



## CRISPR/Cas9 Genome Editing of Human-Induced Pluripotent Stem Cells Followed by Granulocytic Differentiation

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### Abstract

Research on patient-derived induced pluripotent stem cells (iPSCs) could immensely benefit from the implementation of CRISPR/Cas9 genome editing of iPSCs, creating unique opportunities such as the establishment of isogenic iPSC lines for disease modeling or personalized patient-specific drug screenings. Here we describe a stepwise protocol of safe, efficient, and selection-free CRISPR/Cas9-mediated gene correction or knockout in human iPSCs followed by 3D spin-embryoid body (EB)-based hematopoietic/neutrophilic iPSC-differentiation.

**Key words** CRISPR/Cas9, Human-induced pluripotent stem cells, Genome editing, Ribonucleoprotein, iPSC differentiation, Hematopoietic, and neutrophilic differentiation of iPSCs

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## 1 Introduction

iPSC technology is a powerful tool for disease modeling, patient-specific drug screening, and paving the way for personalized medicine [1, 2]. Patient-derived iPSC cells can be used as an excellent tool to model the disease and reveal the key driving pathomechanisms behind it. An important challenge of iPSC technology is the high degree of variability between individual iPSC lines that are mainly due to differences in genetic background and reprogramming history [3]. Therefore, to have an optimal comparison in studies using iPSC, especially disease modeling, the role of the isogenic iPSC lines is indispensable. Combination of CRISPR/Cas9-based gene correction or knockout in human iPSCs (hiPSCs) derived from patients suffering from inherited or acquired hematopoietic disease with subsequent EB-based in vitro hematopoietic

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differentiation is a valuable tool to delineate the role of the gene of interest or gene mutations on hematopoietic differentiation in a fast, efficient, and reproducible manner [4–6].

In this chapter, we first explain in detail our CRISPR/Cas9 gene-editing strategy of iPSCs which is safe, as no viral vectors or plasmids are used, efficient, and selection free. We next describe our 3D spin-embryoid body (EB)-based iPSCs-differentiation protocol, which has many advantages over standard 2D iPSCs-differentiation protocols. The main advantage is the high amounts of HSPCs and mature granulocytes derived from iPSCs (more than  $1 \times 10^6$  cells/10 EBs) and improved functionality of mature cells [4]. Another advantage is that the present protocol is based on the use of STEMdiff APEL medium, a fully defined, serum-free, and animal component free-differentiation medium which is highly versatile and allows the differentiation of cells to multiple hematopoietic lineages [7, 8]. By adapting variable cytokine combinations, our strategy allows efficient generation of not only a high number of CD34<sup>+</sup>/CD45<sup>+</sup> cells and mature granulocytes but also monocytes or megakaryocytes. Furthermore, the current protocol displays high reproducibility between different experiments.

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## 2 Materials

The reagents describing below have been successfully used by our group, but there are other commercially available alternatives that can be used and optimized.

### 2.1 Transfection and Electroporation of iPSCs with Cas9 Ribonucleoprotein (RNP)

1. Guide RNAs for target sequence ordered as lyophilized single-guide RNA with or without 2'-O-methyl 3'phosphorothioate (Integrated DNA Technologies).
2. Alt-R S.p Cas9 Nuclease V3 or Alt-R S.p HiFi Cas9 Nuclease V3 (Integrated DNA Technologies).
3. Ultramer DNA oligos, 4 nm synthesis scale, and standard desalting as purification method (Integrated DNA Technologies).
4. *TransIT*-X2 transfection reagent (Mirus, Madison, USA).
5. Amaxa 4D-Nucleofector system and P3 primary Cell 4D-Nucleofector Kit (Lonza).

### 2.2 iPSCs Maintenance and EB-Based Myeloid Differentiation

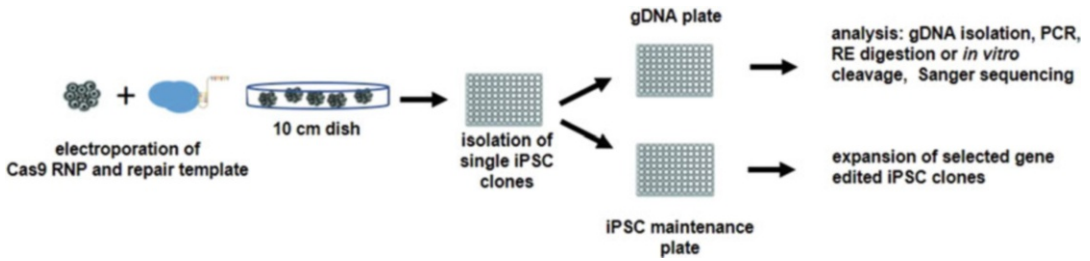
1. StemFlex medium, RevitaCell supplement, and Geltrex LDEV-Free reduced growth factor basement membrane matrix (ThermoFisher Scientific).
2. STEMdiff APEL 2 Medium (Stem Cell Technologies).
3. ROCK inhibitor, FGF2, BMP4, VEGF, SCF, IL3, and G-CSF.

### 3 Methods

#### 3.1 Design, Testing, and Validation of Guide RNA

1. Design guide RNAs using guide RNA designing web tools (e.g., CCTOP, Centre for Organismal Studies Heidelberg):
  - (a) For gene-correction purpose, select 4–6 guide RNAs in a window of 40 nt distance from the desired mutation site with good off-target activity (*see Note 1*).

The optimal window is 10 nt distance. Rank the guide RNAs based on the off-target activity profile.
  - (b) For gene knockout purpose, select 4–6 guide RNAs in the coding sequence of the first or second exon of the gene of choice. Consider all possible transcripts and isoforms for a successful knockout.
2. Cross check the guide RNAs list in the second guide RNA designing website (e.g., DESKGEN).
3. Order the list of guide RNAs as crRNA or sgRNA via provider of choice (e.g., Integrated DNA Technologies, Coralville, USA).
4. Upon receiving the guide RNAs, spin them down briefly and resuspend to the final concentration of 100  $\mu\text{M}$ .
5. For validation of guide RNAs, seed  $10^5$  HEK 293T cells 1 day before transfection in 12-well plate.
6. On the day of transfection, assemble 0.24  $\mu\text{L}$  from each single-guide RNA (100  $\mu\text{M}$  Stock, 24 nM final concentration) with 0.2  $\mu\text{L}$  Cas9 protein (62  $\mu\text{M}$  Stock, 12 nM final concentration) in a sterile tube.
7. Add 100  $\mu\text{L}$  of OptiMEM I Reduced Serum Medium to each tube and mix gently.
8. Add 2  $\mu\text{L}$  *TransIT-X2* transfection reagent (*see Note 2*) to each tube, mix gently, and incubate at room temperature for 20 min.
9. Add the *TransIT-X2*: RNP complexes dropwise to the different areas of the well. Gently rock the plate forth and back and incubate at 37 °C for 48 h.
10. Harvest cells for isolation of genomic DNA and amplify the target genomic area by PCR. Upon successful PCR, send PCR products for Sanger sequencing.
11. Quantify the on-target efficiency of guide RNAs by Tideor ICE web tools [9, 10] and re-rank the guide RNA list based on highest on-target and lowest off-target activity.
12. For gene correction purpose, design the repair template as single-stranded oligonucleotide (ssODN) with homology arms of 50–90 nt surrounding the mutation site. Introduce a silent mutation at the PAM site to protect edited sequence from re-cutting by Cas9 after gene correction. Synthesize or order ssODN as 4 nmol Ultramer DNA oligonucleotide.



**Fig. 1** Scheme of CRISPR/Cas9-based genome editing of human iPSCs

**3.2 Electroporation of iPSCs with Cas9 Ribonucleoprotein (RNP)**

Scheme of CRISPR/Cas9-based genome editing of human iPSCs is shown in Fig. 1.

1. Use the nucleofection protocol for the Amaxa 4D Nucleofection system with P3 Primary Cell 4D-Nucleofector X Kit.
2. For the electroporation of  $10^6$  cells, assemble 3.3  $\mu$ L sgRNA and 15  $\mu$ g Cas9 protein at room temperature for 30 min.
3. For detaching iPSCs, remove the medium and add Accutase, and then incubate until the cells start to detach (for approximately 3–5 min). Keep the number of pipetting to a minimum.
4. Wash cells two times with PBS prior to nucleofection to remove all traces of nucleases present in the culture medium. Centrifuge the cells at  $300 \times g$  for 5 min.
5. Remove the supernatant after centrifugation. For successful electroporation, it is important to completely remove the supernatant.
6. Prepare the nucleofection buffer according to the instructions of the P3 Primary Cell 4D-Nucleofector X Kit. Add ssODN and RNP mix to the nucleofection buffer.
7. Resuspend the cell pellet in the nucleofection buffer-RNP mix and transfer iPSCs suspension to the electroporation cuvette. Try to avoid any air bubble formation.
8. Place the cuvette in the Amaxa 4D Nucleofector and choose the CA-137 program.
9. After electroporation, incubate the cells in the cuvette for 10 min at room temperature.
10. Take up the cells gently in pre-warmed StemFlex medium supplemented with RevitaCell and transfer them to the Geltrex-coated 6-well plate.

**3.3 Clonal Isolation and 96-Well Plate Culture of iPSCs**

1. About 48 h post-electroporation, remove the medium, wash two times with PBS, and add 2 mL Accutase; incubate until the cells start to detach. It is important to have single cells at this step. Transfer the cells in 15 mL tube, centrifuge down at

$300 \times g$  for 5 min, discard the supernatant, and resuspend cells in Stemflex medium supplemented with RevitaCell.

2. Count cells.
3. Transfer 5000 iPSCs (*see Note 3*) to 10 mL Stemflex medium supplemented with RevitaCell, mix with 10 mL pipette few times, and transfer to the Geltrex-coated 10 cm dish. Rock forth and back. Put the dish back into the incubator.
4. Incubate the 10 cm dish for 7 days. Change the medium every 24 h with Stemflex medium without RevitaCell.
5. Coat the flat bottom 96-well plate with 100  $\mu$ L Geltrex for at least 2 h at room temperature. Remove the Geltrex from the wells using a multichannel pump and add 100  $\mu$ L Stemflex medium supplemented with RevitaCell to each well using a multichannel pipette.
6. After 7 days (*see Note 4*), wash the 10 cm dish gently with PBS. Add 10 mL PBS to the dish and incubate at room temperature for 5 min.
7. Put the 10 cm dish under the microscope (*see Note 5*) and using a 100  $\mu$ L pipette that adjusted to 20  $\mu$ L, gently scratch, pick, and transfer single iPS clone to a well of 96-well plate. Pipette few times to dissociate the clone. Pick 96 clones (*see Note 6*).
8. Put the 96-well plate containing 96 single iPSC clones to the incubator for three to four for days.
9. Coat at least 2 96-well plates (use flat bottom plates) with Geltrex (*see Note 7*) before use, remove the coating, and add 100  $\mu$ L StemFlex Media supplemented with RevitaCell.
10. After 3–4 days, remove the media from 96-well plate using a multichannel pump (*see Note 8*), add 40  $\mu$ L Accutase diluted 1:3 with PBS to each well, and incubate at 37 °C for 30 min (*see Note 9*) until the iPSCs start to dissociate.
11. Prepare a sterile reservoir filled with StemFlex Media supplemented with RevitaCell. Using a multichannel pipette, add 60  $\mu$ L media to each well, pipette 4–5 times, and transfer 60  $\mu$ L iPSCs containing media to the exact same position in a new Geltrex-coated 96-well plate. This plate will be used later for genomic DNA isolation (gDNA plate). Using the same tips, transfer the remaining 35  $\mu$ L iPSCs containing media to the exact same position in the second Geltrex-coated 96-well plate. This plate will be stored in the incubator (maintenance plate).

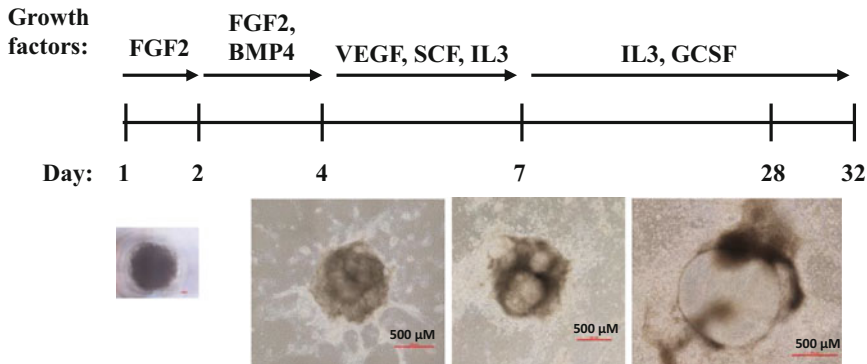
### 3.4 Screening for Gene-Corrected iPSC Clones and Validation of Gene Correction

1. Using a multichannel pump, remove the media from all wells of the gDNA plate and wash each well two times with PBS.
2. Add 30  $\mu\text{L}$  of QuickExtract DNA Extraction solution.
3. Heat the 96-well plate at 65  $^{\circ}\text{C}$  for 6 min and 98  $^{\circ}\text{C}$  for 2 min. The 96-well plate can be directly used as a template DNA in PCR reaction or stored at  $-20^{\circ}\text{C}$ .
4. To amplify the target region, prepare a PCR master mix, pipette it to PCR-specific 96-well plate, and add 2  $\mu\text{L}$  of gDNA prepared in **step 11** of Subheading 3.3.
5. Perform the PCR reaction.
6. For gene correction purpose, use restriction endonucleases enzyme that produces a different pattern of digestion after gene correction (e.g., <http://insilico.ehu.es>) and perform restriction enzyme digestion of each clone.
7. For gene knockout purpose, instead of endonuclease restriction enzyme digestion, perform in vitro Cas9 cleavage using the same sgRNA that has been used for the knockout as following:
  - (a) Prepare sgRNA at a concentration of 10  $\mu\text{M}$  in a sterile microtube by diluting the sgRNA in nuclease-free buffer.
  - (b) Prepare 1  $\mu\text{M}$  RNP complex by adding 1.6  $\mu\text{L}$  Cas9 enzyme (62  $\mu\text{M}$  stock) and 88.4  $\mu\text{L}$  PBS to the microtube containing 10  $\mu\text{M}$  sgRNA.
  - (c) Incubate at room temperature for 15 min.
  - (d) Perform the in vitro digestion reaction by adding 1  $\mu\text{L}$  of 1  $\mu\text{M}$  Cas9 RNP, 1  $\mu\text{L}$  IDT nuclease-free buffer, and 3  $\mu\text{L}$  of unpurified PCR reaction from **step 5** of Subheading 3.4.
  - (e) Incubate the reaction at 37  $^{\circ}\text{C}$  for 1 h.  
 PCR product of knockout candidate iPSC clones should not be digested by Cas9 RNP as the target sequence is already modified.
8. Run the reaction from **steps 6** or **7** on 1% agarose gel and perform Sanger sequencing of the PCR products with a preferred pattern in order to validate gene correction or knock out in the selected iPSC clones.

### 3.5 Hematopoietic and Neutrophilic Differentiation of iPSCs Via Spin-EBs

The scheme of EB-based neutrophilic differentiation is shown in Fig. 2. A list of cytokines used for EB-differentiation is shown in Table 1.

1. Two days before EB formation, seed  $4 \times 10^5$  mitotically inactivated SNL feeder cells on a 3.5  $\text{cm}^2$  cell culture dish.



**Fig. 2** Scheme of EB-based neutrophilic differentiation of human iPSCs

**Table 1**

**List of cytokines used for EB-based hematopoietic iPSCs differentiation**

Cytokine	Stock concentration	Final concentration	Function	Company
BMP4	20 $\mu\text{g}/\text{mL}$	20 $\text{ng}/\text{mL}$	Mesoderm induction	R&D
bFGF (FGF2)	100 $\mu\text{g}/\text{mL}$	20 $\text{ng}/\text{mL}$	Mesoderm differentiation	Peprotech
VEGF	40 $\mu\text{g}/\text{mL}$	40 $\text{ng}/\text{mL}$	Angiogenesis	R&D
SCF	100 $\mu\text{g}/\text{mL}$	50 $\text{ng}/\text{mL}$	Stem cell factor	Peprotech
IL-3	50 $\mu\text{g}/\text{mL}$	50 $\text{ng}/\text{mL}$	Myeloid differentiation	Peprotech
ROCK inhibitor (Y-27632dihydrochloride)	10 $\text{mM}$	10 $\text{nM}$	Inhibition of rho-kinase	Tocris
G-CSF	100 $\mu\text{g}/\text{mL}$	50 $\text{ng}/\text{mL}$	Granulocytic differentiation	Neupogen (Filgrastim)

2. One day before EB formation, seed approximately 50–70% confluent feeder-free iPSCs from one well of a 6-well plate to a 3.5  $\text{cm}^2$  dish with SNL feeder cells in iPSCs maintenance medium supplemented with 20  $\text{ng}/\text{mL}$  bFGF and 10  $\text{nM}$  ROCK inhibitor (*see Note 10*).
3. On the day of EB formation (day 1), precool centrifuge to 4  $^{\circ}\text{C}$ , as it will increase efficiency of compact EB formation.
4. Prepare APEL medium for EB formation. From one 3.5  $\text{cm}^2$  dish, you can produce up to 30 EBs. For 30 EBs, 3 mL of APEL medium supplemented with ROCK (10  $\text{nM}$ ) and bFGF (20  $\text{ng}/\text{mL}$ ) are needed.

5. Remove iPSCs maintenance medium from iPSCs and wash once with 1 mL PBS.
6. Add 1 mL PBS/0.02% EDTA to the 3.5 cm<sup>2</sup> dish with iPSCs for 7 min, remove PBS/EDTA solution, and detach iPSCs in prepared APEL medium.
7. Collect iPSCs in a falcon tube and distribute 100  $\mu$ L per well of iPSCs suspension to a 96-well plate with conical bottom.
8. Centrifuge plate for 5 min at  $435 \times g$  and 4 °C.
9. Incubate the plate for 24 h at 37 °C.
10. On day 2, add BMP4 in 10  $\mu$ L APEL medium per well for a final concentration of 20 ng/mL.
11. On day 3, prepare Geltrex-coated 6-well plates.
12. On day 4, plate EBs on Geltrex-coated wells (10 EBs/well) in 2 mL per well of APEL medium supplemented with VEGF (40 ng/mL), SCF (50 ng/mL), and IL-3 (50 ng/mL).
13. On day 7, change medium to APEL medium supplemented with IL-3 (50 ng/mL) and G-CSF (50 ng/mL). Hematopoietic cells start to appear on day 8.
14. On day 10, add 2 mL of APEL medium supplemented with IL-3 (50 ng/mL) and G-CSF (50 ng/mL).
15. On day 14, take floating cells for further analysis and exchange medium to the fresh APEL medium containing G-CSF (50 ng/mL) and IL-3 (50 ng/mL), 2 mL per well. At this stage, floating cells are mainly CD34<sup>+</sup> hematopoietic stem and progenitor cells that can be used for various downstream analyses such as colony-forming unit (CFU) assay, morphological analysis, or flow cytometry analysis of early hematopoietic markers of hematopoietic stem and progenitor cells (HSPCs) (*see* Subheading 3.6).
16. From day 14, flow cytometry analysis for hematopoietic stem cells (*see* Subheading 3.6) and mature myeloid cells (*see* Subheading 3.7) as well as cell counts of floating cells should be performed every 3–4 days to follow the myeloid maturation process. Floating cells mature stepwise from CD34<sup>+</sup> cells on day 14 to mature CD15<sup>+</sup>CD16<sup>+</sup> neutrophils on day 28–32. The culture medium from day 14 to day 32 consists of APEL medium supplemented with IL-3 (50 ng/mL) and G-CSF (50 ng/mL), 2 mL medium per well.
17. On day 28 and day 32, mature neutrophils are analyzed using multicolor flow cytometry panel for mature myeloid cells (*see* Subheading 3.7 and **Note 11**).



**Table 2**  
**A list of antibodies used for the early panel FACS analysis**

Antibody	Conjugation	Company	Volume in $\mu\text{L}$
CD33	BV421	BioLegend	2
CD45	BV510	BioLegend	2
KDR	AF647	BioLegend	2
CD41a	FITC	BD	0.5
CD235	FITC	BD	0.5
CD34	PE-Cy7	BD	5
CD43	PE	BD	5
7AAD		BD	2

**3.6 Multicolor Flow Cytometry Analysis of the Early Stages of Hematopoietic Differentiation of iPSCs**

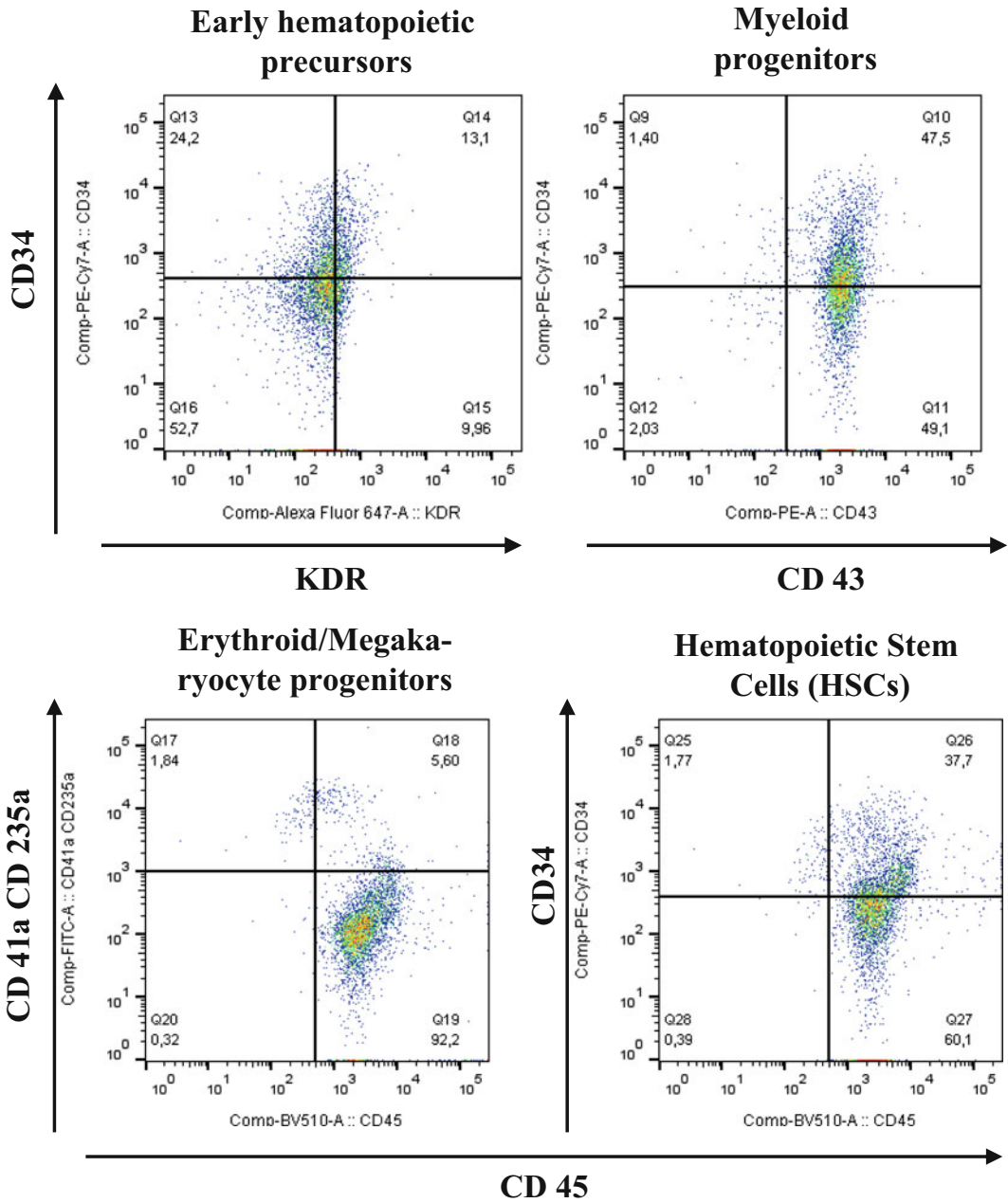
Multicolor flow cytometry analysis for the evaluation of HSPCs in suspension cell fraction of EBs-based hematopoietic differentiation culture of iPSCs is performed on day 14 of culture.

1. Harvest  $6 \times 10^4$  cells in 1 mL of ice-cold PBS and centrifuge for 5 min at  $1200 \times g$ . Usually,  $3 \times 10^4$  cells are used for staining with specific antibody and  $3 \times 10^4$  cells for unstained control.
2. Resuspend cells in 150  $\mu\text{L}$  ice-cold FACS buffer.
3. Add 75  $\mu\text{L}$  of cell suspension into each FACS tube on ice.
4. Add antibody as described in Table 2.
5. Incubate for 15 min on ice and protected from light.
6. Add 500  $\mu\text{L}$  ice-cold FACS buffer to wash stained cells.
7. Centrifuge 5 min at  $435 \times g$  and  $+4^\circ\text{C}$ , and then discard supernatant.
8. Resuspend cell pellet in 100  $\mu\text{L}$  ice-cold FACS buffer.
9. Evaluate stained cells on BD FACS Canto II. Representative images of flow cytometry are shown in Fig. 3.

**3.7 Multicolor Flow Cytometry Analysis of the Late Stages of Myeloid Differentiation of iPSCs**

Multicolor flow cytometry analysis of floating cells derived from EBs-based iPSCs culture in order to evaluate surface expression of myeloid markers is performed on day 28–32.

1. Harvest  $6 \times 10^4$  cells in 1 mL of ice-cold PBS and centrifuge for 5 min at  $12,000 \times g$ . In addition,  $3 \times 10^4$  cells are used for staining with specific antibody and  $3 \times 10^4$  cells for unstained control.
2. Resuspend cells in 150  $\mu\text{L}$  ice-cold FACS buffer.

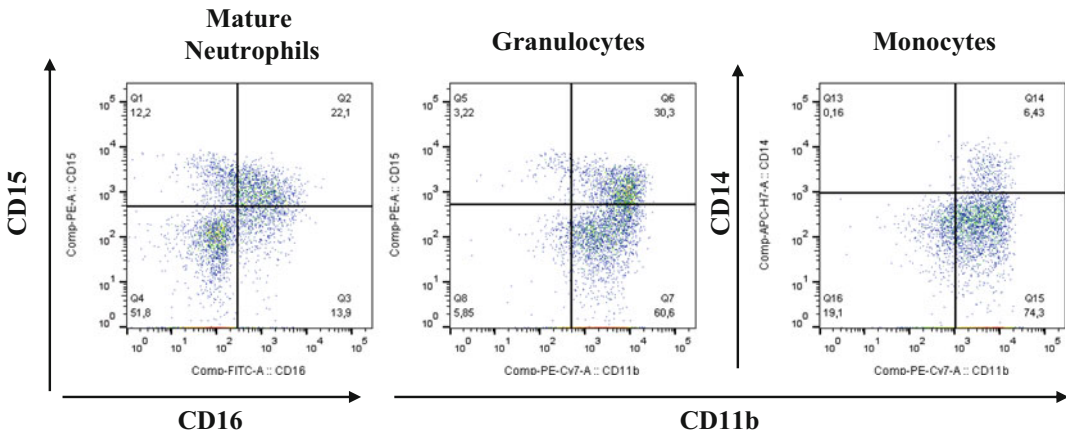


**Fig. 3** Representative images of flow cytometry analysis of early stages of EB-based hematopoietic differentiation of healthy donor-derived iPSCs

3. Add 75  $\mu$ L of cell suspension into each FACS tube to a final volume of 100  $\mu$ L.
4. Add FACS antibodies as described in Table 3.
5. Incubate for 15 min on ice and protect from light.

**Table 3**  
**A list of antibodies used for the late panel FACS analysis**

Antibody	Conjugation	Company	Volume in $\mu\text{L}$
CD33	BV421	BioLegend	2
CD45	BV510	BioLegend	2
CD15	PE	BD	5
CD16	FITC	BD	5
CD11b	PE-Cy7	BioLegend	2
CD14	APC-H7	BD	2
CD177	APC	BioLegend	0.5
7AAD		BD	2



**Fig. 4** Representative images of flow cytometry analysis of late stages of myeloid EB-based differentiation of healthy donor-derived iPSCs

6. Add 500  $\mu\text{L}$  of ice-cold FACS buffer to wash stained cells.
7. Centrifuge 5 min at  $435 \times g$  and  $+4^\circ\text{C}$ , and then discard supernatant.
8. Resuspend cell pellet in 100  $\mu\text{L}$  of ice-cold FACS buffer.
9. Measure stained cells on BD FACS Canto II. Representative images of flow cytometry are shown in Fig. 4.

## 4 Notes

1. Most of the existing off-target prediction methods calculate scores based on the positions of the mismatches to the guide sequence [11]; a good off-target profile is a probability of

having no unintended cut with up to two mismatches (0:0, 1:0, 2:0). Sanger Institute webtool (<https://www.sanger.ac.uk/htgt/wge/>) can be used to assess the off-target profiles of selected sgRNAs.

2. Any RNP-compatible transfection reagent can be used at this step.
3. Cell number should be optimized between 5000 and 50,000 cells for each iPSC line.
4. The incubation time could be up to 14 days due to variations in different iPSC lines.
5. Keep the condition sterile and use a face mask. The probability of cell contamination at this step is high.
6. The number of colonies for picking can be adjusted based on the estimation of the gene modification efficiency in the total cell population.
7. The Geltrex-coated 96-well plates can be stored at 4 °C for up to 7 days.
8. Wash the tips in PBS after each use, to minimize the possibility of clonal cross-contamination.
9. This step could take up to 1 h in case of the high density of iPSC clones. Because diluted Accutase is used at this step, there will be no harm if longer incubation time is needed, but pipetting iPSC clones to accelerate the detachment could kill the cells.
10. To start EB formation, iPSCs should be more than 80% confluent. In this case, no feeder depletion is needed.
11. After day 32 of culture, the number of floating cells drops heavily.

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