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Generation of knockout iPSCs using CRISPR-Cas9 genome editing

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We use this protocol and it's working

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Abstract

This protocol describes the genetic modification of induced pluripotent cells (iPSCs) using CRISPR-Cas9, including synthesis of gRNA plasmids, transfection, selection of clones, and sequencing of genomic DNA to confirm knockout generation. The steps described in this protocol is based on Skarnes et al. (2019) and Fernandopulle et al. (2018).

Attachments



[Rafiq-Protocol-CRISP...](#)

25KB

Materials

Nucleic acid reagents:

-  pSpCas9(BB)-2A-GFP (PX458) addgene Catalog #48138
- **Guide RNAs (gRNAs)** (Designed in CHOPCHOP and ordered from Integrated DNA Technologies)

1. Prepare the following reaction mixture:

	A	B
	Fast AP	3 µl
	Fast Digest BBS1	3 µl
	1x Fast Digest Green	6 µl
	PX458	5 µg
	H2O	(up to 60 µl)

2. Prepare the following reaction mixture:

	A	B
	Sense gRNA (100 µM stock)	1 µl
	Antisense gRNA (100 µM stock)	1 µl
	T4 PNK Buffer	1 µl
	ATP (10 mM)	1 µl
	T4 PNK	0.5 µl
	H2O	5.5µl

3. Prepare the following reaction mixture:

	A	B
	PX458 Vector	50 ng
	gRNA (1:200 Stock)	1 µl
	2X Quick Buffer*	5 µl
	sH2O	1.5 µl



	A	B
	Quick Ligase*	1 μ l
		11 μ l

*Quick Ligation™ Kit (New England Biolabs).

Troubleshooting



Molecular cloning

- 1 Design gRNAs using CHOPCHOP (<https://chopchop.cbu.uib.no/>). Specific gRNAs used in this study can be found in the method section of our manuscript.
- 2 Order gRNAs as complementary single stranded oligonucleotides (Integrated DNA Technologies). The indicated overhanging nucleotides should be included to allow for cloning (Method section of our manuscript).
- 3 Cloning reagents are purchased from Thermo Scientific Fisher, unless stated otherwise.

Digest PX458.

30m

- 4 Prepare the following reaction mixture:

A	B
Fast AP	3 µl
Fast Digest BBS1	3 µl
1x Fast Digest Green	6 µl
PX458	5 µg
H2O	(up to 60 µl)

- 5 Incubate the sample for  00:30:00 at  37 °C .

30m



- 6 Run the sample on the gel, excise the band, perform a gel extraction, and measure the concentration on the Nanodrop.

Anneal and phosphorylate gRNA.

38m

- 7 Prepare the following reaction mixture:

A	B
Sense gRNA (100 µM stock)	1 µl



A	B
Antisense gRNA (100 µM stock)	1 µl
T4 PNK Buffer	1 µl
ATP (10 mM)	1 µl
T4 PNK	0.5 µl
H2O	5.5µl

8 Set up the following parameters on the PCR machine:

38m



- 37 °C 00:30:00
- 95 °C 00:01:00
- 85 °C 00:01:00
- 75 °C 00:01:00
- 65 °C 00:01:00
- 55 °C 00:01:00
- 45 °C 00:01:00
- 35 °C 00:01:00
- 25 °C 00:01:00

Ligate the gRNA and PX458.

5m

9 Prepare the following reaction mixture:

A	B
PX458 Vector	50 ng
gRNA (1:200 Stock)	1 µl
2X Quick Buffer*	5 µl
sH2O	1.5 µl
Quick Ligase*	1 µl
	11 µl

*Quick Ligation™ Kit (New England Biolabs).



- 10 Incubate the transformation for 00:05:00 at Room temperature , then put On ice to chill.

5m



Transformation

1h 32m 45s

- 11 Add 1 μL of the ligation to Oneshot Stable3 bacteria.



- 12 Incubate On ice for 00:30:00 .

30m



- 13 Heat shock in the 42 °C water bath for 00:00:45 .

45s

- 14 Place On ice for 00:02:00 .

2m

- 15 Add 100 μL of super optimal broth with catabolite repression (SOC), and place on 37 °C shaker for 01:00:00 .

1h



- 16 Spread all the bacteria on an Ampicillin plate.

Transfection

- 17 Dissociate 60–70% confluent iPSCs in a 6-well plate according to Method section.

- 18 Transfect cells with 3 μg PX458-gRNA using P3 Primary Cell 4D-Nucleofector™ (Lonza) using CA-137 parameter according to manufacturer's protocol.

- 19 Plate nucleofected cells in geltrex-coated dishes in E8 Flex media containing 10 micromolar (μM) Y-27632 (rock inhibitor).



Cell Sorting

2d

- 20 Select GFP-positive cells by Fluorescence-activated cell sorting (FACS) post-transfection 🕒 48:00:00 2d
- 21 After sorting, plate the cells in Geltrex-coated dish in E8 Flex media containing Y-27632 (rock inhibitor).
- 22 Replace the media with fresh E8 Flex media the next day.

Immunoblotting and Sanger sequencing

- 23 Check wells each day to identify wells with a single colony. Split and expand each clonal well when possible.
- 24 Screen colonies for target protein using immunoblotting or sanger sequencing.
- 25 For sanger sequencing, ICE CRISPR analysis tool (<https://www.synthego.com/products/bioinformatics/crispr-analysis>) was used to analyze the relative contribution of insertions and deletions (INDELS).
- 26 Expand and freeze KO lines.

Protocol references

1. Fernandopulle, M.S., R. Prestil, C. Grunseich, C. Wang, L. Gan, and M.E. Ward. 2018. Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons. *Curr Protoc Cell Biol.* 79:e51.
2. Skarnes, W.C., E. Pellegrino, and J.A. McDonough. 2019. Improving homology-directed repair efficiency in human stem cells. *Methods.* 164-165:18-28.