonies will become large and compact, representing the majority of surface area of the culture dish. In this system, the hESCs are maintained at high density with 300,000–500,000 cells/cm^2 at confluence. In our experiments, we found the optimal split ratio to be 1:3 to 1:4. Using these ratios, the seeding density is approximately 50,000–150,000 cells/cm^2.

Daily Maintenance of Feeder-free StemPro® TARGET™ hESC - BG01v Culture

1. Collect CM from feeders and add bFGF to a final concentration of 4 ng/ml.
2. Feed hESCs with 2–3 ml MEF-CM supplemented with bFGF for each well of 6-well plates every day.
3. Passage when cells are 100% confluent. At this time the undifferentiated cells should represent at least 90% of the surface area.

Constructing the Retargeting Expression Vector with the MultiSite Gateway® Pro 2.0 Vector Module

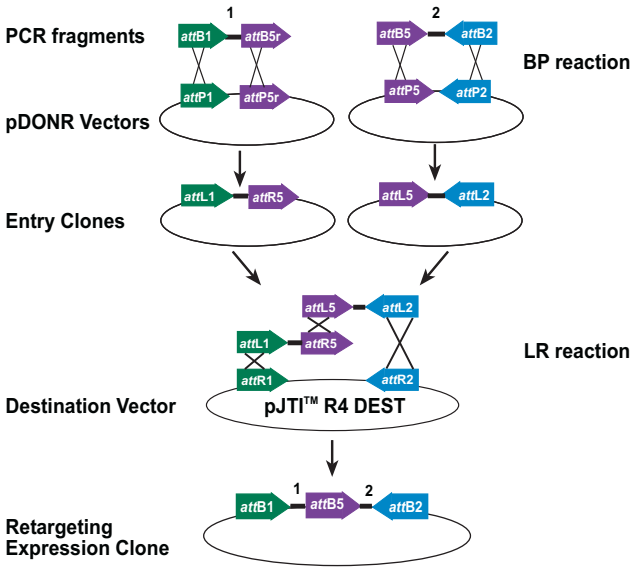
Introduction

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 assembly of more than 3 fragments is an inefficient process, 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 45).

Experimental Outline

Two PCR products flanked by specific *att*B or *att*Br sites and two MultiSite Gateway® Pro Donor vectors, pDONR™ 221 P1-P5r and pDONR™ 221 P5-P2, are used in separate BP recombination reactions to generate two entry clones. The two entry clones and the pJTI™ R4 DEST destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression clone 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 with the MultiSite Gateway® Pro 2.0 Vector Module, continued

MultiSite Gateway® Pro Donor Vectors

The pDONR™ 221 P1-P5r and pDONR™ 221 P5-P2 donor vectors, included in the MultiSite Gateway® Pro 2.0 Vector Module, are used to clone *att*B-flanked PCR products to generate entry clones to facilitate the generation of a retargeting expression construct containing multiple DNA elements. 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, but should not be used to generate multi-fragment entry clones.

pJTI™ R4 DEST Destination Vector

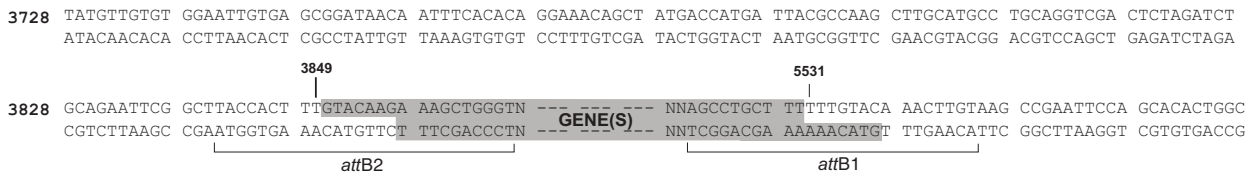
The pJTI™ R4 DEST vector is designed specifically to be used in a MultiSite Gateway® Pro LR recombination reaction to create your retargeting expression clone to site-specifically integrate your multiple DNA elements into the BG01v/R4 genome. The pJTI™ R4 DEST vector contains the human EF1α promoter, which when integrated into the platform line upstream of the *Sh ble* gene by R4 Integrase, results in Zeocin™ resistance of successfully retargeted clones. For a map and a description of the features pJTI™ R4 DEST, see page 38.

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 transferred from the entry clone into pJTI™ R4 DEST by recombination. Non-shaded regions are derived from the pJTI™ R4 DEST vector.

The complete sequence of pJTI™ R4 DEST is available on our website at www.lifetechnologies.com or by contacting Technical Support (see page 45).



Important

Preparation of Plasmid DNA: For retargeting 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, the recommendation would be to have the plasmid DNA 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

Constructing the Retargeting Expression Vector with the MultiSite Gateway® Pro 2.0 Vector Module, continued

Generation of 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. This in turn may lead to inefficient recombination with the pDONR vectors.
- As far as 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, longer incubation times will help. 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 template for PCR to reduce background from inadvertently purified template. The colony should only grow on Kanamycin.

Generation of Retargeting Expression Clones

- Produce clean DNA preparations of the entry clones to be used 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" quality DNA is essential.
- Sequence the entry clones with appropriate primers to ensure 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. The LR clonase 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 for the assembly of greater than 2 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 5-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 on ampicillin and kanamycin plates. True recombinant clones will only grow on ampicillin plates.

Continued on next page

Constructing the Retargeting Expression Vector with the MultiSite Gateway® Pro 2.0 Vector Module, continued

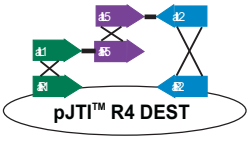
Retargeting Construct Assembly from >2 DNA Fragments

The StemPro® TARGET™ hESC BG01v Kit contains the MultiSite Gateway® Pro 2.0 Vector Module and the pJTI™ R4 DEST vector, which together allow the construction of a 2-fragment retargeting expression construct. For generating expression constructs from up to five individual DNA elements, Life Technologies offers additional MultiSite Gateway® Pro Kits (see page x for ordering information).

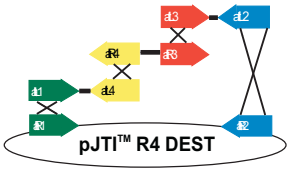
For more information, visit our website at **www.lifetechnologies.com** or contact Technical Support (see page 45).

Schematic for Assembly of Multiple Fragments

The MultiSite Gateway® Pro Kits combined with the pJTI™ R4 DEST vector facilitate rapid and highly efficient construction of retargeting constructs containing your choice of two, three or four separate DNA elements in a defined order and orientation. The schematic below depicts the strategies for the multi-fragment assembly of retargeting constructs with the available pDONR vectors.

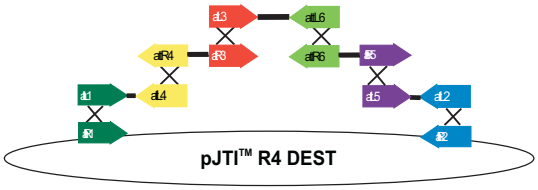


2 fragments



3 fragments

4 fragments



5 fragments

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
5	pDONR221 P1P4, pDONR221 P4rP3r, pDONR221 P3P6, pDONR201 P6rP5r, and pDONR221 P5P2

Culturing StemPro® TARGET™ hESC - BG01v Cells on MEF Feeders

Introduction

You must maintain your StemPro® TARGET™ hESC - BG01v cells on mitotically inactivated Hygromycin-resistant mouse embryonic fibroblast (MEF) feeder cells (MEF feeders) under Hygromycin selection for at least two weeks, and in MEF-conditioned medium (MEF-CM) as a feeder-free culture for at least one passage prior to transfection with your retargeting construct After retargeting, you must plate your transfected cells on MEF feeders to allow them to recover, before starting selection in MEF-CM with Zeocin™. The transformant should be expanded in MEF-CM on Geltrex™-coated dishes under selection before their transfer into StemPro® hESC SFM under selection.

This section provides instructions and guidelines for preparing the MEF feeders, thawing and maintaining StemPro® TARGET™ hESC - BG01v cells (frozen in StemPro® hESC SFM) on MEF feeders, and freezing the hESCs cultured on MEF feeders.

Preparing a MEF Feeder Cell Layer

Follow the protocol below to prepare the matrix (feeder layer) for thawing and establishing StemPro® TARGET™ hESC - BG01v. Cells that are frozen in StemPro® hESC SFM may directly be thawed onto the MEF feeder layer. Use mitotically inactivated MEFs as your feeder cell layer. Both Mitomycin C treatment and irradiation can be used to mitotically inactivate your MEFs.

Materials Needed

To start a MEF feeder cell layer for hESC culture, you will need prepare both MEF and hESC media for which you will need the following reagents (see pages vii–ix for ordering information):

- Mitotically inactivated, Hygromycin resistant MEFs: Order from Millipore or ATCC (see page viii), or generate them as described in **Generating Mitomycin C Treated MEFs** (see Appendix, page 38).
- Dulbecco's Modified Eagle Medium (D-MEM), high glucose with L-glutamine and sodium pyruvate.
- Fetal Bovine Serum (FBS), ES Cell-Qualified.
- MEM Non-Essential Amino Acids Solution 10 mM (100X) (NEAA).
- 2-Mercaptoethanol (1,000X)
- D-MEM/F-12 with GlutaMAX™ (2 mM)
- KnockOut™ Serum Replacement (KSR)
- bFGF: Reconstitute lyophilized human bFGF in sterile D-MEM/F-12 containing 0.1% BSA to 10 µg/ml. Divide stock solution into working aliquots and store at ≤ –20°C.
- Porcine skin gelatin. Prepare 0.1% (w/v) porcine skin gelatin (see page viii) in sterile, distilled water, and sterilize by filtration using a 0.2 micron filter. Store up to 1 year at 4°C.
- 37°C incubator with a humidified atmosphere of 5% CO_2.

Continued on next page

Culturing StemPro® TARGET™ hESC - BG01v Cells on MEF Feeders, continued

MEF Medium

To prepare 500 ml of MEF medium, mix the following reagents:

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.

hESC Medium

To prepare 500 ml of hESC medium, mix the following reagents:

Component	Volume	Final Concentration
D-MEM/F-12 with GlutaMAX™	79 ml	1X
KnockOut™ Serum Replacement (KSR)	20 ml	20%
NEAA (10 mM)	1 ml	0.1 mM
2-Mercaptoethanol, 1,000X (55 mM)	100 µl	55 µM
bFGF (10 µg/ml)	40 µl	4 ng/ml

If stored at 4°C, hESC Medium can be kept for up to 1 week. Pre-heat the medium to 37°C before use.

Gelatin Coated Plates

Coat culture plates for 20–60 minutes at room temperature with sterile 0.1% (w/v) porcine skin gelatin in dH_2O.

Plating MEF Feeder Cell Layer

If you are using commercially available mitotically inactivated MEF, follow the procedure below. For mitotically inactivating your own MEF culture using Mitomycin C, refer to **Generating Mitomycin C Treated MEFs** in the Appendix, page 38.

1. Two days before hESC co-culture, thaw frozen vial of mitotically inactivated, Hygromycin resistant MEFs in a 37°C water bath.
2. Add contents of vial to 5 ml of MEF medium in a 15-ml tube.
3. Centrifuge for 4 minutes at 200 × g.
4. Aspirate medium and re-suspend MEFs in an appropriate volume of MEF medium.
5. Plate MEFs on a 0.1% gelatin-coated culture plate at a density of 30,000/cm^2.
6. One day before hESC co-culture, replace medium with hESC Medium.
7. Next day, the feeder layer is ready to be seeded with StemPro® TARGET™ hESC - BG01v cells in fresh hESC Medium.

Continued on next page

Culturing StemPro® TARGET™ hESC - BG01v Cells on MEF Feeders, continued

Thawing StemPro® TARGET™ hESC - BG01v Cells on MEF Feeders

You may thaw your StemPro® TARGET™ hESC - BG01v cells frozen in StemPro® hESC SFM directly onto the established MEF feeder layer in either 35-mm or 60-mm culture dishes for conditioning prior to transfection. To thaw and establish StemPro® TARGET™ hESC - BG01v cells on MEF feeders:

1. Remove the cryovial of cells from the liquid nitrogen and thaw quickly in a 37°C water bath (to prevent crystal formation).
2. When thawed, immediately transfer cells into a 50-ml tube and add warm hESC Medium **dropwise** up to 10 ml.

   Note: Dropwise addition of pre-warmed hESC Medium during the thawing procedure is crucial.
3. Spin cells down for 4 minutes at 200 × g (1000 rpm).
4. Aspirate supernatant.
5. Resuspend cells in hESC Medium (2 ml for a 35-mm dish)
6. Aspirate feeder layer plates, and plate resuspended StemPro® TARGET™ hESC - BG01v cells on the prepared MEFs.
7. Grow cells in a 37°C incubator with a humidified atmosphere of 5% CO_2. Change the medium every day.
8. When colonies become visible (usually 5–10 days after thawing), passage cells onto Hygromycin-resistant MEF feeders using collagenase (see next page).

Important

You may thaw hESCs frozen in StemPro® hESC SFM directly onto MEF feeders or MEF-CM; however, **if your hESCs were frozen from cultures grown on MEF feeder cells, they must first be thawed and established on MEF feeders.** After your cells have sufficiently recovered and established on 60-mm culture dishes, you may adapt the cells from hESC medium to StemPro® hESC SFM by first passaging them on Geltrex™-coated plates in a feeder-free fashion, then transitioning them to StemPro® hESC SFM, as described in **Expanding Retargeted Clones**, step 4, on page 30.

Materials Needed

For establishing and maintaining your StemPro® TARGET™ hESC - BG01v cells on MEF feeders, you will need to have the following reagents on hand:

- Plates with StemPro® TARGET™ hESC - BG01v cells.
- hESC Medium pre-warmed to 37°C (see page 20 for composition).
- D-MEM/F-12 with GlutaMAX™ (see page vii for ordering information).
- Feeder layer plates with mitotically inactivated MEFs – prepare at least two days in advance (see page 20).
- Collagenase Type IV (see page vii for ordering information).
- Hygromycin B (see page ix for ordering information).
- Disposable, sterile 15-ml tubes.
- 37°C incubator with humidified atmosphere of 5% CO_2.

Continued on next page

Culturing StemPro® TARGET™ hESC - BG01v Cells on MEF Feeders, continued

Passaging StemPro® TARGET™ hESC - BG01v Cells on Hyg-resistant MEF Feeders

The StemPro® TARGET™ hESC - BG01v cells must be maintained on MEF feeders with Hygromycin B selection for at least two weeks prior to transfection.

1. Aspirate culture medium and add 1 ml of 10 mg/ml collagenase solution (see page 9) for every 10 cm^2 of culture vessel surface area. (Alternatively, you may use the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool to cut the cell colonies into pieces; follow the protocol provided with the tool and then proceed to step 4.)
2. Incubate in a 37°C incubator until the edges of colonies curl up (usually less than an hour).
3. Aspirate collagenase solution.
4. Add hESC Medium or 0.1% BSA in D-MEM/F-12.
5. Gently scrape dish using a 5-ml serological pipette and transfer clumps into a 15-ml tube. Do not make the clumps too small; there should be >100 cells per clump.
6. Spin cells down for 2 minutes at 200 × g at room temperature.
7. Gently aspirate media and resuspend the StemPro® TARGET™ hESC - BG01v cells in hESC Medium.
8. Aspirate feeder layer plates, and plate resuspended StemPro® TARGET™ hESC - BG01v cells on the prepared MEFs (passage ratio 1:3 or 1:4).
9. Add a final concentration of 50 µg/ml Hygromycin B (1:1,000 dilution of 50 mg/ml Hygromycin B stock).
10. Grow cells in a 37°C incubator with a humidified atmosphere of 5% CO_2. Change the medium everyday.
11. Feed cells every day and passage by the above protocol whenever required (before colonies start contacting each other; typically every 4–7 days).

Freezing StemPro® TARGET™ hESC - BG01v Cells Cultured on MEF Feeders

Introduction

When freezing StemPro® TARGET™ hESC - BG01v cells that are cultured on MEF feeder cells, we recommend the following:

- Freeze cells at a density of 2–3 × 10^6 viable cells/ml.
- For every 20 cm^2 of cells (one 60-mm dish), prepare 1 ml of MEF Freezing Medium A and 1 ml of MEF Freezing Medium B (see below).
- Bring StemPro® TARGET™ hESC - BG01v cells into freezing medium in two steps, as described in this section.

Materials Needed

- Plates with StemPro® TARGET™ hESC - BG01v cells on MEF feeders.
- hESC Medium (see page 20 for composition).
- Collagenase Type IV working solution (1 mg/ml) in D-MEM/F-12 (see page 9).
- Fetal Bovine Serum, ES Cell-Qualified.
- DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood).
- Disposable, sterile 15-ml conical tubes.
- Sterile freezing vials

Preparing MEF Freezing Medium

Prepare MEF Freezing Medium 1 and 2 immediately before use. Discard any unused medium.

MEF Freezing Medium 1: For every 1 ml, mix together the following in a separate sterile 15-ml tube:

hESC Medium	0.5 ml
Fetal Bovine Serum, ES Cell-Qualified	0.5 ml

MEF Freezing Medium 2: For every 1 ml, mix together the following in a separate sterile 15-ml tube:

hESC Medium	0.8 ml
DMSO	0.2 ml

Keep MEF Freezing Medium 1 at room temperature, and place tube with MEF Freezing Medium 2 on ice.

Continued on next page

Freezing StemPro® TARGET™ hESC - BG01v Cells Cultured on MEF Feeders, continued

Freezing Protocol for StemPro® TARGET™ hESC - BG01v Cells Cultured on MEF Feeders

1. Aspirate culture medium from the cells and add 1 ml of 1 mg/ml collagenase solution for every 10 cm^2 of culture vessel surface area. (Alternatively, use the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool to cut the cell colonies into pieces; follow the protocol provided with the tool and then proceed to step 4.)
2. Incubate in a 37°C incubator until the edges of colonies curl up (usually less than an hour).
3. Aspirate collagenase solution
4. Add hESC Medium (see page 20) or 0.1% BSA in D-MEM/F-12.
5. Gently scrape dish using 5 ml serological pipette and transfer clumps into a 15-ml tube. Try not to make the clumps too small; there should be > 100 cells per clump.
6. Spin cells down for 2 minutes at 200 × g at room temperature.
7. Gently aspirate media and resuspend StemPro® TARGET™ hESC - BG01v cells in Freezing Medium A (e.g., resuspend cells from one 60-mm dish in 1 ml of freezing medium).
8. Add the same volume of Freezing Medium B to cells in a dropwise manner, swirling the tube after each drop.
9. Resuspend the cells by gently pipetting 2–3 times. Aliquot 1 ml of the cell suspension to each freezing vial and store overnight at –80°C in isopropanol chamber.
10. Transfer frozen vials to liquid nitrogen tank for long-term storage.

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

Retargeting StemPro® TARGET™ hESC - BG01v Cells

Introduction

The StemPro® TARGET™ hESC - BG01v platform line is designed for site-specific retargeting mediated by the R4 Integrase, and shows sustained expression and appropriate regulation of the transgenes over long-term culture and random and directed differentiation into a variety of lineages. The MultiSite Gateway® Pro 2.0 vector module, supplied with the kit, allows the assembly of complex genetic elements from two DNA fragments that can efficiently be inserted into the target site on the StemPro® TARGET™ hESC - BG01v genome. This section provides instructions and guidelines on:

- preparing your StemPro® TARGET™ hESC - BG01v platform line for retargeting
- transfection procedure for site-specific integration of genetic elements of interest into the StemPro® TARGET™ hESC - BG01v genome
- selection, expansion, and characterization of the retargeted clones

Growth of StemPro® TARGET™ hESC - BG01v Cells Prior to Transfection

1. Thaw StemPro® TARGET™ hESC - BG01v cells onto MEF feeders in either 35-mm or 60-mm dishes (see page 21 for detailed thawing instructions).
   Note: You may thaw StemPro® TARGET™ hESC - BG01v cells onto MEF feeders regardless of the medium used for their culture prior to freezing.
2. Passage StemPro® TARGET™ hESC - BG01v cells onto Hyg-resistant MEF feeders in 60-mm dishes using collagenase (1 mg/ml) when the colonies become visible, usually 5–10 days after thawing (see page 22 for detailed instructions).
3. Start Hyg selection (10 µg/ml), and keep selection for at least two weeks.
4. Passage cells onto MEF-CM in Geltrex™-coated dishes (feeder-free, see page 14 for detailed instructions) and **stop** Hygromycin selection.
5. Passage StemPro® TARGET™ hESC - BG01v cells once more as a feeder-free culture on MEF-CM and Geltrex™, and proceed to transfection for retargeting.

Continued on next page

Retargeting StemPro® TARGET™ hESC - BG01v Cells, continued

Method of Choice for Transfection

You must use a **high-efficiency transfection method** such as microporation or electroporation to retarget the StemPro® TARGET™ hESC - BG01v platform cell line. The following pages contain two alternative transfection conditions for your convenience.

Important

The following factors are important for a successful retargeting event:

- **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 viii 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 the Vitality of hESCs** on page 42 in the **Appendix**.
- **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.

Materials Needed for Transfection using MP-100 MicroPorator

- StemPro® TARGET™ hESC - BG01v cells (see page 25, step 5).
- Retargeting expression construct generated using MultiSite Gateway® Technology and pJTI™ R4 Int plasmid encoding R4 Integrase.
- 60-mm culture dish containing mitotically inactivated MEF feeders in a 37°C incubator.
- MEF-CM pre-warmed to 37°C.
- TrypLE™ Express Dissociation Enzyme without Phenol Red (see page ix for ordering information).
- D-PBS (Ca- and Mg-free, see page ix for ordering information)
- MP-100 MicroPorator and MicroPorator Kit B (MPK-10096; includes one 100 µl gold plated tip, 20 microporation tubes, 20 ml of Resuspension Buffer R, and 300 ml of Electrolytic Buffer E2) (both available from Digital Bio; refer to www.microporator.com for ordering information).
- LIVE/DEAD® Cell Vitality Assay Kit or Trypan Blue Stain for exclusion counting (see page viii for ordering information).

Continued on next page

Transfection Protocol using MP-100 MicroPorator

1. Turn on MicroPorator; adjust the parameters to 850 V, 30 ms, 1 pulse.
2. Mix 5 µg of retargeting expression vector and 5 µg of pJTI™ R4 Int plasmid in a 1.5-ml microcentrifuge tube for a total of 10 µg DNA per 1×10^6 cells for each transfection.
3. Add 5 ml of pre-warmed MEF-CM (see page 14) to a 60-mm culture dish containing mitotically inactivated MEF feeder layer (see page 19) for each transfection, and put back into 37°C incubator.
4. To harvest target hESC lines, rinse the cells with 2 ml of D-PBS (Ca- and Mg-free), and add 1 ml of TrypLE™ per 60-mm dish. After 4 minutes of incubation at room temperature, gently dislodge cells and transfer to a 15-ml conical tube. Wash the culture dish with at least 2 ml of D-PBS and transfer the wash to the same tube.
5. Set aside a small aliquot of cells for QC, and centrifuge the rest at 200 × g for 4 minutes. During centrifugation, count the cells in the aliquot to determine the resuspension volume.
6. Discard the supernatant and resuspend the cells in Resuspension Buffer R or D-PBS, using 100 µl buffer per 1×10^6 cells. A 60-mm dish should yield about $3–5 \times 10^6$ cells.
7. Combine the DNA (10 µg total, step 2) and 100 µl of resuspended cells (1×10^6 cells) in the same 1.5-ml microcentrifuge tube that contains the DNA.
8. Add 3 ml of Electrolytic Buffer E2 in the microporation tube, pipette 100 µl of the DNA-cell mixture into Gold Tip, and insert Gold Tip into the station.
9. Press the start button, and then the high voltage button to microporate.
10. Immediately transfer microporated cells to MEF-CM/MEF feeder dish from step 3, pre-warmed to 37°C.

Important

After transfection, you must allow the retargeted StemPro® TARGET™ hESC - BG01v cells to recover and the colonies to become well-defined (usually 5 days post-microporation or 2 days post-electroporation) before starting selection with Zeocin™.

Continued on next page

Alternative Transfection Procedure using BTX or Bio-Rad Electroporator

1. Culture feeder-free StemPro® TARGET™ hESC - BG01v cells to 80–90% confluency as described on page 25. Two 60-mm culture dishes usually provide enough cells for one electroporation.
2. Prepare 30 µg total DNA for each electroporation in a 1.5-ml microcentrifuge tube by mixing 15 µg each of the retargeting expression vector and the pJTI™ R4 Int plasmid.
3. For each transformation, add 3 ml of MEF-CM at 37°C to a Geltrex™-coated dish, and put back into 37°C incubator.
4. For each electroporation, add 3 ml of pre-warmed MEF-CM into a 15-ml conical tube and place in a 37°C water bath.
5. To harvest target hESC lines, rinse the cells with 2 ml of D-PBS (Ca- and Mg-free), and add 1 ml of TrypLE™ per 60-mm dish. After 4 minutes of incubation at room temperature, gently dislodge cells and transfer to a 15-ml conical tube.
6. Wash the culture dish with at least 2 ml of D-PBS and transfer the wash to the same tube. Centrifuge to pellet cells.
7. Resuspend the cells in D-PBS to wash and recentrifuge.
8. Remove the D-PBS and resuspend the cell in 800 µl of OptiPro™ SFM.
9. Combine the DNA (10 µg total, step 2) and the resuspended cells (step 8) in the same 1.5-ml microcentrifuge tube that contains the DNA, and transfer the mixture to an electroporation cuvette with a gap of 0.4 cm.
10. Electroporate the cells once using the BTX ECM630 electroporator with the following parameters: 200 V, 10 ms, 2 pulses. If you are using the Bio-Rad Gene pulser II®, the parameters are: 500 V, 250 µF.
11. Immediately transfer the cells into the 15-ml conical tube (prepared in step 4) using a Pasteur pipette and incubate at room temperature for 5 minutes.
12. Transfer the mixture from step 11 into the 60-mm dish (prepared in step 3) and incubate in the 37°C incubator.

Important

After transfection, you must allow the retargeted StemPro® TARGET™ hESC - BG01v cells to recover and the colonies to become well-defined (usually 5 days post-microporation or 2 days post-electroporation) before starting selection with Zeocin™.

Selection and Expansion of Retargeted StemPro® TARGET™ hESC - BG01v Clones

Selecting Retargeted Clones

1. Replace the MEF-CM in your culture dishes with fresh MEF-CM everyday.
2. Each clone recovers at a different rate. Monitor morphology and size of the colonies.
3. When your retargeted StemPro® TARGET™ hESC - BG01v cells have recovered from transfection and the colonies are well-defined, start drug selection with 2.5 µg/ml Zeocin™. If you have used microporation for retargeting, and have been recovering your cells on MEF feeders supplemented with MEF-CM, you should also start Hygromycin B selection to prevent overgrowth of your colonies by MEFs.
   Note: Generally, the cells will have recovered fully 5 days post-microporation or 2 days post-electroporation; however, some microporated clones may take longer to recover.
4. Change MEF-CM containing drug every day. Colonies start appearing as early as day 5 of drug selection. Mark the colonies and observe them for an additional week (total of 12–14 days under selection).

Picking Retargeted Clones

1. Optional: Before picking colonies, add collagenase (1 mg/ml) to the culture dishes containing the transfectants and incubate for 5–15 minutes. This loosens the colonies and makes the subsequent mechanical picking easier.
2. Using a StemPro® EZPassage™ Disposable Stem Cell Passaging Tool (see page vii) or a sharp knife made from a Pasteur pipette, chop the colonies into several pieces and transfer to a well of a 24-well or a 12-well plate containing mitotically inactivated MEF feeder layers and MEF-CM.
3. Check the cells and change the MEF-CM the next day.
4. Start drug selection with Zeocin™ (2.5 µg/ml) and Hygromycin B (10 µg/ml) on the second day after the colonies are picked, and maintain selection for future passages.
   Note: Retargeted clones must be maintained under drug selection at all times.

Continued on next page

Selection and Expansion of Retargeted StemPro® TARGET™ hESC - BG01v Clones, continued

Expanding Retargeted Clones

1. Continue to feed cells using MEF-CM with drug selection. When colonies grown on a 24- or 12-well plate become visible and show growth and expansion (usually after 7–14 days), cells from one well can be passaged onto a 35-mm dish (or a well of a 6-well plate). Passaging should follow the protocol as described in **Passaging StemPro® TARGET™ hESC - BG01v Cells on Hyg-resistant MEF Feeders** on page 22.
2. Freeze at least 4 vials of cells for each clone before conducting any further experiments. Follow the freezing protocol as described in **Freezing StemPro® TARGET™ hESC - BG01v cells Cultured on MEF Feeders** on page 23.
3. Once the retargeted clones become established on 35-mm MEF feeder dishes supplemented with MEF-CM and under drug selection, you may passage them onto 60-mm MEF feeder dishes, and switch from MEF-CM to hESC medium.
4. To adapt cells from hESC medium to StemPro® hESC SFM, you **must** first passage cells onto Geltrex™-coated dishes and grow in feeder-free fashion on MEF-CM for at least one day (refer to **StemPro® TARGET™ hESC - BG01v Culture on MEF-conditioned Medium** on page 14). The confluency of cells at this stage should be between 30–70% (i.e., 1 million to 3 million cells per dish). Next time you change the medium, you may directly replace the MEF-CM with StemPro® hESC SFM and start passaging the StemPro® TARGET™ hESC - BG01v cells in StemPro® hESC SFM as described on page 14

PCR to Screen Retargeting Events

Introduction

Upon retargeting the StemPro® TARGET™ hESC - BG01v 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.

Materials Needed

For ordering information, see page viii.

- Retargeted StemPro® TARGET™ hESC - BG01vs
- Phosphate Buffered Saline (PBS)
- CellsDirect™ Resuspension and Lysis Buffers (Cat. no. 11739-010)
- PCR primers (see below for primer sequences)
- AccuPrime™ *Taq* DNA Polymerase High Fidelity
- Thermocycler
- Water bath or heat block at 75°C

Preparation of Retargeted StemPro® TARGET™ hESC - BG01v Clones 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.
5. Incubate the cell suspension at 75°C for 10 minutes.
6. Centrifuge for 1minute to pellet cell debris.

PCR Targets

Successful retargeting of the StemPro® TARGET™ hESC - BG01v genome introduces the human EF1α promoter upstream of the *Sh ble* gene on chromosome 13, resulting in Zeocin™ resistance of successfully retargeted clones. PCR amplification of the EF1α promoter region in successfully retargeted clones using the recommended nested primers (see the next page for primer sequences) will result in a 296 bp product. The primer sequences for amplifying the Hygromycin resistance gene are given for use in a positive control reaction. You may use the primers for the target site to confirm the presence of the retargeting sequences in the StemPro® TARGET™ hESC - BG01v genome in an optional PCR.

Continued on next page

PCR to Screen Retargeting Events, continued

PCR to Screen for Chromosome 13 Integration

Set up a PCR with the primers and conditions listed below, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page viii for ordering information). Use of nested PCR with primary and secondary reactions is required to eliminate the high background observed with only the primary PCR.

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

PCR Conditions

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	30 seconds	94°C	See table below
Annealing	30 seconds	See table below	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

PCR Target	Primer name	Sequence	Size	PCR conditions	Chr 13	
EF1α-Zeo	ELZP-For	GCCTCAGACAGTGGTTCAAAGTTT	534 bp	58°C, 35 cycles	R4 attL, EF1α-Zeo	Primary
	ELZP-Rev	TGATGAACAGGGTCACGTCGT				
	ELZS-For	GAGCATGCATCTAGTCCAGTGTGG	296 bp	56°C, 35 cycles		Secondary
	ELZS-Rev	CATGGTTTAGTTCCTCACCTTGTCG				
Hyg^R gene	HygPF	ATGAAAAAGCCTGAACTCACC	430 bp	52°C, 35–40 cycles	Hyg control	Primary
	HygPR	ATTGACCGATTCCTTGCG				
	HygSF	AAAAGTTCGACAGCGTCTCC	205 bp	52°C, 30 cycles		Secondary
	HygSR	TCGCTGAATTCCCCAATG				
Target site	13PL1PF	AGTTAAGCCAGCCCCGACAC	592 bp	58°C, 35 cycles	Plus strand, attL	Primary
	13PL1PR	TTTTGGCTACCAGTACTAGGCAGG				
	13PL1SF	CTTGTCTGCTCCCGGCATCC	467 bp	55°C, 30 cycles		Secondary
	13PL1SR	CCCAGCAATGGAGCCTGATT				

Southern Blot Analysis (optional)

PCR is usually sufficient to confirm the presence of the retargeted sequences in the StemPro® TARGET™ hESC - BG01v chromosome 13 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 (Cat. no. 10503-027) to isolate the genomic DNA from the platform cell line.

Characterization of Retargeted Clones

Introduction

While PCR and Southern blot analysis are usually sufficient to confirm the successful retargeting of the StemPro® TARGET™ hESC - BG01v platform cell line, we recommend that you perform quality control assays on retargeted clones to confirm their karyotype and to asses their pluripotency. Guidelines for performing the karyotype analysis and immunocytochemistry are provided below

Karyotype Analysis

1. Passage retargeted clones onto a Geltrex™-coated T-25 cell culture flask in a feeder-free fashion using MEF-CM as you would under normal conditions (see page 14 for culturing hESCs in MEF-CM).
2. Feed the cells with MEF-CM until they are 50% confluent (i.e., the culture is in log phase).
3. Proceed with karyotype analysis using established protocols or send the live culture by overnight delivery service to a karyotype analysis service vendor.
4. If sending to a vendor, fill the culture flask to the top with conditioned medium, tighten the cap and seal with parafilm.

   Note: We recommend Cell Line Genetics as a karyotype analysis service vendor.

   Cell Line Genetics, Suite 254, 510 Charmany Drive, Madison, WI 53719
   Phone: (608) 441-8163; Fax: (608) 441-8162; e-mail: jjohnson@clgenetics.com

Immunocyto-chemistry

Fixing Cells:

1. Remove culture medium and gently rinse 3X with D-PBS without dislodging the cells
2. Fix the cells with 4% fresh **Paraformaldehyde Fixing Solution** (PFA; see **Appendix**, page 44 for recipe) at room temperature for 15 minutes. Rinse 3X with D-PBS.
3. Check for presence of cells and autofluorescence after fixing.
4. Proceed to staining below. You may also store slides for up to 3–4 weeks in D-PBS at 4°C. **Do not** allow slides to dry.

Staining Cells:

5. Incubate cells for 30–60 minutes in blocking buffer (5% serum of the secondary antibody host species, 1% BSA, 0.1% Triton-X in PBS).
6. Remove the blocking buffer and incubate cells overnight at 4°C with primary antibody diluted in 0.1% BSA in PBS. Ensure that the cell surfaces are covered uniformly with the antibody solution.
7. Rinse the wells with PBS.
8. Wash the cells for 5 minutes with blocking buffer (if using a slide, use a staining dish with a magnetic stirrer).
9. Incubate the cells with fluorescence-labeled secondary antibody **in the dark** at 37°C for 30–45 minutes.
10. Wash the cells 3X with PBS and in the last wash counter stain with DAPI (1:1000 diluted) for 5 min and rinse with PBS.
11. If desired, mount with 3 drops of ProLong® Gold antifade reagent per slide and seal with the cover slip (see page viii for ordering information). You may store the slides **in the dark** at 4°C.

Sustained Expression and Appropriate Regulation of Transgenes in Retargeted Clones

Introduction

The images below are included to provide an example of the sustained expression and appropriate regulation of transgenes over long term culture, upon random differentiation, as well as directed induction into specific lineages. For more information on the long term behavior of StemPro® TARGET™ hESC - BG01v cells upon retargeting, and the subsequent active expression of the transgenes in pluripotent as well as in differentiated lineages, refer to Liu *et al.*, 2008.

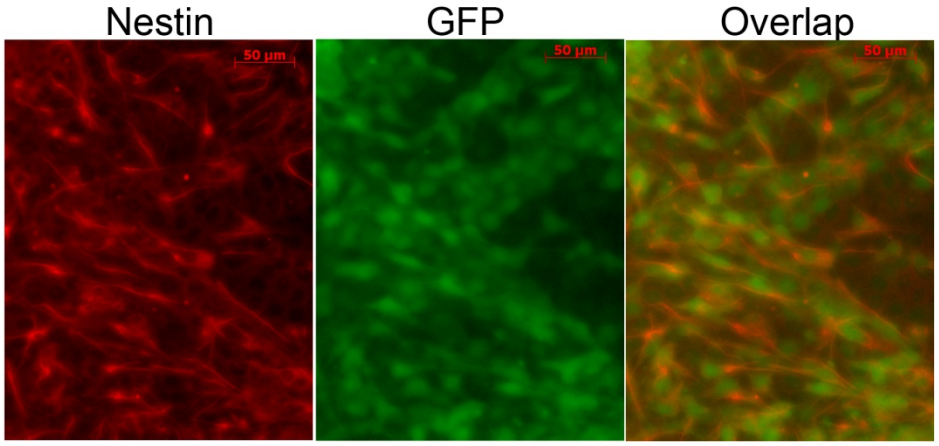


Figure 3. StemPro® TARGET™ hESC - BG01v platform line was retargeted using a GFP cassette driven by a constitutive promoter EF1α. Retargeted cells continue to express GFP after they are differentiated into Nestin+ cells.

Troubleshooting

Introduction

The following tables list some potential problems and possible solutions to help you troubleshoot your hESC cell culture studies retargeting 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 2–3 × 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 StemPro® TARGET™ hESC - BG01v 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 StemPro® TARGET™ hESC - BG01v cells (if thawed on MEF feeders)	Purchase (see page viii) or make a new batch of mitotically inactivated MEFs (see page 40).
MEFs overgrow plate	MEFs not inactivated	Inactivate mitosis in MEFs as described on pages 38–41, or purchase inactivated MEFs (see page viii).

Continued on next page

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 StemPro® TARGET™ hESC - BG01v 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	Cells not thawed and established on StemPro® hESC SFM	Thaw and culture a fresh vial of StemPro® TARGET™ hESC - BG01v cells. Make sure to thaw on StemPro® hESC SFM as described on page 6.
	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 38) new batch of mitotically inactivated MEFs, if necessary. Use Hygromycin resistant MEFs.
	Culture conditions not correct	Thaw and culture fresh vial of StemPro® TARGET™ hESC - BG01v cells. Follow thawing instructions (page 6) and subculture/maintenance procedures exactly.
	Cells overexposed to collagenase	hESCs 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 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 Zeocin™ and/or Hygromycin B is used for selection.	Use 10 µg/ml Hygromycin B and 2.5 µg/ml Zeocin™ for selection.

Continued on next page

Retargeting StemPro® TARGET™ hESC - BG01v Cells

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

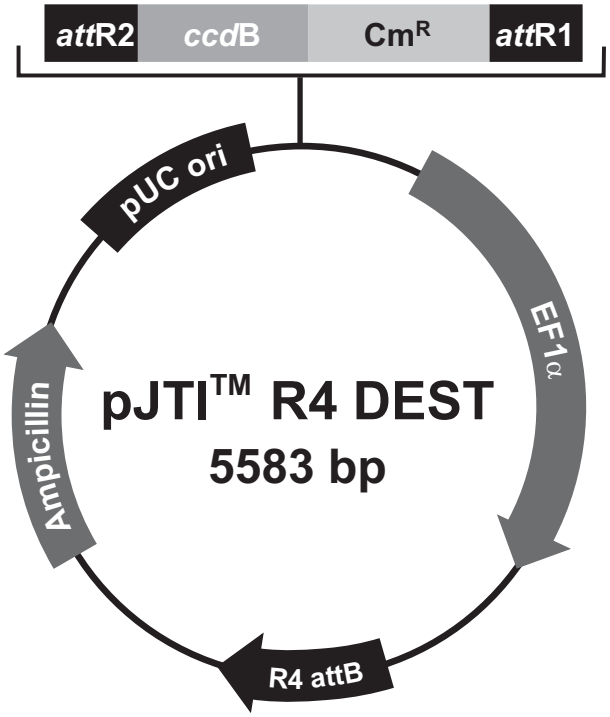
Problem	Cause	Solution
Low survival rate after transfection	Poor DNA quality	The quality of the retargeting construct DNA strongly influences the results of transfection experiments. Use endotoxin-free DNA for all transfections. Make sure that the A260:A280 ratio of the DNA is between 1.8 and 2.0. **Do 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 StemPro® hESC SFM plates prior to transfection	After two weeks of Hygromycin B selection on MEF feeders, StemPro® TARGET™ hESC - BG01v cells must be passaged at least once as a feeder-free culture on MEF-CM and Geltrex™ without drug selection.
	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, or accutase. Pipette cells gently.
	Cells remained too long in electroporation cuvette or the Gold-Tip.	Immediately after electroporation/microporation, transfer cells into pre-warmed medium at37°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 as described on pages 27–28.
	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 as described on pages 27–28.
	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™ 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 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 StemPro® TARGET™ hESC - BG01v genome, and the human EF1α promoter for constitutive expression of Zeocin™ resistance upon successful integration. **The complete sequence of pJTI™ R4 DEST is available from www.lifetechnologies.com or by contacting Technical Support (see page 45).**



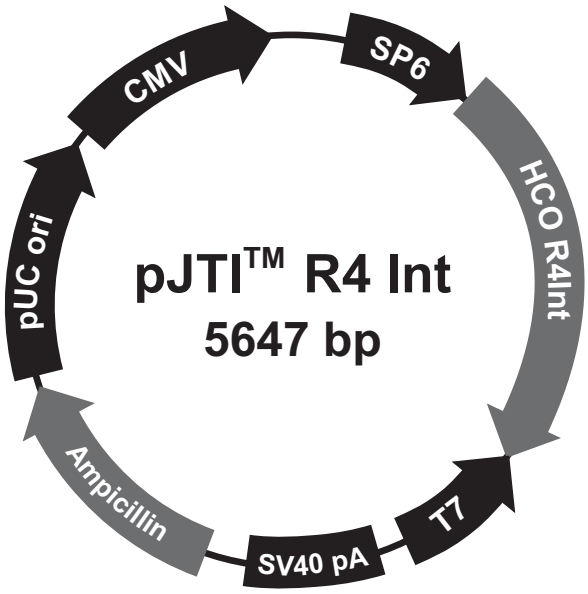
Features of pJTI™ R4 DEST
5583 nucleotides

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 (5647 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 StemPro® TARGET™ hESC - BG01v genome from the pJTI™ R4 DEST retargeting expression construct upon cotransfection of the platform line with both vectors. **The complete sequence of pJTI™ R4 Int is available from www.lifetechnologies.com or by contacting Technical Support (see page 45).**



Features of pJTI™ R4 Int
5647 nucleotides

SP6 promoter: bases 15-34
HCO R4Int: bases 51-1460
T7 promoter: bases 1483-1502 (complementary strand)
SV40 promoter and origin: bases 1889-1970
Ampicillin resistance gene (ORF): bases 2650-3510
pUC origin: bases 3655-4328
CMV promoter: bases 5039-5639

Generating Mitomycin C Treated 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 page 20 for recipe); 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.

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

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 20 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 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 to 5 days after plating for hESC cell culture. The medium should be changed every other day if they are not used immediately.

Assessing the Vitality of hESCs

Introduction

We recommend using the LIVE/DEAD® Cell Vitality Assay Kit, available separately from Life Technologies, to assess the vitality of your hESCs 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 viii.

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).

Optional Quality Control Assays

Introduction

The StemPro® TARGET™ hESC - BG01v platform line was created by the phiC31-mediated site-specific integration of the R4 retargeting sequences into the chromosome 13 of BG01V hESCs. Follow the guidelines below for optional assays to confirm the presence of R4 retargeting site in the correct chromosomal locus on the StemPro® TARGET™ hESC - BG01v genome.

PCR to Screen for Chromosome 13 Integration

Set up a PCR with the following primers and conditions, using AccuPrime™ *Taq* DNA Polymerase High Fidelity (see page viii for ordering information). Use of nested PCR with primary and secondary reactions is required to eliminate the high background observed with only the primary PCR.

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

PCR Conditions

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	30 seconds	94°C	See table below
Annealing	30 seconds	See table below	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

PCR Target	Primer name	Sequence	Size	PCR conditions	Chr 13	
Hyg^R gene	HygPF	ATGAAAAAGCCTGAACTCACC	430 bp	52°C, 35–40 cycles	Hyg control	Primary
	HygPR	ATTGACCGATTCCTTGCG				
	HygSF	AAAAGTTCGACAGCGTCTCC	205 bp	52°C, 30 cycles		Secondary
	HygSR	TCGCTGAATTCCCCAATG				
Target site	13PL1PF	AGTTAAGCCAGCCCCGACAC	592 bp	58°C, 35 cycles	Plus strand, AttL	Primary
	13PL1PR	TTTTGGCTACCAGTACTAGGCAGG				
	13PL1SF	CTTGTCTGCTCCCGGCATCC	467 bp	55°C, 30 cycles		Secondary
	13PL1SR	CCCAGCAATGGAGCCTGATT				

Southern Blot Analysis

Nested PCR is usually sufficient to confirm the presence of the retargeting sequences in the StemPro® TARGET™ hESC - BG01v chromosome 13; however, you may also perform a Southern blot analysis as an additional check to verify that a single copy of the R4 retargeting sequence is integrated into the genome. Use the Southern blot protocol of your choice with a radiolabeled Hyg probe from pCEP4 (Cat. No. V044-50) plasmid digested previously with *Fsp*I and *Sal*I (~1.9 kb). We recommend using the DNAzol® Reagent (Cat. no. 10503-027) to isolate the genomic DNA from the platform cell line.

Recipes

Paraformaldehyde Solution

Top prepare 20% paraformaldehyde (PFA) stock solution:

1. Add PBS to 20 g of EM grade paraformaldehyde (Electron Microscopy Services,
   Cat. no. 19208), and bring the volume up to 100 ml.
2. Add 0.25 ml of 10 N NaOH and heat at 60°C using a magnetic stirrer until completely dissolved.
3. Filter through 0.22 micron filter, and cool on ice. Make sure the pH is 7.5–8.0.
4. Aliquot 2 ml in 15-ml tubes, freeze on dry ice, and store at –20°C.

To prepare 4% PFA for fixing:

1. Add 8 ml PBS into each 15-ml tube containing 2 ml of 20% PFA, and thaw in a 37°C water bath.
2. Once dissolved, cool on ice.

Technical Support

Obtaining Support

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

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- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
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- 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

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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 embryonic 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.

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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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