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Generation of genetically modified pluripotent stem cell lines for disease modeling

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We use this protocol and it's working

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Abstract

We present a unified protocol for the genetic modification of human pluripotent stem cells with CRISPR/Cas9, and the subsequent generation of clonal lines for downstream disease modeling applications. We demonstrate two step cases for generating gene-knockouts, and for homozygous or heterozygous genetic knock-in mutations. Both step cases then converge and adopt the same single-cell cloning method. By presenting the entire procedure, from experimental design to quality control, we are able to highlight the interdependencies between the editing and cloning stages which are often overlooked. Following our protocol the user should hope to acquire high-quality clonal hPSC lines carrying their desired genetic mutation within 6-8 weeks.

Image Attribution

Some images were generated with BioRender.com

Guidelines

The marriage of CRISPR/Cas9 gene-editing with pluripotent stem cell (PSC) differentiation protocols gives researchers the ability to model genetically inherited diseases in potentially any tissue type.

Cas9 induces double-stranded breaks (DSBs) in DNA at a very high efficiency. The DNA binding site is directed by a guide RNA (gRNA) which contains a twenty nucleotide sequence that will bind to complementary DNA sequences when the target sequence contains a protospacer adjacent motif (PAM) sequence immediately upstream on the opposite strand (refer to Figure 1A for a schematic of gRNA, DNA and PAM orientations). The PAM sequence will differ depending on the species of Cas9 being used. The most common is *Streptococcus pyogenes* Cas9 (spCas9) which has an NGG PAM sequence. A gRNA-spCas9 ribonucleoprotein (RNP) complex can therefore induce a DSB anywhere in the genome where there are two successive guanines.

After a DSB is induced, mammalian cells have two ways to repair the break, non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is the predominant form of DNA repair, it fills in the gap between the two ends of a DSB with random nucleotides. If this occurs in an exon of a gene nonsense mutations can occur where the open-reading frame (ORF) is shifted, which almost always leads to loss-of-function of the translated protein. Missense mutations, where one or more codons are changed or lost, are also common, but the ORF is not shifted. HDR involves the copying of a template strand of DNA that is homologous to both ends of the DSB to accurately repair the break. This can be unedited allele on a sister chromosome or exogenous DNA.

Citation

Hsu PD, Lander ES, Zhang F (2014). Development and applications of CRISPR-Cas9 for genome engineering.

<https://doi.org/10.1016/j.cell.2014.05.010>

LINK

Both DNA repair mechanisms can be used to create a desired genetic mutation. As NHEJ often results in protein loss-of-function it is extremely easy to produce genetic knockouts by transfecting cells with just an RNP. To induce precise base-pair edits required to model diseases caused by missense mutations HDR is exploited. RNPs are transfected with an exogenous template in the form of a single-stranded oligodeoxynucleotide (ssODN). The ssODN will contain the desired mutations but otherwise be homologous to the regions flanking the DSB so that it is hopefully incorporated by the HDR machinery. NHEJ occurs more frequently than HDR but they are both inherently random so there has been a collective effort to increase on-target editing efficiency of RNPs, and to increase HDR efficiency for the incorporation of ssODNs.

Many early CRISPR/Cas9 protocols used HEK293 or Jurkat cells to optimise electroporation and on-target editing efficiencies. These cells types are far more robust than PSCs and some of these protocols are not compatible with PSC cell culture best-practice. Fortunately, there is now a plethora of excellent primary research papers and protocols detailing how to perform CRISPR/Cas9 gene-editing in PSCs, many of which were used in the development of this protocol.

We found that many protocols focused on optimizing either the genetic modification or single-cell cloning. There were none that adequately addressed every problem and presented a complete protocol that could guide first-time-users through CRISPR/Cas9 gene-editing of hPSCs. We considered this to be a substantial gap in the literature because there are some inefficient bottlenecks in producing genetically modified hPSC lines. These include low electroporation efficiency, low post-electroporation recovery, variable on-target gene editing efficiency, low rate of HDR for knock-ins, poor single-cell cloning efficiency, and the high proclivity of PSCs for acquiring karyotypic abnormalities under stressed conditions. PSC culture best practice must always be upheld to guarantee that pluripotency and karyotypic stability of edited PSC clones are conserved. Failing to address all of these problems together makes for an expensive, slow, and frustrating experience.

The protocol we present here arose naturally from the desire to produce high-quality PSC lines harbouring either NHEJ-mediated genetic knockouts or HDR-mediated single-base pair mutations. It guides the user through the entire workflow of CRISPR/Cas9 editing, from gRNA design to quality control of genotype-positive lines, highlighting potential pitfalls and alternative methods, hopefully enabling any lab to efficiently generate high quality lines for research use.

The cloning efficiencies in particular that we report (up to 40%) are amongst the highest that we have found in the literature. Comparable to that of Tristan et al. who used a CEPT cocktail supplement. A notable advantage with the our method is that hPSCs do not need to be adapted to CloneR2 supplemented media before performing single-cloning.

Citation

Tristan CA, Hong H, Jethmalani Y, Chen Y, Weber C, Chu PH, Ryu S, Jovanovic VM, Hur I, Voss TC, Simeonov A, Singeç I (2023). Efficient and safe single-cell cloning of human pluripotent stem cells using the CEPT cocktail.

<https://doi.org/10.1038/s41596-022-00753-z>

LINK

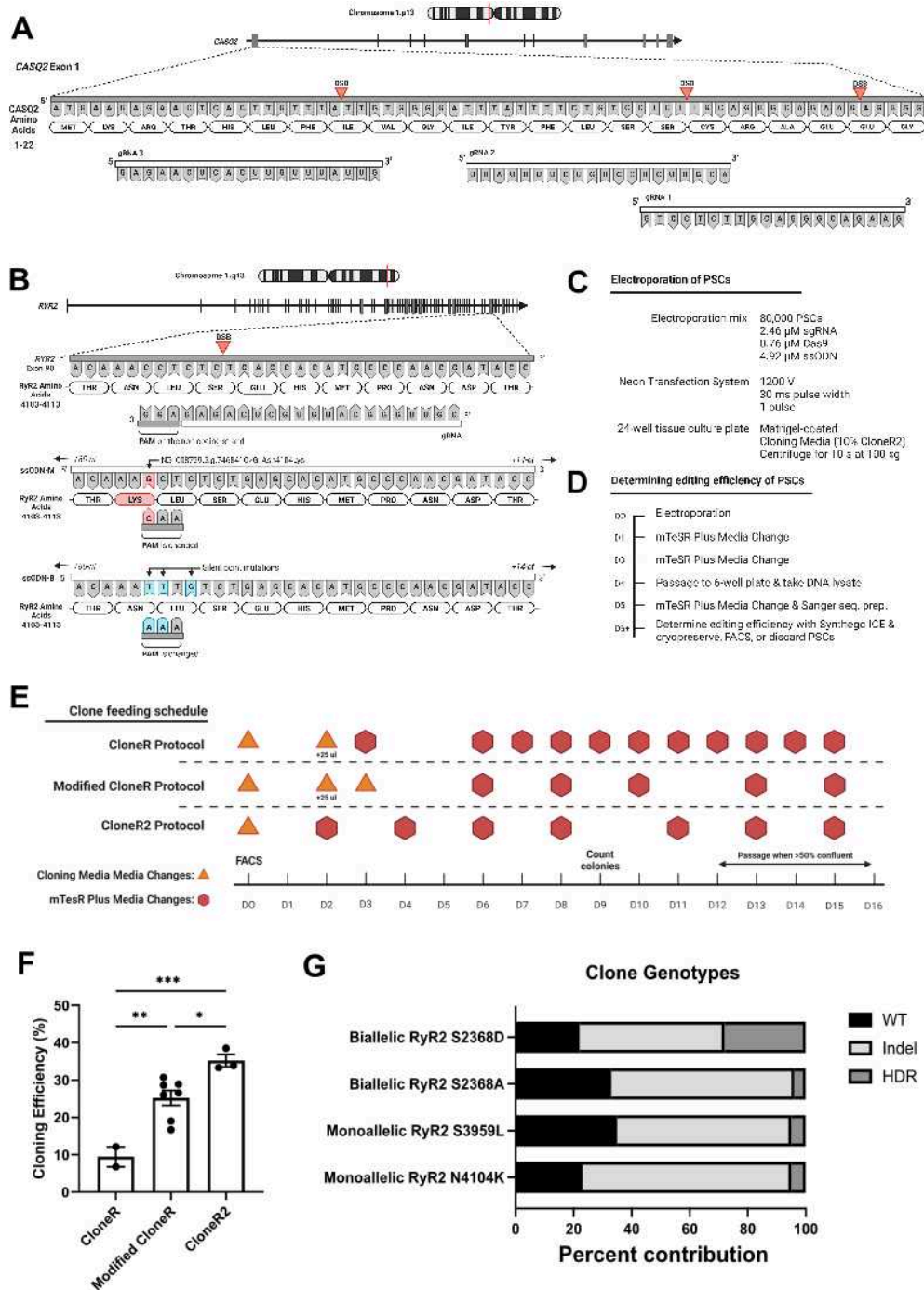


Figure 1

A: Schematic of sgRNA designs for producing a knockout of CASQ2

B: Schematic of sgRNA, ssODN-M and ssODN-B designs and sequence orientation used to produce a heterozygous point mutation in RYR2.

C: Summary of the key parameters used for electroporation of hPSCs for CRIPSR/Cas9 editing.

D: Workflow determining editing efficiency in the bulk population of hPSCs after electroporation with CRISPR/Cas9 components

E: hPSC clone feeding schedules. *CloneR* and *CloneR2* are the protocols according to the manufacturer's instructions. *Modified CloneR* is an alteration to the CloneR manufacturer's instructions that increased cloning efficiency.

F: Corresponding editing efficiencies. Points represent individual cloning experiments and the percentage of wells



across two-to-six 96-well plates that exhibited clonal colonies of hPSCs when plated at a density of 1 cell per well. One-way ANOVA with Tukey post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

G: The percentage of the genotypes for two biallelic and two monoallelic HDR mutations.

Materials

	General Reagents	Manufacturer	Catalogue number
	CloneR or CloneR2	STEMCELL Technologies	cat. no. 05889 (CloneR) or cat. no. 100-0691 (CloneR2)
	Corning Matrigel hESC-Qualified Matrix	Sigma-Aldrich	cat. no. CLS354277
	DMSO	Sigma-Aldrich	cat. no. D2438
	DPBS, no calcium, no magnesium	Gibco	cat. no. 14190444
	Human pluripotent stem cell lines		
	mFreSR	STEMCELL Technologies	cat. no. 05855
	mTeSR Plus	STEMCELL Technologies	cat. no. 100-0276
	Penicillin-Streptomycin	Thermo Fisher Scientific	cat. no. 15140122
	ReLeSR	STEMCELL Technologies	cat. no. 100-0484
	UltraPure DNase/RNase-Free Distilled Water	Invitrogen	cat. no. 10977015

Table of General Reagents

	Equipment	Manufacturer	Catalogue number
	12-Well cell culture plates	Corning	cat. no. 3513
	24-Well cell culture plates	Corning	cat. no. 353047
	6-Well cell culture plates	Corning	cat. no. 353046
	96-Well cell culture plates	Greiner or Corning	cat. no. 655180 (Greiner) or cat. no. 3595 (Corning)
	Automated cell counter		
	Cell Freezing Container	Corning	cat. no. 432138



Equipment	Manufacturer	Catalogue number
Centrifuge tubes, 15 mL	Corning	cat. no. 14-959-53A
Centrifuge tubes, 50 mL	Corning	cat. no. 352070
Coverslips		
Cryopreservation vials	Corning	cat. no. CLS430487
Flow Cytometer (FACS Aria III)	BD Bioscience	
Flow cytometry tubes, 5 mL	Corning	cat. no. 352063
Haemocytometer		
Microcentrifuge tubes (Sterile, Nuclease Free), 1.7 mL		
Microscope		
Neon Transfection System	Thermo Fisher Scientific	cat. no. MPK5000
PCR thermocycler		
T25 cell culture flask	Thermo Fisher Scientific	cat. no. 156367

Table of Equipment

Small molecules, proteins, kits, oligonucleotides	Manufacturer	Catalogue number
ALT-R CRISPR-Cas9 sgRNA, 2 nmol	Integrated DNA Technologies	
CRISPR Revolution sgRNA EZ Kit, 1.5 nmol	SYNTHGO	
DNeasy Blood & Tissue Kit (250)	QIAGEN	cat. no. 69556
Extract-N-Amp Blood PCR Kit	Sigma-Aldrich	cat. no. XNAB2R
Neon Transfection System 10 µl Kit	Thermo Fisher Scientific	cat. no. MPK1025
PCR primers (Single-stranded DNA oligos), 25 nmol	Integrated DNA Technologies	
Platinum Taq DNA Polymerase High Fidelity	Invitrogen	cat. no. 11304029

	Small molecules, proteins, kits, oligonucleotides	Manufacturer	Catalogue number
	QIAquick PCR Purification Kit (250)	QIAGEN	cat. no. 28106
	Sanger sequencing primers (Single-stranded DNA oligos), 25 nmol	Integrated DNA Technologies	
	TrueCut Cas9 Protein v2	Thermo Fisher Scientific	cat. no A36498
	Ultramer DNA Oligonucleotides (ssODNs), 4 nmol	Integrated DNA Technologies	
	Y-27632 (Rock inhibitor)	STEMCELL Technologies	cat. no. 72302

Table of small molecules, proteins, kits, oligonucleotides

	Software and online tools	Manufacturer	Web domain name
	COSMID		https://crispr.bme.gatech.edu/
	CRISP-ID		http://crispid.gbiomed.kuleuven.be/
	CRISPOR		http://crispor.tefor.net/
	IDT Alt-R Custom Cas9 crRNA Design Tool	Integrated DNA Technologies	https://sg.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM
	IDT CRISPR-Cas9 Design Checker	Integrated DNA Technologies	https://sg.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM
	NCBI GenBank		https://www.ncbi.nlm.nih.gov/nucleotide
	SnapGene Viewer	Dotmatics	https://www.snapgene.com/
	SYNTHGO ICE CRISPR Analysis Tool	SYNTHGO	https://www.synthego.com/products/bioinformatics/crispr-analysis
	Synthego CRISPR Design Tool	SYNTHGO	https://design.synthego.com/#
	NCBI BLAST		https://blast.ncbi.nlm.nih.gov/Blast.cgi

Table of software and online tools



Protocol materials

- ✕ Matrigel hESC-qualified (Corning Cat# 354277) **Corning Catalog #354277**
- ✕ UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- ✕ mTeSR plus media kit **STEMCELL Technologies Inc. Catalog #100-0276**
- ✕ CloneR™ 10 mL **STEMCELL Technologies Inc. Catalog #5888**
- ✕ CloneR2 **STEMCELL Technologies Inc. Catalog ## 100-0691**
- ✕ QIAGEN DNeasy Blood and Tissue Kit, 50 rxn **Qiagen Catalog #69504**
- ✕ Platinum Taq DNA Polymerase High Fidelity **Invitrogen - Thermo Fisher Catalog #10966034**

Safety warnings

- ⚠ Perform risk assessment and mitigation as required by your jurisdiction and institute or employer. Refer to the MSDS of each reagent and piece of equipment for additional details. This protocol requires the use of blue-light and/or UV-light gel docks which can cause skin burns and eye damage.

Ethics statement

Prior ethics approval should be obtained before work with human pluripotent stem cell lines. Work with human embryonic stem cells is prohibited in some countries or jurisdictions.

Ethical approval for the generation and/or use of human pluripotent stem cells (hPSCs) were obtained from QIMR Berghofer's Ethics Committee and were carried out in accordance with the National Health and Medical Research Council of Australia (NHMRC) regulations.

hPSCs (ESIBle003-A (RRID:CVCL_7158)) were obtained from WiCell.



Before start

We have designed this protocol to be as approachable as possible. It should be practicable by any lab that already cultures PSCs. This protocol can synergise with other protocols. For example, if completely scarless genome editing is required then refer to Kwart et al.'s protocol for their CORRECT method.

Citation

Kwart D, Paquet D, Teo S, Tessier-Lavigne M (2017)
. Precise and efficient scarless genome editing in stem cells using CORRECT.

<https://doi.org/10.1038/nprot.2016.171>

LINK

Guide RNA design for knockouts

For gene knockouts you use multiple sgRNAs to induce DSBs within a small region of the gene which will be repaired via non-homologous end-joining (NHEJ). Synthego's design tool (available at <https://design.synthego.com/>) is ideal for finding sgRNAs for this purpose. The tool provides sgRNAs that target early exons that are common to as many transcript splice variants as possible to increase the probability of inducing a robust knockout of the gene. It also provides estimation of off-target effects and on-target editing efficiency. For the knockout of CASQ2 100% on-target editing efficiency using Synthego sgRNAs (displayed in Figure 1A), which was confirmed by Sanger sequencing of > 20 clones. We recommend purchasing the top three sgRNAs that the Synthego design tool suggests to produce gene knockouts.

Guide RNA design for knock-ins

Here we summarize some key aspects that should be considered when designing gRNAs as suggested by Kwart et al. gRNAs can be identified by the presence of a PAM motif. Any NGG that appears around your desired mutation is a PAM motif and thus a potential site for an sgRNA. Figure 1B shows the orientation of the sgRNA to the genomic sequence and the ideal orientation of the ssODNs.

The 20-nt sgRNA sequence is sense to the 20-nt DNA sequence upstream of the PAM motif, and therefore binds to the opposing strand (i.e. the one that the PAM motif is not on). Cas9 induces the DSB between base pairs 3 and 4 upstream of the PAM motif. To increase the rate of homozygous HDR mutations the cut-to-mutation distance should be as small as possible, ideally within a few bases. For heterozygous HDR mutations, the cut-to-mutation distance should be between 2 and 26 bp.

Citation

Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, Olsen KM, Gregg A, Noggle S, Tessier-Lavigne M (2016) . Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9.

<https://doi.org/10.1038/nature17664>

LINK

The IDT sgRNA design tool can be used to quickly search for sgRNAs at any genomic locus. It provides an estimation of on-target cutting efficiency and off-target activity which will help picking the most promising gRNA. However, do not be deterred by a low score, having an optimal cut-to-mutation distance is the most important consideration. For example, the sgRNA that was used for the monoallelic RyR2 N4104K mutation (Figure 1B) has a low on-target score of 20 and a low off-target score of 31 (higher is better for both), yet we were still able to produce heterozygous clones at a rate greater than 1% (Figure 1G) with no evidence of off-target editing in any clone.

To functionally validate the on-target activity of candidate sgRNAs, Kwart et al. recommend screening them with a Surveyor nuclease assay. We did no such functional validation of any of our sgRNAs, instead we used Synthego's ICE tool to determine indel and HDR efficiency after electroporation of the bulk PSC population. The indel percentage predicted by this tool provides an indication for efficient on-target activity.

To minimise off-target activity, the sgRNA should differ from all other genomic sequences by at least 3 bp. These differences should be located in close proximity of one another and they should preferably be close to the PAM-proximal side of the sgRNA sequence.

We recommend ordering 2 nmol of Alt-R CRISPR-Cas9 sgRNA from IDT. This amount will be enough for approximately 30 reactions with the 10 µl Neon Electroporation Kit. IDT offers different types and formulations of gRNAs. For laboratory-scale disease modeling applications, single guide RNAs (sgRNAs) are the most economical option.

Donor template design for homology-directed repair

HDR requires the use of a single-stranded oligo DNA nucleotide (ssODN) that acts as a donor DNA repair template. When the ssODN is successfully incorporated, the PAM sequence must be changed to prevent the Cas9 RNP from rebinding and cutting the site again. So, you need to design the ssODN to also alter the PAM sequence. This must be done without inducing a change in the amino acid sequence, and any codon(s) you adjust must also appear elsewhere in the sequence – this is to ensure that the relevant tRNA is available during mRNA translation. It is also important that you do not change the NGG PAM motif to NGA or NAG as these are non-canonical PAM sites for *S.pyogenes* Cas9.



Citation

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F (2013). DNA targeting specificity of RNA-guided Cas9 nucleases.

<https://doi.org/10.1038/nbt.2647>

LINK

There are two types of ssODNs that are used in this protocol:

- ssODN-M induces your mutation of interest and one or more silent blocking mutations that prevent gRNA recognition of the target after they are incorporated.
- ssODN-B only induces one or more silent blocking mutations that prevent gRNA recognition of the target after they are incorporated.

Homozygous mutations only require ssODN-M. Heterozygous mutations can be efficiently achieved with only ssODN-M when the cut-to-mutation distance is 10-26 bp. An ssODN-B should be used for heterozygous mutations where the DSB is less than 10 bp from the target.

The ssODNs are 127-nt long and asymmetric: 91-nt on the PAM-proximal side of the double stranded break and 36-nt on the PAM-distal side. Richardson et al. found that this design yielded higher HDR rates when compared to other similarly sized ssODNs.

Citation

Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE (2016) . Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA.

<https://doi.org/10.1038/nbt.3481>

LINK

If it is not possible to silently change the PAM motif, then editing the bases immediately upstream of the PAM motif has been reported to work as they are the next-most critical for sgRNA recognition of the target site.



Citation

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F (2013). DNA targeting specificity of RNA-guided Cas9 nucleases.

<https://doi.org/10.1038/nbt.2647>

LINK

Primer design for PCR amplification and Sanger sequencing

PCR and Sanger sequencing primer designs generally have the same considerations. Sanger sequencing primers do benefit from the addition of a 2 bp GC clamp on their 3' end, and may have tighter restrictions regarding overall GC content. We were able to use the same primers for PCR and Sanger sequencing for all but one of our on-target and off-target sites.

For the genotyping of clones we recommend using the *Extract-N-Amp Kit for Blood* from Sigma Aldrich. This is a DNA-extraction-straight-to-PCR kit and may require some optimization with your primers. For all other PCR reactions we recommend following your own PCR workflows, we prefer Invitrogen's HiFi Platinum Taq.

For Sanger sequencing, talk to your sequencing facility about any specific primer requirements they may have regarding melting temperature, GC content, primer concentration, and template concentration. Remember to look for hard-to-sequence repetitive sequences surrounding your target site as this may prevent you from sequencing in a particular direction.

Single-cell cloning of PSCs without enrichment or selection

PSCs are not well disposed to single-cell passaging or cloning. These harsh conditions can cause cell-death, perturb their pluripotency and select for karyotypic abnormalities. This necessitates the use of dedicated media supplements like ROCK inhibitor Y-27632 during stressful conditions like FACs. Even with Y-27632, some protocols require the seeding of up to 30 cells per well because survival rates are low, but this then necessitates sub-cloning to ensure any genotype-positive clones arose from single cells. There are more bespoke media supplements, like the CEPT cocktail described by Tristan et al., that further improve efficiency but they can require a PSC adaption period of up to seven passages before single-cell cloning, extending the entire protocol by many weeks.



Citation

Tristan CA, Hong H, Jethmalani Y, Chen Y, Weber C, Chu PH, Ryu S, Jovanovic VM, Hur I, Voss TC, Simeonov A, Singeç I

(2023). Efficient and safe single-cell cloning of human pluripotent stem cells using the CEPT cocktail.

<https://doi.org/10.1038/s41596-022-00753-z>

LINK

In this protocol we demonstrate that it is possible to seed PSCs in 96-well plates at a density of one cell per well and achieve cloning efficiencies of ~30% without prior adaption to the cloning media or single-cell passaging. This was done with mTeSR Plus supplemented with 1X CloneR2, used according to STEMCELL Technologies provided instructions.



Reagent and equipment setup

- 1 **mTeSR Plus Media:** 40 mL of with 2.5% P/S, 10 mL of mTeSR Plus Media Supplement.

 mTeSR plus media kit **STEMCELL Technologies Inc. Catalog #100-0276**

Lot Number

- 2 **Cloning Media:**  22.5 mL of mTeSR Plus Media and  2.5 mL of CloneR or CloneR2.

 CloneR™ 10 mL **STEMCELL Technologies Inc. Catalog #5888**

 CloneR2 **STEMCELL Technologies Inc. Catalog ## 100-0691**

Lot Number

- 3 **sgRNAs:** On receipt dilute to  60 micromolar (μM) in sterile

 UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

in a BioSafety Cabinet under sterile conditions.

- 4 **ssODNs:** On receipt dilute to  100 micromolar (μM) in sterile

 UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

in a BioSafety Cabinet under sterile conditions.

- 5 **PCR and Sanger sequencing primers:** On receipt dilute to 100 μM in

 UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

r then create 10 micromolar (μM) working stocks of the primers also in UltraPure DNase/RNase-Free Distilled Water.

- 6 **Matrigel coating of tissue culture plates and flasks:** Obtain the dilution factor for the lot of  Matrigel hESC-qualified (Corning Cat# 354277) **Corning Catalog #354277** from the certificate of analysis on the Corning website. Thaw the Matrigel on ice and dilute in ice-cold sterile PBS- according to the dilution factor. Coat the surface of the tissue plate or flask with approximately 1 mL per 10 cm² of Matrigel.

PCR and Sanger sequencing primer design

2d

- 7 Search for gene of interest in the NCBI gene database. The URL structure should look like this: <https://www.ncbi.nlm.nih.gov/gene/1832> . Then navigate to 'Genomic regions, transcripts, and products' and follow the 'GenBank' hyperlink next to 'Go to nucleotide.' This will bring up the GenBank entry with all of the aligned features (DNA, mRNA, and amino acid sequences etc.)

? TROUBLESHOOTING Problem: Gene does not appear in GenBank

- 8 Download the .gb file by clicking: Send To ► Complete Record ► File ► Format (GenBank (full)) ► Show GI (ticked).
- 9 Open the .gb file in SnapGene and remove feature annotations that are not needed (e.g.: predicted transcript variants and non-coding RNA). You should be able to see the DNA, mRNA, and protein sequences all aligned.
- 10 Find the locus of your desired mutation.

▲ CRITICAL STEP For gene knockouts complete the sgRNA design first in Step 25 to find the location of the gRNA cut sites. For specific knock-in mutations you are restricted to the locus of your desired mutation.

- 11 Export a 1000 bp sequence into Primer-BLAST that is centred around the gRNA cut site(s).
- 12 Large deletions are possible in CRISPR/Cas9 reactions, so set the range such that the primers will be at least 300 bp from any gRNA cut site.
- 13 Change PCR product size to 300-1000 bp and Max T_m difference to 1.5.



- 14 In Advanced parameters, change Primer GC content (%) to 40-60%, GC clamp to 2.
- 15 Run the program and loosen the above parameters as necessary if no primer pairs are found.
- 16 Add the PCR and Sanger sequencing primers to the design in SnapGene, order them from IDT, and prepare them to 10 µM in Nuclease-free water on receipt.

▲ CRITICAL STEP The same primers can often be used for both PCR and Sanger sequencing. GC content, T_m, and GC clamp constraints are stricter for Sanger sequencing primers. Attempt to use the same primers for both purposes. Design new Sanger sequencing primers if PCR amplification succeeds but sequencing fails.

- 17 Extract genomic DNA from the parental PSC line that will be edited with a QIAGEN DNeasy Blood and Tissue Kit according to manufacturer's instructions.

 QIAGEN DNeasy Blood and Tissue Kit, 50 rxn **Qiagen Catalog #69504**

- 18 Prepare the following PCR reaction. Volumes are for a  25 µL reaction with Invitrogen Platinum Taq DNA Polymerase High Fidelity.



 Platinum Taq DNA Polymerase High Fidelity **Invitrogen - Thermo Fisher Catalog #10966034**

	Number of Samples	Component	Amount (1X)	[Final]	Amount * # Samples
	1				
		Genomic DNA		<500 ng	
		10X High Fidelity PCR Buffer	2.5 µl	1X	2.5
		50 mM MgSO ₄	1 µl	2.0 mM	1
		10 mM dNTP Mix	1 µl	0.2 mM each	1
		Platinum Taq	0.1 µl	1 U / rxn	0.1

	Number of Samples	Component	Amount (1X)	[Final]	Amount * # Samples
		10 μ M Primers	0.5 μ l	0.2 μ M (each)	0.5
		Nuclease-free water	to 25 μ l	-	25

- 19 Load samples into PCR thermocycler and run a program with a gradient annealing temperature of 53°C to 63°C . Note the 68°C Extension temperature which may be lower than other *Taq* polymerases.



Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	3 min	1x
Denaturation	94°C	15 s	40x
Annealing	$53\text{-}63^{\circ}\text{C}$	30 s	
Extension	68°C	1 min / 1,000bp	
Final Extension	68°C	10 min	
Hold	4°C	Infinite	1x

- 20 Load $10\ \mu\text{L}$ of each completed PCR reaction with $2\ \mu\text{L}$ of 5X loading dye on a 2% (w/v) agarose gel mixed with 10,000X SYBR Safe DNA Gel Stain.
- 21 Load a 100 bp ladder and run the gel at 70-90 V until the bottom band of the loading dye reaches 80% of the way down the gel.
- 22 Image the gel on a blue-light gel doc.
- ! CAUTION** Some laboratories may only have access to a UV-light gel doc that can cause burning of the skin and eye-damage. Always wear PPE appropriate for the system you are using.
- 23 From the annealing temperature gradient, identify the annealing temperature that produced the most definitive band with no smearing.
- 24 Perform PCR purification with the QIAquick kit on the best sample and submit it for Sanger sequencing.





- 25 Design three sgRNAs for a gene knockout, homozygous knock-in, or heterozygous knock-in.

STEP CASE

Design of for sgRNAs for a gene knockout

91 steps

1. Use Synthego's design tool available at <https://design.synthego.com/#> to design gRNAs for your target gene.
2. Choose three of the best gRNA designs, add them and their respective cut sites as features in SnapGene Viewer to easily visualise their orientation and proximity to the desired mutation.
3. Crosscheck that the gRNA designs will recognise the sequence of your cell line you identified. As long as the PAM motif is intact the sequence of the gRNAs can be changed to fit your sequence.

Design of sgRNA

1h

- 26 Verify that the gRNAs have a low predicted probability of inducing off-target mutations by using CRISPOR and COSMID. Repeat **Step 25** until you have gRNA designs that have low predicted off-target activity.

For the top five non-overlapping predicted off-target sites from each tool design and order PCR and Sanger sequencing primers according to **Steps 4-9**.

▲CRITICAL STEP As discussed above, off-target activity can be minimised by ensuring that the 20 bp gRNA sequence differs by at least 3 bp to potential off-target sites, and ensuring that those differences occur within 4 bp of each other, and are preferably on the PAM proximal side.



Ordering and preparing sgRNAs

1h

- 27 Order sgRNAs design with Synthego's design tool
- 27.1 From design tool output page, continue with the 3 best gRNA designs
- 27.2 Select Synthetic Modified sgRNA and order 1.5 nmol of each sgRNA
- 28 On receipt, reconstitute in sterile nuclease-free water under sterile conditions as in **Step 3**.

Thawing and expansion of PSCs for electroporation

1w

29 Thaw PSCs into appropriately sized cultureware. Our PSCs are cryopreserved with mFreSR and we thaw 1×10^6 PSCs into one T25 flask in mTeSR Plus supplemented with 10 μ M Y-27632.

▲CRITICAL STEP Use low-passage PSCs that are karyotypically normal.

30 Leave the cells to recover overnight

1d



31 After 24 hours, change the media to mTeSR Plus without Y-27632.

32 Passage the PSCs at least twice before electroporation.

6d

▲CRITICAL STEP Time the passages so that on the day of electroporation you can comfortably harvest at least 8×10^5 PSCs.



Electroporation of PSCs

3h

33 Two hours before electroporation coat two wells in a 24-well plate for each target with ESC-qualified Matrigel and incubate at 37°C for at least one hour. Then aspirate the Matrigel and add 1 mL of Cloning Media to each well.



▲CRITICAL STEP This protocol prepares a double volume electroporation mix to increase the pipetting volumes. Allowing for up to five different mutations to be performed with the prepared cell. You can scale as you see appropriate.

34 In a biosafety cabinet, prepare the Neon Transfection System Pipette Station by loading a new Transfection Tube and adding 3 mL of Buffer E.



▲CRITICAL STEP The Pipette Station and Transfection Tubes can be a source of contamination, especially if you are reusing the tubes. Be careful to maintain sterility of all components.

35 Prepare and conjugate the sgRNA-Cas9 RNP complex for at least 10 minutes at room temperature. The cells will be added in step xx. The ssODNs will be added immediately before electroporation.

	Components	Volume	Final concentration
	60 μ M sgRNA	1.0 μ l	2.5 μ M
	5 mg/mL TrueCut Cas9 Protein v2	1.0 μ l	0.8 μ M

Components	Volume	Final concentration
Buffer R	to 10 μ l	-
Cell suspension	12 μ l	80,000 PSCs

- 36 Remove mTeSR Plus from the PSCs. Wash with  3 mL of PBS- once.
 - 37 Add  2.5 mL of ReLeSR. After 50 seconds remove almost of the the ReLeSR, leaving a thin film on top of the PSCs.
 - 38 Incubate for 6-8 minutes at  37 °C in a 5% CO₂ incubator. The precise time is cell-line dependent. You want to have small clusters of PSCs.
 - 39 Tap the T25 flask once. Use  3 mL of mTeSR Plus to remove PSCs from the T25 and collect in a  15 mL Falcon tube. Do not pipette the PSCs up and down more than once when removing them from the T25 as it breaks up the clusters too much.
 - 40 Count the cells manually with a haemocytometer or a Countess 3 (or equivalent) automated cell counter.
- Cell count 
- 41 Transfer 8×10^5 cells to a  1.5 mL Eppendorf tube or  15 mL Falcon tube if the cell concentration is low. Keep the remaining cells for the DNA extraction to be performed after electroporation.
 - 42 Centrifuge at 300 xg for 3 minutes.





43 Remove the mTeSR Plus, being careful not to disturb the cell pellet. Then add  1 mL of PBS-, carefully resuspend the cell pellet and transfer to  1.5 mL Eppendorf tube if the cells were centrifuged in a  15 mL Falcon tube. Centrifuge again at 300 xg for 3 minutes.

44 Remove the PBS- and resuspend the PSCs in  60 μ L of Resuspension Buffer R. This is enough for 10 electroporation reactions.



▲CRITICAL STEP Work quickly from this point if you are doing multiple electroporation reactions as the PSCs will begin to die.

45 Prepare the final electroporation mix by transferring  12 μ L of the PSC-Buffer R suspension to the  10 μ L of Cas9-gRNA RNP prepared in **Step 35**. Mix gently.

46 If you are doing a HDR-mediated knock-in mutation then add  1.2 μ L of your ssODN-M, for heterozygous mutations add  0.6 μ L each of ssODN-M and ssODN-B.



47 Turn on the Neon Transfection System and enter the following electroporation settings: 1200 V, 1 pulse, 30 ms pulse width.

48 Use the Neon Transfection System Pipette loaded with a 10 μ L tip to aspirate 10 μ L of the electroporation mix.



▲CRITICAL STEP Ensure that there are no bubbles within the tip as this creates sparks. If you see a bubble then dispense the mix back into the Eppendorf tube and aspirate again.

? TROUBLESHOOTING Problem: Too little mix left for two electroporation reactions

49 Load the Pipette and tip into the Transfection Tube in the Pipette Station.

50 Electroporate and look at the tip. You should see bubbles at the lower electrode in the Transfection Tube. If you see a spark proceed as normal. Though sparks are not ideal and will cause cell death and lower transfection efficiency, the reaction can still be successful.

51 Immediately remove the Pipette from the Pipette Station and dispense the cells into one well of the Matrigel-coated 24-well plate.



▲CRITICAL STEP Slowly pipette out the cells while moving around the well in a circular motion. This prevents the PSCs from forming one big cluster which would increase spontaneous differentiation.

- 52 Repeat from **Step 48** for the rest of the electroporation mix. Use the same Neon Tip one more time. For more reactions use a new Neon Tip – each tip can only be used twice before they have to be discarded.
- 53 Place the electroporated PSCs into a 37°C incubator at 5% CO₂ and do not disturb for 24 hours.
- 54 Isolate and purify genomic DNA from the 1×10⁶ PSCs from **Step 41** with a QIAGEN DNeasy Kit (or equivalent) according to the manufacturers' instructions.

Expansion of electroporated PSCs and assessment of editing efficiency

1w 5d

- 55 24 hours after electroporation check the PSC survival rate, distribution, and morphology. Remove the Cloning Media and dead cells. Wash once with PBS-, and then add 1 mL of mTeSR Plus to each well.

? TROUBLESHOOTING Problem: Low cell survival after electroporation

- 56 48 hours after electroporation again check the PSC survival rate and morphology. This is when you should expect to see the greatest amount of cell death now that the Cloning Media has been removed.

? TROUBLESHOOTING Problem: No evidence of on-target cutting

- 57 Perform media changes with mTeSR Plus every two to three days until PSCs are 70% confluent.
- 58 Passage 50% each electroporated PSC population from the 24-well plate to one well of a 6-well plate.
- 59 Isolate and purify genomic DNA from the remaining PSCs in the 24-well plate with a QIAGEN DNeasy Kit (or equivalent) according to the manufacturers' instructions.
- 60 Perform PCR amplification for your target site from the genomic DNA collected from the parental PSCs (**Step 54**) and electroporated PSCs (**Step 59**). For gene knockouts, if you expect ≥30 bp to be deleted then you can run your amplicons on a 3% (w/v) agarose gel



to ascertain if any editing has occurred. Run the gel with a lower voltage (50-70V) to increase resolution.

- 61 Purify the PCR amplicons with the QIAGEN QIAquick kit (or equivalent).
- 62 Prepare and submit all amplicons for Sanger sequencing according to your sequencing facilities requirements. You can sequence the amplicons from either direction.
- 63 When the electroporated PSCs in the 6-well plate reach 50-70% confluency, passage to a T25. The PSCs are ready for single-cell sorting but continue to maintain and passage the PSCs in T25 flasks until the sequencing results return.
- 64 Alternatively, cryopreserve the electroporated PSCs and proceed to single-cell cloning at a later time. We recommend this if it take >7 days to receive Sanger sequencing results or to book FACS equipment.



Note

Cryopreserve PSCs in 1.5 mL cryovials (option A), clonal PSCs can be alternatively be frozen when they have been passaged into 24-well plates (option B)

(A) Cryopreservation in 1.5 mL cryovials.

1. Culture PSCs in a 6-well tissue culture plate or T25 flask until they are ~70% confluent
2. Bring mTeSR Plus to room temperature and begin thawing mFreSR at room temperature. The amount of mFreSR required will depend on the number of PSCs you expect to obtain ~ 1×10^6 PSCs can be frozen in 1 mL of mFreSR in a 1.5 mL cryovial.
▲ CRITICAL STEP mFreSR should remain cold and should complete thawing as the PSCs are finished centrifuging in step Box 1(A)8 below. mFreSR should only be thawed and refrozen once.
3. Passage with ReLeSR.
4. Collect PSCs in 3 mL of mTeSR Plus and count the total number of cells with a haemocytometer or automated cell counter.
5. Round down the count to the nearest million and centrifuge that number of PSCs at 300 xg for 3 mins. While they are centrifuging, label cryovials with the name of the line, passage number, number of cells, the date and your initials and any other details you wish to include.
6. Aspirate the mTeSR Plus from the PSCs. Gently flick the tube to break up the pellet, immediately resuspend in cold mFreSR, and aliquot 1 mL of the suspension into each of the labelled cryovials.
7. Place the cryovials into a Cell Freezing Container and store at -80°C overnight.
8. PSCs can remain at -80°C for up to 6 months. For longer term storage PSCs should be stored in liquid nitrogen.

(B) Cryopreservation in 24-well tissue culture plates.

▲ CRITICAL STEP This method uses a lot of mFreSR so it is very expensive. We only recommend it if you must pause the culturing of the clones and the predicted editing efficiency of the cloned population was high. There are published protocols for cryopreserving PSCs in 96-well plates that you can explore but we have not verified post-thaw viability when doing this.

1. Ensure most PSCs colonies are between 50-90% confluent. Growth rates will be different between PSC clones and you may lose some that are less confluent using this method.
2. Have a sealable polystyrene cooler ready with parafilm to seal it, and sufficient space for it in a -80°C freezer.
3. Aspirate media from the PSCs add ~500 μL of ReLeSR to each well for up to 1 minute.
4. Aspirate almost ReLeSR from each well in the same order it was added, leaving a thin film across the bottom of each well. Ensure that each well was exposed to the high volume of ReLeSR for the same amount of time.
5. Incubate the PSCs in a 37°C , 5% CO_2 incubator for 6-8 minutes. Start thawing mFreSR.
6. Working quickly, resuspend the PSCs in each well with 750-1000 μL of mFreSR.
7. Seal the 24-well plate with parafilm. Place into the polystyrene cooler and seal that with parafilm too. Place the cooler into a -80°C freezer and do not disturb for at least 24 hours.



8. This is not suitable for long term storage. Genotyping of the clones should be completed as soon as possible so genotype-positive clones can be thawed, expanded and cryopreserved in 1.5 mL cryovials.
9. For thawing 24-well tissue culture plates, remove all parafilm and place in a 37°C incubator until mFreSR is thawed. Collect the desired clones with mTeSR Plus. Centrifuge at 300 xg for 3 minutes to remove all mFreSR. Resuspend in mTeSR Plus and plate all of the PSCs into a Matrigel-coated 6-well tissue culture plate.

- 65 When Sanger sequencing results are returned open the .ab1 files with SnapGene Viewer and manually interrogate the traces for sequencing quality (i.e.: shadow peaks and noisy signals). Low quality traces may give inaccurate results when analysed with the ICE tool. Editing should be obvious and start at a sgRNA cut site.
- 66 Use Synthego's ICE tool to determine editing efficiency. Include the ssODN-M sequence for HDR mutations. The tool does not currently support the submission of more than one HDR template. The incident rate for heterozygous mutations can still be ascertained by submitting two samples, each with the ssODN-M or ssODN-B sequences.

? TROUBLESHOOTING Problem: Low on-target cutting

Single-cell sorting of PSCs

- 67 **▲CRITICAL** We only recommend single-cell cloning electroporated PSC populations when your mutation occurs at rate of >1%. An incident rate lower than this means the number of clones that will be produced will be beyond most people's ability to effectively manage.

Single-cell cloning can be repeated with a bulk population of edited PSCs until you acquire enough clones. Remember to always store a vial of your unsorted edited PSCs so that you can produce more clones at any point in the future.

After single-cell cloning you will want at least three clones with the desired genotype. We recommend you aim for five because you will have to discard any that are karyotypically abnormal or have any off-target editing.

- 68 Coordinate with your flow cytometry facility. Acquire necessary approvals and training. Submit relevant booking requests and provide important information for this sort that may help your flow cytometry facility staff.



Note

- Request an hour long booking on the FACS machine.
- No live/dead cell marker.
- 100-130 μm nozzle width to reduce shear stress on the PSCs.
- Receiving vessels are 96-well tissue culture plates.
- Cells will be sorted from Cloning Media.
- Relevant details regarding the PSC line. This will likely differ due to country or region specific regulations, but will usually include:
 1. Origin: human iPSCs or human ESCs
 2. For transgenic PSC lines provide details of the transgene and editing method
 3. For iPSCs include the reprogramming vector (e.g.: non-integrative sendai virus) and how many passages since they were reprogrammed

- 69 Two hours before the sort, coat two 96-well flat-bottom tissue culture plates with ESC-qualified Matrigel for at least one hour at 37°C. Two plates should produce 50 – 70 clones so consider scaling up or down based on the predicted editing efficiency you obtained in **Step 66**. However, culturing and passaging more than 70 clones is extremely time consuming and tedious. We do not recommend doing any more than two plates the first time you perform this protocol.
- 70 Prepare 25 mL of Cloning Media and bring it to room temperature.
- 71 After 1 hour, aspirate the Matrigel from the 96-well plates and add 100 μL of Cloning Media to each well.
- 72 Remove media from the PSCs and wash them with 2.5 mL of PBS-.
- 73 Add 2.5 mL of ReLeSR for 50 seconds, then aspirate it leaving thin film on top of the PSCs. Incubate the PSCs at 37°C in 5% CO₂ incubator for up to 8 minutes. The incubation time will be longer than a standard passage because you need the cells to dissociate more.
- 74 Tap the flask once and collect the PSCs in a 15 mL Falcon tube with 3 mL of Cloning Media. Pipette the PSCs up and down 2 to 5 times to break up the clusters.
- 75 Use a serological pipette to push the PSCs through a 40 μm cell strainer to remove large clumps.
- 76 Count the number of cells with a haemocytometer or automated cell counter.

- 77 If necessary, dilute the PSCs to $5 \times 10^5 - 1 \times 10^6$ cells per mL. Different FACS facilities or equipment may require different cell concentrations. We find with the ARIA Illu that 1×10^6 PSCs in 1 mL of Cloning Media was enough to sort into at least six 96-well plates.
- 78 Take 1 mL of the PSCs in Cloning Media and sort the PSCs into individual wells at a density of 1 cell/well. 
- ▲CRITICAL** The gating strategy must strictly eliminate doublets. Acquire assistance from an experienced user or technical staff if needed.
- 79 Centrifuge the 96-well plates at 100 *xg* for 10 seconds to encourage the PSCs to adhere to the Matrigel.
- 80 Place in a 37°C incubator at 5% CO₂, and do not disturb the cells for 48 hours.

Expansion of clonal colonies of PSCs

2w 2d

- 81 Here the protocol differs depending on whether you are using CloneR or CloneR2 in the Cloning Media. The CloneR protocol [⇒ go to step #88.1](#) presented here is modified from the manufacturer's instructions in that the Cloning Media is left on the colonies for an additional 72 hours after FACS. The CloneR2 protocol [⇒ go to step #88.2](#) follows the manufacturer's instructions. A schematic of the feeding schedule is found in Figure 1E.

81.1 CloneR media changes

(i) Day 2: Add 25 µl of Cloning Media to each well

(ii) Day 3: Perform a full media change with Cloning Media

(iii) Day 6: Perform a full media change with mTeSR Plus

(iv) Day 7+: Perform full media changes with mTeSR Plus every 2-3 days

(v) Day 11: Count the number of colonies. Number the lid of each well with a colony. Perform a full media change with mTeSR Plus on the wells with colonies.

(vi) Day 13: Perform a full media change with mTeSR Plus on the wells with colonies.

[⇒ go to step #89](#)



81.2 CloneR2 media changes

(i) Day 2: Perform a full media change with Cloning Media

(ii) Day 4+: Perform a full media change with mTeSR Plus every 2-3 days

(iii) Day 7: Count the number of colonies. Number the lid of each well with a colony. Perform a full media change with mTeSR Plus on the wells with colonies.

(iv) Day 8+: Perform a full media change with mTeSR Plus every 2-3 days

 [go to step #89](#)

Passaging clonal colonies of PSCs

4h

82 Coat new 96-well plates and 24-well plates with ESC-qualified Matrigel for one hour at 37°C. Each colony will be split into a one well of the new 96-well plates **AND** one well of the new 24-well plates, so ensure you coat enough wells. Coat a few additional wells in case you make a mistake while passaging.



▲CRITICAL Passage the PSC colonies when the average colony size covers at least 25% of the well surface area. Only passage colonies that show typical PSC morphology with no evidence of spontaneous differentiation. Mark poor quality colonies that you do not want to passage on the lid of the plate.

83 After the new plates are coated with Matrigel, add 50 µl of mTeSR Plus to each well of the 96-well plate and 450 µl of mTeSR Plus to each well of the 24-well plate.

84 Label the wells on the lids of the new plates with the same numbers that you gave the colonies.

85 Passage 10-20 colonies at a time.



▲CRITICAL STEP During passaging you must be confident that you are transferring each clone into the correct corresponding wells of the new plates, so take your time, and discard wells if there's any doubt that you made a mistake.

85.1 Aspirate the media, wash the colonies in PBS.

85.2 Working quickly, add ReLeSR to each colony in numerical order. Have a timer on that is counting up or an analogue clock nearby that you can refer to. Use a P1000 pipette set to 1000 μ l and try to dispense 50-100 μ l into each well. The exact volume does not matter, as long as the colony is covered.

85.3 Aspirate the ReLeSR from the colonies again in numerical order.



▲CRITICAL STEP Do not leave the colonies immersed in ReLeSR for more than 50 seconds, so work quickly and after ~40 seconds start aspirating the ReLeSR.

85.4 Incubate the plate for 6-8 minutes in a 37°C incubator at 5% CO₂.

85.5 With a P200 pipette, firmly pipette 100 μ l of mTesR Plus directly onto each colony – again in numerical order – to break them up and bring the PSCs into suspension. The colonies should be large enough to be able to see by eye.



▲CRITICAL STEP Do not try to dislodge the PSCs by tapping the plate this can cause cross-well contamination, and use a different pipette tip each time to prevent cross contamination.

85.6 With a microscope, quickly check if the PSCs have detached in each well. Large colonies may not be completely detached. Pipette up and down a few additional times to break up these colonies.

85.7 Use a P200 pipette to transfer 50 μ l of each clone to the corresponding wells of the new 96-well and 24-well plates. The passaging ratio is deliberately not adjusted for the differences in surface area of the 96-well and 24-well plates because the 96-well plate needs to reach 100% confluency faster.



▲CRITICAL STEP Again, you have to be absolutely sure that you are transferring each clone to the correct wells of the new plates. If there is any doubt then you must discard that clone. Be careful of droplets on the end your pipette tip as these can fall into wells and contaminate other clones.

85.8 Repeat until all colonies are passaged.

85.9 There will likely be residual cells in the original 96-well plates. You can add 100 μ l of mTeSR Plus back into these wells as the cells can re-adhere. This provides a backup for DNA extraction.

86 Centrifuge the plates at 100 $\times$ g for 10 seconds to encourage the PSCs to adhere to the Matrigel.

Maintenance and genotyping of clonal colonies of PSCs

1w

87 Perform a full media change with mTeSR Plus every 2–3 days on all plates. Expect a large amount of cell death and debris after passaging. Consider a PBS- wash for the first media change after passaging to remove all of the debris.

88 Once the clones in the 96-well plate are all 90%+ confluent generate DNA lysates and perform PCR with the Extract-N-Amp Blood PCR Kit. If you are producing gene knockouts and are expecting a ≥ 30 bp section of DNA to be deleted you can run 6 μ l of these reactions on an agarose gel as described above. If you designed an RFLP assay you can perform it now. Otherwise, proceed to genotype with Sanger sequencing.

89 Purify the PCR amplicons with the QIAquick Kit according to the manufacturer's instructions.



▲CRITICAL STEP Elute in nuclease-free water, salts in eluent buffers will interfere with Sanger sequencing.

90 Prepare and submit samples for Sanger sequencing

91 Continue to maintain the clones in the 24-well plates. Discard any clones that show excessive signs of spontaneous differentiation, are growing too slowly, or display atypical PSC morphology. Each clone will grow at different rates and reach confluency at different times. We recommend passaging clones once they reach 70–80% confluency into a new 24-well plate. Keeping them in the 24-well plate format will reduce the cost of maintaining them.



■ PAUSE POINT Cryopreserve the clones in the 24-well plate (Box 1), and thaw them once they have been genotyped. This will be expensive if you are using proprietary freezing media like mFreSR.

92 *Interpretation of Sanger sequencing results.*

HDR edits are easy to see in Sanger chromatograms as anything other than your desired mutation can be discounted. NHEJ-mediated gene knockouts can produce chromatograms that are difficult to interpret with overlapping traces that represent the indels of each allele. The online tool CRISP-ID can assist in the deconvolution complex traces (available at <http://crispid.gbiomed.kuleuven.be/>).



▲CRITICAL STEP For knockouts, ensure that indels result in frameshift mutations, introduction of early stop codons, or deletion of the start codon.

93 **■ PAUSE POINT** Passage clones that you want to keep into 6-well plates and then cryopreserve them once they are ~70% confluent.



Quality control of PSC lines

- 94 **Morphology of PSC lines.** The morphology of PSCs is the fastest way to ascertain whether they have lost their pluripotency. PSC lines should show typical PSC morphology.
- 95 **Checking for clonality.** Sub-cloning and genotyping of at least ten colonies will reveal whether each line is truly clonal.
- 96 **Karyotyping of PSC lines.** Single-cell cloning of PSCs can induce chromosomal abnormalities and rearrangements. We are a proponent of genotyping with array-based methods. Standard G-banding is extremely difficult to perform and interpret, and does not provide adequate resolution. We use the KaryoStat Assay.
- 97 **Sequencing off-target sites.** At a minimum the top 10 predicted off-target sites in the genome should be sequenced. COSMID, CRISPOR, and the design tools that IDT and Synthego use can all provide predicted off-target sites. We sequence the top 5 non-overlapping sequences from two of these tools.
- 98 **Assessing differentiation potential.** The most relevant, and often fastest, qualitative assessment of differentiation potential is to simply try and differentiate each PSC line to your cell type of interest according to a protocol that you are familiar with. Supplement this by assessing expression of pluripotency markers.

Troubleshooting

- 99 **Problem:** Gene does not appear in GenBank
Possible reason: Gene is not fully annotated
Solution: Import the FASTA sequence into Snagene Viewer and annotate manually
- 100 **Problem:** Too little mix left for two electroporation reactions
Possible reason: Neon pipette is inaccurate or inaccurate pipetting of small volumes when making mix
Solution: Prepare 2.5X volume reactions instead; add a small amount of Buffer R to an already prepared mix and try again
- 101 **Problem:** Low cell survival after electroporation
Possible reason: Presence or high concentration of Pen/Strep in Cloning Media
Solution: Remove or reduce the concentration of Pen/Strep. Keep total time that hPSCs are in suspension to less than 15 minutes. Be gentle when passaging and distributing hPSCs
- 102 **Problem:** No evidence of on-target cutting
Possible reason: Poor sgRNA(s) design and binding
Solution: Repeat reaction 1-2 more times to ensure editing is not possible. Redesign sgRNA(s)



- 103 **Problem:** Low on-target cutting
Possible reason: Poor conjugation of sgRNA(s) to Cas9. Poor sgRNA(s)
Solution: Increase ratio of sgRNA to Cas9 from 3:1 to 9:1. Increase total amount of sgRNA-Cas9 RNP. Redesign sgRNA(s).

Citations

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