

## Growth of Human Embryonic Stem Cells in Long-Term Hypoxia

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### Abstract

Human embryonic stem cells (hESCs) hold a great promise for regenerative medicine and tissue engineering. In order to obtain uniform hESC cultures without spontaneous differentiation, which is of interest for basic investigations as well as the development of future therapeutic protocols, it is important that specific culture conditions are adhered to. Here, we describe in detail a procedure for propagation of hESCs that by virtue of exposure of the cultures to low atmospheric oxygen (5%) enables the maintenance of their undifferentiated phenotype in long term. The critical steps and impact of possible modifications on the final outcome are discussed, and useful hints are provided to streamline the troubleshooting.

**Key words:** Human embryonic stem cells, Long-term culture, Hypoxia, Pluripotency, Self-renewal, Manual microdissection

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### 1. Introduction

The embryonic stem cells (ESCs) stand out in that they have practically unlimited life span and have a potential to differentiate into all body tissues, except for placental trophoblasts (1). These properties render ESCs a very valuable source for prospective therapeutic approaches based on cell regeneration and replacement and tissue-engineered spare parts. ESCs are considered especially useful in applications where the lineages follow the endo- and neuroectodermal differentiation pathways, since there is a lack of suitable somatic stem cell associated with these particular specifications. Recently, a promising progress has been done with in vitro production of liver, lung, neural, or insulin-secreting cells (2–5).

The key prerequisites for in vitro propagation of human ESCs (hESCs) are currently well defined; nevertheless, there are a number of alternative protocols. They differ in the use of different

growth factors, media, gaseous environment, and the mode of passaging (6, 7). Attention has to be given to all these factors, since their compound effect constitutes a microenvironmental setting that determines the behavior and long-term properties of a given hESC line. In this regard, differences have been observed between different hESC lines with respect to their biological responses to, for example, trypsinization or dependence on feeder cells (8). It is plausible that such specific features reflect implementation of unique conditions during isolation and growth.

A frequently encountered drawback during hESC culture is the spontaneous differentiation (9, 10). The exact mechanism underlying this phenomenon is not clearly understood, although there is a plethora of evidence linking developmentally important pathways, such as Notch, sonic hedgehog, Wnt, and bone morphogenetic protein-associated signaling, to the control of ESC self-renewal and differentiation (11–14). Recently, it has been demonstrated that mild hypoxic conditions have the capacity to enhance the maintenance of hESC pluripotency by inhibiting the spontaneous differentiation in short as well as long term (15–17), and at least some of the above-mentioned factors appear to be involved (18).

Since the use of hypoxia is a very straightforward and simple approach, it may be of interest to all basic and translational biomedical research as well as industrial applications, where the access to uniform ESC cultures is necessary. It is generally more feasible to expose cultures to short-term hypoxia during the regular passaging intervals. The procedure becomes, however, more difficult when the hypoxic culturing is carried out over extended periods of time and the harmful reoxygenation is to be avoided. In the following sections, we will describe in detail a procedure for long-term maintenance and characterization of hESCs in hypoxic atmosphere.

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## 2. Materials

### **2.1. Growth and Irradiation of Human Foreskin Fibroblasts (HFFs)**

1. HFFs (American Type Culture Collection, CRL-2429).
2. Iscove's Modified Dulbecco's Modified Eagle Medium (IDMEM) (GIBCO/Invitrogen, Carlsbad, CA) is stored at 4°C.
3. Fetal bovine serum (FBS) (GIBCO/Invitrogen) is stored at –80°C and is used to supplement IDMEM in final concentration of 10%.
4. Penicillin/streptomycin 1,000 U/mL/1 mg/mL stock solution mixture (GIBCO/Invitrogen) is stored in aliquots of 10 mL at –20°C.

5. The feeder growth medium, which is fully supplemented IDMEM, is stored at 4°C and is stable for 2 weeks. It is prepared using following volumes to obtain 500 mL:  
448 mL IDMEM  
50 mL FBS  
2 mL penicillin/streptomycin (see Note 1)
6. Phosphate-buffered saline (PBS) (GIBCO/Invitrogen).
7. Trypsin/EDTA blend. Trypsin (GIBCO/Invitrogen) is obtained as a solution of 2.5% in PBS. It is diluted 10 times with PBS and sterile filtered. Aliquots of 10 mL are stored at -20°C. EDTA (anhydrous, crystalline, cell culture tested) (Sigma-Aldrich, Brøndby, Denmark) is dissolved in PBS at a concentration of 0.02%, sterile filtered, and stored in 10 mL aliquots at 4°C. To prepare the blend, trypsin and EDTA solutions are mixed 1:1. The blend is stored at 4°C and is stable for up to a week.
8. Trypan blue solution 0.4%, cell culture tested and sterile filtered (Sigma-Aldrich).
9. Hemocytometer.
10. Tissue culture flasks T-175.
11. Centrifuge tubes (50 mL).
12. 35-mm tissue culture dishes (Cell Bind; Corning, Amsterdam, the Netherlands).
13. Gamma cell irradiator (Gammacell 2000; Mølsgaard Medical, Ganløse, Denmark) (see Note 2).

## **2.2. Propagation of hESCs**

1. Knockout Dulbecco's Modified Eagle Medium (KDMEM) (GIBCO/Invitrogen) is stored at 4°C.
2. Knockout Serum Replacer (GIBCO/Invitrogen) is stored in aliquots of 50 mL at -20°C and is used to supplement KDMEM in final concentration of 20%.
3. L-glutamine 200 mM stock solution (GIBCO/Invitrogen) is stored in aliquots of 10 mL at -20°C and is used to supplement KDMEM in a final 2 mM concentration.
4. Beta-mercaptoethanol 50 mM stock solution (GIBCO/Invitrogen) is stored in aliquots of 200 µL at -20°C and is used to supplement KDMEM in a final 0.1 mM concentration.
5. Nonessential amino acid 10 mM (100×) stock solution (GIBCO/Invitrogen) is stored at 4°C and is used to supplement KDMEM to a final 0.1 mM concentration.
6. Recombinant human bFGF (BioSource Europe, Nivelles, Belgium) is stored in a concentration of 25 µg/µL in aliquots of 50 µL at -80°C and is used to supplement KDMEM in the final concentration of 4 ng/mL.

7. The hESC growth medium is prepared without bFGF and is stored at 4°C for up to 2 weeks. Just prior to use, 0.16 µL of bFGF is added per mL media. The medium is prepared using following volumes to obtain 500 mL:  
387 mL KDMEM  
100 mL Knockout Serum Replacer  
5 mL L-glutamine  
1 mL Beta-mercaptoethanol  
5 mL Nonessential amino acids  
2 mL Penicillin/streptomycin (same stock solution as in Sect. 2.1)
8. Surgical disposable scalpels no. 15 (Aesculap, Tuttlingen, Germany).
9. Stereo microscope in the hypoxia workstation (see Note 3).
10. Xvivo System integrated workbench-incubator glovebox with controllable atmosphere (BioSpherix, Ltd., Redfield, NY) (see Note 4).

### **2.3. Immuno- fluorescence Staining**

1. Hoechst 33342 (Gibco/Invitrogen) is obtained as a solution at 10 mg/mL. The working solution is made ready by diluting 1:100 in PBS to a final 100 µg/mL. The working solution is stable for several months if stored in the dark at 4°C.
2. Phosphate-buffered 4% paraformaldehyde.
3. Bovine serum albumin (BSA) (Standard grade; Europa Bioproducts, Cambridge, United Kingdom) is stored at 4°C. It is used at working concentration of 1%, 2%, or 4%, depending on the assay, by diluting in PBS.
4. Triton X-100 (Sigma-Aldrich) working solution is made freshly by diluting to 0.2% final concentration in 4% BSA.
5. SSEA-1-specific mouse monoclonal antibody (sc-21702; Santa Cruz Biotechnology, Santa Cruz, CA) is supplied at 200 µg/mL and stored at 4°C. Prior to use, it is diluted 200-fold in 1% BSA. It should be used within a couple of hours.
6. Cy-5 goat anti-mouse conjugate (AP130S; Millipore, Bedford, MA) is mixed upon receipt with an equal volume of glycerol and stored at -20°C. Prior to use, the antibody is further diluted 100-fold (total dilution is 200-fold) in 1% BSA and should be used within a couple of hours.
7. Oct4-specific rabbit polyclonal antibody (ab19857; Abcam, Cambridge, United Kingdom) is mixed upon receipt with an equal volume of glycerol and stored at -20°C. Prior to use, it is further diluted 100-fold (total dilution is 200-fold) in 1% BSA and should be used within a couple of hours.

8. FITC-conjugated goat anti-rabbit polyclonal antibody (ab6717, Abcam) is mixed upon receipt with an equal volume of glycerol and stored at  $-20^{\circ}\text{C}$ . Prior to use, the antibody is further diluted 150-fold (total dilution is 300-fold) in 1% BSA and should be used within a couple of hours.
9. Wide-field fluorescence system Axio Observer Z1 integrated with AxioCam MRm camera and controlled by the AxioVision software package (all Carl Zeiss, Göttingen, Germany).

#### **2.4. Real-Time PCR**

1. Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA).
2. iScript cDNA synthesis kit (Bio-Rad Laboratories).
3. iQ SYBR Green Supermix (Bio-Rad Laboratories).
4. Diethylpyrocarbonate (DEPC)-water (Sigma-Aldrich) is stored at  $4^{\circ}\text{C}$ .
5. PCR primers for pluripotency markers Oct4 and Nanog and internal reference 18 S rRNA:
  - Oct4 forward primer: 5'-CTG GTT CGC TTT CTC-3'
  - Oct4 reverse primer: 5'-GGG GGT TCT ATT TGG-3'
  - Nanog forward primer: 5'-AGG AAG AGT AGA GGC-3'
  - Nanog reverse primer: 5'-CAA CTG GCC GAA GAA-3'
  - 18 S rRNA forward primer: 5'-AGG ACC GCG GTT CTA TTT TGT TGG-3'
  - 18 S rRNA reverse primer: 5'-CCC CCG GCC GTC CCT CTT A-3'

Oct4 and Nanog primers are diluted to a working concentration of  $10\text{ pmol}/\mu\text{L}$ ; 18 S rRNA primers are diluted to  $5\text{ pmol}/\mu\text{L}$ . All primers are stored at  $-20^{\circ}\text{C}$ .
6. 96-well translucent plates (iCycler iQ PCR plates; Bio-Rad Laboratories).
7. ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).
8. MyIQ single-color real-time PCR detection system (Bio-Rad Laboratories).

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### **3. Methods**

Good laboratory practice should strictly be observed, especially in all steps requiring sterile handling. All media prepared in-house should be sterile filtered using a  $0.2\text{-}\mu\text{m}$  exclusion cell culture grade filters (TPP, Trasadingen, Switzerland).

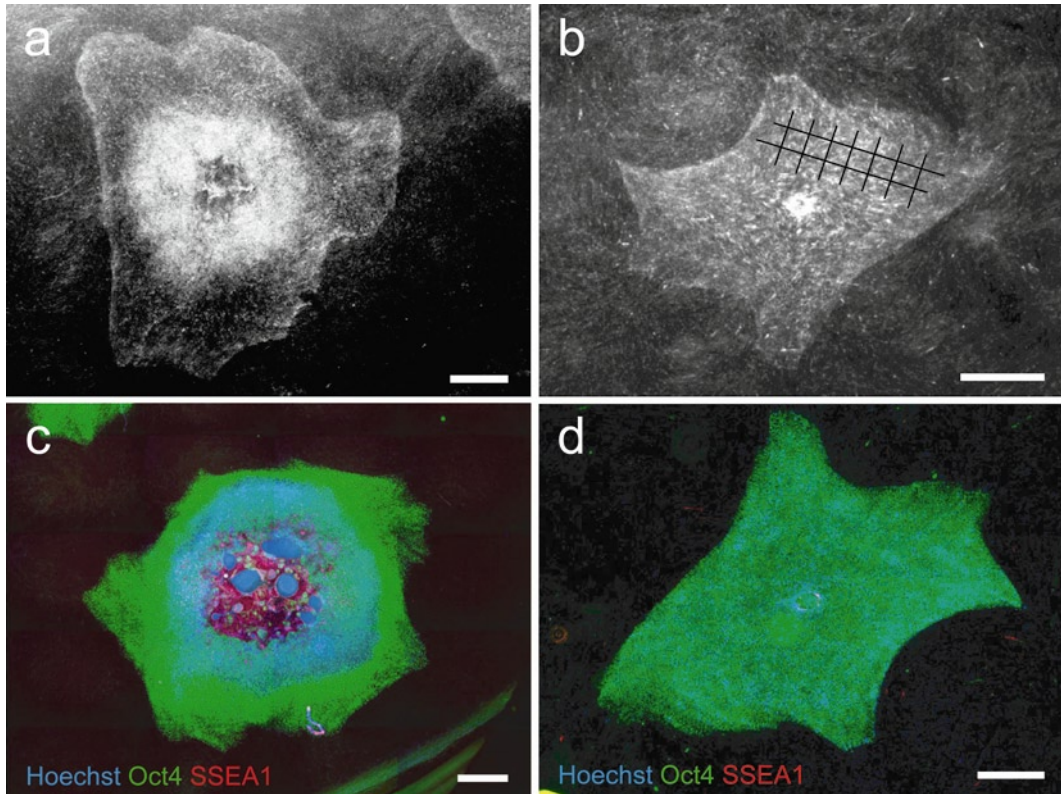


Fig. 1. Effect of hypoxia on the self-renewal of hESCs during long-term cultures. The CLS1 (a and b) and CLS2 (c and d) lines were cultured for 18 months in ambient air conditions (a and c) or hypoxic atmosphere of 5% oxygen (b and d). Four weeks after passaging, the spontaneous differentiation in the central parts of the colonies from normoxic conditions is discernible in the dark field images (a and b) and after immunofluorescence imaging of gene markers (c and d). The grid in (b) indicates the microdissection pattern. The scale bars indicate 1 mm.

### 3.1. Long-Term Maintenance of hESCs in Hypoxia

With regard to the continuous propagation in hypoxic atmosphere, the greatest challenge is to maintain stable gaseous conditions during the subculture steps. This means that the manual microdissection of hESC colonies has to be performed within enclosure with controllable environment, and feeder culture and media have to be properly pre-equilibrated to these conditions. Xvivo System workstation appears especially well suited for this purpose. Different oxygen tensions can be used, but 5% offers the best compromise between the proliferation rate and suppression of spontaneous differentiation. In our hands, the different hESC lines display undiminished replication rate and other properties of ESCs for over 18 months (approx. 25 passages) (Fig. 1).

#### 3.1.1. Preparation of Feeder Cells

A large stock of feeder cells can be prepared and kept frozen at  $-140^{\circ}\text{C}$  (see Note 5). One confluent T175 flask typically yields  $5\text{--}6 \times 10^6$  cells, which is sufficient for eight dishes. In the following sections, we will describe the preparation of approximately 40 dishes of feeder cells.

1. An aliquot of  $2 \times 10^6$  HFFs is thawed and seeded in a T175 flask in 20 mL of feeder growth medium. The cells will reach confluency after approximately 4 days.
2. When the cells reach confluency, the medium is removed, and the cells are washed twice with 10 mL of PBS to prepare for trypsinization.
3. 4 mL of Trypsin/EDTA blend is added and incubated at 37°C.
4. The detachment is assessed visually under the microscope at 3-min intervals and can be aided by manually tapping the flask.
5. When cells have detached, the trypsinization is stopped by adding 12 mL of feeder growth medium.
6. The cells are divided into eight T175 flasks, and the growth medium is added to achieve a total of 20 mL per flask. After approximately a week, the cells will reach confluency and may be subcultured, as described above.
7. Cells from one flask are typically used to continue the propagation of feeder cells and are divided into eight new T175 flasks. For additional expansion of cells, repeat steps 2–7 (see Note 6).
8. Cells from the remaining seven flasks are pooled.
9. An aliquot of the cell suspension is mixed with an equal volume of trypan blue, and the yield of live cells is determined using a hemocytometer.
10. The cells are divided into four 50-mL centrifuge tubes, and the concentration is adjusted with feeder growth medium to  $2.5 \times 10^5$  cells/mL.
11. The cells are irradiated with 35 Gray, and 2.5 mL aliquots are then seeded into 35-mm tissue culture dishes yielding 625,000 cells/dish.
12. The cultures are grown overnight in CO<sub>2</sub> incubator in standard normoxic conditions, after which the medium is changed to 3 mL of hESC growth medium. The cultures are then transferred to incubation chambers with preselected hypoxic atmosphere, and after 2 h, they are ready for transfer of hESCs. The dishes with feeder cells may be used for seeding with hESC for up to 1 week (see Note 7).

### 3.1.2. Passaging of hESCs

1. Every 3–4 weeks, incisions are made in the hESC colonies using a disposable scalpel under the guidance of stereo microscope (50-fold magnification) to demarcate several rectangular subsections of up to  $0.3 \times 0.3$  mm in size. With the tip of the scalpel, the hESC subsections are gently released from the culture dish (Fig. 1b) (see Note 8).

2. Each of the subsections is transferred with a micropipette in 8  $\mu\text{L}$  volume and deposited on the feeder monolayer.
3. Approximately six subsections are seeded in a single dish. After seeding, the dishes are left on the work space for 2 h to allow the subsections to settle onto the feeders, and the dishes are transferred to the incubation chambers. Caution is taken to avoid swirling, which can result in irregular distribution of the colonies.
4. The hESC growth medium is completely replaced twice a week (see Note 9).

### **3.2. Determination of hESC Pluripotency**

The pluripotency status can be assessed by identification of cell surface or intracellular markers by immunostaining or real-time RT-PCR. There are several hESC markers, such as Oct4, Nanog, SSEA3 and 4; in addition, the differentiation marker SSEA1 is routinely used to counterstain for the cells that departed from the hESC pool. The methods in the following text will be exemplified using immunofluorescence staining for Oct4 and SSEA1 and RT-PCR for Oct4 and Nanog. Functional assessment normally also involves differentiation assays that are not dealt within this chapter.

#### **3.2.1. Immunofluorescence Staining**

1. After predetermined period of growth, a portion of 35  $\mu\text{L}$  of Hoechst 33342 reagent is added to the media of the cultures in hypoxia to achieve final concentration of 10  $\mu\text{g}/\text{mL}$ . Gently swirl the culture dishes 3–4 times to aid proper mixing, and incubate them for 30 min.
2. The cultures are removed from hypoxia, the medium is discarded, and the cells are washed with 1 mL of PBS for 5 min at room temperature (RT). All subsequent incubation steps are done in the dark, i.e., with aluminum foil cover over the dishes.
3. The cells are fixed with buffered 4% formaldehyde for 20 min at RT.
4. The cells are washed twice with 1 mL of PBS for 3 min, followed by blocking with 2% BSA for 10 min at RT.
5. The blocking solution is replaced with 0.5 mL of SSEA1 antibody per dish and incubated for 1 h at RT.
6. The primary antibody is removed by washing twice with 1 mL of PBS for 3 min, and the cultures are incubated with 0.5 mL of Cy-5 goat anti-mouse conjugate for 30 min at RT.
7. To detect nucleus-localized Oct4, cell permeabilization is necessary. To accomplish this, wash the cells twice with 1 mL of PBS for 3 min and subsequently incubate them with 1 mL of 0.2% Triton X-100 in 4% BSA for 1 h at 37°C.



8. The detergent is removed by washing twice with 1 mL of PBS for 3 min at RT, and 0.5 mL of Oct4 antibody is added and incubated for 1 h at RT.
9. The unbound antibody is removed by washing twice with 1 mL of PBS for 3 min, and the specific immune complexes are revealed by adding 0.5 mL of FITC-conjugated goat anti-rabbit polyclonal antibody and incubating for 30 min at RT.
10. The dishes are washed twice with 1 mL of PBS for 3 min at RT.
11. After the final washing step, the preparations are preserved in PBS. They can be analyzed immediately or stored at 4°C for at least 2 weeks.
12. The immunofluorescence imaging microscopy is done with the aid of Axio Observer Z1 wide-field fluorescence system. The Mosaic module enables tiling of images to capture whole colony area using three-channel full resolution based on 2.5–10-fold objective magnification.

### 3.2.2. Semiquantitative Real-Time PCR

#### Isolation of RNA and cDNA Synthesis

RNA isolation and cDNA synthesis is performed with Aurum Total RNA Mini and iScript cDNA synthesis kits, respectively, according to manufacturer's instructions. Steps that do not involve the commercial kits are detailed below. For RNA isolation, we have found that five to six colonies will yield sufficient RNA materials for the assay.

1. A 1.5-mL microcentrifuge tube with 1 mL of ice-cold PBS is placed on ice in the hypoxia workstation.
2. The hESC colonies are released gently by using the scalpel. The stereo microscope in the hypoxia workstation is used to control that the embryonic cells are fully separated from underlying layer of feeder cells (see Note 10).
3. The floating cell clumps are recovered in 8  $\mu$ L of medium using a micropipette and transferred to the ice-cold PBS (see Note 11).
4. The cells are centrifuged at  $300 \times g$  for 1 min, and the supernatant is removed. The pellets are resuspended in 350  $\mu$ L of lysis solution from the Aurum RNA Mini Kit supplemented with 1%  $\beta$ -mercaptoethanol, and thoroughly repetitive pipetting is applied until the complete lysis is achieved. The rest of the procedure is carried out as instructed by the manufacturer.
5. The concentration of eluted total RNA is determined spectrophotometrically, and the sample is used immediately for the synthesis of cDNA (see Note 12).
6. To produce cDNA, a reaction mix is prepared containing 100 ng total RNA, 4  $\mu$ L 5 $\times$  iScript reaction mix, 1  $\mu$ L iScript reverse transcriptase, and DEPC-nuclease-free water to a final volume of 20  $\mu$ L. The rest of the procedure is performed as suggested by the manufacturer.

## Amplification Assay

1. Each reaction is performed in duplicate and is set up using 8  $\mu\text{L}$  of cDNA, 13  $\mu\text{L}$  of iQ SYBR Green Supermix reaction components, 0.03 pmol of 18 S primers or 0.192 pmol of gene-specific primers, and water to make the total volume of 25  $\mu\text{L}$ .
2. The amplification is performed using two-temperature cycling consisting of a single annealing/extension step of 30 s at 60°C and a denaturation step of 15 s at 95°C, for a total number of 40 cycles.
3. To confirm the quality of each run, the occurrence of primer dimers is monitored by invoking a melting curve function of the program.
4. The relative transcriptional levels are assessed by extrapolation from amplification of a fourfold dilution series of pooled cDNA.
5. For each sample, the levels of Oct4 and Nanog are normalized to the levels of 18 S rRNA.

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## 4. Notes

1. The final concentration of penicillin and streptomycin is 40 U/mL and 40  $\mu\text{g}/\text{mL}$ , respectively, which is 2.5-fold lower than the standard concentration used for cell culture.
2. If a gamma irradiator is not in-house, blood banks often have one. We have prepared the feeder cells in the centrifuge tubes, transported to blood bank, irradiated, and transported back in about 90 min without noticeable cell death.
3. SteREO Lumar.V12 is due to its motorization especially suitable to be incorporated in the hypoxia workstation. It enables contrasting stereomicroscopy as well as fluorescence imaging.
4. The workstation enables incubation and handling in an uninterrupted hypoxic environment buffered with 5%  $\text{CO}_2$  and nitrogen. Several humidified incubation chambers with variable oxygen concentrations can be used simultaneously.
5. When the cells are received from ATCC, they are thawed and expanded in a T175 tissue culture flask, and designated passage (P) 0. Upon confluency, the cells are trypsinized and divided into 10 T175 tissue culture flasks (P1). When P1 cultures are confluent, the cells are frozen in aliquots of  $2 \times 10^6$  cells. These aliquots can be thawed and expanded to replenish the stock or support passaging of hESCs.
6. We use cells up to P10, after which a new aliquot of frozen cells is thawed and propagated.

7. Culturing the feeders on Cell Bind (Corning) culture dishes enhances integrity of the monolayer. Nevertheless, when the feeder cells are cultured after irradiation for more than 3–4 weeks, there is a tendency for detachment.
8. The number of sections available from a single colony varies depending on its size, but approximately 10–15 can be obtained. The center, which represents the original seed, is usually not selected, neither are sections from the outermost area of the colony. It is not important whether the cuts penetrate deeper in the feeder layer, such that some feeder cells are transferred together with the hESCs.
9. For pre-equilibration, the medium already with bFGF is placed in hypoxia. A maximum of 25 mL is used per T175 cell culture flask for 2 h. The flask is placed horizontally to allow for rapid release of oxygen from the medium.
10. It is quite critical for the quality of RNA that the scrapping of hESCs is achieved without contamination with feeder cells. By placing the scalpel perpendicularly to the colony and very gently scraping while confirming the separation from feeders microscopically, the colonies can be released. The same type of scalpel is used as for passaging of hESCs. This procedure requires some practice, so we recommend including several additional dishes with hESC colonies for training purposes for the first couple of experiments.
11. We try to recover as many pieces of stem cells as possible per 8  $\mu$ L media. We repeat the recovery of stem cells until all the material has been harvested and transferred to the ice-cold PBS.
12. Alternatively, Bioanalyzer (Agilent Technologies, Naerum, Denmark) can be used to measure concentration as well as determine integrity of the RNA.

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