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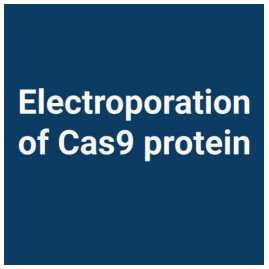
CRISPR editing of TMEM230 and TMEM9/9B genes in H9 ES AAVS-NGN2; Flag-EEA1 cells



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We use this protocol and it's working

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Abstract

This protocol describes a method for either knock out of TMEM230, TMEM9, and TMEM9B as well as knockin of TMEM230X121W in human H9 ES AAVS1-NGN2; Flag-EEA1 (see protocol: dx.doi.org/10.17504/protocols.io.kqdg3×99eg25/v1) cells using CAS9 and electroporation with a NEON system.

Attachments

PDF

Electroporation_of_C...

20KB

Materials

Materials
Cells employed: H9 AAVS1-TRE3G-NGN2 3xFLAG-EEA1 (RRID:CVCL_D1KV)

Electroporation kit: Neon Transfection Kit (Invitrogen, MPK1096B)

Guide
RNAs (sgRNAs) were generated using the GeneArt Precision gRNA synthesis kit
(ThermoFisher Scientific) for the sequences:
$TMEM230^{-/-}$
CCTGAAGGTCAATGTAGCCATCGT,
$TMEM9^{-/-}$
TATCTTTGGTGGCTGTGGTC,
$TMEM9B^{-/-}$
TCTACATCAGGCCCCCGCAC.
$TMEM230^{X121W}$
CTCCTCCTCAGCTATGGGGT,

To generate hESCs homozygous for $TMEM230^{X121W}$ variant, a ssDNA oligo
was included in the electroporation
(CTACCGTGGTTACTCCTATGATGACATTCCAGACTTTGATGACTGGCACCCACCCCATAGCTGAGGAGGAGTCACAGTG
GAACTGTCCCAGCTTTAAGATATCTAGCAGAAACTATAGCTG).

PCR primers for analysis of candidate clones
TMEM230 KO
actgtagctgtgtcagcgtgtt Forward primer
tctggaaggtcttcctgctaac Reverse primer
TMEM9 KO:
tccgactccgtatctctttttc Forward primer
cttgaacctgaaggaagaccac Reverse primer
TMEM9B KO:
ctgggcttaagcagcattttat Forward primer
aaaccatccagaagcagaaaag Reverse primer
TMEM230 X121W
cacctgcgcatcgcttacta Forward primer
ctgcaagctgcagaattcctta Reverse primer

Troubleshooting

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 10m

1. Add 10 µL buffer R [from Neon Transfection Kit (Invitrogen, MPK1096B)] to a sterile 1.5 ml tube.
2. Add 6 µg purified Cas9 protein .
3. Add 1.2 µg sgRNA .
4. Pipet up and down to mix.
5. Let it sit at Room temperature for 00:10:00 .

This is enough for 2 electroporations.

STEP CASE

gRNAs and repair template 16 steps

Create guide RNAs using Geneart Precision gRNA Synthesis kit according to manufacturer instructions (Thermo Fischer, A29377).
TMEM230 knock-out sgRNA target sequence: CCTGAAGGTCAATGTAGCCATCGT
TMEM9 knock-out sgRNA target sequence: TATCTTTGGTGGCTGTGGTC
TMEM9B knock-out sgRNA target sequence: TCTACATCAGGCCCCCGCAC.
TMEM230^{X121W} knock-in sgRNA target sequence:CTCCTCCTCAGCTATGGGGT

Repair template:

To generate hESCs homozygous for TMEM230^{X121W} variant, a ssDNA oligo for homology mediated repair was included in the electroporation (CTACCGTGGTTACTCCTATGATGACATTCCAGACTTTGATGACTGGCACCCACCCCATAGCTGAGGAGGAGTCACAGTGGAACTGTCCCAGCTTTAAGATATCTAGCAGAAACTATAGCTG).

Verify the gene editing of individual clones by sequencing with Illumina MiSeq system.
Use the following primers together with Tracr Fragment PCR template for PCR
Forward primer for TMEM9 KO: TCCGACTCCGTATCTCTTTTTC
Reverse primer for TMEM9 KO: CTTGAACCTGAAGGAAGACCAC

Forward primer for TMEM9B KO: CTGGGCTTAAGCAGCATTTTAT
Reverse primer for TMEM9B KO: AAACCATCCAGAAGCAGAAAAG

Forward primer for TMEM230 KO: CCTGAAGGTCAATGTAGCCATCGT
Reverse primer for TMEM230 KO: TCTGGAAGGTCTTCCTGCTAAC

Forward primer for TMEM230^{X121W} knock-in: CACCTGCGCATCGCTTACTA
Reverse primer for TMEM230^{X121W} knock-in: CTGCAAGCTGCAGAATTCCTTA

2 While waiting for the Cas9 to bind to sgRNA, individualize H9 ES AAVS-NGN2; Flag-EEA1 cells with Accutase.

3 Neutralize Accutase with 5x volume E8 with Rock inhibitor and count cells: 2×10^5 for each transfection.

4 Spin down cells and remove media.
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, one well per transfection. 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 When everything is ready, mix 10 µL - 11 µL of resuspended cells with the Cas9+RNA containing R buffer. The final volume should be in the range of 21 µL - 22 µL .

10 Take up a NEON tip, pipet 10 µL cell protein mix and electroporate.

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

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

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

12 Repeat the same procedure with the same tip and the left over cell mixture.

13 Disperse cells evenly in the well and place cells in a low O2 incubator for 2 days to help maintain viability.

Expansion of clones for analysis by immunoblotting

14 Single-cell sort into 96-well plates coated with matrigel and keep cells in E8 medium + 10% Clone R (STEMCELL Technologies).

15 Put cells into a low-oxygen incubator for 3-4 days until colonies are visible under the microscope, then move cells to a regular incubator. Change media with regular E8 every other day.

16 10-14 days post sorting, split cells in 2 sets: one for genetic test/immunoblotting and the other for expansion. Keep cells in 10µM Rock inhibitor while splitting and consolidate if necessary.

17 Clone screening is done using either:
- PCR-based sequencing for the relevant mutant allele.
- 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.