invitrogen™

BG01V/hOG Cells

Variant hESC hOct4-GFP Reporter Cells

Catalog no. R7799-105

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Version D
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User Manual

Table of Contents

Contents and Storage

Shipping and Storage

This manual is shipped with BG01V/hOG Cells. BG01V/hOG cells are shipped on dry ice. Upon receipt, store in **liquid nitrogen**.

Contents

Storage conditions: Liquid nitrogen

Amount supplied: One vial containing ~2 × 10^6 cells

Composition: 1 ml of cells in Freezing medium (see page 10 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 24).

Item	Quantity	Catalog no.
Collagenase Type IV	1 g	17104-019
DMEM/F12* containing GLUTAMAX™ (2mM)	500 ml	10565-018
Knockout™ Serum Replacement (KSR)	500 ml	10828-028
MEM Non-Essential Amino Acids Solution, 10 mM (100X)	100 ml	11140-050
FGF-Basic (bFGF)	50 µg	PHG0026
STEMPRO® hESC SFM	1 kit	A1000701
2-Mercaptoethanol, 1,000X (55 mM)	50 ml	21985
Dulbecco's Modified Eagle Medium (D-MEM) high glucose with L-glutamine and sodium pyruvate	500 ml	11995-065
Fetal Bovine Serum, ES Cell-Qualified (US)	500 ml	16141-079
Bovine Albumin Fraction V Solution (7.5%)	100 ml	15260-037
Geltrex™	5 ml	12760
DPBS	500 ml	14190
Antibiotic-Antimycotic (100X), liquid	100 ml	15240-062
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
Water, distilled	500 ml	15230-162

Mitomycin C Treated MEFs

Mitomycin C-treated, Hygromycin-resistant MEFs (DR4) are available from ATCC (Cat. no. SCRC-1045.2). Hygromycin-resistant MEFs (DR4) are also available separately from ATCC (Cat. no. SCRC-1045); Mitomycin C is available separately from Sigma, St. Louis (Cat. no. M0503).

Continued on next page

Accessory Products, Continued

Fetal Bovine Serum, ES Cell-Qualified	Invitrogen also provides ES Cell-Qualified Fetal Bovine Serum originating from countries other than the U.S. (go to www.invitrogen.com for information). These can be more appropriate for your situation, and may be used to grow BG01V/hOG Cells.
Porcine Skin Gelatin	Porcine Skin Gelatin can be obtained from Sigma, St. Louis (Cat no. G1890).

Introduction

Introduction

BG01V/hOG human embryonic stem cells (hESCs) are engineered to enable monitoring the pluripotency of hESCs without sacrificing cells. When pluripotent, these cells express Emerald Green Fluorescent Protein (emGFP). Use these cells to assess whether your culture conditions are adequate to keep hESCs undifferentiated, or determine the efficiency of your differentiation assays.

Characteristics of BG01V/hOG Cells

BG01V/hOG cells have the following characteristics:

- Prepared from low-passage (passage 17) parental BG01V cells cultured on murine embryonic feeders (MEFs)
- Pluripotent: can differentiate to representatives of the three primary germ layers
- Express EmGFP when pluripotent; lose EmGFP expression upon differentiation.

BG01V Parental Cell Line

BG01V/hOG Cells are derived from the BG01V hESC 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 murine embryonic feeders (MEFs) 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.

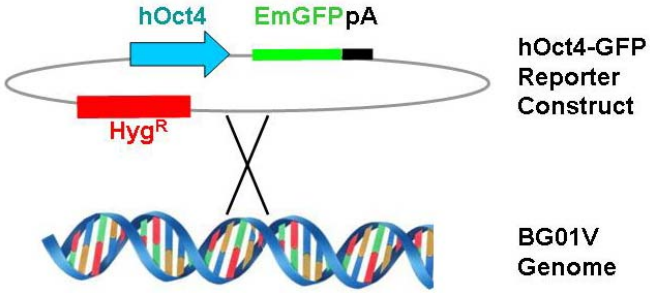
Continued on next page

Introduction, Continued

Generation of BG01V/hOG Cells

We constructed an integration vector containing EmGFP expressed under the direction of the human Oct4 promoter, and followed by the HSV-TK polyadenylation signal. This plasmid was stably integrated into the genome of BG01V 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/hOG.

Note: Since the integration vector used contains a Hygromycin resistance gene (Hyg^R), BG01V/hOG Cells are resistant to Hygromycin B. If you want to stably integrate more genes in these cells, do not use the Hygromycin resistant gene as your selection marker.



GFP Expression Indicates Pluripotency

Engineered cells will express EmGFP under the appropriate conditions when the Oct4 promoter is active. This promoter is only active when hESCs are in the pluripotent state and therefore acts as a sensitive indicator of differentiation (Pan *et al.*, 2002). This tool can be used as a reporter of the cell culture's response to external stimuli including media composition and stress.

Methods

General Information

General Cell Handling

Follow the general guidelines below to grow and maintain BG01V/hOG 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.
- BG01V/hOG Cells may be cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or may be thawed on MEFs and then transitioned to MEF-conditioned media (MEF-CM) or STEMPRO® hESC SFM. Make sure to start preparing the feeder layer two days before thawing BG01V/hOG Cells.
- 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 BG01V/hOG cells to ensure that you have an adequate supply of early-passage cells.
- For general maintenance of cells, pass BG01V/hOG Cells before colonies start contacting each other.
- 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 v for ordering information).

Important

It is very important to strictly follow the guidelines for culturing BG01V/hOG Cells in this manual to keep them undifferentiated.

CAUTION

As with other human cell lines, when working with BG01V/hOG cells, handle as potentially biohazardous material under at least Biosafety Level 1 containment.

Preparing a Feeder Cell Layer

Introduction

BG01V/hOG cells are frozen from cultures grown on mouse embryonic fibroblast (MEF) feeder cells, and should be thawed on feeders. Follow the protocol below to prepare the feeder-layer matrix. 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 v 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 24).
- 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

Continued on next page

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 coculture, 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 coculture, replace medium with hESC Medium
3. Next day, the feeder layer is ready to be seeded with BG01V/hOG Cells in fresh hESC Medium.

Thawing and Establishing Cells

Introduction

BG01V/hOG cells are supplied in a vial containing 1 ml of cells at 2×10^6 viable cells/ml in freezing medium. They are frozen from cultures grown on MEF feeder cells, and must be thawed on MEF feeders. Follow the protocol below to thaw BG01V/hOG Cells directly into hESC Medium in a 35-mm dish.

Materials Needed

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

- BG01V/hOG cells (store frozen cells in liquid nitrogen until ready to use)
- hESC Medium (see page 5 for composition); pre-warm to 37°C before use)
- Feeder layer plates with mitotically inactivated MEFs – prepare at least two days in advance (see page 4)
- Disposable, sterile 50 ml tubes.
- 37°C incubator with humidified atmosphere of 5% CO_2

Thawing Procedure

Store frozen cells in liquid nitrogen until ready to use. To thaw and establish BG01V/hOG Cells:

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 50-ml tube and add warm hESC Medium dropwise up to 10 ml.
3. Spin cells down for 4 minutes at 200 × *g*.
4. Aspirate supernatant
5. Resuspend cells in hESC Medium (2 ml for a 35-mm dish)
6. Aspirate feeder layer plates, and plate resuspended BG01V/hOG 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.

Following thawing, you can continue to culture cells on MEF feeders as described starting on page 8, or you can transition the cells into StemPro® hESC SFM as described starting on page 10.

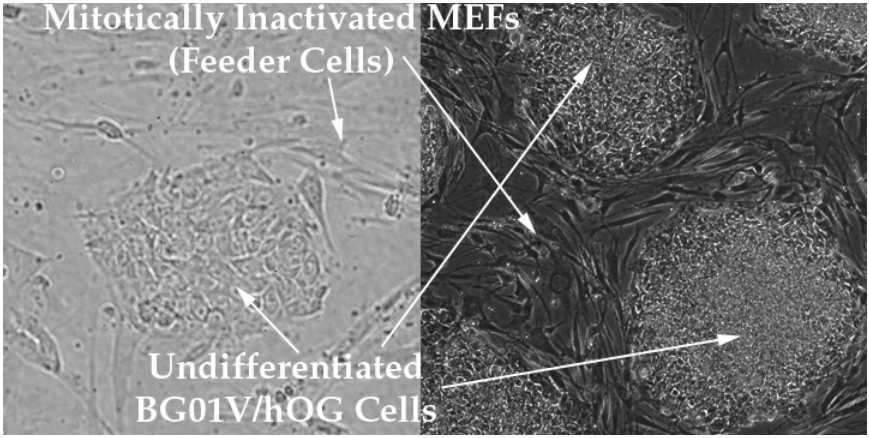
Continued on next page

Thawing and Establishing Cells, Continued

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 25), and grow as colonies (see image below for examples of undifferentiated BG01V/hOG Cells). If not, refer to the **Troubleshooting** section (page 22).
- Before colonies start contacting each other, they should be passed (see next page).



Culturing on MEF Feeders

Introduction

Follow the protocol below to culture BG01V/hOG Cells on feeder layer plates. For culturing cells in serum-free medium without feeders, see page 10.

Important

Before starting experiments, we recommend that you first prepare ample cell stocks, as described in **Freezing Cells** (page 16).

Materials Needed

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

- Plates with BG01V/hOG Cells
- hESC Medium (see page 5 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 4)
- Collagenase Type IV
- Hygromycin B
- Disposable, sterile 15-ml tubes.
- 37°C incubator with humidified atmosphere of 5% CO_2

Important

BG01V/hOG Cells should be cultured in the presence of 50 µg/ml Hygromycin B, on order to prevent losing the GFP expression cassette during prolonged culturing.

Collagenase Preparation

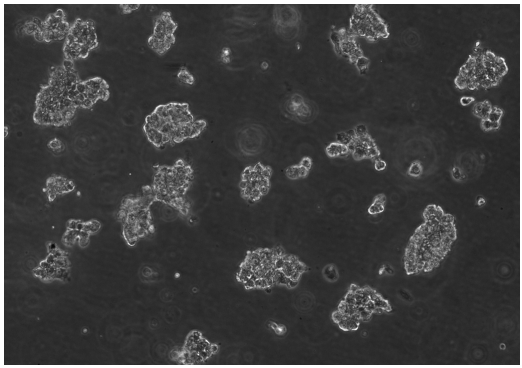
Prepare 1-mg/ml aliquots of collagenase IV in DMEM/F-12. Filter to sterilize and freeze at –20°C.

Continued on next page

Culturing on MEF Feeders, Continued

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 BG01V/hOG Cells in hESC Medium.
8. Aspirate feeder layer plates, and plate resuspended BG01V/hOG 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.

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

Culturing on STEMPRO® hESC SFM

Introduction

STEMPRO® hESC SFM allows you to culture BG01V/hOG Cells in a serum-free medium (SFM) without feeder cells. Follow the protocol in this section to culture BG01V/hOG Cells using STEMPRO® hESC SFM.

Note

BGO1V/hOG Cells are initially frozen down from cells grown on MEF feeders and should be thawed on feeders as described on pages 6–7 before transferring into MEF-conditioned medium (MEF-CM) and then into STEMPRO® hESC SFM, as described in this section.

Frozen stocks may then be prepared from the cells grown on STEMPRO® hESC SFM.

Important

Before starting experiments, we recommend that you first prepare ample cell stocks, as described in **Freezing Cells** (page 10).

Features of the Medium

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.

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Culturing on STEMPRO® hESC SFM,
Continued

Guidelines for SFM Culture

To prevent differentiation and slow growth of BGO1V/hOG 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 Geltrex™ in Mouse Embryonic Fibroblast-Conditioned Medium (MEF-CM). See the protocol in this section for transferring cells from MEF feeders to MEF-CM and then into STEMPRO® hESC SFM.
- **Passaging:** It is critical to achieve high plating/survival of colony pieces. The pieces must be a bit smaller than typical collagenase passaging on Geltrex™/ MEF-CM.
- 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 2.5 to 4×10^5 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

Culturing on STEMPRO® hESC SFM,
Continued

Materials Needed

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

- STEMPRO® hESC SFM, which includes:
  DMEM/F-12 with GLUTAMAX™
  STEMPRO® hESC Supplement
  Bovine Serum Albumin 25% (BSA)
- FGF-basic, 10 µg/ml, prepared as described below
- Collagenase Type IV, prepared as described on next page
- Geltrex™
- DPBS
- 2-Mercaptoethanol
- Hygromycin B
- Culture dishes (60-mm dishes recommended)
- An incubator at 37°C, humidified atmosphere of 5% CO_2 in air

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 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, 1.5 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.

Continued on next page

Culturing on STEMPRO® hESC SFM,
Continued

Preparing Collagenase IV

Prepare 1-mg/ml and 10-mg/ml aliquots of collagenase IV in DMEM/F-12. Filter to sterilize and freeze at –20°C.

Preparing MEF-Conditioned Medium (MEF-CM)

1. Prepare mitotically inactive MEF cells as described on pages 4–5.
2. Change the MEF medium to hESC medium (prepared on as page 5) after a 24-hour incubation
3. Collect medium from the cells every 24 hours and supplement with FGF-basic (prepared as described on the previous page) at a final concentration of 4 ng/ml before using.

Preparing Complete STEMPRO® hESC 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.

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

BG01V/hOG cells should be cultured in the presence of 50 µg/ml Hygromycin B to prevent losing the GFP expression cassette during prolonged culturing.

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Culturing on STEMPRO® hESC SFM,
Continued

Transferring Cells into STEMPRO® hESC SFM

After thawing and establishing BGO1V/hOG cells on MEF feeders as described on pages 6–7, use the following procedure to transfer the cells into STEMPRO® hESC SFM.

1. Aspirate medium from the cells and add 1 ml of 1 mg/ml collagenase solution for every 10 cm^2 of culture vessel surface area.
2. Incubate in a 37°C incubator until the edge of colonies curl up (usually it is less than an hour to see this curling).
3. Aspirate collagenase solution and add MEF-CM supplemented with FGF-basic, as described on page 13.
4. Gently scrape the dish using a 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.
5. Remove the Geltrex™ from a Geltrex™-coated plate by tipping the plate slightly and aspirating the solution. Wash the plate once with DMEM/F12. Take care to avoid drying of the plate surface before plating.
6. Seed the cells onto the plate.
7. Add a final concentration of 50 µg/ml Hygromycin B (1:1,000 dilution of 50 mg/ml Hygromycin B stock).
8. Place the plate with the cells in the incubator. Shake the plate gently to evenly distribute the cells.
9. The next day, feed the cells once more with MEF-CM supplemented with FGF-basic.
10. The following day (Day 3), feed the cells with an equivalent amount of complete STEMPRO® hESC SFM and a final concentration of 50 µg/ml Hygromycin B.
11. Thereafter, continue feeding cells daily with complete SFM and 50 µg/ml Hygromycin B.

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Culturing on STEMPRO® hESC SFM,
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 13). Minimize dwell time.
2. Set up the plate with BGO1V/hOG 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 13).
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.
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.
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 Cultured on MEF Feeders

Introduction

When freezing BG01V/hOG 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 BG01V/hOG 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 v for ordering information):

- Plates with BG01V/hOG Cells on MEF feeders
- hESC Medium (see page 5 for composition)
- Collagenase Type IV working solution (1 mg/ml) in DMEM/F12 (see page 8)
- 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.

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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 5) 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 9 for an example).
6. Spin cells down for 2 minutes at 200 × *g* at room temperature.
7. Gently aspirate media and resuspend BG01V/hOG 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.

Freezing Cells Cultured on SFM

Introduction

When freezing BG01V/hOG Cells cultured on STEMPRO™ hESC SFM, 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 0.5 ml of SFM Freezing Medium 1 and 0.5 ml of SFM Freezing Medium 2.
- Bring BG01V/hOG 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 v for ordering information):

- Plates with BG01V/hOG Cells in SFM
- Wash Medium (see page 13)
- 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 SFM Freezing Medium

Prepare SFM 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 **SFM Freezing Medium 1** needed:

DMEM/F12 with GLUTAMAX™	0.5 ml
Knockout™ Serum Replacement (KSR)	0.5 ml

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

DMEM/F12 with GLUTAMAX™	0.8 ml
DMSO	0.2 ml

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

Continued on next page

Freezing Cells Cultured on SFM, 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 13).
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 BG01V/hOG cells in SFM 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 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.

Expected Results

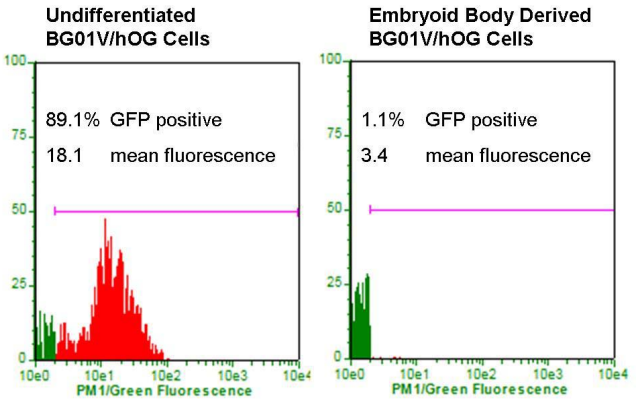
Introduction

Pluripotent BG01V/hOG Cells express EmGFP, but upon differentiation, these cells lose EmGFP expression. In this section, some typical examples are shown to help you interpret your experiments.

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

FACS Analysis

BG01V/hOG human embryonic stem cells differentiate when induced to form embryoid bodies. Embryoid body-derived BG01V/hOG Cells hardly contain any EmGFP positive cells, as determined by FACS analysis.

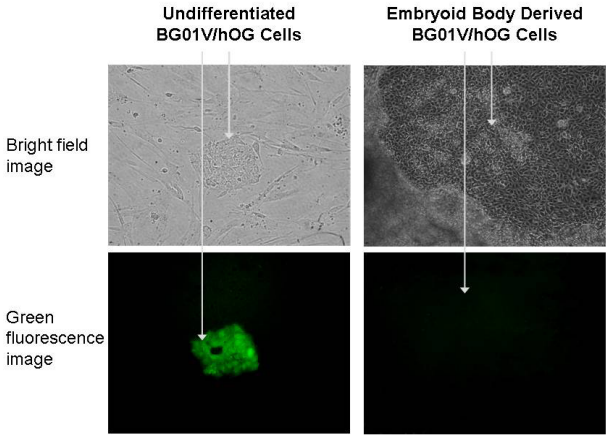


Continued on next page

Expected Results, Continued

Fluorescence Microscopy

EmGFP expression is lost upon embryoid body induced differentiation of BG01V/hOG Cells, as seen in the green fluorescence image below.



Note: If you are unfamiliar with fluorescence microscopy, see page 25.

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 2.5×10^6 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 16) 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 BG01V/hOG 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 $200 \times g$ at room temperature and dilute the cells at higher density.
	MEFs sub optimal and do not support recovery of BG01V/hOG Cells	Purchase (see page v) 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 (see page v)

Continued on next page

Troubleshooting, Continued

Culturing Cells

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

Problem	Cause	Solution
Cells grow slowly	Growth medium not correct	Use correct growth medium.
	bFGF inactive	bFGF is not stable when frequently warmed and cooled Add bFGF to medium just before use, or store medium with bFGF in aliquots at –20°C.
	Cells too old	Use healthy BG01V/hOG 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 feeder layers	Thaw and culture a fresh vial of BG01V/hOG cells. Make sure to thaw on feeder layers as described on page 4.
	Suboptimal quality of feeder layer.	Check the concentration of feeder cells used. Purchase (see page v) or make (see page 24) new batch of mitotically inactivated MEFs. if necessary. Use Hygromycin resistant MEFs.
	Culture conditions not correct	Thaw and culture fresh vial of BG01V/hOG Cells. Follow thawing instructions (page 6) 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 25). Be sure to use an inverted fluorescence microscope for analysis.
	Cells lost GFP expression cassette	Thaw and culture fresh vial of BG01V/hOG Cells. Culture cells in presence of 50 µg/ml Hygromycin B.
	Cells differentiated	See points above

Appendix

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 µm filter. Store up to 1 year at 4°C.

Coat plates for 20 to 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 5)
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 5) 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.

Detecting Fluorescence

Introduction

You may detect EmGFP protein expression directly in undifferentiated BG01V/hOG 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 21.

Technical Support

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

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

Information for European Customers

BG01V/hOG 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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