PEPROTECH®
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Instruction Manual

PeproGrow™ hESC Medium

Expansion and Maintenance Media
for hESCs and iPSCs

Product Use Limitations: Not for human use.

Made in the United States.

Feeder-Free Culture Techniques for Pluripotent Stem Cells using PeproGrow™ hESC Media Products

A. Introduction

PeproTech's PeproGrow™ hESC Media products are comprised of complete, chemically-defined formulations designed for the feeder-free expansion and maintenance of both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). Intended for the culturing of hESCs and iPSCs in the undifferentiated, pluripotent state (Nanog+/Lin28+/Tra-1-60+/SSEA1-/SSEA4+/Oct4+), these formulations demonstrate less than 15% spontaneous differentiation as indicated by immunofluorescent staining and flow cytometry. These proprietary formulations were designed and developed by PeproTech in collaboration with the Stem Cell Training Course at Rutgers University. Each PeproGrow™ hESC Medium Kit includes a bottle of basal medium and a separate, lyophilized component of PeproTech's recombinant growth factors. Additional companion products are available separately and include the Animal-Free Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220), and the Cell Passaging/Non-Enzymatic Detachment Buffer (catalog number CPD-125). PeproTech's Animal-Free Human Vitronectin Matrix and Buffer Kit can be used following either a surface-coating or premix method.

PeproGrow™ hESC Medium Kit is an insulin-free formulation, in which insulin is replaced by a unique activator of similar pathways to eliminate possible interference during the measurement of insulin-production in subsequently differentiated cells.

PeproGrow™ hESC Plus Medium Kit is an insulin-inclusive formulation ideal for use when switching from other insulin-inclusive media products.

B. Materials and Reagents.

1. PeproGrow™ hESC Media Products:

Kit/Components	Catalog Number	Size
PeproGrow™ hESC Medium Kit	**HESC-500**	**500mL**
PeproGrow™ hESC Basal Medium	BM-HESC-500	500mL
PeproGrow™ hESC Growth Factor Component	GF-HESC-500	Vial for 500mL Basal Medium

Kit/Components	Catalog Number	Size
PeproGrow™ hESC Medium Kit	**HESC-100**	**100mL**
PeproGrow™ hESC Basal Medium	BM-HESC-100	100mL
PeproGrow™ hESC Growth Factor Component	GF-HESC-100	Vial for 100mL Basal Medium
PeproGrow™ hESC Plus Medium Kit	**HESCP-500**	**500mL**
PeproGrow™ hESC Plus Basal Medium	BM-HESCP-500	500mL
PeproGrow™ hESC Plus Growth Factor Component	GF-HESCP-500	Vial for 500mL Basal Medium
PeproGrow™ hESC Plus Medium Kit	**HESCP-100**	**100mL**
PeproGrow™ hESC Plus Basal Medium	BM-HESCP-100	100mL
PeproGrow™ hESC Plus Growth Factor Component	GF-HESCP-100	Vial for 100mL Basal Medium

2. Refer to Appendix I for additional materials, reagents, and companion products.

C. Preparation of Medium and Growth Factor Supplement

1. Centrifuge the vial of lyophilized growth factor component prior to opening, and reconstitute with sterile, cell culture grade water.

- **a.** Use 500µl of water for 500mL-size Medium Kits.
- **b.** Use 100µl of water for 100mL-size Medium Kits.

2. For use within two weeks, aseptically add the entire reconstituted growth factor component to the basal medium and mix well by swirling or pipetting. Otherwise, aseptically transfer the necessary volume of basal medium into a sterile polycarbonate bottle or conical-bottom polypropylene tube, aseptically add the necessary proportion of reconstituted growth factor component and mix well by swirling. Filtration is not necessary when prepared aseptically.

3. Label with the date of mixture and newly calculated expiration date (2 weeks from date of mixture). Store at 2°C to 8°C.

4. Remove only the necessary amount of medium for immediate use and warm to room temperature (RT) prior to feeding cells. Where necessary due to time constraints, a working volume can be removed and warmed in a 37°C water bath for approximately 5 minutes.

Storage/Stability: Keep medium in the dark.

Product Form	Temperature	Storage Time
Liquid Basal Medium	2°C to 8°C; Keep in the dark	6 months
Liquid Basal Medium after preparation	2°C to 8°C; Keep in the dark	2 weeks
Growth Factor Component (lyophilized)	-20°C to -80°C 2°C to 8°C	5 years 6 months

**Avoid repeated freeze-thaw cycles.*

D. General recommendations:

- Culturing on uncoated plastic is not recommended. Corning Matrigel® is recommended during initial adaptation; however, other suitable ECM products can be used (See **Appendix II**).
- PeproTech's Animal-Free Recombinant Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220) is recommended to coated standard cell dishes and performs optimally at 5µg/mL.
- PeproTech's Animal-Free Recombinant Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220) can also be used in a premix format, eliminating the need for prior preparation of coated cultureware.
- Enzymatic methods should not be used during cell culture steps with Animal-Free Human Vitronectin Matrix-coated cultureware. PeproTech's osmotically-compatible Cell Passaging/Non-Enzymatic Detachment Buffer (catalog number CPD-125), which only contains PBS, HEPES, and EDTA, should be used instead.
- Accommodation protocols are not necessary when switching from one insulin-inclusive formulation to another but may be necessary when switching from an insulin-inclusive formulation to an insulin-free formulation to avoid issue. Accommodation requirement may also depend on cell type used.
- When an acute method (no accommodation) does not function as expected, then additional accommodation protocols (short or long) may be used. Where accommodation protocols do not function as expected, then a long accommodation can be used in combination with a gradually increasing percentage of new medium (e.g. 100:0, 80:20, 60:40, 20:80, 0:100).
- Refer to **Appendix II** for additional products and information.

E. Preparation of Animal-Free Human Vitronectin Matrix-Coated Cell Cultureware

1. Reconstitute the Animal-Free Human Vitronectin Matrix according to the instructions provided on the product's Certificate of Analysis (CoA) using osmotically-compatible Animal-Free Human Vitronectin Matrix Buffer (PBS + Kolliphor P 188) from PeproTech's Animal-Free Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220).
2. Coat the cell cultureware with 5µg/mL of the reconstituted Animal-Free Human Vitronectin Matrix according to Table 2 below. For other cultureware, adjust the volume in relation to the surface area and ensure surface area is completely covered.

Table 2: Recommended Volumes of Animal-Free Vitronectin Matrix

Dish Size	Surface Area (cm^2)	Volumes (mL)
6 cm dish	21	2.5
10 cm dish	55	5
1 well of a 6-well dish	9.5	1
1 well of a 12-well dish	3.8	0.5
1 well of a 24-well dish	1.9	0.3

NOTE: It is critical that the entire culture surface is covered during coating and/or storage. Do not allow the dish surface to dry out as this may result in suboptimal performance.

3. For same-day use, place dish in a warm humidified cell culture incubator for 2 hours. Otherwise, wrap each dish with Parafilm and store on a flat surface at 4°C for up to one week. When storing at 4°C, a slightly larger volume may be required later where evaporation/condensation is observed on the cell culture dish lid.
4. After incubation, aspirate the Animal-Free Human Vitronectin Matrix solution and immediately add 1-2mL of complete medium (pre-conditioning step) into a well of a 6-well dish. Adjust volumes for other sized dishes/plates. Do not allow surface to dry.
5. Allow medium to warm by returning dish to an incubator.
6. Use dish by end of day.

F. Preparation of Animal-Free Human Vitronectin Matrix Premix Reagent

1. Reconstitute the Animal-Free Human Vitronectin Matrix according to the instructions provided on the product's Certificate of Analysis (CoA) using osmotically-compatible Animal-Free Human Vitronectin Matrix Buffer (PBS + Kolliphor P 188) from

PeproTech's Animal-Free Human Vitronectin Matrix and Buffer Kit (catalog number AF-VMB-220).

2. Passage cells according to normal protocol and resuspend in cell culture medium.
3. Add equivalent volume of reconstituted Animal-Free Human Vitronectin Matrix directly to medium/cell suspension using volume required for coating cultureware in **Table 2.**
4. Gently mix by inverting the tube containing the vitronectin/cell suspension several times (do not shake or vortex).
5. Pipette the suspension into appropriate cultureware.

G. Maintenance of Pluripotent Stem Cells

NOTE: Cultures may appear sparse within a day of thawing or splitting; however, growth should occur rapidly after 3-4 days in culture.

NOTE: Each well should be checked regularly for growth and monitored for bacterial or fungal microorganisms.

1. To maintain cells, medium should be changed daily, except for a "double volume" feed over the weekend.
2. To supplement with a "double volume" feed, passage cells on a Thursday or Friday so that the "double volume" feed occurs when the cells are at a lower density (e.g. cells are passaged and plated into 4mL of medium on a Friday, then on Sunday the medium is aspirated from the plate and fresh medium is added).

H. Thawing Cryopreserved Pluripotent Stem Cells

NOTE: Thaw ultra-frozen samples slowly and consistently to avoid temperature shock and shear stress. Cold and cool solutions can be used for gradual introduction of a newly thawed sample to warmer environments in a controlled manner.

NOTE: Once thawed, DMSO should be diluted out of samples as soon as possible as DMSO is known to exert cytotoxic effects during prolonged exposure. Centrifuge to pellet cells and transfer samples to a suitable matrix-coated culture vessel.

NOTE: When grown with PeproTech's PeproGrow™ hESC Media on Animal-Free Human Vitronectin Matrix-coated substrate and non-enzymatically cryopreserved using PBS/EDTA, cells may be thawed into the PeproGrow™ hESC Media and plated onto Animal-Free Human Vitronectin Matrix-coated substrate.

WARNING: Liquid nitrogen (LN_2) can accumulate in cryovials stored in liquid phase LN_2 and cause an explosion during rapid thawing. Transfer cryovials stored in liquid phase LN_2 to a -80°C freezer overnight to allow for gradual evaporation of any LN_2.

1. In a Biosafety Level 2 (BL2) Laminar Flow hood (BSC), place a 15mL conical tube in a cooled Biocision CoolRack™ (15mL) and add 9mL of cold medium.
2. Remove a vial of hESCs or hiPSCs from vapor phase LN_2 storage and transfer to a floater rack in a 37°C water bath.
3. Observe occasionally and remove the vial when only a small amount of ice remains to ensure average temperature remains cool. Do not shake the vial.
4. Clean the vial with 70% ethanol and transfer to an appropriate rack in a sterile BSC. A cooled Biocision CoolRack® XT CFT24 is recommended to prevent temperature fluctuation. Be careful not to spill cells.
5. Remove 1mL of medium from the 15mL tube prepared in **Step 1** using a disposable, sterile plastic transfer pipette (a 1-2mL serological pipette, or 1000µL sized micropipette, can also be used). Slowly backfill cryovial with gentle stirring.
6. Transfer vial contents to a 15mL conical tube.
7. **OPTIONAL:** Rinse the vial with a second round of 2mL diluted cells/medium from the 15mL tube using the same transfer pipette.
8. If large clumps of cells are observed, transfer vial to a cooled Biocision CoolRack® (15mL) placed in a refrigerator or on ice for 5-10 minutes to allow aggregate to settle. Proceed directly to **Step 11**, skipping centrifugation.
9. For pre-coated cultureware, transfer dishes from the incubator to a BSC and allow to equilibrate to RT. Not required for premix method.
10. Centrifuge cells for 2-5 minutes at 100 x g (times gravity) at 4°C. If using a Biocision CoolRack® (or ice) and the medium is still cool to the touch, spin at RT.
11. Remove supernatant and gently resuspend cells in 2mL of cool medium. Warm by holding in hand for 30 seconds or leaving in rack for 1-2 minutes.
12. Transfer cell suspension to appropriate number of cultureware wells. Use pre-coated plates unless following premix method.

TIP: When plating cells on a 6-well dish, run pipette tip around each well's rim while slowly dispensing cell solution. Use of a 5mL glass serological pipette is recommended here as the smooth bore can decrease shearing stress to cell clumps.

TIP: It is recommended to passage cells at least once prior to use for analytical purposes (immunostaining, flow cytometry, western blot analysis, Q-RT-PCR, etc.); however, cells can be plated directly onto vessels of other sizes at this point. The freeze/thaw process can introduce a relatively noticeable amount of differentiation.

TIP: 24-well dishes are recommended for indirect immunofluorescence techniques, and PBS/EDTA is recommended when passaging onto any dish of 96-wells or more.

I. Passaging Pluripotent Stem Cell Cultures Using PBS/EDTA

NOTE: PBS/EDTA is recommended for removal of cells from culture dishes with high surface tension (e.g. 96-well dishes, or 4-well and 8-well chamber slides). This dissociates colonies into small clumps and ensures an even spreading of cells.

NOTE: Spin-seed cells onto culture vessel's surface at 80-120 x g for 2-5 minutes using the appropriate plate carriers to avoid cell clumping near the center of wells. If appropriate plate carriers are unavailable, use minimal volumes to ensure faster attachment.

NOTE: Cells passaged in the morning should be attached by early afternoon, at which time the low volume of medium can be gently topped off with incubator-warmed, pre-equilibrated medium.

1. Aspirate medium from cell culture(s) and add 1-2mL of PBS/EDTA warmed to 37°C.
2. Transfer plate to an incubator for 3 minutes. Observe and return to incubator as needed. After 5 minutes, previously smooth colonies should be perforated with holes and phase contrast should be much brighter around many of the cells.

TIP: If cell detachment is not observed, quickly but gently aspirate PBS/EDTA. Immediately squirt desired volume of medium onto the cells using a somewhat forceful, circular motion out from center to well wall. Proceed directly to Step 7, skipping centrifugation.

3. Once cells begin detaching, wash cells from the plate by gently adding 3-4mL of culture (or suitable base) medium to each well in a circular motion from well wall to center.
4. Gently transfer cell suspension to 50mL conical tube. A 15mL conical tube can also be used but removing medium/PBS/EDTA solution following centrifugation can prove difficult.
5. Centrifuge for 2-5 minutes at 120 x g at RT.
6. Aspirate supernatant and gently add appropriate amount of medium for given split volume (e.g. when splitting 1 well 1:6, add 6mL of medium and gently titrate using a 5mL glass serological pipette to disaggregate cells into a "sandy-water" consistency.

WARNING: Over-titration of cells can lead to poor results.

7. Gently swirl well contents and transfer needed split volume to a new well/dish using a 5mL serological or 1000µL wide-bore pipette depending on seed volume. A 1000µL pipette is recommended for volumes below 500µL, but cell volume should be aspirated and dispensed slowly to minimize shearing of aggregates.
    - **a.** EXAMPLE: If splitting well 1:4 into a 4mL volume, add 1mL/well.
    - **b.** EXAMPLE: If splitting well 1:8 into a 4mL volume, add 0.5mL/well.

TIP: When using a 6-well dish, distribute the volume around the well rim for even distribution of aggregates; the cells will naturally gravitate towards the wells' center during incubation.

TIP: If cells are dense and the split volume is 3mL, cells should be plated 1:6 to 1:12 using 0.5mL to 0.25mL per well.

1. Once colonies are at optimal density, split cells 1:6 to 1:12 every 5-7 days (i.e. aggregates from 1 well of a 6-well plate can be plated in 6-10 wells). Adjust split ratio accordingly where colonies are too dense or too sparse.

TIP: Early or late passaging can result in decreased attachment or increased differentiation, respectively.

TIP: Ensure newly seeded colonies are distributed evenly to avoid increased differentiation.

NOTE: Results may vary between cell lines and laboratories. Protocol based on H1 hESC and two iPSC lines used by the Stem Cell Training Course at Rutgers University.

J. Freezing Pluripotent Stem Cells with PBS/EDTA

NOTE: Avoid centrifuging cells to remove PBS/EDTA. Instead, follow the protocol below to remove PBS/EDTA before colonies lift from the surface. This method allows cells to cool and settle gently and can permit the passaging of many cell plates/dishes.

1. Aspirate medium from cell culture(s) and add 1-2mL of PBS/EDTA.
2. Transfer plate to an incubator for 3 minutes. Observe and return to incubator as needed. After 5 minutes, previously smooth colonies should be perforated with holes and phrase contrast should be much brighter around many of the cells.
3. **TIP:** If cell detachment is not observed, quickly but gently aspirate PBS/EDTA. Immediately squirt desired volume of medium onto the cells using a somewhat forceful, circular motion out from center to well wall. Proceed directly to **Step 4**.
4. Once cells begin detaching, wash cells from the plate by gently adding 2-4mL of culture (or suitable base) medium to each well in a circular motion from well wall to center.
5. For PBS/ETDA method, transfer cells from multiple wells to a 15mL tube. Place in a cooled Biocision CoolRack® (15mL), or on ice, for 5-15 minutes to allow cells to clump and proceed to **Step 6**, skipping centrifugation.
6. Centrifuge cells for 2-5 minutes at 100 x g at 4°C.
7. Aspirate supernatant and gently add appropriate amount of medium for given volume (e.g. 0.5mL of medium for one well).
8. Add an equal volume of Defined Humanized Freeze Medium (Protocol provided in **Appendix II**) and mix gently by slow inversion.
9. Transfer cells to cryovials (1mL/cryovial) and store overnight at -80°C in a Biocision CoolCell® or suitable alternative (e.g. Mr. Frosty™ Freezing Container).
10. Transfer to LN_2 storage the following day.

Appendix I – PeproGrow™ hESC Media & Companion Products

PeproGrow™ hESC Media & Companion Products

PeproGrow™ hESC Media Products:

Kit/Components	Catalog Number	Size
PeproGrow™ hESC Medium Kit	**HESC-500**	**500mL**
PeproGrow™ hESC Basal Medium	BM-HESC-500	500mL
PeproGrow™ hESC Growth Factor Component	GF-HESC-500	Vial for 500mL Basal Medium
PeproGrow™ hESC Medium Kit	**HESC-100**	**100mL**
PeproGrow™ hESC Basal Medium	BM-HESC-100	100mL
PeproGrow™ hESC Growth Factor Component	GF-HESC-100	Vial for 100mL Basal Medium
PeproGrow™ hESC Plus Medium Kit	**HESCP-500**	**500mL**
PeproGrow™ hESC Plus Basal Medium	BM-HESCP-500	500mL
PeproGrow™ hESC Plus Growth Factor Component	GF-HESCP-500	Vial for 500mL Basal Medium
PeproGrow™ hESC Plus Medium Kit	**HESCP-100**	**100mL**
PeproGrow™ hESC Plus Basal Medium	BM-HESCP-100	100mL
PeproGrow™ hESC Plus Growth Factor Component	GF-HESCP-100	Vial for 100mL Basal Medium

Companion Products

Companion Product/Components	Catalog Number	Size
Animal-Free Human Vitronectin Matrix and Buffer Kit	**AF-VMB-220**	**Kit**
Animal-Free Human Vitronectin Matrix		500µg
PBS + Kolliphor P 188		220mL
Cell Passaging/Non-Enzymatic Detachment Buffer (Contains PBS+HEPES+EDTA)	**CPD-125**	**Kit/125mL**

PeproGrow™ hESC Media Products Chart

Catalog Number	PeproGrow™ hESC Medium Kit HESC-100 / HESC-500	PeproGrow™ hESC Plus Medium Kit HESCP-100 / HESCP-500
Complete	✓	✓
Chemically-Defined	✓	✓
Phenol Red-Free	✓	✓
Serum-Free	✓	✓
Insulin-Free	✓	
Animal-Free		
Xeno-Free		
Protein-Free		
Additional Notes	Includes Basal Medium & Growth Factor Component. Additional companion products available.	Includes Basal Medium & Growth Factor Component. Additional companion products available.

Appendix II – Defined Humanized Freeze Medium

2x Defined Humanized Freeze Medium (10% HSA) Preparation

1. Prepare 20mL DMEM/F12 solution containing 25% HSA.
2. Dissolve 5g of trehalose into 12-15mL DMEM/F12.
3. Fill volume to 40mL using DMEM/F12.
4. Filter using a 0.2-micron SFCA filter unit.
5. Add 10mL sterile DMSO.
6. Aliquot into 15mL conical tubes and freeze at -20°C until use.

Appendix III – Additional Reagents & Materials

Alternative Extracellular Matrix (ECM) Products

- BG-iMatrix-511 (BioGems, RLS11)
- BG-iMatrix-511 Silk (BioGems, RL511S)
- VitroGel 3D (BioGems, V3D)
- VitroGel 3D-RGD (BioGems, V3DR)
- Corning Matrigel®, Synthemax® II-SC

Reagents & Materials for Cell Culture

- PeproGrow™ hESC Media Product
- DMEM/F12, 340mOsm (or equivalent)
- Phosphate Buffered Saline (PBS), 340mOsm
- 0.5mM PBS/EDTA (or PBS/EGTA), pH 7.4, 340mOsm
- Sterile Cell Culture Grade Water (ddH2O)
- DMSO (Sigma, D2650) - Sterilize with syringe-mounted nylon filter and store in polypropylene tube (Do not use polycarbonate tube).
- Human Serum Albumin (HSA) (Sigma, A5843; powder)
- Trehalose (Sigma, T0167)
- HEPES (Corning, 25-060-CI)
- Corning Matrigel® (Corning, 354277; hESC qualified or equivalent)
- Cell Culture Plastics (6-well dishes available from Corning, BD Biosciences, and Sarstedt)

Reagents and Materials for Immunostaining

- Oct4 (1:1000-2000, monoclonal rabbit antibody, Life Technologies, A13998)
- Tra-1-60 (1:200-300, mouse IgM, Life Technologies, 411000)
- Nanog (1: 250; mouse IgG1, Sigma, N3038, clone NNG-811)
- Lin28 (1:500; monoclonal rabbit antibody, Abcam, ab124765)
- DAPI (5 mg/mL stock diluted 1:10000 to 500ng/mL in PBS; Sigma, D9542)
- Alexa Fluor® 568 goat anti-mouse IgM (1:500-1000, Life Technologies, A21043)
- Alexa Fluor® 488 goat anti-mouse IgG1 (1:500, Life Technologies, A21121)

- Goat-anti-rabbit-AlexaFluor 647 (1:500-1000, Life Technologies)
- ProLong® Gold mounting medium (Life Technologies)
- Glass coverslips (12mm)
- Fine forceps
- PBS
- ddH2O
- Normal goat serum (EMD Millipore, S26-100ML)
- Triton™ X-100 (diluted to 10% in PBS)
- Bovine Serum Albumin (BSA) (low endotoxin, Fatty Acid, IgG Free)
- Blocking solution (10% goat serum, 1% BSA, 0.1% Triton X-100, 20mM HEPES in PBS)
- Antibody Solution (0.5x Blocking Solution in PBS)

Reagents and Materials for Flow Cytometry

NOTE: For hiPSCs reprogrammed using Oct4, use Nanog antibody in place of Oct4 antibody.

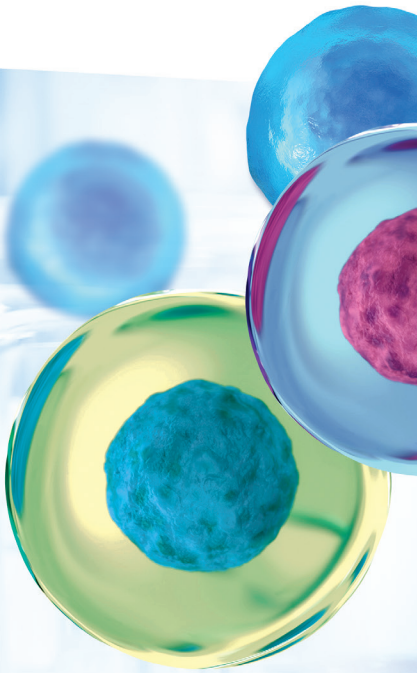
NOTE: Inclusion of a viability dye is optional.

NOTE: Accutase®, Accumx™, or TrypLE™ is needed for efficient dissociation to single-cell hiPSCs/hESCs suspensions.

NOTE: To ensure removal of any large clumps not dissociated via enzymatic activity, filter with a 50mL tube fit with a 40-micron mesh basket.

NOTE: Use of freshly-prepared paraformaldehyde-based fixative, Transcription Factor Fixation/Permeabilization Concentrate (BioGems, 92555-00) and Permeabilization Buffer (10X) (BioGems, 92110-00) is crucial when using cell surface antibodies combined with transcription factor antibodies, such as Oct4 or Nanog. Alternatives, such as Cytofix™ (BD Biosciences, 554655) and BD Perm/Wash™ Buffer (BD Biosciences, 554723), can also be used.

- Human and Mouse Pluripotent Stem Cell Analysis Kit (BD Biosciences)
- Nanog-PerCP-Cy™5.5 (BD Biosciences, 562259)



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