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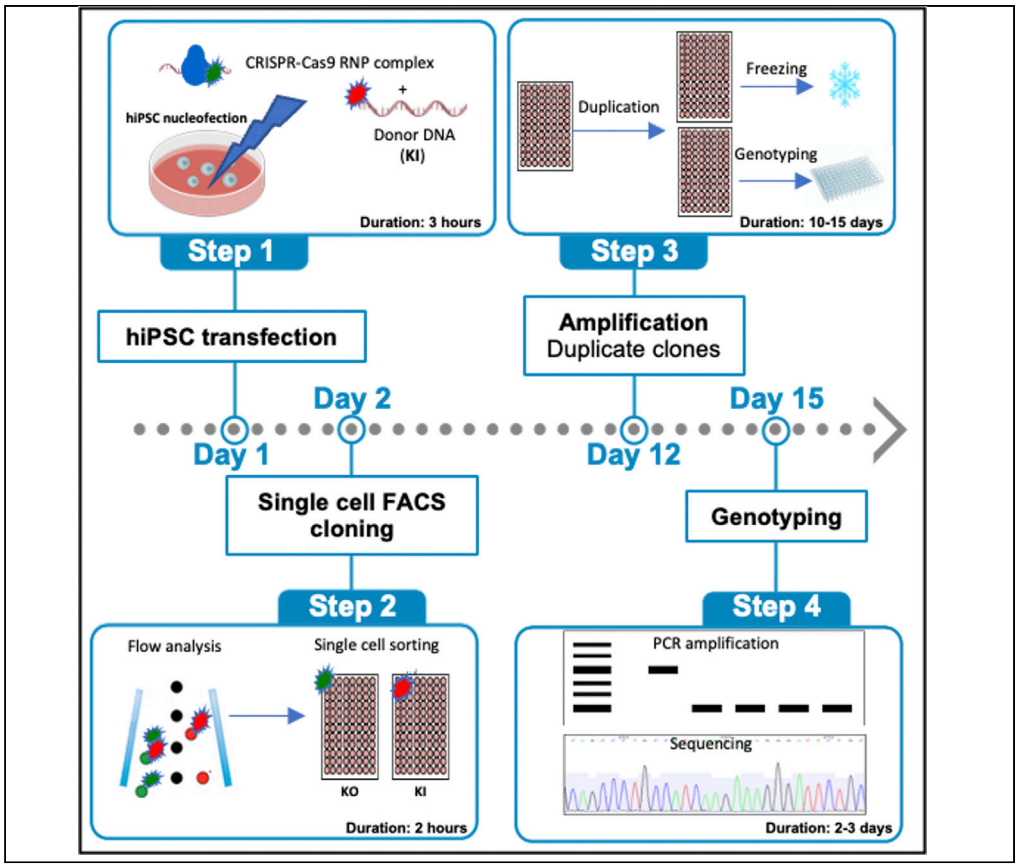
FACS-assisted CRISPR-Cas9 genome editing of human induced pluripotent stem cells



This manuscript proposes an efficient and reproducible protocol for the generation of genetically modified human induced pluripotent stem cells (hiPSCs) by genome editing using CRISPR-Cas9 technology. Here, we describe the experimental strategy for generating knockout (KO) and knockin (KI) clonal populations of hiPSCs using single-cell sorting by flow cytometry. We efficiently achieved up to 15 kb deletions, molecular tag insertions, and single-nucleotide editing in hiPSCs. We emphasize the efficacy of this approach in terms of cell culture time.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generation of knockout and knockin edits in hiPSCs using the CRISPR-Cas9 RNP system

FACS-assisted genome editing of hiPSCs

An optimized approach for culturing and genotyping hiPSC clones

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FACS-assisted CRISPR-Cas9 genome editing of human induced pluripotent stem cells

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SUMMARY

This manuscript proposes an efficient and reproducible protocol for the generation of genetically modified human induced pluripotent stem cells (hiPSCs) by genome editing using CRISPR-Cas9 technology. Here, we describe the experimental strategy for generating knockout (KO) and knockin (KI) clonal populations of hiPSCs using single-cell sorting by flow cytometry. We efficiently achieved up to 15 kb deletions, molecular tag insertions, and single-nucleotide editing in hiPSCs. We emphasize the efficacy of this approach in terms of cell culture time.

For complete details on the use and execution of this protocol, please refer to Canac et al. (2022) and Bray et al. (2022).

BEFORE YOU BEGIN

This protocol describes an efficient methodology for generating knock-out and knock-in clonal populations of human induced pluripotent stem cells (hiPSCs) using CRISPR-Cas9 technology assisted with fluorescent activated cell sorting (FACS). Here, we efficiently achieved up to 15 kb deletions, molecular tag insertions, and single nucleotide editing in hiPSCs as well as in another cell line (i.e., immortalized human hepatocytes (IHH) cells).

To illustrate the biological relevance and efficacy of our protocol, two examples were established: the deletion of the *GPR146* gene and the addition of a HA-tag to the IRX5 protein.

GPR146 is a gene encoding the G protein-coupled receptor 146, which was recently described as a regulator of plasma cholesterol levels in mice (Yu et al., 2019). Generation of hiPSCs-derived hepatocyte-like cells (HLCs), lacking GPR146, will undoubtedly provide a powerful model for further characterization of the molecular mechanism mediated by *GPR146,* which may shed the light on the potential metabolic benefits of inhibiting GPR146 (Yu et al., 2019; Bray et al., 2022).

IRX5, a transcription factor (TF) of the Iroquois homeobox family, has been described as a regulator of the murine cardiac ventricular electrical repolarization gradient (Costantini et al., 2005; Gaborit et al., 2012; Kim et al., 2012). The generation of hiPSCs edited for the *IRX5* gene (*IRX5* KO and HA-tagged *IRX5*) will provide a remarkable resource to further investigate the function of the IRX5 transcription factor in hiPSC-derived cardiomyocytes.

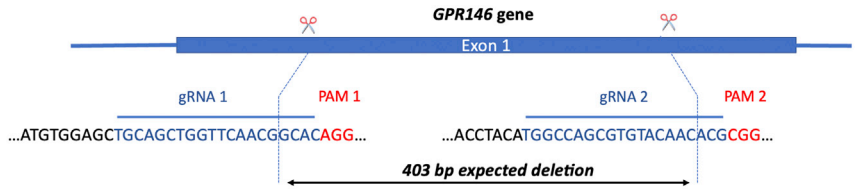

Figure 1. Guide RNAs design for the deletion of *GPR146*

Through our rigorous characterization of the established genetically modified cells (GPR146-KO and IRX5-HA), we confirmed that FACS-assisted CRISPR-Cas9 genome editing of hiPSCs does not alter the genomic stability and integrity and does not affect the pluripotency and differentiation potential of these cells (Bray et al., 2022; Canac et al., 2022).

Institutional permissions

This protocol requires institutional permission for handling genetically modified organism. For this protocol, we received permission from the French ministry of higher education, research and innovation for the use of genetically modified organism in the context of research, development and education (Approval N°8208).

Design and preparation of CRISPR-Cas9 tools

Timing: 1 day

1. Design guide RNAs (gRNAs) using Integrated DNA Technologies (IDT) software or CRISPOR and, if necessary, a donor DNA for gene KI.

   Note: We used the Alt-R CRISPR-Cas9 system (IDT) with the 5′-NGG-3′ nucleotides as a protospacer adjacent motif (PAM) and the high-fidelity Alt-R® HiFi Cas9 Nuclease V3 (IDT) to limit off-target effects.

   Note: Chemical modifications were introduced into the gRNAs to improve their stability against degradation by cellular nucleases. To achieve this, we used two-part gRNAs consisting of a chemically modified target-specific sequence crRNA (Alt-R® CRISPR-Cas9 crRNA XT) complexed with ATTO™ 488 tagged transactivating RNA (Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 488). The introduction of such a fluorescent tag into the CRISPR-Cas9 gRNA enables the selection and enrichment of gene-edited hiPSCs.

   a. To generate KO hiPSCs, design two gRNAs targeting both sides of the genomic region to be deleted as described in Xu et al. (2021) (Figure 1).

   Note: KO can alternatively be designed with a single gRNA causing a frameshift.

   Optional: To attain optimal editing, we tested the performance of gRNAs at each cutting site (Sentmanat et al., 2018). Each gRNA was experimentally tested *in vitro* after the transfection of hiPSCs with each gRNA complexed with Cas9. PCR amplification for the targeted locus was performed followed by heteroduplex formation and T7E1 assay (Mashal et al., 1995) according to the manufacturer's instructions (https://international.neb.com/protocols/2015/03/09/protocol-for-standard-rna-synthesis). Heteroduplexed PCR products were treated with the T7 endonuclease. Then, the precise size and quantity of DNA products were analyzed by automated electrophoresis (TapeStation system, Agilent). This step enables the selective identification of the gRNAs with the highest cutting efficiency. Alternatively, dedicated PCR analysis

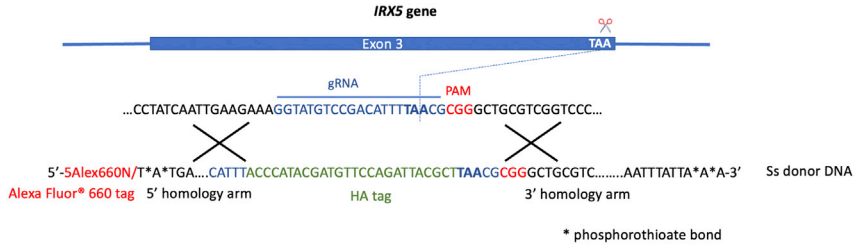

Figure 2. Design of guide RNA and 100 bp single-strand DNA donor for HA tag insertion in the *IRX5* gene

software, such as TIDE or ICE, can be used to determine the performance of gRNAs (Brinkman et al., 2014; Conant et al., 2022).

b. To generate KI hiPSCs, i.e., single nucleotide mutation or molecular tag insertion, design one gRNA, and one donor DNA (Figure 2). In this case, the gRNA cutting site must be close to the insertion point. Indeed, optimal bi-allelic insertion was detected when the gRNA cutting site was up to 10 bp away from the desired target sequence as described in Paquet et al. (2016).

Note: For genomic loci with PAM sequences distant from the insertion point (10–35 bp), homology-directed repair (HDR) efficiency will be reduced with a higher probability to get mono-allelic recombination (Paquet et al., 2016).

To augment the HDR efficiency, based on previous results, we recommend : (1) Using an asymmetric single DNA strand with two phosphorothioate bonds modifications at both 5′- and 3′-ends (Renaud et al., 2016; Richardson et al., 2016; Liang et al., 2017), (2) Having a target nucleotide or molecular tag placed in the middle of the donor DNA with at least 30 nucleotides (nt) homology arms at both sides (Liang et al., 2017), (3) Implementing one Alexa Fluor® 660 fluor tag at the 5′- extremity for the enrichment of hiPSCs transfected with the donor DNA.

Note: The size of the single strand DNA may depend on the manufacturer of the donor DNA sequence and may be limited to 200 nt owing to the addition of the Alexa Fluor® 660.

Optional: Testing multiple template designs is recommended to increase the HDR efficiency.

Optional: The incorporation of silent CRISPR-Cas9 blocking mutations in the PAM sequence or gRNA target sequence may increase HDR accuracy (Paquet et al., 2016).

Optional: The insertion (or removal) of a restriction enzyme site with silent mutations in the donor DNA may facilitate the genotyping of the hiPSC clones by PCR followed by a digestion step using the corresponding restriction enzyme and electrophoresis run. This step may reduce the number of clones that will be subjected to genotyping by Sanger sequencing.

2. Prepare Alt-R CRISPR-Cas9 complexes (IDT) by combining high fidelity Alt-R® *HiFi* Cas9 V3 nuclease with gRNAs as described in the materials and equipment section.

Passaging of hiPSCs

Timing: 2 weeks

3. Perform hiPSCs passaging in feeder-free conditions twice a week, by dissociating the cells into small aggregates, for at least two weeks before cell transfection (Lai et al., 2010; Stover and

Schwartz, 2011). Around three to five confluent wells of a 6-well cell culture plate are needed for one CRISPR experiment.

⚠ CRITICAL: hiPSCs used for CRISPR-Cas9 genome editing should be at very low passage and recently checked for genomic integrity as recently described (Popp et al., 2018; Doss and Sachinidis, 2019).

a. Prepare human embryonic stem cell (ESC) qualified Matrigel® solution at 0.05 mg/mL and coat each well of a 6-well plate with 1 mL Matrigel® solution. Incubate the plate at 37°C for at least 1 h before the cell passaging.

Note: Coating may be alternatively performed one day before the cell passaging.

b. Monitor hiPSCs confluency under inverted microscope. hiPSCs passaging should be done when cells reach 70%–80% confluency.
c. Warm-up the Dulbecco's phosphate buffer solution without Ca^{2+}/Mg^{2+} (DPBS *wo* Ca^{2+}/Mg^{2+}), the non-enzymatic gentle cell dissociation reagent (GCDR) and the hiPSC culturing medium (e.g., StemMACS™ Ips Brew medium) at 21°C for at least 20 min before passaging.

Alternatives: We use StemMACS™ Ips Brew medium as culturing medium but other alternative media, such as mTeSR™1, may be considered.

d. Discard the medium by aspiration.
e. Wash cells with 1 mL DPBS *wo* Ca^{2+}/Mg^{2+} per well, gently tap the plate to flush out dead cells then aspirate the DPBS. Repeat twice.
f. Add 1 mL of GCDR for 2–3 min at 21°C and monitor hiPSCs dissociation under an inverted microscope.

Note: The time for dissociation may vary between hiPSC clones.

g. Discard the GCDR by aspiration as soon as cells start to dissociate from each other but before they fully detach from the plate.
h. Gently flush the dissociated hiPSCs clusters using 1 mL of culturing medium.
i. Adjust the final volume of hiPSCs suspension according to the total number of wells needed.

Note: For routine maintenance, hiPSCs can be splitted twice a week (dilution 1/4 or 1/3).

j. Aspirate Matrigel solution from pre-coated 6-well plates and pipet 1.5 mL of hiPSC suspension per well.
k. Cells were grown at 37°C in humidified atmosphere of 5% CO2 and 4% O2 to prevent spontaneous differentiation (Ezashi et al., 2005) and maintain genetic stability (Forristal et al., 2013).

Alternatives: hiPSCs may alternatively be cultured under normoxic conditions.

l. One day after passaging, renew the medium with 1.5 mL fresh hiPSC culturing medium and then change the medium every two days.

⚠ CRITICAL: Adapting hiPSCs to small aggregate dissociation is a prerequisite for single cell dissociation before hiPSCs transfection and single cell sorting.

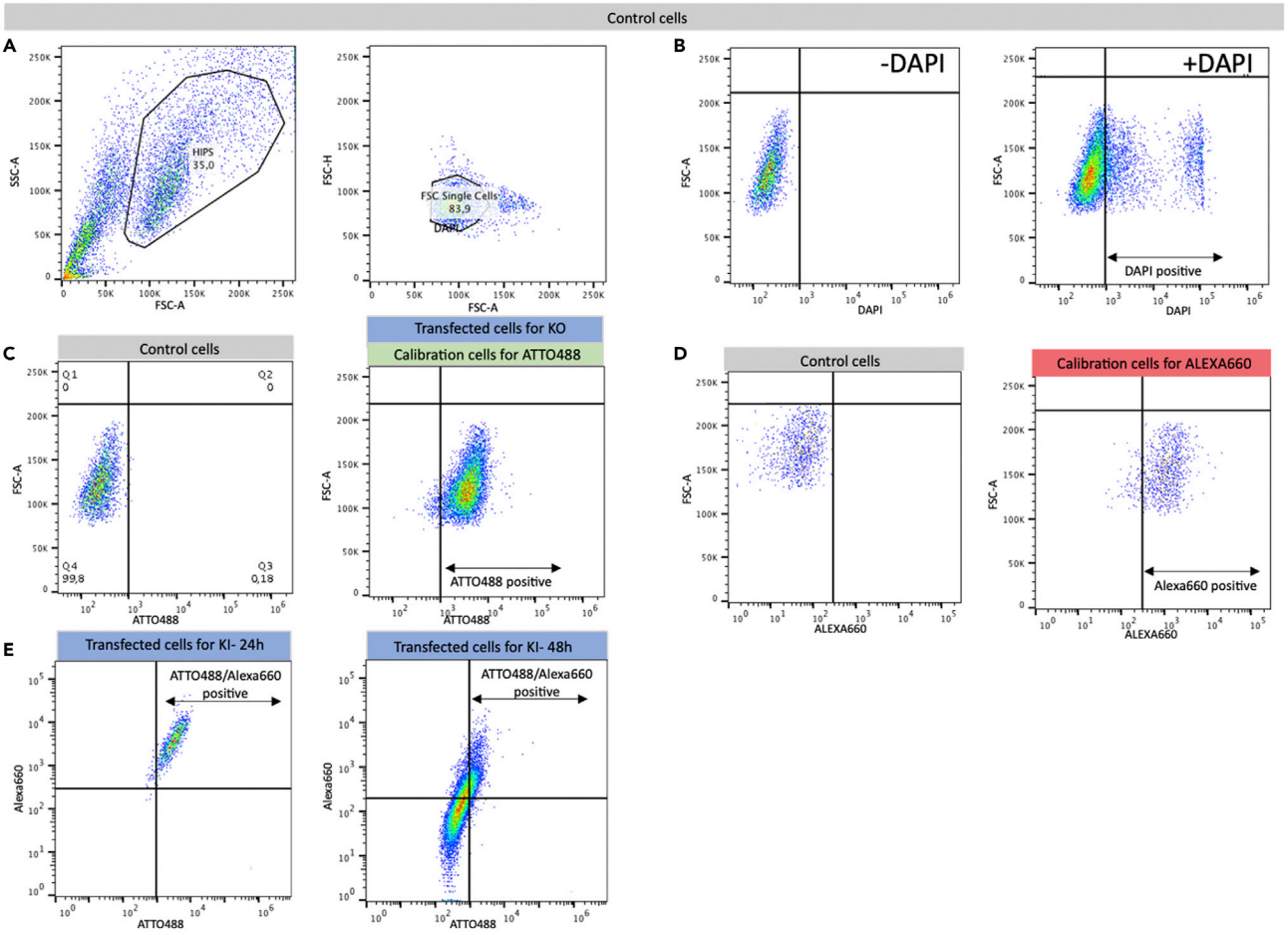

Figure 3. Cell sorting of transfected KO- and KI-hiPSCs

Flow cytometry dot plots showing (A) the total hiPSCs population (left) and the single cell population (right); (B) unstained single hiPSCs (left) and DAPI-stained hiPSCs (right); (C) ATTO488™ signal in control live cells (left) and live cells transfected with CRISPR-Cas9 RNP complex suitable for KO sorting and calibration of KI experiments (right); (D) Alexa Fluor® 660 signal in control live cells (left) and live cells transfected with the donor DNA only for calibration of KI experiments (right); (E) single cells positive for both ATTO488™ and Alexa Fluor® 660 signal suitable for KI sorting, 24 h (left) and 48 h (right) after transfection.

FACS sorter set-up

Timing: 1 h

4. Set-up and perform calibration of the flow cytometry sorter instrument to insure proper single cell analysis and subsequent sorting (in 96-well plate format) (Figure 3). The steps below describe how to calibrate the BD FacsMelody cell sorter instrument.

⚠ CRITICAL: Make sure that the FACS is maintained under aseptic condition.

a. Power ON the cytometer and annex instrumentation.
b. Refill with BD FACSFlow™ and empty the waste tank whenever needed.
c. Perform instrument quality control using BD™ Cytometer Setup and Tracking (CS&T) beads to check the system performance and BD™ Accudrop beads to set an accurate drop delay for cell sorting.
d. Cool down the sample chamber of the sorting system to 4°C.

e. Define the desired parameters of the experiment.
   i. For the generation of KO-hiPSCs, set the 488 nm laser with 527/32 filter for ATTO488™ and the 405 nm laser with 448/45 filter for DAPI fluorochrome detection.
   ii. For the generation of KI-hiPSCs, choose the 640 nm laser with 660/10 filter for the detection of the Alexa Fluor® 660 donor DNA in addition to the set of lasers described for KO-hiPSCs.
f. Load unstained control sample and adjust gates to identify the single-cell populations as shown in Figure 3A.
g. Add DAPI solution (10 ng/mL final concentration) to the unstained control cells and adjust gates to discriminate dead (DAPI positive) and live cells (remain unstained) (Figure 3B).
h. Add DAPI solution (10 ng/mL final concentration) to cells transfected with all CRISPR-Cas9 tools as described in the table "Transfection mixes" in the "materials and equipment" section.
   i. For the generation of KO-hiPSCs, adjust gates to identify the cells that are positive for ATTO488™ and negative for DAPI (Figure 3C).
   ii. For the generation of KI-hiPSCs, first calibrate and adjust gates to identify cells that are positive for ATTO488™ (Figure 3C) and for Alexa Fluor® 660 donor DNA (Figure 3D). Then, load cells transfected with both i.e., ATTO488™ complexes and the Alexa Fluor® 660 donor DNA to identify the double positive cells that are negative for DAPI (Figure 3E).
i. Load the recipient 96-well plate and assure that sorted cells are falling in the middle of the well.
j. Proceed with single cell sorting of KO or KI cells using a minimum flow rate.

Alternatives: If 96-well plate sorting is not available, you may sort cells in bulk and then perform limited dilutions in 96-well plates. Otherwise, you may culture the sorted cells in a Petri-dish and then pick colonies manually into a 96-well plate.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Gentle Cell Dissociation Reagent (GCDR)	STEMCELL Technologies	Cat# 100-0485
DPBS no calcium, no magnesium	Thermo Fisher Scientific	Cat# 14190144
TrypLE™ 1×	Thermo Fisher Scientific	Cat# 12604013
Accutase®	Sigma-Aldrich	A6964
StemMACS™ iPS-Brew XF, human	Miltenyi Biotec	Cat# 130-104-368
mTeSR™1	STEMCELL Technologies	Catalog # 85850
TeSR™-E8™	STEMCELL Technologies	Catalog # 05990
Matrigel® hESC-Qualified Matrix, LDEV-free	Corning	Cat# 354277
CloneR™	STEMCELL Technologies	Cat# 05888
BD FACSFlow™ Sheath Fluid - 20 l	Becton Dickinson	Cat# 342003
BD™ CS&T RUO Beads	Becton Dickinson	Cat# 661415
BD FACS™ Accudrop Beads	Becton Dickinson	Cat# 661612
DAPI	Sigma-Aldrich	Cat# D9542
Y27632	Cell Guidance Systems	Cat# SM02-10
QuickExtract DNA Extraction	Lucigen	QE09050
CryoStor® CS10 cryopreservation medium	STEMCELL Technologies	Cat# 100-1061
Alt-R® S.p. HiFi Cas9 Nuclease V3	Integrated DNA Technologies	Cat# 1081061
Alt-R® A.s. Cas12a (Cpf1) Ultra	Integrated DNA Technologies	Cat# 10001272
Opti-MEM™	Thermo Fisher Scientific	Cat#31985062
Critical commercial assays		
P3 Primary Cell4D Kit P3 Primary Cell 4D-Nucleofector™ with nucleocuvette™ vessel	Lonza	Cat# V4XP-3024
T7 endonuclease I	NEB	M0302S
High Sensitivity D1000 ScreenTape	Agilent	5067–5584

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human iPSC GPR146 WT	L'Institut du Thorax	ITXi001-A
Human iPSC IRX5 WT	L'Institut du Thorax	ITXi002-A
Oligonucleotides		
Alexa Fluor® 660 single strand oligo DNA	Integrated DNA Technologies	5′-Alex660N/T*A*TGAATTGAAGAAAGGTATGT CCGACATTTACCCATACGA TGTTCCAGATTA CGCTTAACGCGGGCTGCGTCGGTCCC GGA CTTTTCTAATTTATTA*A*A-3′ _
Alt-R® CRISPR-Cas9 crRNA XT, IRX5 gRNA	Integrated DNA Technologies	GGTATGTCCGACATTTTAACG
Alt-R® CRISPR-Cas9 crRNA XT, GPR146 gRNA 1	Integrated DNA Technologies	TGGCCAGCGTGTACAACACG
Alt-R® CRISPR-Cas9 crRNA XT GPR146 gRNA 2	Integrated DNA Technologies	*TGCAGCTGGTTCAACGGCAC*
Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 488	Integrated DNA Technologies	N/A
Software and algorithms		
FlowJo™ software	FlowJo (Becton Dickinson)	https://flowjo.com
Custom Alt-R® CRISPR Cas9 guide RNA design tool	Integrated DNA Technologies	https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM
TIDE	Stichting het Nederlands Kanker Instituut	http://shinyapps.datacurators.nl/tide/
ICE	Synthego	https://ice.synthego.com/
CRISPOR	TEFOR infrastructure	http://crispor.org
Other		
6-well cell culture plate	Falcon™	Cat# 353046
96-well cell culture plate	Falcon™	Cat# 353075
96-well PCR plate	Axygen	Cat# 12799438
12-well cell culture plate	Falcon™	Cat# 353043
15 mL tube	Falcon™	Cat# 352096
Malassez counting chamber	Brand™	N/A
70 µm nylon mesh cell strainer	Falcon™	Cat# 352350
Sterile polypropylene Facs tubes	Falcon™	Cat# 352052
FacsMelody cell sorter	Becton Dickinson	N/A
4D-Nucleofector core unit	Lonza	N/A
4200 TapeStation	Agilent	N/A

MATERIALS AND EQUIPMENT

hiPSCs culturing medium

Prepare complete medium by adding StemMACS iPS Brew XF, 50× Supplement to StemMACS iPS-Brew XF, Basal Medium. Mix well and prepare 40 mL aliquots in 50 mL plastic tubes.

Store at 4°C for 2 weeks or freeze at −20°C for long term storage.

Alternatives: Other media such as mTeSR™1 or TeSR™-E8™ can be used but may require some optimizations.

hiPSC single-cell medium

Reagent	Final concentration	Amount
hiPSCs culturing medium	N/A	40 mL
Y27632 (10 mM)	10 µM	40 µL

Prepare fresh medium to be used immediately or store it at 4°C for no longer than 4 days after preparation.

Alternatives: Other commercial solutions designed for hiPSC single cell recovery and survival, such as CloneR™, may be used.

crRNA XT: ATTO™ 488 tracrRNA duplex

Complex the two-part gRNA by combining crRNA XT with ATTO™ 488 tracrRNA at equimolar concentrations. Heat the complex for 5 min at 95°C then allow it cool down on the bench.

Reagent	Final concentration	Amount
Alt-R® CRISPR-Cas9 crRNA XT (200 µM)	100 µM	3 µL
Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 488 (200 µM)	100 µM	3 µL
The crRNA XT:tracrRNA duplex can be stored at −20°C for several months.		

⚠ CRITICAL: Prepare the two-part gRNAs under RNase-free conditions.

CRISPR-Cas9 RNP complex

Form the CRISPR-Cas9 RNP complex by mixing the crRNA XT: ATTO™ 488 tracrRNA duplex with the Alt-R® *HiFi* Cas9 Nuclease V3. Incubate the complex for 20 min at 21°C. Volumes shown below are for one transfection reaction.

⚠ CRITICAL: If two gRNAs are used for the gene KO, complex each gRNA independently with the HiFi Cas9 nuclease. This step is essential to avoid possible affinity competition between the gRNAs and the Cas9 nuclease.

Reagent	Final concentration	Amount
crRNA XT: ATTO™ 488 tracrRNA duplex (100 µM)	34 µM	1,7 µL
Alt-R® S.p. HiFi Cas9 Nuclease V3 (61 µM)	16 µM	1,3 µL
Opti-MEM™	N/A	2 µL

⚠ CRITICAL: Prepare fresh before transfection.

Donor DNA

Dilute the single strand oligo DNA donor with OptiMEM™ at a final concentration of 1 µg/µL.

Transfection solution

The transfection solution of the P3 Primary Cell 4D Kit was prepared following the manufacturer's instructions (https://bioscience.lonza.com/download/product/asset/21093).

The volumes indicated below are for one transfection reaction using a 100 µL Nucleocuvette™ vessel found in the P3 Primary Cell 4D Kit.

Reagent	Amount
P3 Primary Cell Nucleofector Solution	82 µL
Supplement 1	18 µL
Prepare fresh and warm at 21°C for 20 min before use.	

Transfection reaction mixes

Expand hiPSCs in culture to have enough cells for all transfection conditions (see Table 1).

Prepare and use immediately for transfection.

Table 1. Transfection reaction mixes

Modification	Number of 6-wells to prepare	Condition	Number of cells	Number of transfection reaction	Volumes for 1 transfection reaction (uL)			
					CRISPR-Cas9 RNP complex 1 (gRNA 1)	CRISPR-Cas9 RNP complex 2 (gRNA 2)	Donor DNA	Opti-MEM™
KO	4	WT cells	1.10^6	1	–	–	–	5
		KO cells	2.10^6	2	2,5	2,5	–	–
KI	6	WT cells	1.10^6	1	–	–	–	10
		ATTO488 calibration cells	1.10^6	1	5	–	–	5
		Alexa Fluor® 660 calibration cells	1.10^6	1	–	–	5	5
		KI cells	2.10^6	2	5	–	5	–

STEP-BY-STEP METHOD DETAILS

Transfection of hiPSCs with CRISPR-Cas9 tools

Timing: 3 h

This step enables the transfection of hiPSCs with high efficiency and low toxicity using the Alt-R CRISPR-Cas9 tools, yielding more than 70% of transfected live cells. The yield of transfected hiPSCs will provide enough material for cloning 384 hiPSC clones using single cell FACS sorting.

Alternatives: Transfection techniques such as lipofection and magnetofection can be employed instead.

1. Seed hiPSCs for cell transfection.
   a. Expand hiPSCs in culture to get the required number of cells for KO or KI generation (refer to the "transfection reaction mixes" table in the materials and equipment section.
   b. Replace hiPSCs culturing medium with hiPSCs single cell medium.
      i. Aspirate hiPSCs culturing medium.
      ii. Add 1.5 mL of hiPSCs single cell medium.
      iii. Incubate for at least 1 h at 37°C in humidified atmosphere of 5% CO2 and 4% O2.

Note: Pre-treating hiPSCs with 10 μM Y27632 will improve cell viability post transfection.

2. Coat each well of a 6-well plate with 1 mL Matrigel solution for one hour as described earlier. Aspirate the Matrigel solution and add 1.5 mL of hiPSCs single cell medium per well. Refer to the "transfection reaction mixes" table in the materials and equipment section to adjust the total number of wells needs to be prepared.
3. Dissociate hiPSCs as single cells.
   a. Dissociate hiPSCs into single cell suspension as described previously. At this step, hiPSCs should be incubated with the GCDR for 20–30 min to ensure the complete dissociation into single cell suspension.

Note: Pre-treating hiPSCs with the hiPSCs single cell medium (containing Y27632) enhances the viability post transfection and consequently prolongs the dissociation time.

   b. Gently pipet the dissociated hiPSCs using 1 mL of hiPSC single cell medium per well.
   c. Pool the dissociated cells (from 3 to 5 wells) by transferring the cell suspensions into a 15 mL Falcon tube.
   d. Take 50 μL of the cell suspension and proceed to cell counting using the Malassez counting chamber.

Optional: Use trypan blue staining to observe dead cells appearing in blue.

e. After counting, collect the suitable volume of the cell containing 1.10^6 cells for one transfection reaction and transfer into a 1.5 mL microcentrifuge tube. Prepare the total number of tubes needed according to the "transfection reaction mixes" table in the materials and equipment section.
f. Centrifuge the 1.5 mL microcentrifuge tubes at 100 × g for 5 min at 21°C.
g. Discard the cell supernatant.

4. Transfection of hiPSCs with CRISPR Cas9 RNP complex and donor DNA.
   a. Add 100 µL of the transfection reaction mix per transfection reaction to each cell pellet from step 3.
   b. Add CRISPR Cas9 tools according to the "transfection reaction mixes" table.
   c. Gently pipet up and down until the cell pellet completely mixes with the CRISPR Cas9 tools.
   d. Transfer the cell suspension into a nucleocuvette™ vessel.
   e. Proceed immediately with the nucleofection using Program CA-137 on a 4D-Nucleofector Core Unit.
   f. Remove transfected cells from the nucleocuvette™ using a pipet provided by the nucleofection kit. Seed the transfected cells drop by drop in one Matrigel-pre-coated well of the 6-well plate containing 1.5 mL of hiPSCs single cell medium.
   g. Incubate for 24 h at 37°C in a humidified atmosphere under hypoxic condition of 4% O2.

   Alternatives: HiPSCs can be incubated under normoxic conditions.

   h. Proceed to single cell FACS sorting.

   Optional: Single cell FACS sorting may be performed 48 h post transfection. In this case, replace hiPSCs single cell medium with hiPSCs culturing medium the day after transfection.

hiPSCs single-cell sorting

Timing: 2 h

Single cell FACS sorting of transfected hiPSCs is a strategy that allows (1) enrichment of hiPSCs susceptible to carrying genomic modifications and (2) clonal isolation/selection of hiPSCs. Since single cell culturing of hiPSCs is critical, a yield of 50% viability is expected after single cell sorting.

5. Prepare 96-well cell culture plates for hiPSCs single cell sorting.
   a. Coat four 96-well cell culture plates with 50 µL of Matrigel solution per well.
   b. Incubate the 96-well plates at 37°C for at least one hour.
   c. Remove Matrigel solution by aspiration and then add 100 µL of hiPSC single cell medium per well.
6. Single cell dissociation of transfected hiPSCs.
   a. Dissociate transfected hiPSCs into single cells as described in Passaging of hiPSCs by incubating hiPSCs with GCDR for 20–30 min.
   b. Add 1 mL of hiPSC single cell medium and gently pipet the dissociated hiPSCs.
   c. Transfer dissociated hiPSCs into a 15 mL tube.
   d. Centrifuge the dissociated hiPSCs at 100 × *g* for 5 min at 4°C.
   e. Discard the supernatant and resuspend the cell pellet with 500 µL of hiPSC single cell medium.
   f. Place the 15 mL tube on ice until single cell FACS sorting.

   Alternatives: The use of enzymatic dissociation solutions such as Accutase® or TrypLE™ will accelerate the dissociation process. However, a dramatic decrease in clone recovery will be encountered after single cell FACS sorting.

7. Single cell FACS sorting.
   a. Filter the cell suspension through a 70 μm nylon mesh cell strainer.

   Note: Filtering the cell suspension will prevent clogging of the FACS sorter if hiPSCs are not fully dissociated.

   Alternatives: 37 μm or smaller nylon mesh cell strainer can be used, but might not be sufficient to get the cell suspension out of cells doublets. In this case, you may use single cell FACS gating to distinguish cell doublets.

   b. Transfer the cell suspension into a 5 mL sterile polypropylene tube suitable for cytometry.
   c. Perform calibration and adjust gates as described in the FACS sorter set-up section.
   d. Analyze the transfected cells to sort them and proceed with single cell sorting.
   e. Insert the four 96-well plates into the cytometer one at a time and sort one cell per well.
   f. Incubate the 96-well plates immediately at 37°C in a humidified atmosphere under hypoxic condition (4% O2).

Culture, amplification, and freezing of hiPSCs clones

Timing: 10–15 days

This step presents an optimized strategy for short-term culture of a large number of hiPSCs clones, suitable for genotyping and further amplification of genetically modified clones.

8. Keep the hiPSCs clones in culture for a total of ten days by changing the medium with 100 μL fresh medium every two days. Use hiPSCs single cell medium during the first week after sorting and then switch to hiPSCs culturing medium for the last 3 days.

   ⚠ CRITICAL: Keep hiPSCs clones for two to three days after single cell sorting without changing media.

   Note: Use a multichannel pipette.

   Note: Make sure to use different pipette tips between the different wells to avoid cross-contamination.

   Note: Increase the culture time in hiPSCs culturing medium if hiPSCs clones grow slowly.

9. If the hiPSC clones form large colonies, dissociate and duplicate the clones in two wells of two separate 96-well cell culture plates.
   a. Coat 96-well cell culture plates with 50 μL of 0.05 mg/mL Matrigel® solution per well and incubate at 37°C for one hour. Prepare 2 plates for each plate of clones.
   b. Remove the Matrigel solution by aspiration and fill each well with 100 μL of hiPSCs single cell medium.
   c. Wash the hiPSCs clones twice with 100 μL of DPBS *wo* Ca^{2+}/Mg^{2+}, then dissociate them as described in Passaging of hiPSCs using 100 μL of GCDR per well for 5–10 min.
   d. Remove GCDR by aspiration as soon as the cells start to dissociate from each other, and before they completely detach from their support.

   Note: Not all clones may dissociate at the same time. Keep in mind that dissociation continues after aspirating the GCDR if cells are left without medium for a short time. Note that clones may be left without solution or medium for 5–10 min.

Alternatives: Do not detach all hiPSCs clones at the same time.

e. Detach cells by pipetting up and down with 100 μL of hiPSCs single cell medium.

Note: Observe under the microscope to verify if the cells have detached well.

f. Transfer 50 μL of cell suspension into two wells of two distinct 96-well plates.
g. Incubate at 37°C in a humidified atmosphere under hypoxia (4% O2) for 24 h.
h. Replace the medium with 100 μL of hiPSCs culturing medium every two days.

Note: Cells usually reach confluency two to three days after duplication.

Note: If the duplicated clones are too small, leave the cells in hiPSCs single cell medium after duplication.

10. When confluent, lyse the cells from one 96-well plate for genotyping.
    a. Wash the wells twice with 100 μL DPBS *wo* Ca^{2+}/Mg^{2+}.
    b. Add 50 μL of QuickExtract DNA extraction solution to a confluent well.

Note: Reduce the volume of QuickExtract DNA extraction solution to 20 μL if the cells are not confluent.

    c. Scrape the cells at the bottom of the well using a pipette tip.

Note: Use a multichannel pipette.

    d. Seal the 96-well plate with parafilm and freeze at −20°C for further DNA extraction.
    e. Proceed with DNA extraction.
11. Freeze the cells of the second 96-well plate for further amplification of the genetically-engineered clones of interest.
    a. Wash the wells twice with 100 μL DPBS *wo* Ca^{2+}/Mg^{2+} per well.
    b. Dissociate the hiPSCs clones in 100 μL GCDR per well for 5–10 min.
    c. Remove GCDR by aspiration before the cells completely detach from their support.
    d. Detach the cells by pipetting up and down with 100 μL of CryoStor® CS10 cryopreservation medium.
    e. Seal the 96-well plate with parafilm and quickly transfer it to a −80°C freezer.

⚠ CRITICAL: This step carries the risk of cross-contamination. Handle the plates with care when sealing and transporting to the freezer.

Pause point: Cells may be **stored at -80°C for at least 3 months.**

DNA extraction for genotyping of hiPSCs clones

Timing: 1 h

DNA extraction from hiPSCs clones will provide sufficient material for genotyping and identification of genetically-modified hiPSCs clones.

12. DNA extraction.
    a. Thaw the 96-well plate prepared for genotyping on ice.
    b. Transfer the DNA cell extracts into a 96-well PCR plate using a multichannel pipette.

c. Proceed with DNA extraction in a thermocycler using the following program: 65°C for 15 min, 68°C for 15 min and 98°C for 10 min.
d. Proceed directly to genotyping as described in Bray et al. (2022) and Canac et al. (2022).

Pause point: DNA may be stored at -20°C for several weeks.

Thawing and amplification of genetically modified hiPSCs clones

Timing: 1–2 weeks

13. After genotyping, thaw and amplify the genetically-modified hiPSCs clones of interest.
    a. Determine the position of the hiPSCs clones of interest in the 96-well plate.
    b. For one hiPSC clone to be amplified, coat a well of a 12-well microplate with 0.5 mL of Matrigel® solution at 0,05 mg/mL for at least one hour.
    c. Aspirate the Matrigel® solution and fill the well with 1 mL of hiPSCs single cell medium.
    d. Thaw the 96-well plate prepared for clonal amplification by transferring the plate directly from the −80°C freezer to a 37°C incubator.

Note: Do not use a water bath to thaw the cells to limit the risk of contamination.

    e. Transfer the 100 µL thawed cell suspension directly into the 12-well plate.
    f. Incubate at 37°C in a humidified atmosphere under hypoxia of 4% O2 for 24 h.
    g. Replace the hiPSCs single cell medium with hiPSCs culturing medium.
    h. Refresh the medium every two days and amplify the cells for banking.

Note: Check under the microscope if cells were collected from the 96-well plate.

Optional: If some cells remain attached to the bottom of the 96-well plate, you may add hiPSCs single cell medium directly into the 96-well culture plate and keep the plate in culture for clone amplification.

EXPECTED OUTCOMES

HiPSCs have the potential to provide differentiated cells for a wide range of applications, including disease modeling, drug testing for precision medicine, high-throughput drug discovery in a patient-specific manner, and cell-based therapies. The development of genome editing technologies has made the insertion or correction of mutations, the insertion of molecular tags or even gene depletions increasingly feasible.

However, genome editing in hiPSCs remained challenging, mainly due to their low transfection efficiency (Chatterjee et al., 2011), high activity of the cell cycle regulator p53 (Ihry et al., 2018), and anoikis, a type of apoptosis that occurs in anchorage-dependent cells when they detach from the surrounding extracellular matrix (Wang et al., 2009). Several technical solutions have been developed that have paved the way for more efficient genome editing and cloning of hiPSCs. These include the development of RNP systems that facilitate the delivery of the Cas9 enzyme and gRNA in hiPSCs. In addition to this advantage, the RNP system provides transient genome editing with high efficacy and lower risk of off-target compared to plasmids or mRNA (Zhang et al., 2021). Second, the availability of specific commercial solutions specifically designed for the survival of single hiPSCs has facilitated the cloning of hiPSCs, e.g., CloneR™ or Y27632 (Lai et al., 2010). In line with these advances, many protocols for efficient genome editing in hiPSCs have been published (Byrne and Church, 2015; Yumlu et al., 2017, 2019; Geng et al., 2020; Zhong et al., 2020; Xu et al., 2021; Zhang et al., 2021). For instance, Xu et al. recently published an optimized procedure, very close

to our approach, for electroporation of the RNP CRISPR-Cas9 system into hiPSCs, followed by bulk analysis of the efficiency of KI, and subsequent sub-cloning and expansion of hiPSCs. As already described in other protocols, our approach presents three major optimized steps for efficient genome editing of hiPSCs, namely, transfection, single cell cloning and amplification. However, our approach differs from others in that we use a combined FACS approach for both enrichment of hiPSCs susceptible to carry genome editing events and direct single cell cloning of hiPSCs at the same time. This FACS-assisted approach simplifies the single cell cloning by implementing the use of pipetting robots to reduce the time required for medium change.

As described previously (Yumlu et al., 2017; Xu et al., 2021), we confirmed that the use of Y27632, a widely used inhibitor of Rho-associated kinase, definitely overcame some causes of limited gene editing efficiency in hiPSCs while improving the survival of hiPSCs after single cell dissociation for transfection and cloning purposes.

Genetic abnormalities and loss of pluripotency can occur after genome editing and single cell culturing of hiPSCs. Quality control of CRISPR-Cas9-modified hiPSCs is required before they can be employed in research and is a prerequisite for publication in Lab Resource papers or depositary in hiPSCs banks. The European Bank for Induced Pluripotent Stem Cells (EBiSC) has collected hiPSC lines generated from patients with genetic diseases and healthy donors from all over Europe. To ensure consistently high quality hiPSCs, EBiSC has established a list with a set of rapid testing techniques for hiPSCs quality control (O'Shea et al., 2020). Indeed, this latter concept can be applied to CRISPR-Cas9- derived hiSPCs. For instance, hiPSCs may be tested for (1) their pluripotency status and differentiation potential by qPCR or flow cytometry (2) genetic stability, e.g., by molecular karyotyping, and (3) cell line identity through copy number variation (CNV) analysis.

The hiPSC lines generated by our protocol were characterized to meet the quality control standards required for publication in Lab Resource papers. In-depth studies on the genome stability and pluripotency of hiPSCs showed no adverse effects for our procedure (Roudaut et al., 2021; Bray et al., 2022; Canac et al., 2022). Off-target analysis by PCR sequencing for the 5 most likely off-target sites revealed that our procedure did not generate off-targets (Bray et al., 2022; Canac et al., 2022).

However, some technical issues may limit the desired outcomes at each step of the protocol, depending on the targeted gene, hiPSCs lines, and the desired modification. In the troubleshooting section, we provide some useful tips to avoid potential pitfalls.

QUANTIFICATION AND STATISTICAL ANALYSIS

The efficiency of recovering edited KO or KI hiPSC clones may vary depending on several factors, such as: the gRNA on-target score, the type of genomic edition per se (homozygous *versus* heterozygous KO or KI), and the susceptibility of individual hiPSCs to grow as single cells after FACS sorting.

To generate the *GPR146*-KO hiPSCs, we used two gRNAs for deleting G*PR146*. Both gRNAs were selected according to their on-target potential and off-target risk provided by the gRNA design software. After single cell FACS sorting, we were able to obtain and freeze 184 clones from 384 sorted clones, representing a cloning yield of 47%. Of these clones, 8.1% were edited with 6.5% having heterozygous editing and 1.6% carrying KO on both alleles. Only one clone was GPR146 KO homozygous (0.54%), and the others were heterozygous compound. This lower editing rate compared to a previous study (Skarnes et al., 2019) is likely due to the fact that gRNAs were not selected for their cutting efficiency *in vitro* prior to their transfection into hiPSCs. Indeed, we used our protocol for a 292 bp deletion in *VHL* using two gRNAs that were experimentally selected for their *in vitro* cutting efficiency. After genotyping, 65% editing rate was achieved, of which 40% were heterozygous and 25% were KO on both alleles. Among these double KO, 16% were compound heterozygous and 9%

homozygous. These statistical results confirmed the need to experimentally test the on-target score of gRNAs to achieve a higher yield of modified hiPSC clones as described in Kwart et al. (2017).

On the other hand, we conducted another KO project involving deletions in regulatory regions implicated in cardiac arrhythmias. Herein, we found that the editing efficiency was inversely proportional to the size of the deletion. For deletions of 250 bp, 1,000 bp, 10 Kb, and 15 Kb, we obtained success rates of 5,2%, 4.5%, 2.2% and 1.7% respectively. This observation highlights the need to increase the number of clones to be screened as the size of the deletion increases.

For the IRX5 HA tag insertion project, we sorted 384 clones and recovered 112 clones after duplication and freezing, representing only 29% cloning efficiency. The HDR rate was 5.4% with 4.5% KI for one allele and one homozygous clone (0.9%). This is consistent with HDR scores from previous study (Byrne and Church, 2015), but still lower than other studies (Xu et al., 2018; Skarnes et al., 2019). As suggested earlier, testing gRNAs efficiency would likely have increased the KI success rate.

LIMITATIONS

Targeted gene may directly impact the efficiency of the protocol. For example, we deleted *PCSK9* in a hiPSC line, which was recently described as a regulator of cell proliferation in hiPSCs (Roudaut et al., 2021). We demonstrated that deletion of PCSK9 strongly reduced cell proliferation. That being so, obtaining homozygous clones for *PCSK9* deletion required more attention as we observed a decrease in cell recovery after single cell cloning. Thereby, we succeeded in deleting *PCSK9* in hiPSC clones thanks to a longer culture time with Y27632.

As described previously, insertion of a molecular tag or single nucleotide mutation required a gRNA having a cutting site within 10 bp from the insertion point (Paquet et al., 2016). We observed partial recombination of the donor DNA or bp insertions or deletions when the gRNA was too far from the insertion point. The efficiency of this protocol for KI clearly depends on the availability of gRNAs to cut as close as possible to the insertion point. Other Cas9 enzymes with other PAM sequences, e.g., CRISPR-Cas12a (Zetsche et al., 2015), and if available as RNP system, could be tested in case no gRNAs with NGG PAM in the desired region of interest are identified.

TROUBLESHOOTING

Problem 1

The high mortality of hiPSCs encountered after transfection or the low frequency of transfected hiPSCs as assessed by flow cytometry (step 1 in transfection of hiPSCs with CRISPR-Cas9 tools).

Potential solution

Efficient transfection relies on the balance between cell density and the quantity of transfected components for editing. For example, cellular toxicity was noticed when the amount of donor DNA for HDR was more than 7 μg per transfection reaction. Moreover, we also realized that the quality/state of cell passaging significantly affected the transfection of cells. As described previously, cells should be in the logarithmic growth phase with minimal signs of differentiation or cell death (Xu et al., 2021). Possible solutions to such high cell mortality after transfection and low transfection frequency could be:

- Reduce the concentration of transfected CRISPR Cas9 RNP and the concentration of donor DNA. For instance, test multiple donor DNA concentrations ranging from 2 to 5 μg for every 1.10^6 of cells to be transfected. After 24 h, evaluate the transfection efficiency by flow cytometery to determine the optimal concentration.
- Test other transfection programs available for Nucleofection (Lonza).
- Use commercial solutions such as CloneR™ to recover cells after transfection.

If none of the above suggestions worked out in fixing the problem(s), you may attempt to transfect another hiPSC line, or you may transfect the hiPSCs with a small GFP plasmid to verify the functionality of your editing components.

Problem 2

Cells didn't attach after single cell FACS sorting (step 5 in hiPSCs single cell sorting).

Potential solution

Single cell FACS sorting is a stressful step for live cells and precautions should be taken to reduce cell death during this step:

- Reduce the time during which hiPSCs stay dissociated, e.g., by setting up and calibrating the cytometer before starting the dissociation of the hiPSCs to be sorted.
- Sort the cells 48 h after transfection instead of 24 h.
- Use non-enzymatic solutions for single cell dissociation of hiPSCs prior to FACS sorting, i.e., GCDR. We observed a sharp decrease in single cell FACS sorting rate when enzymatic solutions were used.
- Use commercial solutions such as CloneR™, which are specifically designed to boost the survival of hiPSCs after single cell dissociation.
- Perform the initial single cell FACS optimization steps using non-transfected hiPSCs.
- Test other cloning strategies if flow cytometry is too deleterious for hiPSCs, such as limiting dilutions or cloning after plating hiPSCs with feeder cells.

Problem 3

Single hiPSCs after FACS sorting are not proliferating (step 8 in culture, amplification and freezing of hiPSCs clones).

Potential solution

The targeted gene might be an essential gene for hiPSCs. As described above under the "limitations" part, some specific gene deletions may directly affect the proliferation of hiPSCs.

- Use commercial solutions such CloneR™ to boost the growth of single cells after sorting.
- Owing to the fact that hiPSCs grow faster when they are plated in small clumps and not as single cells, try not to sort them into single cell per well, however sort them at a density of 6–10 cells per well. Then proceed with successive enrichments of edited sub-populations.
- Change your CRISPR-based strategy by using, for example, an inducible CRISPR system (Ishida et al., 2018) or a CRISPR interference system (Larson et al., 2013).

Problem 4

Absence of editing (step 12 in DNA extraction for genotyping of hiPSCs clones and in before you begin).

Potential solution

As described earlier, the efficiency of editing largely depends on the type of modification itself, i.e., KO *vs.* KI, as well as on the availability of efficient cutting gRNAs in the target region. If no editing is achieved, explore the following possible solutions:

- The gRNA does not cut and thus other gRNA candidates should be tested. You may use the T7 endonuclease assay or specific PCR analysis software, e.g., ICE (Conant et al., 2022) or TIDE (Brinkman et al., 2014), to identify efficient gRNAs cutting candidates.
- To find more suitable gRNAs, try other PAM sequences and their respective Cas enzyme, e.g., Type V CRISPR–Cas12a, which is also available as an RNP system (Zetsche et al., 2015).

- No HDR events while the gRNA showed cutting efficiency. As described previously, the design of the HDR template may directly affect the KI efficiency (Paquet et al., 2016; Richardson et al., 2016; Liang et al., 2017). Try different HDR templates and, if possible, include a silent mutation that affects PAM to increase KI efficiency (Paquet et al., 2016).
- Genome editing is lethal: perform genotyping on the transfected hiPSCs bulk to determine if any cutting events were occurring before the single cell cloning step. Lyse the cells within 24 h post transfection and perform a T7 endonuclease assay or TIDE/ICE analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pr. Bertrand Cariou (Bertrand.cariou@univ-nantes.fr).

Materials availability

Generated *GPR146* KO (ITXi001-A-1) and IRX5 HA (ITXi002-A-3) hiPSC lines are not banked in any cell repository but details on the generated cell lines are available in the database https://hpscreg.eu/cell-line.

Data and code availability

This study did not generate or analyze any datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.C. and K.S.T.; methodology, A.C., A.L., and A.T.; investigation, A.C., A.L., A.G., R.C., L.B., and M.B.; writing – original draft, A.C.; writing – review & editing, A.C., S.I., R.C., M.B., A.R., B.C., and C.L.M.; funding acquisition, K.S.T., A.R., C.L.M., and B.C.; supervision, K.S.T., A.R., B.C., C.L.M., N.G., G.L., J.B., and B.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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