invitrogen™

STEMPRO® BG01v/EG Cells

Variant hESC EF1α-EmGFP Reporter Cells

Catalog no. R7799-205

Version B
30 September 2008
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User Manual

Table of Contents

Contents and Storage

Shipping and Storage

This manual is shipped with STEMPRO® BG01v/EG Cells. STEMPRO® BG01v/EG cells are shipped on dry ice. Upon receipt, store in **liquid nitrogen**.

Contents

Storage conditions: Liquid nitrogen

Amount supplied: One vial containing ~3 × 10^6 cells

Composition: 1 ml of cells in Freezing Medium (see page 12 for composition).

Handle 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.

Accessory Products

Additional Products

For more information about the following products, refer to our Web site (www.invitrogen.com) or call Technical Support (see page 17).

Item	Quantity	Catalog no.
STEMPRO® hESC SFM Kit	1 kit	A1000701
Collagenase Type IV	1 g	17104-019
Knockout™ Serum Replacement (KSR)	500 ml	10828-028
FGF-Basic (bFGF)	50 µg	PHG0026
2-Mercaptoethanol, 1,000X (55 mM)	50 ml	21985
Geltrex™ hESC-qualified	5 ml	A10480-02
KnockOut™ DMEM (500 ml)	500 ml	10829-018
KnockOut™ DMEM/F-12	500 ml	12660-012
CELLstart™	2 ml	A10142-01
DPBS	500 ml	14190
Hygromycin B	20 ml	10687-010
STEMPRO® EZPassage™ Disposable Stem Cell Passaging Tool	10 disposable tools	23181-010
STEMPRO® EZChek™ Human Tri-Lineage Multiplex PCR Kit	100 reactions	23191-050
ESC Antibodies		
SSEA-1	100 µg	41-1200
SSEA-3	100 µg	41-4400
SSEA-4	100 µg	41-4000
Tra-1-60	100 µg	41-1000
Tra-1-81	100 µg	41-1100
Sonic Hedgehog (Shh)	100 µg	435800
CD9	100 tests	AHS0907
E-Cadherin (Clone: ECCD-2)	100 µg	13-1900

Introduction

Introduction

StemPro® BG01v/EG human embryonic stem cells (hESCs) are engineered to express Emerald Green Fluorescent Protein (EmGFP) when pluripotent and during and after differentiation, for the easy monitoring of these cells in different environments (*e.g.*, in co-cultures or after transplantation) and with different treatments.

Characteristics of StemPro® BG01v/EG Cells

StemPro® BG01v/EG cells exhibit the following characteristics:

- Prepared from BG01v cells that have been engineered to contain a chromosomal target for the R4 integrase
- Pluripotent: can differentiate to representatives of the three primary germ layers
- Express EmGFP when pluripotent and during and after differentiation

BG01v and BG01v/R4 Cells

StemPro® BG01v/EG Cells are derived from the BG01v human embryonic stem cell line (ATCC No. SCRC-2002). BG01v cells in turn are a variant with abnormal karyotype of the wild-type, parental hESC line BG01 (Mitalipova *et al.*, 2003; Plaia *et al.*, 2006). BG01v cell colonies grow on serum-free medium or MEF feeder cells with uniform morphology and are easy to culture at a predictable growth rate. BG01v cells stain positive for pluripotency markers and alkaline phosphatase activity. BG01v cells are pluripotent and can differentiate to representatives of all three primary germ layers.

For the development of the EG 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. The resulting cell lines contained an R4 *attB* site placed upstream of a selectable marker lacking a promoter 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. We confirmed that these cells were pluripotent and able to differentiate into representatives of all three primary germ layers. The resulting cell line was called BG01v/R4.

Continued on next page

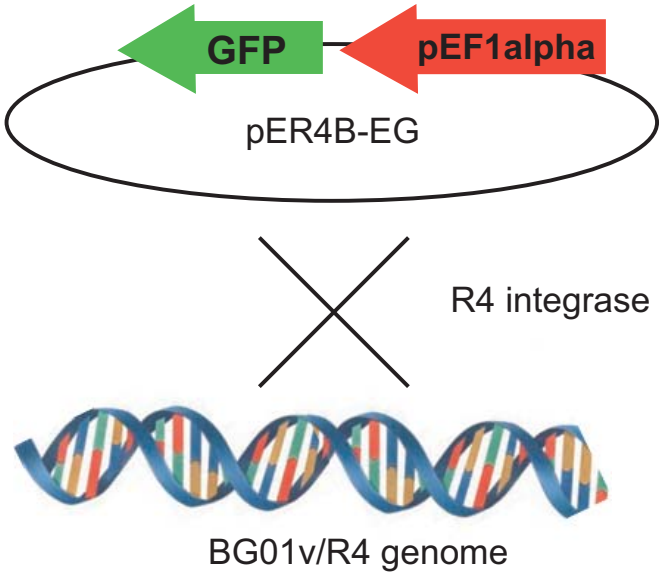
Introduction, Continued

Generation of STEMPRO® BG01v/EG Cells

First, we used phiC31 integrase to deliver a chromosomal target for a second integrase, R4, into BG01v cells, as described on the previous page. We then constructed a vector containing EmGFP expressed under the direction of the human EF1α promoter. This plasmid was stably integrated into the genome of the engineered BG01v/R4 cells.

We selected a clone expressing EmGFP, which was tested extensively to make sure it was pluripotent and able to differentiate to representatives of the three primary germ layers. The resulting cell line was called BG01v/EG.

Note: STEMPRO® BG01v/EG Cells are resistant to Hygromycin B and Zeocin. If you want to stably integrate more genes in these cells, do not use Hygromycin B or Zeocin for selection.



Methods

General Information

General Cell Handling

Follow the general guidelines below to grow and maintain StemPro® BG01v/EG Cells.:

- **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.
- StemPro® BG01v/EG Cells should be thawed on StemPro® hESC SFM
- StemPro® BG01v/EG Cells may be cultured on StemPro® hESC SFM or a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or MEF-conditioned media (MEF-CM).
- Before starting experiments, be sure to have cells established (at least 5 passages) and also have some frozen stocks on hand. We recommend using early-passage cells for your experiments (below 30 passages). Upon receipt of the cells from Invitrogen, grow and freeze multiple vials of the StemPro® BG01v/EG cells to ensure that you have an adequate supply of early-passage cells.
- 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 vi for ordering information).

Important

It is very important to strictly follow the guidelines for culturing StemPro® BG01v/EG Cells in this manual to keep them undifferentiated.

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

Continued on next page

General Information, Continued

STEMPRO® hESC SFM

Traditional hESC culture methods require the use of mouse or human fibroblast feeder layers, which are labor-intensive and hard to scale. In addition, it is difficult to maintain hESCs in an undifferentiated state due to the undefined conditions on feeder cultures. STEMPRO® hESC SFM, available separately from Invitrogen, enables culture of hESCs in serum-free medium (SFM) without feeder cells (see page vi for ordering information).

Go to www.invitrogen.com/stempro/hesc for an instructional video on how to use STEMPRO® hESC SFM.

Features of STEMPRO® hESC SFM

STEMPRO® hESC SFM has been extensively tested and proven to have the following characteristics:

- Supports hESC growth for up to 80 passages, maintaining the ability of hESCs to differentiate into all three germ line lineages without any signs of karyotypical abnormalities.
- Maintains pluripotency in multiple hESC lines, including BG01v cells.
- Supports scale-up production of hESCs to over 1×10^9 cells while maintaining pluripotency.
- No need to maintain feeder cells or produce feeder-conditioned medium.
- More reproducible results due to steady growth factor levels.

Thawing and Establishing Cells

Introduction

STEMPRO® BG01v/EG cells are supplied in a vial containing 1 ml of cells at 3×10^6 viable cells/ml in freezing medium. They are frozen from cultures established on STEMPRO® hESC SFM and should be thawed on the same medium. Follow the protocol in this section to thaw cells.

Materials Needed

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

- STEMPRO® BG01v/EG cells (store frozen cells in liquid nitrogen until ready to use)
- STEMPRO® hESC SFM Kit, which includes:
  DMEM/F-12 with GLUTAMAX™
  STEMPRO® hESC Supplement
  Bovine Serum Albumin 25% (BSA)
- Geltrex™ hESC-qualified
- 2-Mercaptoethanol
- Culture dishes (60-mm dishes recommended)
- Disposable, sterile 15-ml tubes.
- 37°C incubator with humidified atmosphere of 5% CO_2

Preparing Geltrex™ Aliquots

Thaw the Geltrex™ bottle at 2–8°C overnight and prepare 1-ml aliquots of Geltrex™ in 50-ml conical tubes. Store the tubes at –20°C.

Coating Plates with Geltrex™

1. Thaw a 1-ml tube of Geltrex™ at 2–8°C.
2. Remove DMEM/F-12 from 2–8°C storage and add 29 ml of cold DMEM/F-12 to the 1 ml of Geltrex™. Mix gently.
3. Cover the whole surface of each culture plate with the Geltrex™ solution (1 ml for a 35-mm dish, 2 ml for a 60-mm dish).
4. Seal each dish with parafilm to prevent drying, and incubate 1 hour at 37°C.
5. Transfer each dish to a laminar flow hood and allow it to equilibrate to room temperature (about 1 hour) before using.

The Geltrex™-coated dish may be stored at 2–8°C for up to 1 week.

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Thawing and Establishing Cells, Continued

Storage and Handling of SFM Supplement

- STEMPRO® hESC SFM supplement is supplied as a frozen sample. Thaw 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).

Preparing Complete SFM

Thaw STEMPRO® hESC Supplement in 37°C water bath (minimize dwell time), and prepare according to the table below:

Component	Final conc.	For 500 ml	For 100 ml
DMEM/F-12 with GLUTAMAX™ (1X)	1X	454 ml	90.8 ml
STEMPRO® hESC SFM Growth Supplement (50X)	1X	10 ml	2 ml
25% BSA	1.8%	36 ml	7.2 ml
FGF-basic (10 µg/ml)	8 ng/ml	400 µl	80 µl
2-Mercaptoethanol (55 mM)	0.1 mM	909 µl	182 µl

Storing Complete Medium

Store complete STEMPRO® hESC SFM at 2–8°C in the dark for up to 7 days. **Add 2-Mercaptoethanol daily during storage,** at volumes listed in the table above.

Thawing Procedure

To thaw and establish STEMPRO® BG01v/EG Cells:

1. Warm an appropriate amount of complete STEMPRO® hESC SFM to 37°C in a water bath. Minimize dwell time.
2. Remove a cryovial of cells from the liquid nitrogen and thaw quickly in a 37°C water bath (to prevent crystal formation).
3. When thawed, immediately transfer cells from the cryovial into a 15-ml tube.
4. Rinse the cryovial with 1 ml of warm complete STEMPRO® hESC SFM and transfer this dropwise to the 15-ml tube. **Note:** Be careful to add the media slowly to the cells.
5. Repeat the previous step 2–3 times.

Procedure continued on next page

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Thawing and Establishing Cells, Continued

Thawing Procedure, continued

Procedure continued from previous page

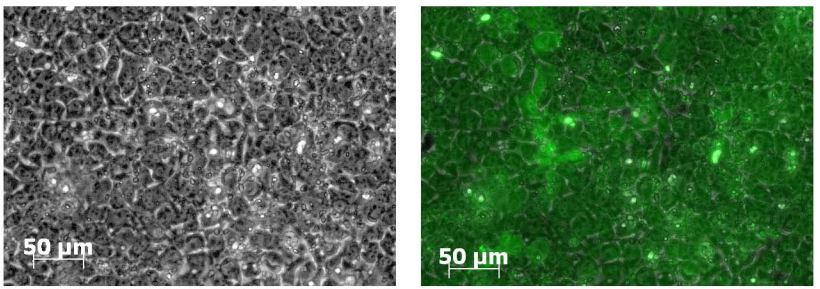
6. Spin cells down for 2 minutes at 200 × *g*.
7. Aspirate the supernatant, and gently resuspend the cells in warm complete STEMPRO® hESC SFM (2 ml for a 35-mm dish).
8. Remove a Geltrex™-coated plate from 2–8°C storage and tip slightly to aspirate the Geltrex™ solution. Immediately plate the cells. Do not allow the surface to dry out before plating.
9. Mix the plate gently to evenly spread out clumps and place the plate in a 37°C incubator with a humidified atmosphere of 5% CO_2. Change the medium every day.

Following thawing, you can culture cells as described starting on page 8.

Judging Thawed Cells

Observe colonies recovered at day 5 after thawing, to assess growth rate and differentiation state, keeping the following in mind:

- Cells should be undifferentiated. They should express EmGFP (if you are unfamiliar with fluorescence microscopy, see page 17), and grow as colonies (see image below for examples of undifferentiated STEMPRO® BG01v/EG Cells). If not, refer to the **Troubleshooting** section (page 15).
- Before colonies start contacting each other, they should be passed (see next page).



STEMPRO® BG01v/EG cells 2 days after thawing. Left: White light image. Right: White light image overlapped with GFP image.

Subculturing Cells

Introduction

This section describes how to culture STEMPRO® BG01v/EG Cells in serum-free medium (SFM) without feeder cells. To culture cells on MEF feeder cells, see the Appendix starting on page 20.

Subculture cells when needed (before colonies start contacting each other), typically every 4-7 days.

Important

Before starting experiments, we recommend that you first prepare ample cell stocks, as described in **Freezing Cells** (page 12).

Guidelines for SFM Culture

Go to www.invitrogen.com/stempro/hesc for an instructional video on how to use STEMPRO® hESC SFM.

To prevent differentiation and slow growth of STEMPRO® BG01v/EG 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. Cells that have been maintained on MEF feeders should be transferred to Mouse Embryonic Fibroblast-Conditioned Medium (MEF-CM) before being transitioned into STEMPRO® hESC SFM.
- Some **cell death** at passaging is normal, but wide-scale cell death (*i.e.*, <20% survival) indicates poor passaging.
- **Timing of passaging**. *Critical:* the cultures need to grow to near-confluence, *i.e.*, a day or two after the colonies are just touching, cultures should be harvested. This usually results in a cell density of 1–2 × 10^5 cells/cm^2 at time of harvest.
- Do not over-expose cells to **collagenase**; we recommend 3 minutes at most, even with lower amounts of collagenase.
- **Density**: The cultures must be maintained at a high density (200+ colonies in a 60-mm dish).
- hESCs grown in culture are always under selection pressure of **proliferation vs. 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

Subculturing Cells, Continued

Materials Needed

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

- Complete STEMPRO® hESC SFM (see preparation instructions on page 6)
- FGF-basic, 10 µg/ml, prepared as described below
- Collagenase Type IV, prepared as described below
- Geltrex™-coated plates (see preparation instructions on page 5)
- 25% BSA (provided with the STEMPRO® hESC SFM Kit)
- DMEM/F-12 with GLUTAMAX™ (provided with the STEMPRO® hESC SFM Kit)
- DPBS
- Hygromycin B
- Culture dishes (60-mm dishes recommended)
- An incubator at 37°C, humidified atmosphere of 5% CO_2 in air

Complete STEMPRO® hESC SFM

See preparation instructions on page 6. Remember to add 2-Mercaptoethanol daily during storage, at volumes listed in the preparation table.

Preparing FGF-basic

Prepare 10 µg/ml FGF-basic in DMEM/F-12 with 0.1% BSA. Aliquot 80 µl per tube and freeze at –20°C.

Preparing Collagenase IV

Prepare 10-mg/ml aliquots of collagenase IV in DMEM/F-12. Filter to sterilize and freeze at –20°C.

Preparing Wash Medium

For the wash medium, prepare 0.1% BSA in DMEM/F-12 with GLUTAMAX™ (use the 25% BSA provided in the kit).

Important

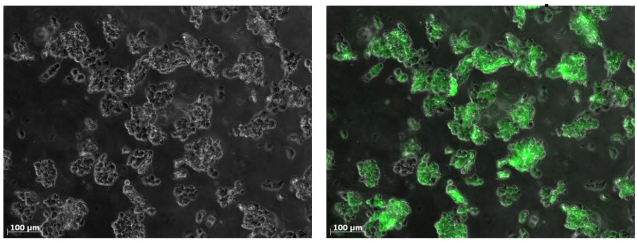
STEMPRO® BG01v/EG cells should be cultured in the presence of 10 µg/ml Hygromycin B to prevent losing the EmGFP expression cassette during prolonged culturing.

Continued on next page

Subculturing Cells, Continued

Passaging Cells

1. In a 37°C water bath, warm appropriate amounts of 10-mg/ml collagenase solution, complete STEMPRO® hESC SFM, and wash medium (prepared as described on page 9). Minimize dwell time.
2. Set up the plate with STEMPRO® BG01v/EG cells on a dissecting microscope in a biosafety cabinet or laminar flow 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 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 7.)
5. Leave for 3 minutes to dislodge cells from the substrate.
6. Remove collagenase and rinse with DPBS
7. Add 3 ml of wash medium (0.1% BSA in DMEM/F-12 with GLUTAMAX™; see page 9).
8. Gently scrape the dish using a sterile 1000-µl pipette tip.
9. Gently transfer the cell clumps using a 5-ml pipette and place into a 15-ml tube (see below for images of clumps at passaging).



Left: White light image of cells at passage. Right: White light image overlapped with GFP image.

10. Wash plate with 3 ml of wash medium and add to the tube.
11. Spin cells at 200 × *g* for 2 minutes at room temperature.

Procedure continued on next page

Continued on next page

Subculturing Cells, Continued

Passaging Cells, continued

Procedure continued from previous page

12. Gently aspirate the media and flick the tube to loosen cells.
13. Gently resuspend the cells in warm complete STEMPRO® hESC SFM using a 1-ml or 5-ml serological pipette.
14. Remove the Geltrex™ from a Geltrex™-coated plate by tipping the plate slightly and aspirating the solution. Immediately plate the cells. Do not allow the surface to dry out before plating.
15. Mix plates gently to evenly spread out clumps and place the plate in a 37°C incubator at with 5% CO_2 in air.
16. Each day, gently change the media to remove excess cells and provide fresh nutrients.
17. Observe cells every day and passage by the above protocol whenever required (about every 5–7 days).

Freezing Cells

Introduction

When freezing STEMPRO® BG01v/EG Cells cultured on STEMPRO® hESC SFM, we recommend the following:

- Freeze cells at a density of 3×10^6 viable cells/ml.
- For every 20 cm^2 of cells (one 60-mm dish), prepare 0.5 ml of Freezing Medium 1 and 0.5 ml of Freezing Medium 2.
- Bring STEMPRO® BG01v/EG 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 vi for ordering information):

- Plates with STEMPRO® BG01v/EG Cells in SFM
- Wash Medium (see page 9)
- DMEM/F-12 with GLUTAMAX™
- Collagenase Type IV working solution (10 mg/ml) in DMEM/F12
- 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 Freezing Medium

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

1. In a sterile 15-ml tube, mix together the following for every 0.5 ml of **Freezing Medium 1** needed:

DMEM/F12 with GLUTAMAX™	0.25 ml
Knockout™ Serum Replacement (KSR)	0.25 ml

2. In another sterile 15-ml tube, mix together the following for every 0.5 ml of **Freezing Medium 2** needed:

DMEM/F12 with GLUTAMAX™	0.4 ml
DMSO	0.1 ml

3. Place tube with Freezing Medium 2 on ice and leave Freezing Medium 1 at room temperature.

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Freezing Cells, Continued

Freezing Cells 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 DPBS.
4. Add 3 ml of wash medium (0.1% BSA in DMEM/F-12 with GLUTAMAX™; 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® BG01v/EG cells in Freezing Medium 1 at room temperature (use 0.5 ml of Freezing Medium 1 for one 60-mm dish).
10. Add the same volume of cold 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.

Expected Results

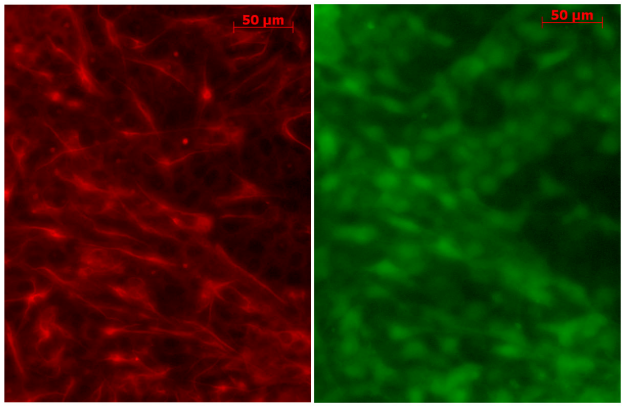
Introduction

EmGFP expression has been tested in STEMPRO® BG01v/EG cells that have been differentiated down various pathways. While we have not tested all possible pathways, EmGFP expression has been preserved in the pathways we have tested.

Note: Suggestions for differentiation protocols are available from www.invitrogen.com/stemcells; click on the section **protocols**.

Example of Differentiated Cells

EmGFP expression is retained after EG cells have differentiated into Nestin+ cells, as shown in the images below.



Left: Nestin staining. **Right:** GFP imaging.

Note: If you are unfamiliar with fluorescence microscopy, see page 17.

Troubleshooting

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 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 (starting on page 12) exactly. Slow freezing and fast thawing are key. 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® BG01v/EG 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 2 minutes at 200 × *g* at room temperature and dilute the cells at higher density.
	MEFs sub optimal and do not support recovery of STEMPRO® BG01v/EG Cells	Purchase or make (see page 24) a new batch of mitotically inactivated MEFs.
MEFs overgrow plate	MEFs not inactivated	Inactivate mitosis in MEFs as described on page 24, or purchase inactivated MEFs.

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Troubleshooting, Continued

Culturing Cells

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

Problem	Cause	Solution
Cells grow slowly	Growth medium not correct	Use correct growth medium.
	FGF-basic inactive	FGF-basic is not stable when frequently warmed and cooled Add FGF to medium just before use, or store medium with FGF in aliquots at –20°C.
	Cells too old	Use healthy STEMPRO® BG01v/EG cells, under passage 30; do not overgrow.
	Cells too diluted	Spin down cells for 2 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 correctly	STEMPRO® BG01v/EG cells are frozen on STEMPRO® hESC SFM. We recommend thawing on STEMPRO® hESC SFM as described on page 5.
	Culture conditions not correct	Thaw and culture fresh vial of STEMPRO® BG01v/EG Cells. Follow thawing instructions (page 5) and subculture procedures (page 8) exactly.
No fluorescence signal detected	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter sets for detection of fluorescence (see page 17). Be sure to use an inverted fluorescence microscope for analysis.
	Cells lost GFP expression cassette	Thaw and culture fresh vial of STEMPRO® BG01v/EG Cells. Culture cells in presence of 10 µg/ml Hygromycin B.
	Cells differentiated	See points above

Appendix

Detecting Fluorescence

Introduction

You may detect EmGFP protein expression directly in STEMPRO® BG01v/EG Cells by fluorescence microscopy or other methods that use light excitation and detection of emission. See below for recommended fluorescence microscopy filter sets.

Filters for Use with EmGFP

The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein such as the Omega XF100 filter set for fluorescence microscopy.

The spectral characteristics of EmGFP are listed in the table below:

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509

For information on obtaining these filter sets, contact Omega Optical, Inc. (www.omegafilters.com) or Chroma Technology Corporation (www.chroma.com).

Fluorescence Microscope

You may view the fluorescence signal of EmGFP in cells using an inverted fluorescence microscope with FITC filter or Omega XF100 filter (available from www.omegafilters.com) for viewing cells in culture or a flow cytometry system.

Color Camera

If desired, you may use a color camera that is compatible with the microscope to photograph the cells. We recommend using a digital camera or high sensitivity film, such as 400 ASA or greater.

What You Should See

See the Expected Results Section, page 14.

Preparing a Feeder Cell Layer

Introduction

STEMPRO® BG01v/EG cells are frozen on STEMPRO® hESC SFM and should be thawed on same. After thawing, cells may be cultured on either SFM or MEF feeder cells. To culture STEMPRO® BG01v/EG cells on MEFs, follow the instructions in this section. Use mitotically inactivated MEFs to prevent overgrowth of the hESCs. Both Mitomycin C and irradiation methods can be used to mitotically inactivate your MEFs.

Materials Needed

Have the following reagents on hand before beginning (see page vi for ordering information):

- Mitotically inactivated, Hygromycin resistant MEFs. Order from ATCC (SCRC-1045.2), or generate them as described in **Generating Mitomycin C Treated MEFs** (page 20).
- Dulbecco's Modified Eagle Medium (D-MEM) high glucose with L-glutamine and sodium pyruvate.
- Fetal Bovine Serum, ES Cell-Qualified.
- MEM Non-Essential Amino Acids Solution 10 mM (100X) (NEAA).
- 2-Mercaptoethanol, 1,000X
- DMEM/F12 with GLUTAMAX™ (2 mM)
- Knockout™ Serum Replacement (KSR)
- bFGF. Reconstitute lyophilized human bFGF in sterile, DMEM/F12 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 (Sigma Cat no. G1890) in sterile, distilled water, and sterilize by filtration using a 0.2 µm filter. Store up to 1 year at 4°C.
- 37°C incubator with a humidified atmosphere of 5% CO_2

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Preparing a Feeder Cell Layer, Continued

Preparing MEF Medium

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

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

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

Preparing hESC Medium

To prepare 100 ml hESC Medium, mix following reagents:

Volume	Reagent	Final Concentration
79 ml	DMEM/F12 with GLUTAMAX™	1x
20 ml	Knockout™ Serum Replacement (KSR)	20%
1 ml	NEAA (10 mM)	0.1 mM
100 µl	2-Mercaptoethanol, 1,000X (55 mM)	55 µM
40 µl	bFGF (10 µg/ml)	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.

Preparing Gelatin Coated Plates

Coat plates for 20–60 minutes at room temperature with 0.1% gelatin in dH_2O.

Plating Feeder Layer

1. Two days before hESC co-culture, plate 30,000/cm^2 of mitotically inactivated mouse embryonic fibroblasts on a 0.1% gelatin-coated culture plate in MEF medium.
2. One day before hESC co-culture, replace medium with hESC Medium
3. Next day, the feeder layer is ready to be seeded with STEMPRO® BG01v/EG Cells in fresh hESC Medium.

Culturing Cells on MEF Feeders

Introduction

Follow the protocol below to culture STEMPRO® BG01v/EG Cells on feeder layer plates. For culturing cells in serum-free medium without feeders, see page 8.

Important

Before starting experiments, we recommend that you first prepare ample cell stocks, as described in **Freezing Cells** (page 22).

Materials Needed

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

- Plates with STEMPRO® BG01v/EG Cells
- hESC Medium (see page 19 for composition); pre-warm to 37°C before use)
- DMEM/F12 with GLUTAMAX™
- Feeder layer plates with mitotically inactivated MEFs – prepare at least two days in advance (see page 17)
- Collagenase Type IV
- Hygromycin B
- Disposable, sterile 15-ml tubes.
- 37°C incubator with humidified atmosphere of 5% CO_2

Important

STEMPRO® BG01v/EG Cells should be cultured in the presence of 10 µg/ml Hygromycin B, on order to prevent losing the GFP expression cassette during prolonged culturing.

Collagenase Preparation

Prepare 1-mg/ml aliquots of collagenase IV in DMEM/F-12. Filter to sterilize and freeze at –20°C.

Note: 1-mg/ml aliquots of collagenase IV are usually 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 Cells

1. Aspirate culture medium 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 edge of colonies curl up (usually less than an hour).
3. Aspirate collagenase solution.
4. Add hESC Medium or 0.1% BSA in DMEM/F12.
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. See below for an example of acceptable clumps.
6. Spin cells down for 2 minutes at 200 × *g* at room temperature.
7. Gently aspirate media and resuspend the STEMPRO® BG01v/EG Cells in hESC Medium.
8. Aspirate feeder layer plates, and plate resuspended STEMPRO® BG01v/EG Cells on the prepared MEFs (passage ratio 1:3 or 1:4).
9. Add a final concentration of 10 µg/ml Hygromycin B (1:5,000 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.

Feed cells every day and passage by the above protocol whenever required (before colonies start contacting each other; typically every 4–7 days).

Freezing Cells Cultured on MEF Feeders

Introduction

When freezing STEMPRO® BG01v/EG Cells that are cultured on MEF feeder cells, we recommend the following:

- Freeze cells at a density of 2.5×10^6 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® BG01v/EG 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 vi for ordering information):

- Plates with STEMPRO® BG01v/EG Cells on MEF feeders
- hESC Medium (see page 19 for composition)
- Collagenase Type IV working solution (1 mg/ml) in DMEM/F12 (see page 20)
- 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 Freezing Medium

Prepare Freezing Medium A and B immediately before use. Discard any unused medium.

1. In a sterile 15-ml tube, mix together the following for every 1 ml of **Freezing Medium A** needed.

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

2. In another sterile 15-ml tube, mix together the following for every 1 ml of **Freezing Medium B** needed:

hESC Medium	0.8 ml
DMSO	0.2 ml

3. Place tube with Freezing Medium B on ice and leave Freezing Medium A at room temperature.

Continued on next page

Freezing Cells Cultured on MEF Feeders,
Continued

Freezing 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 edge of colonies curl up (usually less than an hour).
3. Aspirate collagenase solution
4. Add hESC Medium (see page 19) or 0.1% BSA in DMEM/F12.
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 clumps (see page 21 for an example).
6. Spin cells down for 2 minutes at 200 × *g* at room temperature.
7. Gently aspirate media and resuspend STEMPRO® BG01v/EG 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 at –80°C overnight 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.

Generating Mitomycin C Treated MEFs

Introduction

If you are culturing STEMPRO® BG01v/EG cells on a feeder layer of MEFs, use mitotically inactivated feeders to prevent overgrowth of the hESCs. Both Mitomycin C and irradiation methods can be used to mitotically inactivate your 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 µm filter. Store up to 1 year at 4°C.

Coat plates for 20–60 minutes at room temperature with 0.1% gelatin in dH_2O.

Preparing Mitomycin C

Prepare 10 µg/ml Mitomycin C in MEF medium; filter sterilize and store at -20°C until use. Good for 2 weeks at 4°C.

Obtaining MEFs

Obtain Hygromycin resistant MEFs (DR4) from ATCC (Cat. no. SCRC-1045).

Mitomycin C Treatment

Use the procedure below to generate mitotically inactivated MEFs (DR4 strain):

1. Culture MEFs in MEF medium (see page 19)
2. Inactivate by treating MEFs with 10 µg/ml mitomycin C for 2 to 3 hours at 37°C.
3. Wash cells four times with Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14190-144)
4. Trypsinize cells with 0.05% Trypsin-EDTA (Cat no. 25300-054)
5. Plate MEFs at a density of 3×10^4 cells / cm^2 of culture surface area in MEF medium (see page 19) with 2.5 ml per well of a gelatin-coated 6-well dish.
6. 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.

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Purchaser Notification

Information for European Customers

STEMPRO® BG01v/EG Cells (variant hESC hOct4-GFP Reporter Cells) are genetically modified and carry a GFP reporter 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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