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CRISPR editing of CHMP2B gene in KOLF2.1J AAVS-NGN2 iPSCs

Electroporation of Cas9 protein

Forked from CRISPR editing of TMEM230 and TMEM9/9B genes in H9 ES AAVS-NGN2; Flag-EEA1 cells

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes a method for either knock out of CHMP2B, as well as knockin of CHMP2B Q165X or CHMP2B I29V in human KOLF2.1J AAVS1-NGN2 iPSCs; (see protocol: dx.doi.org/10.17504/protocols.io.kqdg3×99eg25/v1) cells using either CAS9 or Cpf1 and electroporation with a NEON system.

Attachments

PDF

Electroporation_of_C...

20KB

Materials

Materials

Cells employed: KOLF2.1J AAVS1-TREG3-NGN2 iPSCs (CVCL-D1J6)

Electroporation kit: Neon Transfection Kit (Invitrogen, MPK1096B)

Reagents

	Y-27632 Dihydrochloride (ROCK inhibitor)	Biozol	S1049

Gene editing enzyme

Cas9-NLS	QB3 MacroLab; UC Berkeley	

gRNA synthesis

	GeneArt Precision gRNA Synthesis Kit	Thermo Fisher Scientific	A29377

Oligonucleotides

	gRNA for creation of CHMP2B-/-	IDT	GCCAAACAACTTGTGCATC TACGG
	gRNA for creation of CHMP2B Q165X/+	IDT	ATCAAGAACTTGATTCACA ATATC
	gRNA for creation of CHMP2B I29V/I29V	IDT	AGAGTTACGAGGTACACAG A
	Ultramer homolog arms for CHMP2B Q165X	IDT	ccaactaagaaaagatgatgttcat acctttccagaaatttcaattccaat CtcatcaagaacttAattcacaata

			tcctggctttcttcttcgtcatcagaaccgtcaaagatgtc
	Ultramer homolog arms for CHMP2B I29V	IDT	ctcctagatgtaataaaggaacagaatcgagagttacgaggtacacagagAgctataGtcagagatcgagcagctttagagaaacaagaaaaacagctggtaagtag
	CHMP2B-/- genotyping forward PCR primer	IDT	5'-AAGAAAATGGCCAAGATTGGTA
	CHMP2B-/- genotyping reverse PCR primer	IDT	5'-CCATCTTCATTTGGGAATTCAT
	CHMP2B Q165X genotyping forward PCR primer	IDT	5'-TTGATGACATCTTTGACGGTTC
	CHMP2B Q165X genotyping reverse PCR primer	IDT	5'-GAAATAAAAACCATGCACCTCC
	CHMP2B I29V genotyping forward PCR primer	IDT	5'-GGTTTCTTTTGTGATTCTCCTAG
	CHMP2B I29V genotyping reverse PCR primer	IDT	5'-CATGTGCCTTCTTCCTAGTTAGC

Safety warnings

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

Before start

Use ThermoFisher Kit to directly electroporate ESCs with Cas9 protein and sgRNA.
Works better than plasmid transfection.

1 Add 10 µL buffer R to a sterile 1.5 ml tube. Add 6 µg purified Cas9 or Cpf1 protein (2mg/ml). Then add 1.2 µg sgRNA Pipet up and down to mix. Let it sit at Room temperature for 00:10:00. This is enough for 2 transfections (== two 24 well). Buffer R is a reagent in the electroporation kit: Neon Transfection Kit (Invitrogen, MPK1096B). In the case of knock-in mutations, also add 1 microgram of the homology mediated repair template. 10m

STEP CASE

gRNAs and repair template 19 steps

CHMP2B knock-out sgRNA target sequence: GCCAAACAACTTGTGCATCTACGG

CHMP2B Q165X knock-in sgRNA target sequence: ATCAAGAACTTGATTCACAATATC

CHMP2B I29V knock-in sgRNA target sequence: AGAGTTACGAGGTACACAGA

To generate hESCs homozygous for CHMP2B Q165X variant, a ssDNA oligo for homology mediated repair was included in the electroporation (ccaactaagaaaagatgatgttcatacctttccagaaatttcaattccaatCtcatcaagaacttAattcacaatatcctggctttcttcttcgtcatcagaaccgtcaaagatgtc).

To generate hESCs homozygous for CHMP2B I29V variant, a ssDNA oligo for homology mediated repair was included in the electroporation (ctcctagatgtaataaaggaacagaatcgagagttacgaggtacacagagAgctataGtcagagatcgagcagctttagagaaacaagaaaaacagctggtaagtag).

Employ Geneart Precision gRNA Synthesis kit according to manufacturer instructions (Thermo Fischer, A29377)
Use the following primers together with Tracr Fragment PCR template for PCR
Forward: 5'-TAATACGACTCACTATAGAGTAGAACGTGAGAGGCTCA
Reverse: 5'-TTCTAGCTCTAAAACTGAGCCTCTCACGTTCTACT

2 While waiting for the Cas9 to bind to sgRNA, individualize KOLF2.1J AAVS1-NGN2 iPS cells with Accutase. Neutralize Accutase with 5x volume E8 with Rock inhibitor.

3 Count cells. You will need 2×10^5 for each transfection.

4 Spin down cells. Let it sit for a while so all the residue media can go down to the bottom of the tube. If the residue media is too much, take it out with a P200 pipet.

5 Resuspend cells to a concentration of 2×10^5 per 5 µl (ie 4×10^7 per ml) using buffer R.

Note

You don't have to take all the residue media off but you will need to take into account the volume of residue media so you are not too much off.

6 Prepare a 24 well matrigel coated plate. Add 0.5 mL - 1 mL E8+ rock inhibitor (1:1000) to the wells you will use. Add Human Serum Albumin (1:2500) to each well. Each transfection goes into one well. Typically, we perform 2-3 transfections per gRNA-Cas9 complex.

7 Wipe the Neon pipet station with EtOH and place it inside the hood.

8 Add 3 mL electrolytic buffer (buffer E) to the neon tube. Place the tube inside the station. You should feel a click before the tube is securely seated in the station.

9 Use program 13 from the optimization tab for electroporation parameter. Program 9 should also work.

10 When everything is ready, mix 10 µL - 11 µL of resuspended cells with the Cas9 (or Cpf1)+RNA containing R buffer. The final volume should be in the range of 21 µL - 22 µL .

11 Take up a NEON tip, pipet 10 µL cell protein mix and electroporate with program 13.

Note

It is important to pipet slowly to avoid air bubble formation. It is also important to insert the pipet slowly into the station, especially during the end of the insertion when you will feel a click. I normally help the pipet down slowly during the clicking so there is no sudden movement of the tip, which might create tiny air bubbles.

12 If you see air bubble in the tip, take it out, push everything out of the tip and re-pipet the mixture.

13 If you see sparking during the electroporation, your efficiency will reduce significantly.

14 Once electroporation is complete, push everything into one well of a 24 well plate. Do not pipet up and down with Neon tip.

15 Repeat the same procedure with the same tip and the left over cell mixture. This is just a replicate.

16 Disperse cells evenly in the well and place cells in a low O2 incubator.

17 Put electroporated cells into low oxygen incubator for 2 days to help maintain viability.

Expansion of clones for analysis by immunoblotting or Sequencing

18 Sort cells into single wells of 96 well plates and keep cells in E8 medium + 10% Clone R2 (STEMCELL Technologies), and put cells into a low-oxygen incubator for 3-4 days till colonies are visible under the microscope, then move cells to a regular incubator. Change media with regular E8 every other day.

18.1 10-14 days post sorting, split cells in 2 sets; 1 set for genetic test/or immunoblotting and the other for expansion. Keep cells in 10µM Rock inhibitor and 12.5µg/ml human serum albumin(HSA) while splitting. Consolidate cells while splitting if necessary.

18.2 Clone screening is done using either PCR-based sequencing for the relevant mutant allele (primers for CHMP2B deletion and point mutants are provided in Materials) or by immunoblotting for knock-out clones if suitable antibodies are available, or by mass spectrometry is antibodies are not available. PCR primers for each of the alleles targeted in this protocol are provided in the material section.