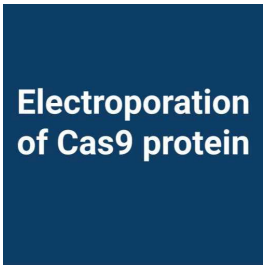
Aug 21, 2024

CRISPR tagging of the TMEM192 gene with 3xHA in H9 ES AAVS-NGN2 cells for Lyso-IP



Forked from CRISPR tagging of the EEA1 gene in H9 ES cells for Endo-IP

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ASAP Collaborative Rese…

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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 knockin of a 3X HA tag onto the C-terminus of the TMEM192 gene in human H9 ES AAVS1-NGN2 cells using CAS9 and a homologous recombination template

Attachments

PDF

Electroporation_of_C...

20KB

Materials

H9 ES cells (WiCell) AAVS-NGN2 [see **dx.doi.org/10.17504/protocols.io.br9em93e**]

gRNA are 3xHA repair template19 steps

sgRNA target sequence: AGTAGAACGTGAGAGGCTCA

PCR primers for generating TMEM192-3xHA C-terminal-Knockin
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

Synthesize gRNA using T7 polymerase as described by manufacturer

For more information on the constructs used, see: https://elifesciences.org/articles/72328#content

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 protein (2mg/ml). Then add 1.2 µg sgRNA and 1 microgram TMEM192-3xHA homology arm plasmid (Addgene#175777). Pipet up and down to mix. Let it sit at Room temperature for 00:10:00. This is enough for 2 transfections (== one 6 well). 10m

STEP CASE

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2 While waiting for the Cas9 to bind to sgRNA, individualize H9 ES AAVS-NGN2 cells with Accutase. Neutralize Accutase with 5x volume E8 with Rock inhibitor.

3 Count cells. You will need $2x10^5$ 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 $2x10^5$ per 5 µl (ie $4x10^7$ 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 HAS (1:2500) to each well.

Each transfection goes into one well.

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+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 repipet 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

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 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 SDS extracts from each well are subjected to SDS-PAGE (18% gel) and analyzed by immunoblotting with anti-TMEM192 antibodies (such as # PA5-118314 from ThermoFisher). For further validation, samples can also be probed with anti-HA antibodies (Biolegend; #901513). HA tagging causes a shift in the apparent molecular weight of TMEM192.