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Protocol for assembly of a serine integrase-based platform for functional validation of genetic switch controllers in eukaryotic cells-Human

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Spotlight series

**Marco Oliveira**

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Protocol status: Working

We use this protocol and it's working

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Keywords: genetic switch controllers in eukaryotic cell, serine integrase, genetic switch controller, platform for functional validation, eukaryotic cell, protocol for assembly, serine, integrase, functional validation

Abstract

This protocol describes the assembly of a serine integrase-based platform for functional validation of genetic switch controllers in eukaryotic cells in human.

Attachments



pbt9ca8tp.docx

83KB



Materials

Biological materials

Obtaining nonactivated human T lymphocytes from PBMCs.

This protocol describes the isolation of human peripheral blood mononuclear cells (PBMCs) from leukocyte filters. Usually, 5×10^7 to 3×10^8 cells are obtained from a leukocyte filter from a healthy blood donor donation.

! CAUTION Universal precautions must be taken, experiments must be carried out in (at least) category 2 biological safety cabinets, and appropriate personal protection equipment should be used.

! CAUTION Informed consent must be obtained for the use of human blood samples.

! CAUTION Experiments with human materials must conform to all relevant institutional and governmental ethics regulations, and appropriate informed consent must be obtained for the use of human blood or patient-derived materials.

hES/NSC cell lines

Human embryonic stem (hES) cells called BR1 and human induced pluripotent stem (hiPS) cell-derived neural stem cell (NSC) lines (the cell lines were supplied by Dr. Stevens Rehen of D'Or Institute for Research and Education (iDOR), Rio de Janeiro, Brazil)

▲CRITICAL Use the BR1 cell line up to 40 passages of cells and NSCs line using up to 20 passages cells.

Reagents

Medium and supplements for PBMC

-  RPMI 1640 Medium **Thermo Fisher Catalog #11875101**
-  FBS **Gibco - Thermo Fisher Scientific Catalog #12657-029**
-  Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #15140122**
-  L-Glutamine (200 mM) **Gibco - Thermo Fisher Scientific Catalog #25030081**
-  Recombinant Human IL-2 GMP Protein, CF **R&D Systems Catalog #202-GMP-01M**

PBMC separation reagents

-  PBS (Phosphate-Buffered Saline) Tablets **Thermo Fisher Catalog #003002**
- Ficoll-Paque PLUS density gradient media (GE Healthcare, cat. no. GE17-1440-02)



-  Trypan Blue solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #72-57-1**
- Alcohol 70% for material sterilization
-  eBioscience® 7-AAD Viability Staining Solution **Thermo Fisher Catalog #00-6993-50**

Medium and supplements for HEK293T cell line

-  DMEM, powder, high glucose **Gibco - Thermo Fisher Scientific Catalog #12100046**
-  FBS **Gibco - Thermo Fisher Scientific Catalog #12657-029**
-  Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #15140122**
-  Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**
-  HEPES 1M **Thermo Fisher Scientific Catalog #15630080**
-  MEM Amino Acids Solution (50X) **Thermo Fisher Catalog #11130051**
-  MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**
-  MEM Vitamin Solution (100X) **Thermo Fisher Catalog #11120052**
-  2-mercaptoethanol **Gibco - Thermo Fisher Scientific Catalog #21985023**

Growth medium and supplements for hECs

-  mTeSR™1 500 mL Kit **STEMCELL Technologies Inc. Catalog #85850**
- Advanced™ DMEMF-12 (Thermo Fisher Scientific, cat. no. 12634)
- Neurobasal® Medium and Neural Induction Supplement (NIS) (Gibco, cat. no. 12634)
-  DMEM/F-12, GlutaMAX® supplement **Thermo Fisher Catalog #10565018**

Enzymes, growth factors and chemicals for hECs

-  ACCUTASE™ **STEMCELL Technologies Inc. Catalog #07920**
-  ROCK Inhibitor (Y-27632) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #SCM075**

Other reagents and chemicals

- HEK293T transfection reagents using HBS and CaCl₂
-  HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H3375**
-  Calcium chloride dihydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C5080**
- Na₂HPO₄ - Sodium Phosphate dibasic heptahydrate (Sigma-Aldrich, cat. no. 30413)
-  Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3014**
- 1S electroporation buffer:



-  Potassium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #104936**
-  Magnesium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #105833**
-  di-Sodium hydrogen phosphate dodecahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #106579**
- 120mM NaH₂PO₄ (VETEC, cat. no. 001236)
- 50mM Sodium Succinate (Merck, cat. no. s6638601)
- For 1SM electroporation buffer add
- 25mM Mannitol (VETEC, cat. no. c000197)

▲**CRITICAL** Before preparing electroporation buffers, mix Na₂HPO₄/NaH₂PO₄ (phosphate buffer) and adjust the pH to 7.2 using 1 M NaOH or 1 M HCl. Use the electroporation buffer aliquots only once. Electroporation buffer aliquots (1 mL) can be stored at -20 °C for up to 3 months.

Vectors for transfection: The pT2/CAGGS-GFP plasmid was kindly provided by Dr. Sang Wang Han (UNIFESP, Brazil), and the pT3-Neo-EF1a-GFP plasmid was ordered from Addgene (Addgene, no. 69134)

 Geltrex LDEV Free hESC Quality 5 ml **Thermo Fisher Scientific Catalog #A1413302**

Equipment

- Centrifuge for 50 mL tubes and microtubes (Thermo Centrifuge C3Ri, cat. no. 1115774)
- Multifunction Centrifuge (Thermo Centrifuge, Jouan B4i, cat. no.11175671)
- Biological Safety Cabinet Class 2
- Nucleofector IIb (Lonza, cat. no. AAB-1001)
- Tissue culture incubator at 5% CO₂, 21% O₂ at 37°C.
- FACSCalibur® (BD Bioscience)
- Evos XL Cell Imaging System (Thermo Fisher Scientific).

Equipment	
Corning® 75cm ² U-Shaped Canted Neck Cell Culture Flask with Plug Seal Cap	NAME
Cell Culture Flask	TYPE
Corning	BRAND
430720U	SKU
https://ecatalog.corning.com/life-sciences/b2b/UK/en/Surfaces/Advanced-Cell-Culture-Surfaces/Corning%C2%AE-and-Costar%C2%AE-Cell-Culture-Flasks/p/430720U	LINK

Equipment	
Costar® 12-well Clear TC-treated Multiple Well Plates, Bulk Pack, Sterile	NAME
Cell culture plate	TYPE
Costar	BRAND
3512	SKU
https://ecatalog.corning.com/life-sciences/b2b/NO/en/Microplates/Assay-Microplates/96-Well-Microplates/Costar%C2%AE-Multiple-Well-Cell-Culture-Plates/p/3512	LINK

Equipment	
PIPETTE, 5 ML, GRADUATED 1/10 ML, STERILE, BULK PACKAGING, 25 PCS./BAG	NAME
Sterile Syringe	TYPE
Greiner Bio-One	BRAND
606107	SKU
https://shop.gbo.com/en/netherlands/products/bioscience/liquid-handling/serological-pipettes/606107.html	LIN K

Equipment	
PIPETTE, 10 ML, GRADUATED 1/10 ML, STERILE	NAME
Sterile Syringe	TYPE
Greiner Bio-One	BRAND
607180	SKU
https://shop.gbo.com/en/netherlands/products/bioscience/liquid-handling/serological-pipettes/607180.html?sword_list%5B0%5D=607180%2C&no_cache=1	LI NK

Equipment	
PIPETTE, 25 ML, GRADUATED 2/10 ML, STERILE	NAME
Sterile Syringe	TYPE
Greiner Bio-One	BRAND
760180	SKU
https://shop.gbo.com/en/netherlands/products/bioscience/liquid-handling/serological-pipettes/760180.html?sword_list%5B0%5D=760180&no_cache=1	LI NK

Equipment	
TUBE, 50 ML, PP, 30/115 MM, CONICAL BOTTOM	NAME
Conical Sterile Polypropylene Centrifuge Tubes	TYPE
Greiner Bio-One	BRAND
210261	SKU
https://shop.gbo.com/en/netherlands/products/bioscience/tubes-beakers/15ml-cellstar-polypropylene-tube/210261.html?sword_list%5B0%5D=210261&no_cache=1	LI NK

- Sterile Polypropylene Microtubes, 1.5 mL (Greiner Bio-One, cat. no. 616275)
- Barrier Pipette tips, 10 µL, 20 µL, 200 µL, 1000 µL (KASVI cat. no. K8-10F-1, K8-20F-1K8-200F-1, K8-1000F-1)

Equipment	
PIPETMAN L P2L, 0.2-2 µL, Metal Ejector	NAME
Metal Ejector	TYPE
Gilson	BRAND
FA10001M	SKU
https://gb.gilson.com/GBSV/pipetman-l-p2l-0-2-2-micro-l-metal-ejector.html	LINK

Equipment	
PIPETMAN L P10L, 0.5-10 µL, Metal Ejector	NAME
Metal Ejector	TYPE
Gilson	BRAND
FA10002M	SKU
https://gb.gilson.com/GBSV/pipetman-l-p10l-1-10-micro-l-metal-ejector.html	LINK

Equipment	
PIPETMAN L P200L, 20-200 µL, Metal Ejector	NAME
Metal Ejector	TYPE
Gilson	BRAND
FA10005M	SKU
https://gb.gilson.com/GBSV/pipetman-l-p200l-20-200-micro-l-metal-ejector.html	LINK

Equipment	
PIPETMAN L P1000L, 100-1000 µL, Metal Ejector	NAME
Metal Ejector	TYPE
Gilson	BRAND
FA10006M	SKU
https://gb.gilson.com/GBSV/pipetman-l-p1000l-100-1000-micro-l-metal-ejector.html	LINK

PBMC electroporation

Equipment	
Ingenio 0.2 cm Cuvettes 50 pk	NAME
Electroporation Cuvette	TYPE
Mirus Bio	BRAND
MIR 50121	SKU
https://www.mirusbio.com/product/ingenio-electroporation-accessories-50-pk-0-2-cm-cuvettes-for-ezporator-and-lonza-nucleofector-ii-2b/	LIN K

For hES/NSC cells

Equipment	
Costar® 24-well Clear TC-treated Multiple Well Plates, Bulk Pack, Sterile	NAME
Tissue-culture plates	TYPE
Costar	BRAND
CLS3527	SKU
https://ecatalog.corning.com/life-sciences/b2b/NL/en/Microplates/Assay-Microplates/96-Well-Microplates/Costar%C2%AE-Multiple-Well-Cell-Culture-Plates/p/3527	LI NK

Equipment	
Corning® 60 mm TC-treated Culture Dish	NAME
Tissue-culture treated culture dishes	TYPE
Corning	BRAND
CLS430166	SKU
https://ecatalog.corning.com/life-sciences/b2b/NL/en/Surfaces/Advanced-Cell-Culture-Surfaces/Corning%C2%AE-Treated-Culture-Dishes/p/430166	LI NK

Equipment	
Corning® 100 mm TC-treated Culture Dish	NAME
Tissue-culture treated culture dishes	TYPE
Corning	BRAND
CLS430167	SKU
https://ecatalog.corning.com/life-sciences/b2b/NL/en/Surfaces/Advanced-Cell-Culture-Surfaces/Corning%C2%AE-Treated-Culture-Dishes/p/430167	LI NK

Equipment

Corning® Small Cell Scraper

NAME

Cell Scraper

TYPE

Corning

BRAND

CLS3010

SKU

<https://ecatalog.corning.com/life-sciences/b2b/NL/en/Cell-Culture/Cell-Culture-Accessories/Cell-Scrapers/Corning%C2%AE-Cell-Scrapers-and-Lifters/p/3010>

LI
NK

Software

TreestarFlowJo Version 10 (<http://www.flowjo.com/>).

Reagent Setup

hEC/NSC cell lines

Thaw Geltrex™ Matrix solution undiluted vial at 4 °C overnight on ice. Transfer aliquots of adequate volume into Eppendorf tubes. The aliquots can be stored at –20 °C until the expiration date. Thaw aliquots on ice and dilute with 1% DMEM/F-12 and GlutaMAX™ Supplement medium.

Neural expansion medium (NEM): 50% v/v Advanced™ DMEM/F-12, 50% v/v Neurobasal® Medium, 2% Neural Induction Supplement (NIS). NEM medium can be stored at 4 °C for up to 1 month.

Prepare Y-27632 stock solution in aliquots of 10 mM in PBS. Stored at –20 °C for 1 year.

Buffer FACS for hECs/NSCs: DPBS without CaCl₂ and MgCl₂, 1% fetal bovine serum, fresh.

PBST (0.2% Tween 20-PBS)

Add 0.2% (vol/vol) Tween 20 to PBS. Store the buffer at room temperature for 1 year.



- ▲CRITICAL For a high transfection efficiency, at the electroporation step, the final volume of buffer + plasmid should be 100 μ L. Do not mix less than 85 μ L of buffer because it drops transfection efficiency.
- ▲CRITICAL Ficoll-Paque and PBS for PBMC separation must be at room temperature before use.
- ▲CRITICAL Antibiotic addition immediately after electroporation will cause intense cell death.



PBMC isolation ● Timing 1.5 h

25m

- 1 After performing the sterilization procedure in the safety cabinet, couple the syringe containing  20 mL PBS to the leukocyte filter, cut the filter tube extremity, and put it into a 50 mL tube. 

Note

! CAUTION Blood can spill over from the tube, always maintain hypochlorite solution to clean up the drops.

- 2 Press the syringe to wash the leukocyte filter. Repeat this step if more cells are needed.

- 3 With a 25 mL serological pipette, add ~  35 mL of blood at the top of the 10 mL tube containing Ficoll-Paque at  Room temperature .  

Note

▲ CRITICAL STEP Add the blood on top of the Hystopaque very carefully. Do not take more than 20 minutes with blood on top of Ficoll; otherwise, the red blood cells will start to fall into the gradient. If the blood shakes and mixes with Ficoll-Paque, separation will not occur.

▲ CRITICAL Do not shake or disturb the gradient between the Ficoll and blood.

- 4 Centrifuge at  800 x g, Room temperature, 00:20:00 , in a swinging-bucket rotor, without breaking. 

Note

▲ CRITICAL STEP Centrifuge must be at low acceleration and without a break setting; otherwise, the separation gradient will be lost.  

- 5 With a 10 mL serological pipette, very carefully remove the PBMC “white ring” on top of the Ficoll-Paque and put it into a fresh tube.

**Note**

! CAUTION Avoid removing and placing the pipette several times as it will disturb the gradient and could contaminate the leukocytes with other fractions.
▲ CRITICAL STEP Remove only the white ring containing the leukocytes without disturbing the red blood cell at the bottom. Additionally, avoid the solution above the white ring because it contains platelets and soluble factors that can change culture conditions. If the person is not experienced, add more than 10 mL of Ficoll-Paque to increase the distance between the leukocytes and the red blood cells.

- 6 To wash the cells, complete the volume to  50 mL with PBS and centrifuge at  400 x g, 10°C, 00:05:00 . Remove the supernatant.

5m



- 7 Repeat the wash step twice.



- 8 Resuspend the cells in  5 mL -  10 mL PBS and maintain them  On ice .

- 9 Count the cells with a Neubauer chamber by adding Trypan blue solution at 4% for cell viability staining.

**Note**

PAUSE POINT The cells can be placed on ice for up to 2-3 hours.

PBMC electroporation ● Timing 1 h

5m

- 10 Add 10×10^6 cells per electroporation reaction separating in individual microtubes.



- 11 Centrifuge the tubes at  400 x g, 10°C, 00:05:00 .

5m



- 12 Remove all supernatant with a pipette and resuspend the cells in a total of  100 μ L of 1SM electroporation buffer + plasmids (integrase + target) per reaction.





- 13 Immediately insert the cells in the cuvette and electroporate using program U-014 on Nucleofector 2b. 

Note

▲ CRITICAL Electroporate and remove the cells from the cuvette quickly to avoid cell death. Wait 2 minutes for new cell electroporation.

- 14 Very carefully, resuspend the cells in warm RPMI/FBS 20% (no antibiotics) and plate them in total  500 μ L in a 12-well plate. After 16 hours, add  500 μ L RPMI/FBS 10% + P/S antibiotics + 50 U/mL IL2 + L-Glutamine.  

Note

▲ CRITICAL STEP If T-cell activation is needed, it should be done at this moment.

Integrase activity evaluation in PBMCs at 2 h

25m

15

Note

Integrase activity was evaluated by flow cytometry 24 h after transfection. For optimization, several time points should be evaluated. In our hands, 72 h after electroporation, eGFP expression achieved its maximum (1-35% of positive cells).

Collect the cells and place them in a microtube.

- 16 Centrifuge at  400 x g, 00:05:00 . Remove the supernatant. 

- 17 Wash the cells with  1 mL of cold PBS. If DNA will be extracted, separate an aliquot at this time. 

- 18 Repeat the centrifugation 2X to wash the cells. 

**Note**

If antibody staining will be performed, it should take place at this moment.

- 19 Resuspend the cells in  300 μL of cold PBS and place them in the flow cytometer tub. 

Note

▲ CRITICAL Fixation buffers based on formaldehyde should not be used because formaldehyde decreases GFP fluorescence.
! CAUTION Fixation buffers should not be used in case of cell viability staining with 7-AAD or PI (Propidium Iodide).

- 20 Add  5 μL of 7-AAD and incubate at  4 °C for  00:20:00 . 

Note

▲ CRITICAL Keep the cells in the dark after staining. Do not wash cells after the addition of the 7-AAD staining solution.   

- 21 Acquire at least 10,000 cells at the viable gate to evaluate eGFP expression. For a higher sensitivity, acquire more cells.

Note

▲ CRITICAL STEP Acquire a nonstained GFP-negative tube as a negative control for the gating strategy. Acquire a 7-AAD -stained GFP negative tube as a negative control for GFP expression for the gating strategy. Acquire a 7-AAD -negative and GFP-positive tube (with the GFP control plasmid as pT2-GFP) for GFP-positive staining and gating strategies.

HEK293T transfection with Calcium Phosphate ● Timing 1 h

- 22 Plate 4×10^6 HEK293T cells per well of a 75 cm^2 plate at least 16 h before transfection in DMEM complete medium. 

**Note**

▲ CRITICAL Cells should be less than 80% confluent, or transfection efficiency will decrease.

- 23 Replace the medium before transfection, adding only  10 mL of DMEM complete medium.



- 24 Mix the plasmids (integrase + targets,  5 µg each) to  500 µL of 2X CaCl₂.

**Note**

! CAUTION CaCl₂ must be freshly prepared or maintained frozen in single-use aliquots.

- 25 Next, agitate CaCl₂ using a vortex at full speed and add  500 µL of the HBS solution drop-by-drop very slowly. Make bubbles on the solution with a Pasteur pipette. Let it rest for an instant.

**Note**

▲ CRITICAL STEP HBS solution should be at pH 7.1 at the transfection of HEK293T cells. Any small change will have a great impact on transfection efficiency.

- 26 Very carefully add drop-by-drop at the cells covering all the flask with circular movements avoiding two drops at the same spot. Mix the solution with ∞ movement and place the cells in the incubator.

**Integrase activity evaluation in HEK293T cells.● Timing 2h**

- 27 Integrase activity was evaluated by flow cytometry 24 h after transfection. For optimization, several time points should be evaluated. In our hands, 72 h after electroporation, eGFP expression achieved its maximum (1-35% of positive cells).

Cell expansion of the hES cell line-Passage cells

39m

- 28 Culture hES cells in a 100-mm culture dish until the cells cover the dish area at 60-70% confluence. This occurs at approximately 5-6 days.



**Note**

▲ **CRITICAL** Start the enzymatic dissociation with high-quality hES colonies. The spontaneous differentiation of the colonies should be removed before the split.
! **CAUTION** For 60-70% confluence, consider a split ratio of 1:5.

29 Prior to starting the dissociation, coat the fresh 100-mm culture dishes with 6 mL/dish of cold Geltrex™ and place it into the incubator at  37 °C for at least  00:30:00 .

30m

30 Remove Geltrex™, and immediately add 9 mL/dish of fresh growth mTeSR™ 1 medium to prevent drying.



31 Place the fresh culture dishes into the incubator at  37 °C .

32 Carefully aspirate the old growth medium from the cell culture dish.

33 Wash the dish with  4 mL of DPBS without calcium and magnesium (DPBS-/-) and remove immediately.



34 Add  4 mL of Accutase™ into the 100-mm culture dish, ensure that cover of dish area.



35 Incubate the cell suspension at  37 °C for  00:05:00 .

5m

Note

! **CAUTION** Monitoring the cell viability and morphology changes, it should be observed cells round up into the colonies while remaining attached to the surface of the dish. If necessary, repeat step 35.
! **CAUTION** Dissociate hES cells into small clusters rather than single-cell suspensions at each passage.



36 Add  3 mL of DMEM/F12 and gently triturate the clusters across the dish surface.





- 37 Scrape the attached cells using a cell scraper.
- 38 Transfer the unattached cell suspension into a 15 mL conical tube containing  3 mL of DMEM/F12 using a 5 mL serological pipette. 
- 39 Spin down at  100 x g, 25°C, 00:04:00 . 

- 40 Aspirate and discard the supernatant.
- 41 Resuspend the cell pellet with  5 mL of fresh growth mTeSR™ 1 Medium using a 5 mL serological pipette.
- 42 Add the cell suspension 1 mL/dish into fresh 100-mm culture dishes prepared previously at step 28-31. 
- 43 Incubate the cells at  37 °C , 95% O₂, 5% CO₂ in humidified air. 
- 44 After 24 h, replace the old growth medium with fresh mTeSR™ 1 medium.
- 45 Split the cells every 5-6 days.

Maintenance of hES cell culture ● Timing 5 min daily

- 46 Carefully aspirate the old growth medium from the cell culture dish.
- 47 Replace with  10 mL of fresh growth mTeSR™ 1 Medium.
- 48 Incubate the cells at  37 °C , 95% O₂, and 5% CO₂ in humidified air until 60-70% confluent. 



- 49 Replace the medium daily.



Note

! CAUTION After 10 passages to ensure the pluripotency status and genetic integrity of hES cells

Cells preparation for electroporation ● Timing 6 d

- 50 Replace the old growth mTeSR™1 Medium for 6 days.

Note

! CAUTION After 80% confluence, the number of cells/dish should be increased to approximately 8-10 x 10⁶, sufficient for 8-10 electroporation reactions.

hES cell electroporation ● Timing 1 h

- 51 (Optional) Prior to starting dissociation, add iROCK ([IM] 10 micromolar (μM)) in the 100-mm cell culture dish for  01:00:00 .

1h



- 52 Coat the fresh 12-well plate with  6 mL of cold Geltrex™ (0.5 μL/well) as described in steps 29-31.

- 53 Aspirate Geltrex™ and add 0.5 μL/well of antibiotic-free fresh mTeSR™1 Medium with iROCK ([IM] 10 micromolar (μM)).



- 54 Carefully aspirate the old growth medium from the cell 100-mm culture dishes.

- 55 Wash with 4 mL/dish of DPBS-/- and remove immediately.



- 56 Add  4 mL /dish of Accutase™, ensure that cover of dish area.





57 Incubate the cell suspension at  37 °C for 5-10 minutes.



Note

! CAUTION The spontaneous differentiation of the colonies should be removed before the split.

! CAUTION During the incubation with Accutase™ monitoring the morphology of colonies, it should be observed separated round cells by microscopy. Dissociate hES cells into single-cell suspensions and avoid clusters. If necessary, repeat step 30.

▲ CRITICAL STEP High transfection efficiency is dependent on high-quality hES colonies as well as efficient single-cell enzymatic dissociation.

58 Add  3 mL /dish of DMEM/F12 and scrape the attached cells using a cell scraper.



59 Gently triturate the clusters across dish surface.

60 Transfer the unattached cell suspension using a 5 mL serological pipette into a 50 mL conical tube containing  10 mL of DMEM/F12.



61 Spin down at  100 x g, 25°C, 00:04:00 .

4m



62 Remove the supernatant and wash with  10 mL of DPBS-/-.

63 Count the cells with Trypan blue dye exclusion.

Note

▲ CRITICAL STEP An ineffective transfection is observed using lower cell density or low cell viability.

64 Spin down at  100 x g, 25°C, 00:04:00 .

4m



65 Aspirate and discard the supernatant.

**Note**

▲ **CRITICAL STEP** Calculate the total volume needed to resuspend the hES cells. A single electroporation reaction requires 1×10^6 viable hES cells. It should be transferred 2 mL per electroporation reaction (i.e., For 3 electroporation reactions, add a total volume of 6 mL into a tube.)

66 Resuspend the cells with ice-cold DPBS-/-.

67 Gently mix the suspension cells and transfer 2 mL/15 mL conical tube (1×10^6 cells per tube).

68 Spin down at  100 x g, 4°C, 00:04:00 .



4m



69 Remove the supernatant and resuspend the pellet with 1SM electroporation buffer mix with the plasmids in a total volume of  100 μ L .

**Note**

▲ **CRITICAL STEP** For transient expression, hES cells can be electroporated with 12 μ g of pIE plasmids and 8 μ g of pSG.

70 Immediately transfer the suspension cells into the 0.2 cm electroporation cuvette, preventing bubbles.

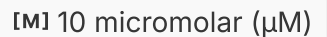
71 Electroporate the cells by using the Nucleofector 2B program A-23.

**Note**

▲ **CRITICAL STEP** Electroporation frequently leaves considerable cell death.



72 Immediately add  1 mL of fresh mTeSR™1 Medium with iROCK ( 10 micromolar (μM)) into the electroporation cuvette using a micropipette with a 1000 μL tip to avoid low cell viability. 

73 Immediately transfer the cell suspension into a 15 mL conical tube containing 0.5 mL of antibiotic-free fresh mTeSR™1 Medium with iROCK ( 10 micromolar (μM)).  

Note

▲ CRITICAL STEP Repeat steps 69-72 for each single electroporation reaction. It is important to transfect one reaction at a time. A long period of cell incubation with the electroporation buffer can affect cell viability.

74 Gently mix the cell suspension and transfer into three wells (0.5 mL/well) of the coated Geltrex™ 12-well plate previously prepared in steps 51-52. 

75 Incubate the cells at  37 °C , 95% O₂, 5% CO₂ in humidified air. 

76 After 24 h, replace the old growth medium with antibiotic-free fresh mTeSR™1 medium.

Postelectroporation ● Timing 2 d

77 After 48 h of transfection, evaluate the integrase activity by flow cytometry.

Preparation of sample and flow cytometry ● Timing 2 h

78 Harvest cells using Accutase™ as described in step 24.

79 Remove the old growth medium and wash with  0.5 mL /well of DPBS-/-, remove immediately.

80 Add  0.4 mL /well of Accutase™. 



81 Incubate the cells in the incubator at 37 °C for 5-10 minutes.



Note

! CAUTION Monitoring the morphology of colonies, it should be observed individual round cells by microscopy. Dissociate hES cells into single-cell suspensions, avoiding clusters. If necessary, repeat step 81.

82 Add 0.5 mL /well of DMEM/F12 into a 15 mL conical tube containing 5 mL of DMEM/F12.



83 Spin down at 100 x g, 25°C, 00:04:00 .

4m



84 Aspirate and discard the supernatant.

85 Wash the cells with 2 mL of ice-cold DPBS-/-.



86 Spin at 100 x g, 4°C, 00:04:00 .

4m



87 Remove the supernatant and resuspend the cells in 2 mL of ice-cold DPBS-/-.

88 Count the cells with Trypan blue dye exclusion.

89 Transfer 1×10^5 cells/tube for flow cytometry analysis.

90 Keep the cell suspension in the original 15 mL conical tube and collect the pellet for DNA extraction.



91 Spin at  100 x g, 4°C, 00:04:00 .

4m



92 Resuspend the cells (1×10^5 cells/tube) in  300 μL of ice-cold buffer for FACS.

93 Add  5 μL of 7-AAD into a tube with the cells and incubate at
 Room temperature for  00:10:00 .

10m



Note

- ▲ CRITICAL STEP After incubation, store the tubes at 4 °C protected from light prior to analysis.
- ▲ CRITICAL STEP Acquire at least 10,000 events at the viable gate to evaluate eGFP expression.

Cell expansion of the NSC cell line ● Timing 14-20 d-Passage cells ● Timing 30 min

94 Culture NSC cells into a 60-mm culture dish until the cells cover the dish area, at 85-90% confluence. It is approximately 6-7 days.



Note

- ▲ CRITICAL STEP Start enzymatic dissociation with high-quality NSC cells.
- ▲ CRITICAL STEP For over confluence consider a split ratio of 1:8 to 1:10. NSCs can be seeded at a density of 0.5×10^5 cells/cm². For the 60-mm culture dish, consider an area of 20 cm².

95 Prior to starting the dissociation, coat the fresh 60-mm culture dishes with 3 mL/dish of cold Geltrex™ and place it into the incubator at  37 °C for at least  00:30:00 .

30m

96 Remove Geltrex™, and immediately add  2 mL /dish of fresh NEM medium to prevent drying.



97 Place fresh culture dishes into the incubator at  37 °C .



98 Carefully aspirate the old medium from the cell culture dish.

99 Wash the dish with  2 mL of DPBS-/-, remove immediately.



100 Add  2 mL of Accutase™ into the 60-mm culture dish, ensure that cover of dish area.



101 Incubate the cell suspension at  37 °C for 3-5 minutes.



Note

▲ CRITICAL STEP Monitoring the cell viability and morphology changes, it should be observed that cells round up while remaining attached to the surface of the dish. If necessary, repeat step 101.

▲ CRITICAL STEP Dissociation of NSC cells into single-cell suspensions is required at each passage.

102 Add  3 mL of DMEM/F12 and gently triturate the clusters across the dish surface.



103 Transfer the unattached cell suspension into a 15 mL conical tube containing  3 mL of DMEM/F12 using a 5 mL serological pipette.



104 Spin down at  300 x g, 25°C, 00:04:00 .

4m



105 Aspirate and discard the supernatant.

106 Wash with  5 mL of DMEM/F12 using a 5 mL serological pipette.



107 Count the cells with Trypan blue dye exclusion.



108 Spin down at  300 x g, 25°C, 00:04:00 .

4m



109 Resuspend the cell pellet with fresh growth NEM medium using a 5 mL serological pipette.



Note

! CAUTION Calculate the total volume needed to resuspend the NSC cells. Consider seed 1×10^6 cells/dish in 1 mL.

110 Add the cell suspension at 1 mL/dish into fresh 60-mm culture dishes previously prepared at steps 94-97. Should have a total volume of 3 mL/dish.



111 Incubate the cells at  37 °C , 95% O₂, 5% CO₂ in humidified air.



112 After 24 h, replace the old growth medium with  3 mL of fresh NEM medium.

113 Split the cells every 6 days.

Maintenance of NSC cell culture for 5 min daily

114 Carefully aspirate the old growth medium from the cell culture dish.

115 Replace with  4 mL of fresh growth NEM medium.

116 Incubate the cells at  37 °C , 95% O₂, and 5% CO₂ in humidified air until they reach 80-90% confluent.



117 Change the growth medium every 2 days.



**Note**

▲ **CRITICAL STEP** After 10 passages to ensure the pluripotency status and genetic integrity of NSC cells

Cells preparation for electroporation ● Timing 6 d

- 118 Replace the old growth NEM media for 6 days.

**Note**

! **CAUTION** After 90-95% confluence, the number of cells/dish should be increased to approximately $8-12 \times 10^6$, sufficient for 8-12 electroporation reactions.

NSC cell electroporation ● Timing 1 h

- 119 (Optional) Prior to starting dissociation, add iROCK ([IM] 10 micromolar (μM)) into 60-mm culture cells dish for 01:00:00 .

1h



- 120 Coat the fresh 12-well plate with 6 mL of cold Geltrex™ (0.5 μL /well) as described in steps 95-97.

- 121 Aspirate Geltrex™ and add 0.5 μL /well of antibiotic-free fresh NEM medium with iROCK ([IM] 10 micromolar (μM)).



- 122 Carefully aspirate the old growth medium from the cell 60-mm culture dishes.

- 123 Wash with 2 mL /dish of DPBS-/-, and remove immediately.



- 124 Add 2 mL /dish of Accutase™, ensure that cover of dish area.



- 125 Incubate the cell suspension at 37 °C for 3-5 minutes.



**Note**

! CAUTION Discard the cells if spontaneous differentiation is observed.

▲ CRITICAL STEP High transfection efficiency is dependent on high-quality NSCs as well as efficient single-cell enzymatic dissociation.

▲ CRITICAL STEP During incubation with Accutase™ monitor the morphology of colonies by microscopy. Cells must be rounded. Dissociate NSC cells into single-cell suspensions. If necessary, repeat step 125.

▲ CRITICAL STEP Ineffective transfection is observed in the presence of cluster cells.

126 Add  2 mL /dish of DMEM/F12 and gently triturate the clusters across the dish surface.



127 Remove unattached cells using a 5 mL serological pipette.

128 Transfer the cell suspension into a 50 mL conical tube containing  10 mL of DMEM/F12.

129 Spin down at  300 x g, 25°C, 00:04:00 .

4m



130 Remove the supernatant and wash with  5 mL of DPBS-/-.



131 Count the cells with Trypan blue dye exclusion.

132 Spin down at  300 x g, 25°C, 00:04:00 .

4m

Note

! CAUTION Calculate the total volume needed to resuspend the NSC cells. A single electroporation reaction requires 1x10⁶ viable NSCs. It should be transferred 2 mL per electroporation reaction (i.e., For 3 electroporation reactions, add a total volume of 6 mL into a tube.)

▲ CRITICAL STEP An ineffective transfection is observed using lower cell density or low cell viability.





133 Resuspend the cells with ice-cold DPBS-/-.

134 Gently mix the suspension cells and transfer 2 mL/15 mL conical tube. 

135 Spin down at  300 x g, 4°C, 00:04:00 .

4m



136 Remove the supernatant and resuspend the cells with 1S electroporation buffer mix with the plasmids in a total volume of  100 µL . 

Note

▲ CRITICAL STEP For transient expression, NSC cells can be electroporated with 12 µg of pIE plasmids and 8 µg of pSG.

137 Immediately transfer the suspension cells into the 0.2 cm electroporation cuvette, avoid bubbles. 

138 Electroporate the cells by using the Nucleofector 2D program A-33. 

Note

! CAUTION Cell death is frequently observed after electroporation.

139 Immediately add  1 mL of fresh NEM media with iROCK ( 10 micromolar (µM)) into the electroporation cuvette using a micropipette with a 1000 µL tip to avoid low cell viability. 

140 Immediately transfer the cell suspension into a 15 mL conical tube containing  0.5 mL of antibiotic-free fresh NEM media with iROCK ( 10 micromolar (µM)). 

**Note**

▲ **CRITICAL STEP** Repeat steps 136-138 for each single electroporation reaction. It is important to transfect one reaction for a specified time. A long period of cell incubation with the electroporation buffer can affect cell viability.

141 Gently mix the cell suspension and transfer into three wells (0.5 mL/well) of the coated Geltrex™ 12-well plate previously prepared in step 28.



142 Incubate the cells at 37 °C , 95% O₂, 5% CO₂ in humidified air.



143 After 24 h, replace the old growth medium with antibiotic-free fresh NEM medium.

Note**? TROUBLESHOOTING****Postelectroporation ● Timing 2 d**

144 After 48 h of transfection, the integrase activity can be evaluated by using flow cytometry.

Preparation of sample and flow cytometry ● Timing 2 h**22m**

145 Harvest cells using Accutase™.

146 Remove the old growth medium and wash with 0.5 mL/well of DPBS-/-, remove immediately.

147 Add 0.4 mL /well of Accutase™.



148 Incubate the cells in the incubator at 37 °C for 5-10 minutes.





Note

▲ **CRITICAL STEP** Monitor the morphology of colonies by microscopy. Rounded cells must be observed.

149 Add  0.5 mL /well of DMEM/F12 into a 15 mL conical tube containing  5 mL of DMEM/F12.



150 Spin down at  300 x g, 25°C, 00:04:00 .

4m



151 Wash the cells with ice-cold DPBS-/-. 

152 Spin at  300 x g, 4°C, 00:04:00 .

4m



153 Remove the supernatant and resuspend the cells in  2 mL of ice-cold DPBS-/-. 

154 Count the cells with Trypan blue dye exclusion.

155 Transfer 1×10^5 cells/tube by flow cytometry analyses.

156 Keep the cell suspension in the original 15 mL conical tube and collect the pellet for DNA extraction.

157 Spin at  300 x g, 4°C, 00:04:00 .

4m



158 Resuspend the cells (1×10^5 cells/tube) in  300 μ L of ice-cold buffer for FACS.



159 Add  5 μL of 7-AAD into a tube with the cells and incubate at  Room temperature for  00:10:00 .

10m



Note

- ▲ CRITICAL STEP After incubation, store the tubes at 4 °C protected from light prior to analysis.
- ▲ CRITICAL STEP Acquire at least 10,000 events at the viable gate to evaluate eGFP expression.