**Human induced Pluripotent Stem Cells Protocols**

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**Abbreviations**

|  |  |
| --- | --- |
| **C**B | cord blood |
| cDNA | complementary DNA |
| CDM | chemically defined media |
|  |  |
| **E**DTA | ethylene diaminetetraacetic acid |
| EPC | endothelial precursors cells |
|  |  |
| **G**MP | good manufacturing practice |
|  |  |
| **h**ESCs | human embryonic stem cells |
| hiPSCs | human induced pluripotent stem cells |
|  |  |
| **I**RR MEFs | irradiated mouse embryonic fibroblasts |
|  |  |
| **M**EFs | mouse embryonic fibroblasts |
| MNCs | mononuclear cells |
|  |  |
| **N**PC | no program control |
|  |  |
| **O**SKM | OCT4, SOX2, KLF4, c-MYC |
|  |  |
| **P**B | peripheral blood |
| PBMCs | peripheral blood mononuclear cells |
| PCR | polymerase chain reaction |
| p100 | 100mm cell culture Petri dish |
| P/S | Penicillin/streptomycin |
| PVA | Polyvinyl Alcohol |
|  |  |
| **R**eV | retrovirus |
| RBCs | red blood cells |
|  |  |
| **S**eV | Sendai virus |

# I. Introduction

**1. Induced Pluripotent Stem Cells (iPSCs) – a general overview**

In August 2006 Shinya Yamanaka and colleagues at Kyoto University, Japan have demonstrated that murine embryonic fibroblast can be reprogrammed to induced pluripotent stem (iPS) cells by over-expression of a combination of four transcription factors: OCT4, SOX2, KLF4, c-MYC (OSKM) using retroviral system [1]. In November 2007, a milestone was achieved by creating iPSCs from adult human cells; two independent research teams' studies were released - one in [*Science*](http://en.wikipedia.org/wiki/Science_(journal)) by [James Thomson](http://en.wikipedia.org/wiki/James_Thomson_(cell_biologist)) at [University of Wisconsin-Madison](http://en.wikipedia.org/wiki/University_of_Wisconsin%E2%80%93Madison) [3] and another in [*Cell*](http://en.wikipedia.org/wiki/Cell_(journal)) by Shinya Yamanaka and colleagues at Kyoto University, Japan [2]. With the same principle used earlier in mouse models, Yamanaka had successfully reprogrammed human fibroblasts into pluripotent stem cells using the same four genes: *OCT3/4, SOX2, KLF4,* and *C-MYC* with a [retroviral](http://en.wikipedia.org/wiki/Retrovirus) system. Thomson and colleagues used *OCT4,* *SOX2, NANOG*, and a different gene *LIN28* encoding RNA binding protein, using a [lentiviral](http://en.wikipedia.org/wiki/Lentivirus) system. Since this pioneering discovery several methods to deliver those factors have been applied including genome-integration and integration-free methods. Subsequently, combinations of different reprogramming factors have been investigated in order to increase the efficiency and reduce the expression of oncogenes like c-MYC or KLF4.

Several human postnatal somatic cell types have been successfully reprogrammed to induced pluripotent stem cells (iPSCs) including skin fibroblasts, blood mononuclear cells (MNCs) [6,7], endothelial precursors cells (EPCs) derived from peripheral blood [5], exfoliated renal epithelial cells present in urine [9]. Blood mononuclear cells offer several advantages compared with skin fibroblasts. They are easily isolated from umbilical cord blood (CB) or adult peripheral blood (PB), and can be used fresh or after freezing. A short culture allows for more efficient reprogramming, with iPSC colonies forming from blood MNCs in 14 days, compared with 28 days for age-matched fibroblastic cells. The advantages of briefly cultured blood MNCs may be due to favorable epigenetic profiles and gene expression patterns. Blood cells from adults, especially non-lymphoid cells that are replenished frequently from intermittently activated blood stem cells, are short-lived *in vivo* and may contain less somatic mutations than skin fibroblasts, which are more exposed to environmental mutagens over time.

This new technology offers great promise for novel approaches in regenerative medicine, including human cell-based assays for target validation, lead development screens, efficacy and toxicity testing, functional pharmacogenomics, and personalized medicine. On the other hand, generation, maintenance and differentiation of iPSCs according to Good Manufacturing Practice (GMP) will form the basis for the potential clinical application of cell replacement therapies. However, pitfalls of iPSCs technology, including the potential for genetic and epigenetic abnormalities, tumorigenicity, and immunogenicity of transplanted cells, need to be considered before applying iPSC-based cell therapy.

**2. Induced Pluripotent Stem Cells (iPSCs) properties**

Human induced pluripotent stem cells similarly to human embryonic stem cells (hESCs) are capable of self-renewal and differentiation into three germ layers: mesoderm, neuroectoderm, endoderm. However, due to their inherent capacity for differentiation, the maintenance of undifferentiated cultures of human iPSCs is not as simple as growing other types of mammalian cells.

While the development and optimization of iPSCs derivation techniques and culture conditions will be an ongoing concern of this research field, the following collection of protocols represent the current reprogramming techniques and culture conditions based on Standard Operating Procedures (SOPs) developed at Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge.

**3. Notes on hiPSCs Culture**

• iPSCs can be maintained on a layer of mitotically inactive mouse embryonic fibroblasts (MEFs), in media consisting of 20% KSR iPSCs medium: Advanced DMEM:F12, 20% KSR, 4ng/ml bFGF, 2mM glutamine, 0.1mM non-essential amino acids, 50units/ml penicillin and 50μg/ml streptomycin, 0.1mM ß-Mercaptoethanol

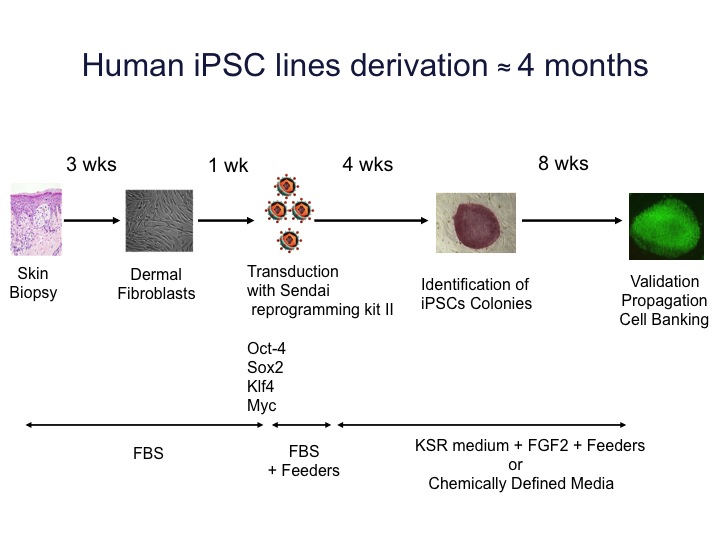
• iPSCs cultures are split via bulk passaging with Dispase and Collagenase, followed by dissociation into 100-200 cell clumps, which remains the most effective approach for maintenance of undifferentiated cultures and reduce cells death during passaging. Overtly differentiated colonies should still be removed prior to each passage, to maintain the general undifferentiated state of the culture. This requires competency in recognizing the morphology of differentiated cells/colonies and the capacity to remove them from the culture. Ideally, this is performed by mechanically excising the differentiated regions from the dish, using a dissecting microscope within a laminar flow cabinet.

• In case of a culture with a high level of spontaneous differentiation instead of bulk passaging it is recommended to break up undifferentiated colonies by microdissection and transfer pieces into a new dish.

• Cultures may be maintained on a high or low density of MEFs, which results in different colony morphologies. iPSCs grow as domed colonies on high density MEFs, but colonies are flat and individual cells exhibit prominent nucleoli on low density MEFs. Both these colony types express pluripotent markers and can effectively differentiate. We culture cells on low density MEFs.

• Cryopreservation and thawing of hiPSCs is not an efficient process, with poor survival being a major issue. It is not uncommon for only a few colonies to proliferate after a thaw.

**4. Human induced pluripotent stem cell lines derivation – an outline**

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# II. Feeder-dependent iPSCs protocols

**1. Isolation of fibroblast cells from 2 mm human skin biopsies**

This protocol allows efficient isolation of skin fibroblast cells from 2 mm round human skin biopsies, taken from upper arm. Fibroblasts are derived from mechanical dissected skin biopsies. Timing for isolation of fibroblast is about 4 weeks. In order to keep a small master cell bank expansion of isolated fibroblasts is also described in this protocol. Expansion should take around 3 weeks.

1.1. Day -1

1.1.1. Provide dermatologist/nurse with a 50ml falcon tube with 20ml of

MEF media, store in the fridge before use. Sterilise forceps and 6 cover slips per biopsy.

1.2. Day 0

1.2.1. MEF media should be removed from the fridge one hour prior to

arrival of skin punch and kept at room temperature. Skin punch samples should be transported at room temperature in the provided 50ml falcon tube.

1.2.2. Clean the work surface of the laminar flow cabinet with 1% Trigene

and 70% ethanol also wipe the 50ml tubes (containing the biopsies) and media bottles with 70% ethanol and place them in the hood.

1.2.3. Gently remove biopsy sample from tube with 1ml Gilson pipette and

place in the centre of a 60mm plate with a drop of MEF media

1.2.4. Using sterile forceps and scalpel remove the fatty layer and cut the

skin layer into 5-7 pieces. Mince the biopsies to increase surface area contact.

1.2.5. Add 5 to 7 drops of MEF media (well separated from each other)

onto another 60mm plate and place the minced biopsy piece on to each drop.

1.2.6. Carefully place a sterile cover slip onto the pieces to hold them in

place against the bottom of the plate. Add a few drops of MEF media to the surroundings of the cover-slips to prevent samples from drying out due to evaporation and incubate at 37°C, 5% CO2.

1.2.7. On day 2 post-isolation, check biopsy for signs of contamination.

Carefully add a drop of MEF media onto each cover slip (or by the side) to minimize moisture loss. It is very important not to dislodge the cover slips in the first week.

1.2.8. On day 5 aspirate MEF media (taking care not to dislodge the

cover slips) and replace with a few drops of fresh MEF media and repeat every 4-5 days. For the first two weeks add only few drops (0.5-1ml) of MEF media and then 1.5-2ml from second week onwards.

1.2.9. Outgrowths of primary skin cells should appear 5 days post-isolation

1.2.10. Morphology and growth of cells, and the presence of any microbial

contaminants, should be checked regularly under inverted microscope.

1.2.11. A dense fibroblast outgrowth of cells appears (around some 3-4

explants) after 23-26 days. The cells can be passage at this stage. Do not seed fibroblasts at low densities, since this leads to a lag in proliferation.

1.3. Day 23 – 26 (Passaging of skin fibroblasts)

1.3.1. Aspirate MEF media and wash once with D-PBS. Use sterile forceps

to lift the cover slip. Carefully transfer cover slips into a 6-well plate and add 2.0ml trypsin to underside of the cover slip and incubate for 5 min at 37°C. Fibroblasts will probably adhere to the cover slip. Add 1.5ml trypsin to 60mm plate and incubate for 5 min at 37°C.

1.3.2. Using a 5ml pipette add 1.5ml of MEF media to the 60mm plate

and 2.0ml to the 6-well plate to inactive the trypsin. Disperse all the cells by aspirating up and down gently, and by rinsing the bottom of the dish. Collect cells in a 15ml Falcon tube.

1.3.3. Centrifuge cells at 300g at room temperature for 3 min and aspirate

supernatant

1.3.4. Add 5ml MEF media to the cells, resuspend and plate onto

either T25 flask or T75 flask, depending on number of fibroblasts (passage 1).

1.3.5. A week later or when cells have reached confluency split fibroblasts in

1-3 ratio (passage 2). Wash flask with D-PBS, remove the D-PBS and add trypsin to cover bottom of flask. Incubate flask for 5 min at 37°C. Centrifuge cells at 300g at room temperature for 3 min and aspirate supernatant. Resuspend cells and plate.

1.3.6. A week later or when cells have reached confluency split fibroblasts in

1-3 ratio (passage 3).

1.3.7. A week later or when cells have reached confluency harvest the

fibroblasts. Split cells and count.

1.3.8. Prepare cryovials with 1x106 cells per vial in freezing media: 90%

FBS and 10% DMSO. Place cryovials in a Mr Frosty, and transfer to 80°C freezer for 24hrs. Next day transfer cryovials to liquid nitrogen for long-term storage.

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Autoclave |
| Containment Level 2 Lab (CL2) |
| Incubator at 37°C, 5% CO2 |
| Stereo microscope |
| Inverted microscope |
| **Materials** |
| 15ml Falcon tube |
| T25, T75, T225 culture flasks |
| 60mm plates |
| Forceps |
| Scalpels |
| Cover slips |
| **Solutions and Reagents** |
| Fetal Bovine Serum (Invitrogen Ref.16000-044) |
| MEF media see Media and Solutions Recipes |
| Dimethyl Sulfoxide - DMSO (Sigma Ref. D-2650) |

**2. Culture and passaging fibroblasts**

## Fibroblasts require passaging once 80-90% confluent and media changing every 2-4 days to maintain good condition and avoid overgrowth.

2.1. Aspirate media, wash once with D-PBS, cover cells with 0.05% trypsin

2.2. Return flask to incubator and incubate for 5 min at 37°C

2.3. Prepare 15ml conical tube with 10ml of MEF media at room tem. (serum

present in media inactivates trypsin)

2.4. Once cells have detached transfer them into 15ml falcon tubes containing

MEF media

2.5. Centrifuge cells at 256g (radius 16.9) for 3min and discard the supernatant

2.6. Resuspend cell pellet in 1ml of MEF media using 1ml tip and subsequently

add 9ml of MEF media, mix and count cells

2.6.1. For transduction plate required amount of cells into one well of 6-well

plate in a final volume of 2ml per one well of 6-well plate

2.6.2. For maintenance split cells in 1-3 up to 1-6 ratio depending on their

confluency

2.7. Return cells to incubator

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Incubator at 37°C, 5% CO2 |
| Bench-top centrifuge |
| Inverted microscope |
| **Materials** |
| 15ml Falcon tube |
| T25, T75 culture flasks |
| **Solutions and Reagents** |
| D-PBS (life tech Ref. 14190-094) |
| MEF media see Media and Solutions Recipes |
| 0.05% Trypsin-EDTA 1x (life tech Ref. 25300-054) |

**Required Resources:**

**3. Reprogramming of human skin fibroblasts with retroviral vectors**

We are using commercially available monocistronic iPS reprogramming kit from Vectalys, consisting of four retroviral vectors encoding: OCT4, SOX2, KLF4, v-MYC. All genes are expressed under CMV promoter, which in the context of pluripotency is undergoing silencing mediated by trimethylation of Lysine 9 on Histone H3 (H3K9me3) followed by CpG methylation [14].

## The disadvantage of retroviral reprogramming method:

Retroviruses backbone will insert into a host cell’s genome upon transduction with the risk of creating genetic anomalies. Furthermore, stable integrated transgene can be reactivated and transcribed. This can interfere with differentiation and greatly increases the risk of tumors.

**Note:** Work with viral vectors has to be carried out in Containment Level 2 Lab (CL2) till day 5 following transduction.

3.1. Day -1

3.1.1. Passage fibroblasts as described in section - Culture and passaging

fibroblasts. Seed 100 000 cells per one well of 6-well plate in MEF media without P/S per line

3.1.2. Incubate plate in the incubator at 37°C, 5% CO2 for 24 hours

3.2. Day 0 Transduction

3.2.1. Thaw the required amount of retrovirus acc. to calculations below

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Factor | Titer TU/ml  - current batch | volume for MOI 10 per one transduction | volume for MOI 50 per one transduction | Number of cells per one well of 6-well plate |
| Oct4 | 1.2x109 | 0.84 | 4.2 | 100 000 |
| Sox2 | 1.3x109 | 0.77 | 3.85 | 100 000 |
| Klf4 | 5.6x109 | 0.20 | 1 | 100 000 |
| v-Myc | 7.8 x108 | 1.28 | 6.4 | 100 000 |

MOI – Multiplicity of infection is the ratio of the number of infectious virus particles to the number of target cells present in a defined space.

For example for MOI 10 you will need 10 particles of virus per one cell.

Calculation for Oct4 for the MOI 10 per 1x 100 000 cells where the titer of virus = 1.2 x 1000 000 TU/μl

For MOI 10 take 1 x 1000 000 TU per 1x 100 000 cells

1.2 x 1000 000 TU – 1μl

1 x 1000 000 TU – xμl > x= 0.833333μl

3.2.2. For transduction of one cell line prepare 50ml falcon tubes with 2ml of

MEF media without P/S and add 2μl of polybrene-hexadimethrine bromide [10mg/ml] to obtain final concentration of [10μg/ml] and the calculated concentrations of viruses

3.2.3. Aspirate media from the fibroblasts seeded on the day before

transduction and add MEF media containing polybrene-hexadimethrine and viruses. Return the plate to the incubator set at 32°C, 5% CO2 and incubate overnight

3.3. Day 1

3.3.1. After 24 hours aspirate media, wash cells one time with 2ml D-PBS

and add 2ml fresh MEF media without P/S

3.3.2. Incubate plate for next four days at 37°C, 5% CO2

3.4. Day 4 (Preparation of feeders)

3.4.1. Plate feeders on 100mm plate according to section 4 -

Preparation of feeder plates

3.5. Day 5

3.5.1. On day 5 following transduction trypsinize cells according to section - Culture and splitting fibroblasts and seed all the transduced cells on the prepared 100mm feeder plate. Add 8ml of MEF media

3.6. Day 7 onwards

3.6.1. On day 7 following transduction aspirate media and replace with KSR

media

3.6.2. Observe and feed daily with 7-9ml of fresh KSR media

3.6.3. Expect colonies to appear between day 12 and 32

**Required Resources:**

|  |
| --- |
| **Equipment** |
| CL2 lab |
| Laminar flow cabinet |
| Incubator at 37°C, 5% CO2 |
| **Materials** |
| Filter tips |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| Monocistronic retrovirus iPS reprogramming kit Vectalys |
| MEF media see section Media and Solutions Recipes |
| KSR media see section Media and Solutions Recipes |
| polybrene -hexadimethrine bromide [10mg/ml] |
| 100mm feeder plate on day 5 following transduction |

**4. Preparation of feeder plates**

The quality of feeders is absolutely crucial to maintain iPSCs in undifferentiated state. Therefore, we recommend to culture iPSCs using commercially available irradiated MEFs (IRR MEFs) to start with.

4.1. Coat 100mm plates with 0.1% gelatin

4.2. Thaw as many vials as appropriate for the number and density of feeder layer

plates required (Table 4.1). One vial of CF1 IRR MEFs from GlobalStem

contains 4-5 x106 cells

4.3. Thaw cells quickly in a 37°C water bath, gently shaking

4.4. Transfer cells dropwise to a 50ml conical tube containing 10ml MEF media

and centrifuge at 200g for 4 min.

4.5. Resuspend cells in 50ml MEF media and transfer 10ml media per 100mm

feeder plate. It corresponds to 1x106 cells per 100mm plate. Incubated at 37°C, 5% CO2 overnight

4.6. You can seed iPSCs onto prepared 100mm feeder plates up to 3 days

following seeding MEFs

Table 4. 1 Densities for MEFs on Different Culture Dishes

|  |  |  |  |
| --- | --- | --- | --- |
| Cell culture vessel | Surface area in mm2 | # of MEFs | Optimal volume for plating [ml] |
| **Dishes/plates** |  |  |  |
| 35mm plate | 962 | 0.15 x 106 | 2 |
| 60mm plate | 2.827 | 0.4 x 106 | 3 |
| 100mm plate | 7.854 | 1.0 x 106 | 10 |
| **Multi-well plates** |  |  |  |
| 6-well plate | 962 | 0.15 x 106 | 2-5 |
| 12-well plate | 401 | 0.05 x 106 | 0.5-2 |
| 24-well plate | 200 | 0.025 x 106 | 0.5-1 |

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Incubator at 37°C, 5% CO2 |
| Centrifuge |
| **Materials** |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| MEF media see Media and Solutions Recipes |
| KSR media see Media and Solutions Recipes |
| CF1 IRR MEFs (GlobalStem Ref. GSC-6001G ) |

**5. Picking hiPSCs**

Picking of single iPSCs colonies enables separation of clonal population of cells for further culture and propagation

5.1. Aspirate MEF media from 12-well feeder plate, wash with D-PBS and add

0.5ml KSR media containing 10μM ROCK inhibitor/Y-27632 to each well, return to incubator

5.2. Assess the culture to identify undifferentiated colonies or colony regions from

differentiated colonies. Colonies ready to pick should be visible by eye

5.3. Using 20μl pipette set to 15μl encircle colony so that you are sure that you are

picking individual colony. Subsequently, gently grid the undifferentiated colony into clumps consisting of 200-300 cells

5.4. Lift individual undifferentiated iPSCs fragmented colony and transfer to one

well of 12-well feeder plate with KSR media containing 10μM ROCK inhibitor/Y-27632

5.5. Incubate plate in 37°C, 5% CO2. Leave undisturbed for 24 hours

5.6. Check cells after 24 hours, top up with 0.5 ml fresh KSR media without

ROCK inhibitor

5.7. Change media daily with KSR media without ROCK inhibitor

5.8. After 7-10 day colonies will become large enough to be split

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Stereo microscope |
| Inverted microscope |
| **Materials** |
| 100mm plate with colonies ready to pick |
| 12-well feeder plate |
| 20μl pipette and sterile tips |
| **Solutions and Reagents** |
| D-PBS (life tech Ref. 14190-094) |
| KSR Media see section Media and Solutions Recipes |
| ROCK inhibitor/Y-27632 see section Inhibitors Recipes |

**6. Passaging hiPSCs**

Human induced pluripotent stem cells need to be passaged in order to avoid overgrowth and to maintain them in undifferentiated state.

**Optional:** Before splitting, remove differentiated colonies under a microscope in sterile conditions (i.e. via slow-vacuum aspiration or pipette scraping). Be careful not to leave plate out too long so that cells do not dry out if using vacuum method. Please see below Fig.1 for details.

6.1. Wash iPSCs once with 2ml of D-PBS

6.2. Add 1ml of Collagenase IV and 1ml of Dispase per well of a 6-well plate

and incubate at 37ºC in the incubator for up to one hour (expect to see visible curling or thickening of colonies around the edges, which leads to detachment of the colonies).

6.3. Prepare 15ml falcon tube containing 5ml of DMEM/F12 media per each

split line and add the detached colonies from one well to one falcon tube. Do not pipette at this point just wash by inverting falcon tube or tap cells!

6.4. Let colonies to sediment and carefully aspirate off media, so that the colonies

are left at the bottom of the falcon.

6.5. Wash one more time with 7ml DMEM/F12 and allow colonies to sediment.

6.6. While waiting for colonies to sediment, wash feeder plates with 2ml of D-PBS

and add 1ml of KSR media, return to the incubator

6.7. Once colonies have sedimented at the bottom of the falcon tubes aspirate as

much media as possible, and add 1ml of KSR media. Triturate colonies to get to medium-small fragments (~50-200 cells per clump) using 1ml pipette and add 2ml of KSR media.

**Note:** Avoid over-triturating, which may cause cell death, especially when colonies are broken down to single cell suspensions.

6.8. Plate 1ml into one well of a 6 well plate with feeders that was pre-washed

with D-PBS and contains 1ml of KSR media

6.9. Examine microscopically the size and density of cell clumps seeded in

the 6-well plate. There should be up to 15 cell clumps per one well of 6-well plate

## Fig.6.1. Removal of differentiated colonies using the stereomicroscope. (A) hESC colonies viewed under a stereo-microscope. Differentiated colony areas appear white (arrow), while undifferentiated colonies are opaque. (B) Differentiated areas are excised and discarded before bulk passaging. Adapted from online methods.

## 

B

A

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Stereo microscope |
| Inverted microscope |
| **Materials** |
| 6-well feeder plate |
| 50ml Falcon tubes |
| 15ml Falcon tubes |
| **Solutions and Reagents** |
| DMEM/F12 |
| KSR media see section Media and Solutions Recipes |
| D-PBS (life tech Ref. 14190-094) |
| Dispase (Invitrogen Ref.17105-041) - resuspend 0.5g Dispase in 500ml DMEM/F12, mix well store in +4C up to 4 weeks |
| Collagenase IV (Invitrogen Ref. 17104-019) |

**7. Freezing iPSCs**

## It is important to store cells for future studies, cryopreservation also ensures the availability of back-up cells in case of contamination, loss of cell supply and for repetition of experiments. It is optimal to cryopreserve cells when they are at their maximum growth rate. It is common practice to create a master bank consisting of 2 to 20 vials of the cell line.

## It is important to freeze iPSC at different stages of derivation. Derivation is a long process; back-up cells may prove invaluable in the event that cells are lost later in the derivation process. We recommend freezing cells at passage 4 (p4), p6, p9, p12.

7.1. Passage cells according to section - Passaging iPSCs, however in this case

triturate the colonies 5-10 times

7.2. Prepare freezing media and cryovials

7.2.1. Mix 0.1ml DMSO and 0.9ml KOSR per vial (multiply by the number

of vials you wish to freeze)

**Note:** DMSO is toxic open only in the laminar flow cabinet! Wear gloves and dispose tubes and tips after all residual DMSO has evaporated in the cabinet.

7.2.2. Label cryovials with (i) for induced PS cells, name of the cell line from

which they were derived, passage number, date (dd-mm-yy), derivation method (SeV/ Epi/retro-MOI)

For example **(i) AT54 - p9 -12 04 13 - SeV MOI 3**

7.3. Aspirate media from iPSCs pellet and carefully add 1ml of freezing media per

vial and mix gently once

**Note:** Work as fast as possible – DMSO is toxic for cells and cells should not be in

contact with freezing medium longer than 3 min before transferring them into -80C

7.4. Place the cryovials in the Mr Frosty.

7.5. Transfer Mr Frosty to -80°C freezer and store overnight

7.6. After 24 hrs transfer cryovials into the liquid nitrogen for long-term storage

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Inverted microscope |
| **Materials** |
| Mr Frosty (isopropanol-containing freezing container) |
| Cryovials |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| DMSO (Sigma Ref. D-2650) |
| KOSR (Invitrogen Ref.10828-028) |

**8. Thawing iPSCs**

## Thawing cryopreserved cells enables re-establishment cells in culture after long-term storage.

8.1. Set up 2x 15 ml Falcon tubes.

8.1.1. To tube 1 add 1ml of pre-warmed KSR media

8.1.2. To tube 2 add 9ml of pre-warmed KSR with 10μM final concentration of Y-27632 (ROCK inhibitor)

8.2. Partially thaw the frozen vial of iPS cells at 37ºC in water-bath until there is a

small piece of ice remaining. Spray the vial with 70% ethanol.

8.3. Taking 1ml of media at a time from tube 2, slowly add the pre-warmed media

drop-wise to the vial and transfer the liquid content with cells into tube 1. Repeat until all 9ml used.

8.4. Spin at 200g for 3 min

8.5. Meanwhile, wash one well of a 6-well feeder plate with D-PBS. Add 1ml KSR

media containing 10μM ROCK inhibitor.

8.6. Aspirate media from the spun down tube 1, and gently resuspend the pellet

with 1ml of KSR media containing 10μM ROCK inhibitor/Y-27632. Transfer cells to one well of a 6-well feeder plate, return to incubator

8.7. Change media after 24-48 hours with fresh KSR media with ROCK

inhibitor

**Note:** Do not add ROCK inhibitor to subsequent feedings.

8.8. Change media daily. Expect colonies to emerge between day 7-15 following

thawing

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Bench-top centrifuge |
| Incubator 37°C, 5% CO2 |
| **Materials** |
| 6-well feeders plate |
| 15ml Falcon tube |
| **Solutions and Reagents** |
| Y-27632 (Sigma Aldrich Cat # Y0503) |
| KOSR Media see Media SOP# |

**9. Non-integrating method of reprogramming human skin fibroblasts**

**with episomal plasmids**

Genomic integration of transgenes increases the risk of tumor formation and mortality in chimeric and progeny mice derived from induced pluripotent stem cells (iPSCs). Therefore, integration-free human iPSCs have been generated using several methods, including adenovirus, Sendai virus, the ‘piggyBac’ system, minicircle vector, episomal vectors, direct protein delivery and synthesized mRNA.

## This Protocol describes integration-free method of reprogramming human skin fibroblasts based on episomal delivery of four Yamanaka reprogramming factors – OCT4, SOX2, KLF4 AND l-MYC with addition of Lin28 and sh-p53.

In the original report describing episomal plasmid reprogramming, the authors used seven factors, including POU5F1 (also known as OCT3/4), SOX2, KLF4, MYC (also known as c-MYC), NANOG, LIN28A (also known as LIN28) and SV40 large T antigen (SV40LT), in three different vector combinations6 (T1–T3 combinations; Fig. 1a). Subsequently, it has been reported that iPSC generation is markedly enhanced by p53 suppression, and L-Myc is more potent and specific than c-Myc during human iPSC generation.

## We use three episomal plasmids generated by Yamanaka et al, available from Addgene via following Accession codes: 27076 (pCXLE-hOCT3/4), 27077 (pCXLE-hOCT3/4-shp53-F), 27078 (pCXLE-hSK), 27079 (pCXLE-hMLN), 27080 (pCXLE-hUL), 27081 (pCXLE-Fbx15-cont2) and 27082 (pCXLE-EGFP). (Fig.1). All genes are expressed under CAG promoter, which is not silenced in the context of pluripotency, therefore several passages of cells need to be performed in order to get rid of plasmids.

Fig.1 Episomal expression vectors. *CAG*, CAG promoter; *WPRE*, woodchuck hepatitis post-transcriptional regulatory element; and *pA*, polyadenylation signal.



[*Yamanaka S*](http://www.ncbi.nlm.nih.gov/pubmed?term=Yamanaka%20S%5BAuthor%5D&cauthor=true&cauthor_uid=21460823) Nat Methods. 2011 May;8(5):409-12

9.1. Culture of cells before Nucleofection

9.1.1. Cells should be passage 2 - 4 days before Nucleofection

9.1.2. Do not use cells after passage 14 for Nucleofection

9.1.3. Optimal confluency before Nucleofection: 80 - 90%

9.2. Preparation for Nucleofection

9.2.1. Thaw a single vial of each episomal plasmid (see Table.1), measure

DNA concentration using Nanodrop and adjust concentration of each plasmid to 1μg/1μl with tissue culture water for example water for embryo transfer

9.2.2. Unpack Lonza Nucleofection kit, which is stored in the fridge and

add supplement to P2 solution (once the supplement is added to the solution, it is stable up to 3 months at 4ºC)

9.2.3. Pre-warm RPMI to 37ºC in water-bath

9.2.4. Prepare 12-well plate with 1ml of MEF media without (P/S) per well

9.2.5. Make sure that there is a proper setting for the plate map on the Amaxa

machine – for the somatic human fibroblasts use program EO 114, solution P2, include on your map -no program control (NPC) - Table.2.

Table. 1

|  |  |  |
| --- | --- | --- |
| **Episomal plasmid** | **Total amount of plasmid per**  **100 000 cell** | **Number of cells** |
| (3) L-Myc, Lin28 | 1μg | 100 000 |
| (4) Sox2, Klf4 | 1μg | 100 000 |
| (6) Oct4, sh against p53 | 1μg | 100 000 |

Table. 2. Plate Map - 1 x 16-well

|  |  |  |
| --- | --- | --- |
|  | P2 solution |  |
|  | 1 | 2 |
| A | EO 114 | EO 114 |
| B | EO 114 | EO 114 |
| C | EO 114 | EO 114 |
| D | EO 114 | EO 114 |
| E | EO 114 |  |
| F | EO 114 |  |
| G | EO 114 |  |
| H | NPC |  |

9.3. Passaging fibroblasts for Nucleofection

9.3.1. Aspirate media from the fibroblasts, wash once with D-PBS and cover

with 0.05% trypsin and incubate for 5min at 37ºC

9.3.2. Transfer cells to a 15ml conical tube with 10ml of RT MEF medium

without P/S

9.3.3. Take an aliquot of cells for counting

9.3.4. Based on the cell count split cells into falcon tubes so that you have the

desired number of cells in each tube after spinning i.e. 100 000 cells per Nucleofection

9.3.5. Centrifuge cells at 178g for 3min and discard the supernatant

9.4. Nucleofection

9.4.1. Resuspend cell pellet in 20μl P2 solution with supplement added and

subsequently add prepared episomal DNA

9.4.2. Immediately transfer cells in solution into one well of Nucleofection

strip, make sure that there is no air bubbles and pulse

9.4.3. Remove cells from the machine and leave at RT for 10 min

9.4.4. Add 80μl pre-warmed at 37°C RPMI media (10%FBS) and leave 10min in the incubator at 37°C to recover cells

9.4.5. Transfer cells into one well of 12-well plate with 1ml of MEF media

without P/S

9.4.6. Return cells to incubator

9.5. After 12-24h replace MEF media with MEF media containing P/S

9.6. Leave cells undisturbed for four days and change media on day 4

following Nucleofection

9.7. On day 6 following Nucleofection prepare 100mm feeder plate

according to the section-Preparation of feeder plates

9.8. On day 7 following Nucleofection transfer all cells from one well of

12-well plate into the 100mm feeder plate according to section-Culture and passaging fibroblasts

**Note**: In case you have very low cell density (around 50% cell death) pool two wells of 12-well plate into single 100mm plate

9.9. Next day change media into KSR media with P/S and subsequently

feed cells daily with KSR media with P/S

9.10. Colonies should appear around day 21 following Nucleofection

**Required Resources:**

|  |
| --- |
| **Equipment** |
| CL2 lab |
| Laminar flow cabinet |
| Lonza electroporation system Amaxa 4D |
| Nanodrop |
| Incubator at 37°C, 5% CO2 |
| **Materials** |
| 15ml Falcon tubes |
| **Solutions and Reagents** |
| Episomal plasmids - Addgene Accession codes: 27076 (pCXLE-hOCT3/4), 27077 (pCXLE-hOCT3/4-shp53-F), 27078 (pCXLE-hSK), 27079 (pCXLE-hMLN), 27080 (pCXLE-hUL), 27081 (pCXLE-Fbx15-cont2) and 27082 (pCXLE-EGFP) in concentration 1μg/1μl |
| MEF media see section Media and Solutions Recipes |
| KSR media see section Media and Solutions Recipes |
| RPMI media see section Media and Solutions Recipes |
| 100mm feeder plate |
| Nucleofection kit (Lonza) |

**10. Non-integrating method of reprogramming skin fibroblasts with Sendai Virus vectors**

**Note:** Work with viral vectors has to be carried out in Containment Level 2 Lab (CL2) till day 5 following transduction.

10.1. Day -1

10.1.1. Passage fibroblasts as described in section - Culture and passaging

fibroblasts. Seed 400 000 cells per one well of 6-well plate in MEF media without P/S per line

10.1.2. Incubate plate in the incubator at 37°C, 5% CO2 for 24 hours

10.2. Day 0 Transduction

10.2.1. Thaw the required amount of Sendai virus based on the calculation as

shown below

Multiplicity of infection is the ratio of the number of infectious virus particles to the number of target cells present in a defined space.

For example for MOI 1 you will need 1 particles of virus per one cell.

Calculation for Oct4 for the MOI 1 per 500 000 cells where the titer of virus = #TU/μl

For MOI 1 take 500 000 TU per 500 000 cells

# TU – 1μl

500 000 TU – xμl

x= μl

|  |  |  |  |
| --- | --- | --- | --- |
| Factor | Titer TU/ml  - current batch | volume for MOI 1 per one transduction | Number of cells per one well of 6-well plate |
| Oct4 |  |  | 500 000 |
| Sox2 |  |  | 500 000 |
| Klf4 |  |  | 500 000 |
| c-Myc |  |  | 500 000 |

10.2.2. Aspirate media from the fibroblasts seeded on the day before

transduction and add MEF media containing viruses. Return the plate to the incubator set at 37°C, 5% CO2 and incubate overnight

10.3. Day 1

10.3.1. After 24 hours aspirate media, wash cells once with 2ml D-PBS

and add 2ml fresh MEF media without P/S

10.3.2. Incubate plate for next four days at 37°C, 5% CO2

10.4. Day 4

10.4.1. Plate feeders on 100mm plate according to section 4 -

Preparation of feeder plates

10.5. Day 5

10.5.1. On day 5 following transduction trypsinize cells according to section –

Culture and splitting fibroblasts and seed all the transduced cells on the prepared 100mm feeder plate. Add 8ml of MEF media

10.6. Day 7 onwards (KSR media)

10.6.1. On day 7 following transduction aspirate media and replace with KSR

10.6.2. Observe and feed daily with 7-9ml of fresh KSR media

10.6.3. Expect colonies to appear between day 20 and 42 following transduction

**Required Resources:**

|  |
| --- |
| **Equipment** |
| CL2 lab |
| Laminar flow cabinet |
| Incubator at 37°C, 5% CO2 |
| **Materials** |
| Filter tips |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| CytotuneTM- iPS Sendai Reprogramming kit (Life Tech A1378001) |
| MEF media see section Media and Solutions Recipes |
| KSR media see section Media and Solutions Recipes |
| 100mm feeder plate on day 5 following transduction |

**11. Isolation of peripheral blood mononuclear cells (PBMCs) for**

**reprogramming**

One of the advantages of using blood mononuclear cells as a starting population of cells for reprogramming is the fact that they can be easily isolated from peripheral blood. This protocol describes the isolation of PBMCs from blood via gradient centrifugation from 50ml of peripheral blood. Subsequently, cells can be used for reprogramming or freeze down until further use.

11.1. Collect 50ml of peripheral blood into Sodium Citrate tubes. Invert tube

8-10x and keep upright at room temperature

11.2. Invert ficoll bottle several times to mix prior to withdrawal. In sterile hood

transfer 15ml of ficoll to 50ml falcon tubes

11.3. Dilute blood 1:1 with D-PBS and slowly layer 25ml on top of the ficoll

11.4. Centrifuge 30 min at 500g at RT with the accelerator and BRAKE OFF

**(ideally within 2 hrs of collection)**

11.5. Following density gradient centrifugation, carefully collect MNC layer using a

Pasteur pipette into a 50ml falcon tube. Use a new falcon tube for every blood tube. Top up the volume to 50ml with D-PBS. Invert several times and spin down at room temperature for 20 minutes at 300g

11.6. Aspirate supernatant and resuspend pellet in 10ml of sterile D-PBS

Take 10ul of cell suspension, dilute in 490ul 1% acetic acid solution and lyse RBCs. Count cells using trypan blue

11.7. Expect to obtain ~60-90x106 cells from 50ml

11.8. Spin cells at 300g for 5 min. Freeze down ~2.5 x106 cells/vial and (Use

90% KOSR, 10% DMSO for freezing medium)

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Phase contrast microscope |
| Laminar flow cabinet |
| Bench-top centrifuge |
| Incubator at 37°C, 5% CO2 |
| **Materials** |
| Blood collected in Sodium Citrate tubes (Vacuette from Greiner bio-one, coagulation with sodium citrate 3.2%, 455322) |
| Sterile plastic trasnfer pipettes |
| Cell counter |
| 50ml falcon tubes |
| **Solutions and Reagents** |
| Ficoll Paque Premium GE healthcare (17-5442-02) density 1.077g/ml |
| D-PBS (without Ca2+ and Mg2+, Sigma, D8537) |
| 1% Acetic Acid in water |
| Trypan Blue |
| Knockout ™ Serum Replacement (Invitrogen, 10828-028) |
| DMSO (Sigma, D2650) |

**12. Non-integrating method of reprogramming PBMC with Sendai**

**Virus (SeV)**

This protocol allows generation of integration-free iPSCs from peripheral blood. Peripheral blood mononuclear cells (PBMCs) are cultured to expand the erythroblast (EB) population. They are then used to derive iPSCs using four recombinant Sendai viral vectors, expressing the four reprogramming factors: OCT4, SOX2, KFL4 and c-MYC.

12.1. Day -9

12.1.1. Thaw 1 vial of frozen PBMCs (2x106) into 10ml of **SS** medium and

centrifuge at 300g for 5 min

12.1.2. Resuspend pellet in 2ml of **EM** and transfer to one well of a 12-well

plate, incubate at 37°C, 5% CO2

12.2. Day -6 and Day -3

**Note:** Minimise disturbing cells when replacing media

12.2.1. Carefully transfer cells in suspension to 15ml falcon tube and add 2ml

of **SS** medium. In order to avoid cells left in the plate of drying off add 1ml of **EM** per one well of 12-well plate

12.2.2. Spin cells at 300g for 5 minutes

12.2.3. Resuspend cells in 1ml of fresh **EM** and carefully transfer to the same

well of 12-well plate (2ml is a total volume in one well of 12-well plate)

12.2.4. Continue to culture at 37°C, 5% CO2

12.3. Day 0 Transduction

12.3.1. Transfer cells to sterile 15ml conical tube and wash well 2x with 1ml

of **SS**medium to collect adherent cells

12.3.2. Count cells

12.3.3. Spin down 5x105 cells in 15ml conical tube and add 0.3ml of fresh

**EM**

12.3.4. Defrost Sendai Virus vectors (SeV) expressing OCT3/4, SOX2, KLF

and c-MYC, according to manufacturer’s protocol. Use MOI 6 for each cell line

12.3.5. Prepare 0.3ml of fresh EM plus viruses at MOI 6 with 4μg/ml

of polybrene -hexadimethrine bromide and transfer to one well of a 12-well plate. Incubate overnight at 37°C and 5% CO2.

12.4. Day 1

12.4.1. Collect and spin cells at 300g in a conical tube for 5 min. Aspirate the

supernatant and resupend cells in 2ml of fresh **EM**. Incubate at 37°C and 5% CO2

12.5. Day 2

12.5.1. Plate MEFs onto 0.1% gelatine 100mm plate according to section-Preparation of feeder plates

12.6. Day 3

12.6.1. Collect cells into 15ml conical tube and spin at 300g for 5min.

12.6.2. Resuspend cells in 7ml of KSR mediaenriched for growth factors and

Ascorbic Acid see section - Media and Solutions Recipes and transfer to 100mm feeder plate

12.7. Day 5

12.7.1. Feed cells every other day with 7ml of KSR media

12.8. ~ Day 9 – Day 12

12.8.1. Once small colonies appear, feed cells daily with 8ml of KSR media

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Inverted microscope |
| Laminar flow cabinet |
| Bench top centrifuge |
| Incubator at 37°C, 5% CO2 |
| **Materials** |
| 12-well plates |
| 15ml conical tubes |
| 100mm plates |
| **Solutions and Reagents** |
| KSR Media see Media and Solutions Recipes |
| MEF Media see Media and Solutions Recipes |
| Expansion Media (EM) see Media and Solutions Recipes |
| StemSpan Media (SS) see Media and Solutions Recipes |
| 0.1% Gelatine see Media and Solutions Recipes |
| polybrene -hexadimethrine bromide [10mg/ml] |
| bFGF in house |
| MEFs (GlobalStem) |
| StemSpan H3000 (StemCell Technologies Ref. 9850) |
| Recombinant human IL-3 (Invitrogen Ref. PHC0035) |
| Recombinant human IGF-1 (Miltenyi Ref. 130-093-885) |
| Recombinant human SCF(Miltenyi Ref. 130-096-692) |
| Recombinant human EPO (R&D Systems 287-TC-500) |
| Dexamethasone (Sigma Ref. D8893-1MG) |
| L-Ascorbic Acid (Sigma Ref. A4544-25G) |
| CytotuneTM- iPS Sendai Reprogramming kit (Life Technologies Ref. A1378001) |

**III. Chemically defined media protocols**

**1. Passaging human induced pluripotent stem cells using EDTA method - iPSCs**

1.1. Coating 6-well plate

1.1.2 Dilute Vitronectin XF in D-PBS (0.2ml of Vitronectin in 4.8ml of D-PBS)

1.1.3 Gently mix the Vitronectin solution and immediately use the diluted Vitronectin for coating - use 1ml per 1 well of 6-well plate

1.1.4 Incubate for 1 hour at room temperature

1.1.5 After incubation tilt the plate on the side and aspirate the excess of Vitronectin, let it settle for one minute add 1ml of E8 media, return to incubator

1.2. Passaging iPSCs

1.2.1. Wash iPSCs with D-PBS

1.2.2. Add 1ml of 0.5mM EDTA per 1 well of a 6-well plate and incubate at room temperature for 2-6 minutes. When the cells start to separate and round up, and the colonies will appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel

1.2.3. Aspirate EDTA and add 1ml of E8 media per one well of 6-well plate

1.2.4. Prepare 15ml falcon tube containing 2ml of E8 medium per each passaged line and transfer the detached colonies from one well to one falcon tube using 1ml pipet. Sometimes colonies do not detach entirely and you will need to lift them by using pressure to wash them away with media.

1.2.5. Let colonies to sediment and carefully aspirate off media, so the colonies stay at the bottom of the falcon, this will help to get rid of single cells

1.2.6. Add 1ml of E8 media to the pellet

Note: Avoid triturating since that will lead to cell death, especially when colonies are broken down to single cell suspensions.

1.2.7 Plate 0.1ml up to 1ml depending on the cells split ratio. Top up with E8 accordingly in order to obtain 2ml of media per one well of 6-well plate.

1.2.8. Check under the microscope for the size and density of cell clumps seeded in the 6-well plate. There should be up to 15 cell clumps of similar size per one well of 6-well plate

1.3. Next day check if all cells did attach, depending on percentage of attached cells - top up or fully change media and return to incubator

1.4. Change media daily and split cells after five days.

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Inverted microscope |
| Incubator at 37ºC |
| **Materials** |
| 6-well plate |
| 50ml Falcon tube |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| Essential 8 media (life tech Cat. No. A1517001) |
| Vitronectin XF (STEMCELL/Primorigen Biosciences, Cat.No. 07180) store at LN and defrost aliquot over night at 4°C before using it |
| D-PBS, (life tech Ref: 14190-094) |
| 0.5 M EDTA, pH 8.0 (life tech Cat. no. 15575-020) |

**IV. Validation methods**

**1. Detection of expression of endogenous pluripotency markers versus transgenes via reverse transcription followed by Q-PCR**

1.1. Passage cells according to section - Passaging iPSCs, however in this case

do not triturate the colonies

1.2. Aspirate media from iPSCs pellet collected from one confluent well of

a 6-well plate and lyse with 250 lysis buffer. Snap-freeze or use fresh for RNA isolation according to manufactures protocols (GenElute mammalian total RNA miniPrep kit from Sigma Ref. RTN70-1KT)

1.3. Use 500ng of DNase treated RNA for reverse transcription according to

SuperScript (R)II Reverse Transcriptase manufactures protocol (Life Tech. Ref. 18064071). Always prepare RT- control, i.e. reaction without Reverse Transcriptase, which needs to be run in parallel with the samples.

**Note**: To confirm that your cDNA samples are DNA-free always run Q-PCR with PBDG primers set and one transgenes primer set using RT- control as a template in order to confirm that your cDNA samples are DNA-free. If you have no amplification on your RT- control template you can proceed with setting up your plate using RT+ cDNA

* On each 96-well plate you need to include the positive control (H9 or iPSCs derived from WT donor cells) as well as your experimental samples – for endogenous expression
* Each cDNA needs to be run in duplicate on the plate.
* Each experiment needs to be set up to run the reference gene (PBDG) along with other genes of interest.

1.4. Prepare MasterMix for Q-PCR according to the table below

|  |  |
| --- | --- |
| Reagent | **Volume per well** |
| F primer [5μM] | 0.6μl |
| R primer [5μM] | 0.6μl |
| Sensi Mix (2x] | 7.5μl |
| Nuclease free water | 1.3μl |
| Total volume of the MasterMix | 10μl |

1.5. Dilute cDNA 30x in sterile water and mix 5μl of cDNA with 10μl of

MasterMix

1.6. Run Q-PCR with primers set for detection: endogenous genes encoding

pluripotency markers, retroviral transgenes or episomal transgenes, respectively

|  |  |  |
| --- | --- | --- |
| **Primer**  **for detection endogenous**  **genes** | **Sequence** | **Annealing Tem** |
| Oct4 F | CCTCACTTCACTGCACTGTA | 56°C |
| Oct4 R | CAGGTTTTCTTTCCCTAGCT | 56°C |
| Sox2 F | ATGTCCCAGCACTACCAGAG | 56°C |
| Sox2 R | GCACCCCTCCCATTTCCC | 56°C |
| Myc F | CTGAAGAGGACTTGTTGCGGAAAC | 56°C |
| Myc R | TCTCAAGACTCAGCCAAGGTTGTG | 56°C |
| Klf4 F | GGTCGGACCACCTCGCCTTACAC | 56°C |
| Klf4 R | CTCAGTTGGGAACTTGACCA | 56°C |
| DNMT3B F | ATAAGTCGAAGGTGCGTCGT | 56°C |
| DNMT3B R | GGCAACATCTGAAGCCATTT | 56°C |
| Rex1F/ZFP42 | CCGAGACCACGTCTGTGCGG | **64°C** |
| Rex1R/ZFP42 | AGCGCTTTCCGCACCCTTCA | **64°C** |
| PBDG F | GGAGCCATGTCTGGTAACGG | 56°C |
| PBDG R | CCACGCGAATCACTCTCATCT | 56°C |

|  |  |  |
| --- | --- | --- |
| **Primer for**  **detection**  **retroviral**  **transgenes** | **Sequence** | **Annealing Tem** |
| Oct4 F | CCTCACTTCACTGCACTGTA | 60°C |
| Klf4 F | GATGAACTGACCAGGCACTA | 60°C |
| Sox2 F | CCCAGCAGACTTCACATGT | 60°C |
| v-Myc F | AAGAGGACTTGTTGCGGAAA | 59°C |
| GeneralTrans  3' Rev | TCCTGTCTTTAACAAATTGGACT |  |

|  |  |  |
| --- | --- | --- |
| **Primer**  **for detection**  **episomal**  **transgenes** | **Sequence** | **Annealing Tem** |
| Oct3/4 (p) F | CAT TCA AAC TGA GGT AAG GG | 56°C |
| Oct3/4 (p) R | TAG CGT AAA AGG AGC AAC ATA G | 56°C |
| Sox2 (p) F | TTC ACA TGT CCC AGC ACT ACC AGA | 56°C |
| Sox2 (p) R | TTT GTT TGA CAG GAG CGA CAA T | 56°C |
| Klf4 (p) F | CCA CCT CGC CTT ACA CAT GAA GA | 56°C |
| Klf4 (p) R | TAG CGT AAA AGG AGC AAC ATA G | 56°C |
| L-Myc (p) F | GGC TGA GAA GAG GAT GGC TAC | 56°C |
| L-Myc (p) R | TTT GTT TGA CAG GAG CGA CAA T | 56°C |

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers**  **for detection**  **Sendai virus**  **transgenes** | **Sequence** | **Annealing**  **Temperature** | **Product size** |
| Oct3/4 (p) F | CCC GAA AGA GAA AGC GAA CCA G | 55°C | 483bp |
| Oct3/4 (p) R | AAT GTA TCG AAG GTG CTC AA | 55°C |
| Sox2 (p) F | ATG CAC CGC TAC GAC GTG AGC GC | 55°C | 451bp |
| Sox2 (p) R | AAT GTA TCG AAG GTG CTC AA | 55°C |
| Klf4 (p) F | TTC CTG CAT GCC AGA GGA GCC C | 55°C | 410bp |
| Klf4 (p) R | AAT GTA TCG AAG GTG CTC AA | 55°C |
| c-Myc (p) F | TAA CTG ACT AGC AGG CTT GTC G | 55°C | 532bp |
| c-Myc (p) R | TCC ACA TAC AGT CCT GGA TGA TGA TG | 55°C |
| SeV F | GGA TCA CTA GGT GAT ATC GAG C | 55°C | 181bp |

**2. *In vitro* differentiation of iPSCs into three germ layers**

In order to validate differentiation capacity of iPSCs, we perform *in vitro* differentiation of iPSCsinto three germ layers: endoderm, mesoderm, neuroectoderm. [10,11,12,13]

2.1. Preparation of 0.1% gelatine coated 12-well plates used for seeding iPSCs for

differentiation

2.1.1. Prepare 3-wells of 12-well plate for differentiation into each lineage.

Cover 12-well plate with 0.1% gelatine and incubate for 20 min at room temperature, subsequently aspirate gelatine, let plate to settle for 3 min and cover plate with MEF media overnight, (you can keep gelatine coated plates up to 7 days).

2.2. Prepare cells according to section - Passaging iPSCs

2.3. Aspirate media from sedimented iPSCs at the bottom of Falcon tube and

carefully add 3ml of:

CDM-PVA media containing:

1μl Activin [10μg/ml] /ml

3μl FGF2 [4μg/ml] /ml

Aspirate MEF media from 0.1% gelatine coated plates and wash once with D-PBS before seeding iPSCs in CDM-PVA

Plate 1ml into well of 12-well plate and incubate overnight

2.4 **Differentiation into Endoderm**

2.4.1 Day 1 - after 24 hrs aspirate media and add

1ml per well of CDM-PVA containing:

10μl Activin [10μg/ml] /ml

20μl FGF2 [4μg/ml] /ml

1μl BMP4 [10μg/ml] /ml

1μl Ly [10mM] /ml

1μl CHIR [3mM] /ml

2.4.2 Day 2 - after 24 hrs aspirate media and add 1ml per well of CDM-

PVA media containing:

10μl Activin [10μg/ml]/ml

20μl FGF2 [4μg/ml]/ml

1μl BMP4 [10μg/ml]/ml

1μl Ly [10mM]/ml

2.4.3 Day 3 - after 24 hrs aspirate medium and add 1ml per well of RPMI

media containing:

10μl Activin [10μg/ml] /ml

20μl FGF2 [4μg/ml] /ml

2.4.4 After 24 hrs aspirate media and fix with 4% paraformaldehyde

2.5 **Differentiation into Mesoderm**

2.5.1 Day 1 - after 24 hrs aspirate media and add 1ml per well of 12-well

plate of CDM-PVA containing:

10μl Activin [10μg/ml] /ml

5μl FGF2 [4μg/ml] /ml

1μl BMP4 [10μg/ml] /ml

1μl Ly [10mM] /ml

1.66μl CHIR [3mM] /ml

Change media one more day

2.5.2 After 2 days of differentiation aspirate medium and fix with 4%

paraformaldehyde

2.6 **Differentiation into Neurectoderm**

2.6.2 After 24 hrs aspirate medium and add 1ml per well of 12-well plate:

CDM-PVA containing:

1μl SB [10μM]/ml

3μl FGF2 [4μg/ml] /ml

1.5μl Noggin [100μg/ml]/ml

Change medium daily for 10-12 days

2.6.3 After 10-12 days of differentiation aspirate medium and fix cells with

4% paraformaldehyde

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Inverted microscope |
| **Materials** |
| 12-well plate |
| parafilm |
| 50ml Falcon tube |
| **Solutions and Reagents** |
| 16% Paraformaldehyde methanol free, Ultra Pure (Polysciences CAS Number: 50-00-06) |
| 0.1% Gelatine (Millipore cat# ES-006-B) |
| D-PBS, RT and ice-cold (life tech Ref: 14190-094) |
| MEF media see Media and Solutions Recipes |
| CDM-PVA media see Media and Solutions Recipes |
| RPMI media see Media and Solutions Recipes |
| Activin in house [10μg/ml] |
| BMP4 in house [10μg/ml] |
| bFGF in house [4μg/ml] |
| CHIR (Stemgent cat. 04-0004) |
| Ly 294002 (Promega cat. 1201) |
| Noggin (R&D cat.33-44NG/CF), SB431542 |
| SB431542 (Tocris bioscience cat.1614) |
| B27 in house [50x] |

**3. Detection of differentiation markers by immunostaining**

3.1. Fix cells with 4% paraformaldehyde

Note: Paraformaldehyde is very toxic, use only in laminar flow cabinet! Wear gloves and dispose tubes and tips after all residual paraformaldehyde was evaporated in the laminar flow cabinet.

3.1.1. Dilute 16% paraformaldehyde in D-PBS to obtain 4%

paraformaldehyde

3.1.2. Aspirate media and cover cells with 4% paraformaldehyde and

incubate for 20 minutes at room temperature in the laminar flow hood (

Do not wash cells with D-PBS)

3.1.3. Aspirate 4% paraformaldehyde and wash cells 3x with ice-cold

D-PBS, 5 min each wash, do not shake at this point

3.2. Cover cells with D-PBS and proceed with immunostaining

3.3. Blocking and permeabilization

Note: Permeabilization is only required for intra-cellular epitopes, do not use it for extra-cellular epitopes. Milder permeabilization can be performed with 0.14% saponin

3.3.1. Prepare blocking solution by mixing 10ml D-PBS, 1ml Serum + 10μl

Triton).

3.3.2. Cover cells with 0.5ml of D-PBS + 0.1% Triton X-100 per well of 12-

well plate

3.3.3. Incubate at room temperature for 20 minutes to one hour

3.4. Incubation with Primar Ab (can be overnight)

3.4.1. Prepare Primary Ab Dilution in Dako Diluent according to the table

below. Use 300μl of solution per well of 12-well plate

You can also use 1% serum (or BSA), 0.1% Triton X-100 in D-PBS for incubation with Primary Ab

3.4.1. Aspirate blocking solution and cover cells with Primary Ab Dilution

3.4.2. Incubate cells for 1 hour at room temperature or overnight at 4ºC

3.5. Wash step I

3.5.1. Aspirate Primary Ab Dilution and Cover cells with D-PBS, shake

gently for 5 minutes and aspirate, repeat 2x

3.6. Incubation with Secondary Ab

Note: Protect cells from light during incubation with Secondary Abs.

3.6.1. Prepare Secondary Ab Dilution

1-1000 > 4μl Ab - 4000μl (Dako Diluent) per 1well of 12-well plate and multiply by number of wells you wish to stain

1-2000 > 2μl Ab - 4000μl (Dako Diluent) per 1well of 12-well plate and multiply by number of wells you wish to stain

3.6.1. Aspirate D-PBS and cover cells with Primary Ab Dilution

3.6.2. Incubate cells for 30 minutes up to 2 hours at room temperature

3.7. Wash step II

3.7.1. Aspirate Secondary Ab Dilution and cover cells with D-PBS – In order

to visualize cells (DNA) add Dapi in first wash in dilution Dapi 1μl -10 000μl D-PBS

3.7.2. Shake for 2 minutes and aspirate D-PBS, repeat 2 x 5 minutes each wash

3.7.3. Cover cells with D-PBS and protect from light

3.8. Capture images using fluorescent microscopy or store plates protected from

light for documentation

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Fluorescent microscope |
| Shaker to wash 12-well plate |
| **Materials** |
| strippets |
| **Solutions and Reagents** |
| D-PBS (life tech Ref: 14190-094) |
| Tween (Fisher Scientific Ref. BP 337-500) |
| Antibody Diluent (Dako Ref. S3022) |
| Donkey Serum (AbD Serotec, Ref. C06SB), Goat Serum (AbD Serotec, Ref. C07SA) |
| 16% Paraformaldehyde methanol free, Ultra Pure (Polysciences CAS Number: 50-00-06%) |
| DAPI (Sigma Ref. D9542) |

|  |  |  |  |
| --- | --- | --- | --- |
| **Primary Ab** | **Clonality/**  **animal where the Ab**  **were raised** | **Dilution** | **Supplier/Catalogue Number** |
| **Pluripotency**  **markers** |  |  |  |
| hNanog | PolyIgG Goat | 1:100 | R&D AF1997 |
| hSox2 | PolyIgG Goat | 1:200 | R&D AF2018 |
| hTRA-1-60 | MonoIgM Mouse | 1:100 | Santa Cruz sc-21705 |
| hOct-3/4 (C-10) | MonoIgG Mouse | 1:100 | Santa Cruz sc-5279 |
| **Endoderm**  **markers** |  |  |  |
| hGATA-4 | MonoIgG Mouse | 1:100 | Santa Cruz sc-25310 |
| FoxA2/hHNF3b | PolyIgG Goat | 1:100 | R&D AF2400 |
| hSox17 | PolyIgG Goat | 1:200 | R&D AF1924 |
| **Mesoderm**  **markers** |  |  |  |
| hBrachyury | PolyIgG Goat | 1:100 | R&D AF2085 |
| hEomes | PolyIgG Rabbit | 1:600 | Abcam ab23345 |
| **Neuroectoderm**  **markers** |  |  |  |
| Nestin | MonoIgG Mouse | 1:100 | Abcam ab22035 |
| hSox1 | PolyIgG Goat | 1:100 | R&D AF3369 |
| hPax6 | PolyIgG Rabbit | 1:100 | CambridgeBioScience  PRB-278P-100 |

**V. Targeting of Human induced Pluripotent Stem Cells and H9 Embryonic Stem Cells**

**1. Knock-in/Knock-out via donor vector mediated homologous recombination facilitated by TALENs/CRISPRs - iPSCs**

1.1. Culture of cells before nucleofection

1.1.1. Cells should be split every fifth day with a sub-cultivation ratio of 1:3 to 1:10.

EDTA is used for splitting iPSCs grown on Vitronectin.

1.1.2. Prepare 2x106 cells per one nucleofection reaction, which corresponds to one confluent plate.

1.1.3. 12-24hrs before nucleofection change media to E8 P/S free media, containing ROCK inhibitor at the final concentration of 10μM and keep this until 12-24 hours post-nucleofection

1.1.4. 24 hrs before nucleofection prepare 6-well DR4 feeder plate

1.2. Preparation for nucleofection

1.2.1. Thaw a single vial of each plasmid, measure DNA concentration using Nanodrop and adjust concentration of each plasmid to 1μg/1μl with D-PBS

1.2.2. Unpack Lonza Nucleofection kit, which was stored at 4°C and add the whole supplement to the P3 solution (once the supplement is added to the solution, it is stable up to three months at 4°C). Keep required volume for the experiment at room temperature (100μl per nucleofection)

1.2.3. Prepare 50ml E8 P/S free media with 10μM ROCK inhibitor at room temperature

1.2.4. Change media in your 6-well feeder plate by replacing MEF media with 2ml of room temperature E8 P/S free media with 10μM ROCK inhibitor

1.2.5. Set up parameters for nucleofection on the machine according to the table below. Use 300μl of solution per well of 12-well plate

Table. 1 Plate view

|  |  |  |
| --- | --- | --- |
|  | P3 solution | |
|  | 1 | 2 |
| A | CA-137 | CA-137 |
| B | NPC | NPC |
| C |  |  |
| D |  |  |
| E |  |  |
| F |  |  |
| G |  |  |
| H |  |  |

NPC = no program control

Table. 2 Amounts of DNA required for transduction of 2x106 cells

|  |  |  |  |
| --- | --- | --- | --- |
| **Factors** | **Concentration of plasmid** | **Volume added [µl]** | **Number of iPS cells per nucleofection** |
| **TALEN exp** | | | |
| For TALEN | 2 [µg/µl] | 1 | 2x106 |
| Rev TALEN | 2 [µg/µl] | 1 |
| EF1-alfa puromycine | 2 [µg/µl] | 1 |
| EF1-alfa neomycine | 2 [µg/µl] | 1 |
| **CRISPR exp** | | | |
| Cas9 | 2 [µg/µl] | 1 | 2x106 |
| guide RNA | 2 [µg/µl] | 1 |
| EF1-alfa puromycine | 2 [µg/µl] | 1 |
| EF1-alfa neomycine | 2 [µg/µl] | 1 |

1.3. Nucleofection procedure

1.3.1. Detachment of cells prior to Nucleofection

1.3.1.1. Culture cells on Vitronectin until the day of Nucleofection

1.3.1.2. Wash cells with D-PBS and subsequently add 1-2ml of Accutase, incubate for about 5min in the incubator at 37°C till cells start to round up (optionally you can also lift cells with Collagenase/Accutase, wash 2x pelleted colonies and afterwards suspend them in Accutase solution)

1.3.1.3. Remove cells from the incubator and triturate colonies into 3-4 cells clumps

1.3.1.4. Resuspend cells in appropriate volume of media (depending on number of samples) and take aliquot for counting

Note: Do not triturate cells into single cell suspension, make sure that cells remain in small clumps of 3-4 cells per clump. Single cells do not form colonies as efficiently as clumps of few cells.

1.3.2. Nucleofection

1.3.2.5. Count dissociated cells

1.3.2.6. Pellet required amount of cells at 115g for 3min

1.3.2.7. Make mastermix containing 100μl P3 solution and DNA plasmids per one cuvette

1.3.2.8. Resuspend cells in the mastermix, transfer to the cuvette and pulse

1.3.3. Post-Nucleofection

1.3.3.9. Incubate cells for 5min at room temperature

1.3.3.10. Add 500μl room temperature E8 P/S free media with 10μM ROCK inhibitor and leave 5min

1.3.3.11. Transfer 600μl from each well to one well of 6-well feeder plate so that you have in total 2,6ml of media per one well of 6-well plate

1.3.3.12. Incubate cells over night and change media 12-24hrs post-nucleofection 48hrs post-nucleofection start antibiotic selection by adding: 4μl G418 - Neomycine [50mg/ml], 1μl Puromycine [1mg/ml] (selection within donor vectors)

1.3.3.13. Colonies ready to pick should be visible by eye after 5-10 days

1.3.3.14. Transfer picked colonies into 12-well plate coated with FBS/MEF media with 1ml of CDM-BSA media added per well

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Lonza electroporation system - Amaxa 4D system |
| Nanodrop |
| Incubator at 37°C 5% CO2 |
| **Materials** |
| 6-well plates |
| 15ml/50ml Falcon tubes |
| **Solutions and Reagents** |
| D-PBS (life tech Ref: 14190-094) |
| Nucleofection kit - P3 solution Lonza (Ref. V4XP-3012) |
| DR4 MEFs - Applied StemCell (Ref. ASF-1013) |
| Essential 8 media Life tech (Ref. A1517001) |
| StemPro® Accutase® Cell Dissociation Reagent (Ref. A11105-01) Life Technologies |
| ROCK inhibitor/Y-27632 see SOP2 Inhibitors Recipes |
| Vitronectin XF - Stemcell technologies/Primorigen Biosciences (Ref. 07180) |

**2. Knock-out using Cas9- guide RNA – H9 cells**

2.1. Culture of cells before nucleofection

2.1.1. H9 cells should be split once per week with a sub-cultivation ratio of 1:3 to 1:10.

D-PBS is used for splitting iPSCs grown on FBS/MEF media coated plates.

2.1.2. Prepare 2x106 cells per one nucleofection reaction, which corresponds to one confluent plate.

2.1.3. 12-24hrs before nucleofection change media to CDM-BSA P/S free media, containing ROCK inhibitor at the final concentration of 10μM and keep this until 12-24 hours post-nucleofection

2.1.4. 24 hrs before nucleofection prepare 6-well DR4 feeder plate

2.2. Preparation for nucleofection

2.2.1. Thaw a single vial of each plasmid, measure DNA concentration using Nanodrop and adjust concentration of each plasmid to 1μg/1μl with D-PBS

2.2.2. Unpack Lonza Nucleofection kit, which was stored at 4°C and add the whole supplement to the P3 solution (once the supplement is added to the solution, it is stable up to three months at 4°C). Keep required volume for the experiment at room temperature (100μl per nucleofection)

2.2.3. Prepare 50ml CDM-BSA P/S free media with 10μM ROCK inhibitor at room temperature

2.2.4. Change media in your 6-well feeder plate by replacing MEF media with 2ml of room temperature CDM-BSA P/S free media with 10μM ROCK inhibitor

2.2.5. Set up parameters for nucleofection on the machine according to the table below. Use 300μl of solution per well of 12-well plate

Table. 1 Plate view

|  |  |  |
| --- | --- | --- |
|  | P3 solution | |
|  | 1 | 2 |
| A | CA-137 | CA-137 |
| B | NPC | NPC |
| C |  |  |
| D |  |  |
| E |  |  |
| F |  |  |
| G |  |  |
| H |  |  |

NPC = no program control

Table. 2 Amounts of DNA required for transduction of 2x106 cells

|  |  |  |  |
| --- | --- | --- | --- |
| **Plasmid** | **Concentration of Cas9- guide RNA plasmid** | **Volume added [µl]** | **Number of iPS cells per nucleofection** |
|  | | | |
| Cas9- guide RNA | 2 [µg/µl] | 1 | 2x106 |
| Cas9- guide RNA | 8 [µg/µl] | 4 |
| Cas9- guide RNA | 16 [µg/µl] | 8 |

2.3 Nucleofection procedure

2.3.1. Detachment of cells prior to Nucleofection

2.3.1.1. Culture cells on FBS/MEF media coated plates until the day of Nucleofection

2.3.1.2. Wash cells with D-PBS and subsequently add 1-2ml of PBS, incubate for about 5min in the incubator at 37°C till cells start to detach

2.3.1.3. Remove cells from the incubator and triturate colonies into 3-4 cells clumps

2.3.1.4. Resuspend cells in appropriate volume (depending on number of samples) and take aliquot for counting

Note: Do not triturate cells into single cell suspension, make sure that cells remain in small clumps of 3-4 cells per clump. Single cells do not form colonies as efficiently as clumps of few cells.

2.3.2. Nucleofection

2.3.2.5. Count dissociated cells

2.3.2.6. Pellet required amount of cells at 115g for 3min

2.3.2.7. Make mastermix containing 100μl P3 solution and DNA plasmids per one cuvette

2.3.2.8. Resuspend cells in the mastermix, transfer to the cuvette and pulse

2.3.3. Post-Nucleofection

2.3.3.9. Incubate cells for 5min at room temperature

2.3.3.10. Add 500μl room temperature CDM-BSA P/S free media with 10μM ROCK inhibitor and leave 5min

2.3.3.11. Transfer 600μl from each well to one well of 6-well feeder plate so that you have in total 2,6ml of media per one well of 6-well plate

2.3.3.12. Incubate cells over-night and change media 12-24hrs post-nucleofection, 48hrs post-nucleofection start antibiotic selection by adding: 4μl G418 - Neomycine [50mg/ml], 1μl Puromycine [1mg/ml] (selection within donor vectors), keep cells in selection for two days

2.3.3.13. Following two days of selection media proceed with changing media daily using CDM-BSA P/S free media

2.3.3.14. Colonies ready to pick should be visible by eye after 5-10 days

2.3.3.14. Transfer picked colonies into 12-well plate coated with FBS/MEF media with 1ml of CDM-BSA media added per well

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Lonza electroporation system - Amaxa 4D system |
| Nanodrop |
| Incubator at 37°C 5% CO2 |
| **Materials** |
| 6-well plates |
| 15ml/50ml Falcon tubes |
| **Solutions and Reagents** |
| D-PBS (life tech Ref: 14190-094) |
| Nucleofection kit - P3 solution Lonza (Ref. V4XP-3012) |
| DR4 MEFs - Applied StemCell (Ref. ASF-1013) |
| Essential 8 media Life tech (Ref. A1517001) |
| StemPro® Accutase® Cell Dissociation Reagent (Ref. A11105-01) Life Technologies |
| ROCK inhibitor/Y-27632 see SOP2 Inhibitors Recipes |
| Vitronectin XF - Stemcell technologies/Primorigen Biosciences (Ref. 07180) |

**3. Knock-out using CRISPRs – guide RNA containing vectors and single strand oligonucleotides – H9 cells**

3.1. Culture of cells before nucleofection

3.1.1. H9 cells should be split once per week with a sub-cultivation ratio of 1:3 to 1:10.

D-PBS is used for splitting iPSCs grown on FBS/MEF media coated plates.

3.1.2. Prepare 2x106 cells per one nucleofection reaction, which corresponds to one confluent plate.

3.1.3. 12-24hrs before nucleofection change media to CDM-BSA P/S free media, containing ROCK inhibitor at the final concentration of 10μM and keep this until 12-24 hours post-nucleofection

3.1.4. 24 hrs before nucleofection prepare 6-well DR4 feeder plate

3.2. Preparation for nucleofection

3.2.1. Thaw a single vial of each plasmid, measure DNA concentration using Nanodrop and adjust concentration of each plasmid to 1μg/1μl with D-PBS

3.2.2. Unpack Lonza Nucleofection kit, which was stored at 4°C and add the whole supplement to the P3 solution (once the supplement is added to the solution, it is stable up to three months at 4°C). Keep required volume for the experiment at room temperature (100μl per nucleofection)

3.2.3. Prepare 50ml KSR P/S free media with 10μM ROCK inhibitor at room temperature

3.2.4. Change media in your 6-well feeder plate by replacing MEF media with 2ml of room temperature KSR P/S free media with 10μM ROCK inhibitor

3.2.5. Set up parameters for nucleofection on the machine according to the table below. Use 300μl of solution per well of 12-well plate

Table. 1 Plate view

|  |  |  |
| --- | --- | --- |
|  | P3 solution | |
|  | 1 | 2 |
| A | CA-137 | CA-137 |
| B | NPC | NPC |
| C |  |  |
| D |  |  |
| E |  |  |
| F |  |  |
| G |  |  |
| H |  |  |

Table. 2 Amounts of DNA required for transduction of 2x106 cells

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Plasmid** | **Concentration of** Cas9- guideRNA **plasmid** | **Concentration of oligonucleotide** | **Volume added [µl]** | **Number of iPS cells per nucleofection** |
|  | | | | |
| Cas9- guide RNA  pSp**Cas9n**(BB)-2A-Puro (PX462)  (Plasmid #48141)  or pSp**Cas9**(BB)-2A-Puro (PX459)  (Plasmid #48139) | 5 [µg/µl] |  | 0.5  1  3  depends on the gene | 2x106 |
| single strand oligonucleotide  - IDT (integrated DNA technologies) |  | 100pmol |  |

3.3 Nucleofection procedure

3.3.1. Detachment of cells prior to Nucleofection

3.3.1.1. Culture cells on FBS/MEF media coated plates until the day of Nucleofection

3.3.1.2. Wash cells with D-PBS and subsequently add 1-2ml of PBS, incubate for about 5min at 37°C till cells start to detach

3.3.1.3. Remove cells from the incubator and triturate colonies into 3-4 cells clumps

3.3.1.4. Resuspend cells in appropriate volume (depending on number of samples) and take aliquot for counting

Note: Do not triturate cells into single cell suspension, make sure that cells remain in small clumps of 3-4 cells per clump. Single cells do not form colonies as efficiently as clumps of few cells.

3.3.2. Nucleofection

3.3.2.5. Count dissociated cells

3.3.2.6. Pellet required amount of cells at 115g for 3min

3.3.2.7. Make mastermix containing 100μl P3 solution and DNA plasmids per one cuvette

3.3.2.8. Resuspend cells in the mastermix, transfer to the cuvette and pulse

3.3.3. Post-Nucleofection

3.3.3.9. Incubate cells for 5min at room temperature

3.3.3.10. Add 500μl room temperature KSR P/S free media with 10μM ROCK inhibitor and incubate 5min

3.3.3.11. Transfer 600μl from each well to one well of 6-well feeder plate so that you have in total 2, 6ml of media per one well of 6-well plate

3.3.3.12. Incubate cells over night and change media 12-24hrs

post-nucleofection, 48hrs post-nucleofection start antibiotic selection by adding: 4μl G418 - Neomycine [50mg/ml], 1μl Puromycine [1mg/ml] (selection within donor vectors), keep cells in selection for two days

3.3.3.13. Keep cells in selection media and proceed with changing media daily using KSR P/S free media without antibiotic selection

3.3.3.14. It takes up to 5-7 days for the colonies to form, start picking once they are visible by eye

3.3.3.14. Transfer picked colonies into 12-well plate coated with FBS/MEF media with 1ml of CDM-BSA media added per well

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Lonza electroporation system - Amaxa 4D system |
| Nanodrop |
| Incubator at 37°C 5% CO2 |
| **Materials** |
| 6-well plates |
| 15ml/50ml Falcon tubes |
| **Solutions and Reagents** |
| D-PBS (life tech Ref: 14190-094) |
| pSp**Cas9**(BB)-2A-Puro (PX459)  (Plasmid #48139) Addgene |
| Nucleofection kit - P3 solution Lonza (Ref. V4XP-3012) |
| Page Ultramer DNA oligo (IDT; Integrated DNA Technologies) |
| DR4 MEFs - Applied StemCell (Ref. ASF-1013) |
| Essential 8 media Life tech (Ref. A1517001) |
| StemPro® Accutase® Cell Dissociation Reagent (Ref. A11105-01) Life Technologies |
| ROCK inhibitor/Y-27632 see SOP2 Inhibitors Recipes |
| Vitronectin XF - Stemcell technologies/Primorigen Biosciences (Ref. 07180) |

**4. DNA extraction for genotyping**

4.1. Label the appropriate number of tubes containing 50 μl of QuickExtract Solution

4.2. Place one sample in each tube, for example: 1-3 single iPSCs colony picked from a plate

4.3. Vortex for 15 sec

4.4. Transfer the tube to 65°C and incubate for 6 minutes (15 min. for fingernails)

4.5. Vortex for 15 sec

4.6. Transfer the tube to 98°C and incubate for 2 min

4.7. Store the DNA at –20°C, or at –70°C for long-term storage

4.8. Use 1μl of the extracted DNA for each PCR amplification

4.9. Run 30μl of your PCR product on the gel to check the amplicon size or proceed with sequencing (for sequencing please go to www.gatc-biotech.com)

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| Vortex |
| **Materials** |
| RNase free eppendorfs |
| RNase free/DNase free filter tips |
| **Solutions and Reagents** |
| D-PBS (life tech Ref: 14190-094) |
| QuickExtract Solution (Ref Nr.QE09050) |
| RNase-free water (Ambion) |

**VI. Media and Solutions Recipes**

**Note:** Media should be filtered before use via Filter System 0.22μm PES Corning and store at 4°C

|  |  |  |  |
| --- | --- | --- | --- |
| **MEF Medium (500 ml)** | **Stock conc** | **Final conc** | **Volume** |
| Advanced DMEM F12 |  |  | 450ml |
| FBS |  | 10% v/v | 50ml |
| L-Glutamine | 100x | 2mM | 5ml |
| 2-Mercaptoethanol | 98% | 0.007% | 3.5μl |
| P/S (optional) | 100x | 1% | 5ml |

|  |  |  |  |
| --- | --- | --- | --- |
| **StemSpan Medium (SS) 50ml** | **Stock conc** | **Final conc** | **Volume** |
| StemSpan H3000 |  |  | 50ml |
| Pen/Strep | 100x | 1% | 500μl |

|  |  |  |  |
| --- | --- | --- | --- |
| **Expansion Medium (EM) 10ml** | **Stock conc** | **Final conc** | **Volume** |
| StemSpan H3000 |  |  | 10ml |
| Pen/Strep | 100x | 1% | 100μl |
| Ascorbic Acid (AA) | 10mg/ml | 50μg/m | 50μl |
| **(Growth factors)** |  |  |  |
| SCF | 50μg/ml | 50ng/ml | 10μl |
| IL-3 | 10μg/ml | 10ng/ml | 10μl |
| EPO | 2U/μl | 2U/ml | 10μl |
| IGF-1 | 100μg/ml | 40ng/ml | 4μl |
| Dexamethasone\* | 1mM | 1μM | 10μl |

\*Keep dexamethasone protected from light (discard every 2 weeks)

|  |  |  |  |
| --- | --- | --- | --- |
| **iPSCs Medium - (KSR) 500ml** | **Stock conc** | **Final conc** | **Volume** |
| DMEM/F12 |  |  | 400ml |
| KOSR |  | 20% v/v | 100ml |
| L-Glutamine | 100x | 2mM | 5ml |
| MEM-Non Essential AminoAcids (MEM-NEAA) | 100x | 1% | 5ml |
| 2-Mercaptoethanol | 98% | 0.007% | 3.5μl |
| P/S (optional) | 100x | 1% | 5ml |
| bFGF (FGF2) \*\* | 4μg/ml | 4ng/ml | 500μl |

\*\* Add just before starting to use media as bFGF is unstable in culture media

|  |  |  |  |
| --- | --- | --- | --- |
| **KSR with Ascorbic Acid and Growth factors** | **Stock conc** | **Final conc** | **Volume** |
| KSR medium |  |  | 10ml |
| Pen/Strep (optional) | 100x | 1% | 100μl |
| Ascorbic Acid (AA) | 10mg/ml | 50μg/m | 50μl |
| **(Growth factors)** |  |  |  |
| SCF | 50μg/ml | 50ng/ml | 10μl |
| IL-3 | 10μg/ml | 10ng/ml | 10μl |
| EPO | 2U/μl | 2U/ml | 10μl |
| IGF-1 | 100μg/ml | 40ng/ml | 4μl |
| Dexamethasone\* | 1mM | 1μM | 10μl |

|  |  |  |
| --- | --- | --- |
| **Freezing Medium (10 ml)** | **Final conc** | **Volume** |
| KOSR | 90% v/v | 9ml |
| DMSO | 10% v/v | 1ml |

|  |  |  |  |
| --- | --- | --- | --- |
| **Differentiation Medium (500ml)-**  **Chemically Defined Media with Polyvinyl Alcohol (CDM-PVA)** | **Stock conc** | **Final conc** | **Volume** |
| IMDM |  |  | 250ml |
| F-12+ GlutaMAX-1 |  |  | 250ml |
| Concentrated Lipids |  |  | 5ml |
| 1-Thioglycerol | 97% |  | 20μl |
| Insulin | 10mg/ml | 7μg/ml | 350μl |
| Transferrin | 30mg/ml | 15μg/ml | 250μl |
| PVA\*\*\*\* | 5% w/v | 0.1% w/v | 10ml |
|  |  |  |  |

\*\*\*\*In order to obtain CDM-PVA media add 10ml of PVA stock [0.05g/ml] to the 500ml of CDM media and filter

|  |  |  |
| --- | --- | --- |
| **PVA stock [0.05g/ml]** | **Final conc** | **Volume/**  **Amount** |
| PVA (powder) | 0.05g/ml | 10g |
| Water for Embryo Transfer |  | 200ml |

Note: solubility of PVA is around [50mg/ml]**,** mix on the shaker using magnetic stirrer, set temperature to 100ºC and bring to boil

|  |  |  |  |
| --- | --- | --- | --- |
| **RPMI Medium (500ml)** | **Stock conc** | **Final conc** | **Volume** |
| RPMI 1640 +Glutamax |  |  | 500ml |
| B27 | 50x | 1x | 10ml |
| NEAA | 100x | 1% | 5ml |
| P/S (optional) | 100x | 1% | 5ml |

|  |  |  |
| --- | --- | --- |
| **Gelatine (500ml)** | **Final conc** | **Volume/**  **Amount** |
| Water for Embryo transfer |  | 500ml |
| Gelatine | 0.1% w/v | 0.5g |

Note:Mix, heat up to 60ºC and filter, store at room temperature

|  |  |  |
| --- | --- | --- |
| **Dispase [1mg/ml] (500ml)** | **Final conc** | **Volume/**  **Amount** |
| Advanced DMEM F12 |  | 500ml |
| Dispase | 0.1% w/v | 0.5g |

Note: Mix well, filter and store at 4ºC up to four weeks

|  |  |  |  |
| --- | --- | --- | --- |
| **Collagenase (500ml)** | **Stock conc** | **Final conc** | **Volume/**  **Amount** |
| Advanced DMEM F12 |  |  | 400ml |
| KOSR |  | 20% v/v | 100ml |
| L-Glutamine | 100x | 1% | 5ml |
| β-Mercaptoethanol | 98% | 0.007% | 3.5μl |
| Collagenase IV | powder | 0.1% w/v | 0.5g |

Note: Mix well, filter and store at 4ºC

|  |  |  |  |
| --- | --- | --- | --- |
| **H9 (500ml)-**  **Chemically Defined Media with BSA** | **Stock conc** | **Final conc** | **Volume** |
| IMDM |  |  | 250ml |
| F-12+ GlutaMAX-1 |  |  | 250ml |
| Concentrated Lipids |  |  | 5ml |
| 1-Thioglycerol |  |  | 20µl |
| Insulin |  |  | 350µl |
| Transferrin |  |  | 250µl |
| BSA |  | 0.5 % w/v | 2.5g |
| P/S (optional) |  | 1% | 5ml |

Add extra bFGF (1.5ml of 4ug/ml stock), Insulin (0.5ml), Activin (0.5ml)

|  |  |  |  |
| --- | --- | --- | --- |
| **Amanda’s media**  **(500ml)** | **Stock conc** | **Final conc** | **Volume** |
| DMEM/F12 |  |  | 500ml |
| Sodium Bicarbonate 7.5% solution |  |  | 3.6ml |
| L-Ascorbic Acid 2-Phosphate Sesquimagnes\*\* | 100x | 1% | 5ml |
| Insulin-Transferrin- Selenium | 100x | 2% | 10ml |
| Activin in house | 10ug/ml | 15ng/ml | 750ul |
| bFGF in house | 4ug/ml | 15ng/ml | 1875ul |
|  |  |  |  |
| P/S (optional) |  | 1% | 5ml |

\*\*In order to obtain 100x L-Ascorbic Acid 2-Phosphate Sesquimagnes dissolve 1g in 156ml Water for Embryo Transfer. Filter and freeze as 5ml aliquots

|  |  |  |  |
| --- | --- | --- | --- |
| **In-house E8 media (500ml)** | **Stock conc** | **Final conc** | **Volume** |
| DMEM/F12 |  |  | 500ml |
| Sodium Bicarbonate 7.5% solution |  |  | 3.6ml |
| L-Ascorbic Acid 2-Phosphate Sesquimagnes\*\* | 100x | 1% | 5ml |
| Insulin-Transferrin- Selenium | 100x | 2% | 10ml |
| bFGF in house | 4ug/ml | 25ng/ml | 3125ul |
| TGF-β1 | 1.74ug/ml | 1.74ng/ml | 500ul |
|  |  |  |  |
| P/S (optional) |  | 1% | 5ml |

\*\*In order to obtain 100x L-Ascorbic Acid 2-Phosphate Sesquimagnes dissolve 1g in 156ml Water for Embryo Transfer. Filter and freeze as 5ml aliquots

## To prepare 10mM SB solution reconstitute 10mg of SB in 2.38ml of DMSO, aliquot and store in -20ºC

## To prepare 0.1μg/μl Noggin solution reconstitute 50μg of Noggin in 500μl of PBS/0.01% BSA, aliquot and store at 4ºC

## To prepare 3mM CHIR99021 solution reconstitute 5mg of CHIR99021 in 3.58ml of DMSO, aliquot and store in -20ºC

## To prepare 10mM Y-27632 solution reconstitute 5mg of Y-27632 in 1.5ml of embryo transfer water, aliquot and store in -20ºC

## To prepare 10mM Ly 294002 solution reconstitute 5mg of Ly 294002 in 1.625 ml of DMSO, aliquot and store in -20ºC

**Required Resources:**

|  |
| --- |
| **Equipment** |
| Laminar flow cabinet |
| **Materials** |
| Filter system 0.22μm PES (Corning Ref.31097) |
| Filter tips |
| **Solutions and Reagents** |
| DMEM: (Life Technologies Ref. 11965-118) |
| Advanced DMEM/F12: (Invitrogen Ref.11330-057) |
| Defined Fetal Bovine Serum FBS: (Hyclone Ref. SH30070.01) |
| Water for Embryo Transfer (Sigma Ref. W1503) |
| Dimethyl Sulfoxide - DMSO (Sigma Ref. D-2650) |
| Fetal Bovine Serum - FBS (Invitrogen Ref.16000-044) |
| Knockout Serum Replacement - KOSR (Invitrogen Ref.10828-028) |
| L-Glutamine (Invitrogen Ref.25030-156) |
| MEM-Non Essential Aminoacids – MEM-NEAA (Invitrogen Ref.11140-050) |
| 2-Mercaptoethanol 98% (Sigma Ref. M3148-100M) |
| Penicillin/streptomycin - P/S (Invitrogen Ref.15140-155) |
| bFGF (Bio-Techne 233-FB - 5mg) |
| Gelatine (Sigma Ref.G1890-100G) |
| Collagenase IV (Invitrogen Ref. 17104019) |
| Dispase (Invitrogen Ref.17105-041) |
| IMDM (Life Technologies Ref. Nr 31765027) |
| Conc. Lipids (Life Technologies. Ref. Nr 11905031) |
| Thioglycerol MTG (Sigma Ref. Nr M6145-100ML) |
| Transferrin (Roche Ref. Nr 652202) |
| DMEM/F12 (Life Technologies Ref: 11330-032) |
| Sodium Bicarbonate 7.5% solution (Life Technologies Ref: 25080094) |
| L-Ascorbic Acid 2-Phosphate Sesquimagnes (Sigma Ref: A8960) |
| Insulin-Transferrin- Selenium (ITS-G) (100x) (Life Technologies Ref: 41400045) |
| Activin in house [10ug/ml] |
| Recombinant human TGF-β1 (Bio-Techne Ref: 240-B/CF) |
| StemSpan H3000 (StemCell Technologies Ref. 9850) |
| Recombinant human IL-3 (Invitrogen Ref. PHC0035) |
| Recombinant human IGF-1 (Miltenyi Ref. 130-093-885) |
| Recombinant human SCF (Miltenyi Ref. 130-096-692) |
| Recombinant human EPO (R&D Systems 287-TC-500) |
| Dexamethasone (Sigma Ref. D8893-1MG) |
| L-Ascorbic Acid (Sigma Ref. A4544-25G) |
| Ly 294002 (Promega Ref. 1201) |
| Y-27632 (ROCK) (Sigma Aldrich Ref. Y0503) |
| CHIR99021 (Selleck chemicals Ref. S1263) |
| SB 431542 (Tocris bioscience Ref. 614) |
| Noggin (R&D Ref. 33-44NG) |

**VII. References**

1. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Takahashi K, Yamanaka S Cell. 2006 Aug 25;126(4):663-76.

2. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Kazutoshi Takahashi, Koji Tanabe, Mari Ohnuki, Megumi Narita, Tomoko Ichisaka, Kiichiro Tomodaand Shinya Yamanaka. *Cell*, 30 November 2007, [Vol. 131, Issue 5](http://www.cell.com/issue?pii=S0092-8674(07)X0731-1), 861-872

3. Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells [Junying Yu](http://www.sciencemag.org/search?author1=Junying+Yu&sortspec=date&submit=Submit), [Maxim A. Vodyanik](http://www.sciencemag.org/search?author1=Maxim+A.+Vodyanik&sortspec=date&submit=Submit), [Kim Smuga-Otto](http://www.sciencemag.org/search?author1=Kim+Smuga-Otto&sortspec=date&submit=Submit), [Jessica Antosiewicz-Bourget](http://www.sciencemag.org/search?author1=Jessica+Antosiewicz-Bourget&sortspec=date&submit=Submit), [Jennifer L. Frane](http://www.sciencemag.org/search?author1=Jennifer+L.+Frane&sortspec=date&submit=Submit), [Shulan Tian](http://www.sciencemag.org/search?author1=Shulan+Tian&sortspec=date&submit=Submit), [Jeff Nie](http://www.sciencemag.org/search?author1=Jeff+Nie&sortspec=date&submit=Submit), [Gudrun A. Jonsdottir](http://www.sciencemag.org/search?author1=Gudrun+A.+Jonsdottir&sortspec=date&submit=Submit), [Victor Ruotti](http://www.sciencemag.org/search?author1=Victor+Ruotti&sortspec=date&submit=Submit), [Ron Stewart](http://www.sciencemag.org/search?author1=Ron+Stewart&sortspec=date&submit=Submit), [Igor I. Slukvin](http://www.sciencemag.org/search?author1=Igor+I.+Slukvin&sortspec=date&submit=Submit), [James A. Thomson](http://www.sciencemag.org/search?author1=James+A.+Thomson&sortspec=date&submit=Submit). *Science,* 21 December 2007: Vol. 318 no. 5858

4. A more efficient method to generate integration-free human iPS cells. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa, M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. Nat Methods. 2011 May;8(5):409-12

5. [A practical and efficient cellular substrate for the generation of induced pluripotent stem cells from adults: blood-derived endothelial progenitor cells.](http://www.ncbi.nlm.nih.gov/pubmed/23283547) Geti I, Ormiston ML, Rouhani F, Toshner M, Movassagh M, Nichols J, Mansfield W, Southwood M, Bradley A, Rana AA, Vallier L, Morrell NW. *Stem Cells Transl Med.* 2012 Dec;1(12):855-65.

6. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. [Chou BK](http://www.ncbi.nlm.nih.gov/pubmed?term=Chou%20BK%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Mali P](http://www.ncbi.nlm.nih.gov/pubmed?term=Mali%20P%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Huang X](http://www.ncbi.nlm.nih.gov/pubmed?term=Huang%20X%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Ye Z](http://www.ncbi.nlm.nih.gov/pubmed?term=Ye%20Z%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Dowey SN](http://www.ncbi.nlm.nih.gov/pubmed?term=Dowey%20SN%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Resar LM](http://www.ncbi.nlm.nih.gov/pubmed?term=Resar%20LM%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Zou C](http://www.ncbi.nlm.nih.gov/pubmed?term=Zou%20C%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Zhang YA](http://www.ncbi.nlm.nih.gov/pubmed?term=Zhang%20YA%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Tong J](http://www.ncbi.nlm.nih.gov/pubmed?term=Tong%20J%5BAuthor%5D&cauthor=true&cauthor_uid=21243013), [Cheng L](http://www.ncbi.nlm.nih.gov/pubmed?term=Cheng%20L%5BAuthor%5D&cauthor=true&cauthor_uid=21243013). *Cell Research* 2011 Mar;21(3):518-29

7. The majority of the in vitro erythroid expansion potential resides in CD34(-)cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. [van den Akker E](http://www.ncbi.nlm.nih.gov/pubmed?term=van%20den%20Akker%20E%5BAuthor%5D&cauthor=true&cauthor_uid=20378567), [Satchwell TJ](http://www.ncbi.nlm.nih.gov/pubmed?term=Satchwell%20TJ%5BAuthor%5D&cauthor=true&cauthor_uid=20378567), [Pellegrin S](http://www.ncbi.nlm.nih.gov/pubmed?term=Pellegrin%20S%5BAuthor%5D&cauthor=true&cauthor_uid=20378567), [Daniels G](http://www.ncbi.nlm.nih.gov/pubmed?term=Daniels%20G%5BAuthor%5D&cauthor=true&cauthor_uid=20378567), [Toye AM](http://www.ncbi.nlm.nih.gov/pubmed?term=Toye%20AM%5BAuthor%5D&cauthor=true&cauthor_uid=20378567). *Haematologica* 2010 Sep; 95(9):1594-8.

8. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. [Leberbauer C](http://www.ncbi.nlm.nih.gov/pubmed?term=Leberbauer%20C%5BAuthor%5D&cauthor=true&cauthor_uid=15358620), [Boulmé F](http://www.ncbi.nlm.nih.gov/pubmed?term=Boulm%C3%A9%20F%5BAuthor%5D&cauthor=true&cauthor_uid=15358620), [Unfried G](http://www.ncbi.nlm.nih.gov/pubmed?term=Unfried%20G%5BAuthor%5D&cauthor=true&cauthor_uid=15358620), [Huber J](http://www.ncbi.nlm.nih.gov/pubmed?term=Huber%20J%5BAuthor%5D&cauthor=true&cauthor_uid=15358620), [Beug H](http://www.ncbi.nlm.nih.gov/pubmed?term=Beug%20H%5BAuthor%5D&cauthor=true&cauthor_uid=15358620), [Müllner EW](http://www.ncbi.nlm.nih.gov/pubmed?term=M%C3%BCllner%20EW%5BAuthor%5D&cauthor=true&cauthor_uid=15358620). *Blood* 2005 Jan 1; 105(1):85-94

9. Generation of human induced pluripotent stem cells from urine samples. [Zhou T](http://www.ncbi.nlm.nih.gov/pubmed?term=Zhou%20T%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Benda C](http://www.ncbi.nlm.nih.gov/pubmed?term=Benda%20C%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Dunzinger S](http://www.ncbi.nlm.nih.gov/pubmed?term=Dunzinger%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Huang Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Huang%20Y%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Ho JC](http://www.ncbi.nlm.nih.gov/pubmed?term=Ho%20JC%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Yang J](http://www.ncbi.nlm.nih.gov/pubmed?term=Yang%20J%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Wang Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Wang%20Y%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Zhang Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Zhang%20Y%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Zhuang Q](http://www.ncbi.nlm.nih.gov/pubmed?term=Zhuang%20Q%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Li Y](http://www.ncbi.nlm.nih.gov/pubmed?term=Li%20Y%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Bao X](http://www.ncbi.nlm.nih.gov/pubmed?term=Bao%20X%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Tse HF](http://www.ncbi.nlm.nih.gov/pubmed?term=Tse%20HF%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Grillari J](http://www.ncbi.nlm.nih.gov/pubmed?term=Grillari%20J%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Grillari-Voglauer R](http://www.ncbi.nlm.nih.gov/pubmed?term=Grillari-Voglauer%20R%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Pei D](http://www.ncbi.nlm.nih.gov/pubmed?term=Pei%20D%5BAuthor%5D&cauthor=true&cauthor_uid=23138349), [Esteban MA](http://www.ncbi.nlm.nih.gov/pubmed?term=Esteban%20MA%5BAuthor%5D&cauthor=true&cauthor_uid=23138349). *Nat Protoc.* 2012 Dec; 7(12):2080-9. doi: 10.1038/nprot.2012.115

10. [Activin/Nodal signaling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors.](http://www.ncbi.nlm.nih.gov/pubmed/21630377) Brown S, Teo A, Pauklin S, Hannan N, Cho CH, Lim B, Vardy L, Dunn NR, Trotter M, Pedersen R, Vallier L. *Stem Cells*. 2011 Aug; 29(8):1176-85.

11. [Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells.](http://www.ncbi.nlm.nih.gov/pubmed/19688839) Vallier L, Touboul T, Brown S, Cho C, Bilican B, Alexander M, Cedervall J, Chandran S, Ahrlund-Richter L, Weber A, Pedersen RA. *Stem Cells*. 2009 Nov; 27(11):2655-66

12. [Differentiation of human embryonic stem cells in adherent and in chemically defined culture conditions.](http://www.ncbi.nlm.nih.gov/pubmed/18770639) Vallier L, Pedersen R. *Curr Protoc Stem Cell Biol*. 2008 Mar; Chapter 1:Unit 1D.4.1-1D.4.7

13. [Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm.](http://www.ncbi.nlm.nih.gov/pubmed/18022151) Smith JR, Vallier L, Lupo G, Alexander M, Harris WA, Pedersen RA. *Dev Biol*. 2008 Jan 1;313(1):107-17

14. Comparative study of mouse and human feeder cells for human embryonic stem cells. Eiselleova L(1), Peterkova I, Neradil J, Slaninova I, Hampl A, Dvorak P. Int J *Dev Biol.* 2008;52(4):353-63. doi: 10.1387/ijdb.082590le.

15. Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells.

[Meilinger D](http://www.ncbi.nlm.nih.gov/pubmed?term=Meilinger%20D%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Fellinger K](http://www.ncbi.nlm.nih.gov/pubmed?term=Fellinger%20K%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Bultmann S](http://www.ncbi.nlm.nih.gov/pubmed?term=Bultmann%20S%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Rothbauer U](http://www.ncbi.nlm.nih.gov/pubmed?term=Rothbauer%20U%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Bonapace IM](http://www.ncbi.nlm.nih.gov/pubmed?term=Bonapace%20IM%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Klinkert WE](http://www.ncbi.nlm.nih.gov/pubmed?term=Klinkert%20WE%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Spada F](http://www.ncbi.nlm.nih.gov/pubmed?term=Spada%20F%5BAuthor%5D&cauthor=true&cauthor_uid=19798101), [Leonhardt H](http://www.ncbi.nlm.nih.gov/pubmed?term=Leonhardt%20H%5BAuthor%5D&cauthor=true&cauthor_uid=19798101). *EMBO Rep.* 2009 Nov;10 (11):1259-64

16. Beers J, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nat Protoc.* 2012 Nov;7(11):2029-40. PubMed PMID:23099485

17. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R.

*Nat Biotechnol*. 2009 Sep;27(9):851-7. PMID: 19680244

18. [A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.](http://www.ncbi.nlm.nih.gov/pubmed/22745249) Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. Science. 2012 Aug 17;337(6096):816-21. doi: 10.1126/science.1225829. PMID: 22745249

19. Genome engineering using the CRISPR-Cas9 system. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F *Nat Protoc.* 2013 Nov;8(11):2281-308. doi: 10.1038/nprot.2013.143