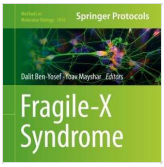

Sep 02, 2021

3.4 Genome Editing with CRISPR/Cas9

Book Chapter

In 1 collection

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ABSTRACT

This is part 3.4 of the "Induced Neurons for the Study of Neurodegenerative and Neurodevelopmental Disorders" collection of protocols.

Collection Abstract: Patient-derived or genomically modified human induced pluripotent stem cell s (iPSCs) offer the opportunity to study neurodevelopmental and neurodegenerative disorders. Overexpression of certain neurogenic transcription factors (TFs) in iPSCs can induce efficient differentiation into homogeneous populations of the disease-relevant neuronal cell types. Here we provide protocols for genomic manipulations of iPSCs by CRISPR/Cas9. We also introduce two methods, based on lentiviral delivery and the piggyBac transposon system, to stably integrate neurogenic TFs into human iPSCs. Furthermore, we describe the TF-mediated neuronal differentiation and maturation in combination with astrocyte cocultures.

ATTACHMENTS

Sauter2019_Protocol_InducedNeuronsForTheStudyOfNeu.pdf

DOI

dx.doi.org/10.17504/protocols.io.bqhemt3e

EXTERNAL LINK

https://link.springer.com/protocol/10.1007/978-1-4939-9080-1_9

PROTOCOL CITATION

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COLLECTIONS

Induced Neurons for the Study of Neurodegenerative and Neurodevelopmental Disorders

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Human induced pluripotent stem cells, Nucleofection, PiggyBac transposon, Lentiviral transduction, CRISPR/Cas9, Transcription factor-mediated neuronal differentiation, Astrocyte coculture

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MATERIALS TEXT

2. Materials

2.4 Genome Editing with CRISPR/Cas9

1. Cas9-sgRNA construct, such as pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid #62988) or pSpCas9(BB)- 2A-GFP (PX458) (Addgene plasmid #48138), which is expressing both sgRNA together with the gRNA scaffold as well as the staphylococcus pyogenes Cas9 [10]. Store at **-20 °C**.
2. [M]**10 x T4 Ligation Buffer (New England Biolabs)**. Store at **-20 °C**.
3. T4 Polynucleotide Kinase (New England Biolabs). Store at **-20 °C**.
4. BbsI restriction enzyme ([M]**10 U/µl**) (Thermo Fisher Scientific). Store at **-20 °C**.
5. Buffer G (Thermo Fisher Scientific). Store at **-20 °C**.
6. Calf Intestinal Alkaline Phosphatase (CIP) (New England Biolabs). Store at **-20 °C**.
7. Electrophoresis gel and chamber.
8. Gel extraction kit, such as QIAquick® Gel Extraction Kit (Qiagen).
9. Ligation kit, such as Mighty Mix ligation kit (Clontech). Store at **-20 °C**.
10. Chemically competent bacteria (e.g., Stbl3). Store at **-80 °C**.
11. S.O.C. medium. Store at **4 °C**.
12. LB-Antibiotics plates with [M]**100 µg/mL ampicillin**. Store at **4 °C**.
13. Miniprep kit, such as QIAprep® Spin Miniprep Kit (Qiagen).
14. Sequencing primer, such as 5'-TTTCTTGGGTAGTTTGCAGTTTT-3'. Dilute in ddH_2O to a concentration of [M]**10 Micromolar (µM)** and store at **-20 °C**.
15. 4D-Nucleofector™ System (see Materials section in protocol 3.2).

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

NB Introduction, Notes, and References are in the Collection Guidelines tab

3.4 Genome Editing with CRISPR/Cas9 1d 7h 52m 45s

1 Design a sgRNA for the locus of interest using designing tools (such as http://crispr.mit.edu/, *see* **Note 23**) [12].

2 Order two oligos representing the sgRNA with a design as follows: top oligo—CACC(G)[20 N of sgRNA], bottom oligo—AAAC[20 N reverse complement of sgRNA](C) [10] (*see* **Note 24**).

3 35m

Anneal the oligos in a thermocycler using **2 µl top oligo**, **2 µl bottom oligo**, **2 µl 10× T4 Ligation Buffer**, T4 Polynucleotide Kinase, and **13 µl ddH2O** and program the thermocycler with **00:30:00** at **37 °C**, **00:05:00** at **95 °C**, a ramp down to **25 °C** (ramp rate of 5 °C/min) and hold at **4 °C**. The oligo hybrid can directly be used for cloning into the linearized PX459 or PX458 vector.

4

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Cut and dephosphorylate the vector using **2 µl BbsI** with **3 µg vector** and fill *up to 50 µl* reaction volume with ddH_2O (minimum of 0.5 µl BbsI for 5 µg of vector).

5 1h 15m

Incubate for **01:00:00** at **37 °C** and optionally heat-inactivate the digestion for **00:15:00** at **65 °C** .

6 Gel-purify the vector by running on an agarose gel and extracting the band using a gel extraction kit, such as QIAquick® Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The vector can be stored at **-20 °C** for several months.

7 30m

Ligate sgRNA insert and vector in a 10 µl reaction with **100 ng vector** , **2 µl oligo hybrid (1:250 diluted)** , **5 µl Mighty Mix** , and ddH_2O. Incubate the mixture for **00:30:00** at **16 °C** .

8 Transform **5 µl reaction product** into chemically competent bacteria, such as Stbl3. The rest of the reaction can be stored for up to 2 weeks.

9 32m 45s

Thaw the bacteria for ~ **00:10:00** **On ice** , add the ligation reaction, stir carefully and incubate **On ice** for **00:05:00** – **00:10:00** . Perform a heat shock for **00:00:45** at **42 °C** transfer the sample back to ice for **00:02:00** – **00:05:00** . Add **300 µl S.O.C. medium** and shake the bacteria at **300 rpm, 37°C, 01:00:00** .

10 1h

Plate the bacteria on LB-plates with **100 µg/mL ampicillin** and incubate **Overnight** at **37 °C** and proceed with the plasmid preparation using a Miniprep kit such as the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's guidelines.

11 Check the construct for correctness using the respective sequencing primer and expand the DNA to a high concentration stock.

Optionally: Test the cutting efficiency with a T7 endonuclease assay [13] (*see* **Note 25**).

12 If homologous recombination of a disease correction or knockin of a reporter is the aim of the gene editing, a donor construct has to be provided in addition to the Cas9-sgRNA vector(s). The tag or the gene for the knockin should be framed by regions of the locus in approximately the same length as the part that is transferred into the gene (such as ~1000 bp for incorporation of an antibiotic resistance with promoter) and provided as a linearized DNA fragment. Alternatively, 5–25 bp homology can be used with microhomologymediated end-joining [14].

13 Electroporate **10 µg vector** encoding for the correct sgRNA and optionally ~ **5 µg donor construct** into iPSCs (*see* materials and steps in protocol 3.2). Seed the cells into two wells of a 6-well plate.

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14 Using the PX459 vector, it is advisable to use [M]**0.5 µg/mL** – [M]**0.8 µg/mL puromycin** for **12:00:00** – **16:00:00** for the next night after electroporation in combination with ROCKi. 1d 4h

15 Keep ROCKi for 2 days and check if you obtained single cells that grow to small colonies. Once the colonies are visible by eye, check half of a colony for integration or knockout of the target gene by (colony) PCR with primers binding around or in the locus that you tested for specificity before (using control DNA). Transfer the other part of each colony not used for PCR to a new well (of a 48-well plate). These are the monoclonal knockout lines usable if the PCR shows the respective shifts.

16 Once a potentially positive clone has been detected, sequence the locus using Sanger sequencing, optimally with subcloning of the PCR product into a carrier vector (for instance using TOPO cloning).

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