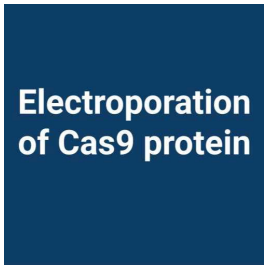
Aug 13, 2024

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

Forked from Electroporation of Cas9 protein into human pluripotent stem 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 knockin of a 3X flag tag onto the N-terminus of the EEA1 gene in human H9 ES cells using CAS9 and an ultramer oligonucleotide homologous recombination template

Attachments

PDF

Electroporation_of_C...

20KB

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. 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

gRNA are FLAG repair template 19 steps

sgRNA target sequence: gtggtggttaaaccatgtta
Ultramer for generating 3xFLAG-3xggs-EEA1
gcagggtctggagagtcaccgcggcggcgccgggtggtggttaaaccatgGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAG
GATGACGATGACAAGGGCGGATCCGGGGGAAGCGGCGGATCCttaaggaggattttacagagggtaagagagtgaaaccgtctttctggcagcacgtg

PCR primers for generating EEA1-N-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'-TAATACGACTCACTATAG gtggtggttaaaccatgtta
Reverse: 5'-TTCTAGCTCTAAAAC taacatggtttaaccaccac

Synthesize gRNA using T7 polymerase as described by maufacturer

2 While waiting for the Cas9 to bind to sgRNA, individualize 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 sequencing by MiSeq

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 sequencing (Miseq) 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 Sequencing by Miseq (Illumina):

- Make lysate by incubating cells in PBND solution (50mM KCl, 10mM Tris-HCl, pH 8.3, 2.5mM $MgCl_2$-$6H_2O$, 0.45% NP-40, 0.45% Tween-20) with 0.2% protease K (Qiagen) for 5 min at 37°C, and transfer lysate to a PCR plate and run the program:

  55°C for 30min
  99°C for 15min
  Hold at 4°C

- Perform the first round of PCR to amplify the region of interest using gene-specific primers

  For each sample, we do a 10 μl PCR.

  2x Master Mix (NEB Next Ultra II Q5): 5 μl
  Primer 1 [5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTaccccgagtagtgagtggc](100 μM): 0.07 μl
  Primer 2 [5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcacgtgctgccagaaagac] (100 μM): 0.07 μl
  Water: 3.86 μl
  Lysate: 1 μl

  Run a 20-cycle PCR using appropriate annealing temp and extension time using the following sequence.

1. 98°C, 30 sec
2. 98°C, 10 sec
3. 64°C, 15 sec
4. 72°C, 30 sec
5. Go to b for additional 19 cycles
6. 72°C, 2 min
7. 4°C, hold

Note: Primer 1 and Primer 2 amplify the genomic region of interest. Each primer contains partial Illumina adaptor serving as anchor sites for the 2nd round of PCR.

- Perform the second round of PCR by universal primers that contains attachment site for the flow cells and index sequences

  For each sample, we do a 10 μl PCR.

  2x Master Mix (NEB Next Ultra II Q5): 5 μl

Primer CDI 666 (100 uM): 0.1 µl
Primer index (10 µM): 1 µl
Water: 2.9 µl
Product from 1st round of PCR: 1 µl
Run a 20-cycle PCR using appropriate annealing temp and extension time.
Subject to MiSeq using manufacturers protocols (MiSeq Kit #MS-102-3001).