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CRISPR-Cas9 Genomic Insertion into the AAV Safe Harbor Site by Electroporation of iPSC

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We use this protocol and it's working

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Abstract

This protocol provides methods for mutagenizing human induced pluripotent stem cells (iPSC) by inserting sequences of interest into the AAV Safe Harbor site (AAVSH). We custom-designed a gRNA targeting the AAVSH site within the first intron of the *PPP12CR1* gene. Insertion into this site interrupts the gRNA targeting sequence, making it resistant to further cleavage. This protocol describes the electroporation of a cas9 protein complexed with this gRNA, along with a drug-selectable donor plasmid, to provide a functional example for integration of any desired sequences into AAVSH site. We found that electroporation of precomplexed gRNA-Cas9 protein produces efficient editing (60-90%) with acceptable cell viability (greater than 50% survival).

As an example of our strategy, we inserted a neomycin resistance gene into the AAVSH site. The homologous recombination donor plasmid, available from AddGene, includes insertion sequences flanked by two homology arms, each with ~800 bp of sequence from the *PPP12CR1* intron adjacent to the target sequence. Following the left homology arm are: (1) a splice acceptor, (2) a T2A ribosome skipping sequence, (3) a Neo^R/Kan^R drug-resistance sequence, and (4) a poly(A) signal. Once inserted into the AAVSH site, exon 1 of the *PPP12CR1* gene is spliced to the splice acceptor, producing an mRNA that provides G418 resistance. Any other payload could be delivered using a similar strategy.

Materials

- DMEM Medium (Gibco #11965092)
- Alt-R Cas9 Electroporation Enhancer, 100 μ M (Electroporation Enhancer, IDT #10759164)
- Alt-R S.p.Cas9 Nuclease V3, 10 μ g/ μ L (Cas9, IDT #1081059)
- HDR enhancer (IDT #10007910)
- Plasmid pAAVS1-sA-neoR (AddGene #**240383**)
- StemMACS iPS-Brew XF medium (iPS-Brew; Miltenyi #130-104-368)
- CloneR2 (StemCell Technologies #100-0691)
- MatriGel (Corning #354277)
- Geneticin (G418 Sulfate; Gibco #10131035)
- Accutase (StemCell Technologies #07922, #07920)
- DPBS (Gibco #14190144)
- CEPT (prepared as described, Chen et al., 2021)¹
- Small activating RNA (AsCrRNA; IDT 5'-acagtggggccactagggacagg-3')
Matches chr19:55,115,754-55,115,776 in the GRCh38 human genome. This is complementary to the *PPP12RC1* AAVSH locus. Alternatively a full-length, custom gRNA could be used.
- Trans-Activating CRISPR- RNA (tracrRNA), 100 μ M (IDT #1073190)
- 0.4% Trypan Blue Solution (Invitrogen #T10282)
- Neon NxT Reagents (Invitrogen, #N1025B), including: Neon NxT Resuspension T Buffer (#A5677701); and Neon NxT Electrolytic E2 Buffer (#A56753-01)
- pMAX-GFP, (AddGene #**177825**)
- Quick-gDNA Microprep kit (Zymo # D3021)
- Diagnostic PCR primers (Life Technologies):
 - UninsSH_F, 5'-ccggttaatgtggctctggt-3'
 - UninsSH_R, 5'-aaaactgacgcacggaggaa-3'
 - InsSH_F, 5'-ccggaactctgccctctaac-3'
 - InsSH_R, 5'-gatcccatcacgctctcgag-3'

Safety warnings

-  All cell culture work should be performed in an approved type II biosafety cabinet. Biosafety level 2 procedures should be followed for all human cells.

Ethics statement

Work with human iPSC cultures must have prior approval from your Institutional Biosafety Committee (IBC) and your Embryonic Stem Cell Research Oversight (ESCRO) committee and/or Institutional Review Board (IRB) for human subjects research.



Before start

1. Prepare Geneticin stock solution (50 mg/mL)
2. Dilute AsCrRNA and tracrRNAs with RNase-free, sterile water to make 100 μ M solutions.
3. Dilute MatriGel 1:1 (v/v) with DMEM and freeze in 1 mL aliquots.
4. Dilute thawed MatriGel aliquot 1:35 (v/v) with DMEM to prepare working stock solution. The final concentration may vary by lot. Adjust as needed. Store at 4°C for up to 2 weeks.
5. Add 0.5 mL of diluted Matrigel to each well of a 24 well plate. Incubate at 37°C in a CO₂ incubator for at least 1 hour up to overnight.
6. Prepare a mixture of post-electroporation medium (per well):
 - a. 449.6 μ L iPS-Brew
 - b. 0.5 μ L CEPT
 - c. 50 μ L Clone R2
 - d. 0.35 μ L HDR Enhancer
7. Remove the MatriGel-coated 24-well plate from the 37°C incubator. Aspirate the MatriGel from the wells.
8. Add the medium mixture to the plate per desired condition.
9. Place the plate in the CO₂ incubator for 1 hour
10. Anneal the AsCrRNA and tracrRNA. Pool equal volumes of AsCrRNA and tracrRNA for a total concentration of 50 μ M. Heat the solution at 95°C for 5 minutes in a thermal cycler. Cool to room temperature on the bench.
11. Prepare the RNP mixes in a PCR tube strip in the biosafety cabinet. Keep the individual CRISPR reagents on ice. For each well:
 - a. 2.72 μ L cas9 protein (10 μ g/ μ L)
 - b. 1.04 μ L Alt-R Electroporation Enhancer
 - c. 2.8 μ L pAAVS1-sA-neoR Plasmid (714 ng/ μ L, total of 1000 ng)
 - d. 4 μ L annealed AsCrRNA + tracrRNA (50 μ M)
 - e. 1 μ L pMAX GFP (500 ng/ μ L), optional, for electroporation efficiency)

Keep the RNP mixes at room temperature for approximately 20-30 minutes. To incorporate a nonelectroporated condition as negative control for drug selection, prepare mix for at least one additional well.

Harvesting iPSC

- 1 Prepare a microcentrifuge tube with 10 μ L of 0.4% Trypan Blue Solution.
- 2 Harvest cells from the desired iPSC cell line, grown in 6 well plates.
 - 2.1 Aspirate medium from desired well.
 - 2.2 Using pipette aid, wash the well with 2 mL of DPBS.
 - 2.3 Add 1 mL Accutase to detach the cells.
 - 2.4 Incubate for 5 minutes in the 37°C incubator.
 - 2.5 Remove the plate from the incubator and check under a microscope to see if cells are detaching. Gently tap the sides of the plate to facilitate detachment.
 - 2.6 Add 1 mL of iPS-Brew plus 1:1000 (v/v) CEPT and triturate the cells to form a single cell suspension.
 - 2.7 Transfer the cell solution to a 15 mL conical tube.
- 3 Add 10 μ L cell solution from the 15 mL conical tube to the prepared microcentrifuge tube with Trypan blue solution and gently pipette up and down to mix.
- 4 Add 10 μ L trypan-stained cell solution to each side of a Countess slide.
- 5 Enable Rapid Capture on the home screen of the Countess.





- 6 Insert the slide into the Countess instrument. Record the viable cell count from both sides of the Countess slide. Average the two amounts to determine the viable cell count.
- 7 Calculate the volume of cells needed to obtain 400,000 cells per condition . Pipet the required volume of counted cells to a new 15 ml tube containing 5 mL DBPS. 400K cells is enough for one transfection and one non-electroporated control.
- 8 Centrifuge the harvested cell solution at 300 x g for 3 minutes. Remove the supernatant from the 15 mL conical tube, ensuring that the pellet remains intact.
- 9 Add Neon NxT Resuspension T Buffer, 8.44 μ L per electroporation, to the cell pellet and gently pipette up and down. The total volume combined with the RNP mix will total 20 μ L per electroporation.
- 10 Retrieve the PCR tube strip containing the CRISPR reagents. Add 8.44 μ L cells in T Buffer (400,000 cells) to each compartment of the strip tube containing the pre-aliquoted CRISPR Reagents (20 μ L total).



Electroporation

- 11 Set up Neon NxT device.
 - 11.1 Place a Neon NxT Tube in pipette station.
 - 11.2 Add 2 mL of Neon Buffer E2 to the center of the Neon Nxt Tube within the pipette station.
- 12 Create a protocol on the Neon screen:
 - 12.1 Select 24-well plate type.
 - 12.2 The optimal electroporation parameters for iPSC lines are 1200V, 30 ms, 1 pulse but may need further optimization based on cell type or line.
 - 12.3 Select Save Protocol.
- 13 Prepare the Neon pipette and tips.





- 14 Using the Neon pipette with a 10 μ L tip attached, withdraw 10 μ L of cell solution from a tube in the prepared strip.
- 15 Electroporate:
 - 15.1 Select the saved protocol from Library > Plate Library.
 - 15.2 Place the pipette in the column pipette station.
 - 15.3 Tap Electroporate.
 - 15.4 The device will indicate whether electroporation was successful with a check mark. If not, put cell solution back in the strip tube it was withdrawn from, and slowly withdraw cell solution again with an effort not to produce bubbles and try again.
- 16 Add the electroporated cells to a well containing prewarmed post electroporation medium in a 24-well plate.
- 17 Repeat steps 13 to 16 for the remainder of experimental conditions.
- 18 For non-electroporated controls:
 - 18.1 Using the Neon pipette, withdraw 10 μ L of the remaining cell solution from the PCR strip tube.
 - 18.2 Add directly to a well labeled "Non-electroporated."
- 19 Place plate in 37°C incubator.



Drug Selection



- 20 Begin selection two days after electroporation. Evaluate cell survival and electroporation efficiency by viewing GFP in a fluorescent microscope.
- 21 Add Geneticin (G418) to iPS-Brew in a 1:100 dilution by adding 2 mL of iPS-Brew to a 15 mL conical tube and adding 20 μ L Geneticin stock solution (50 mg/mL; final concentration 500 μ g/mL).
- 22 Remove medium from plate. Feed the wells to be selected with 1 mL of the iPS-Brew + Geneticin. Control wells are fed with iPS-Brew alone to demonstrate continued viability without selection.
- 23 Repeat drug selection 2-3 times over approximately one week. Cease drug selection upon determination of complete cell death in the GFP-only control well.
- 24 Allow the selected well to expand in the absence of G418 to confluency for passaging.

Screening

- 25 Passage drug-resistant cells to a new well using Accutase. To evaluate successful recombination into the AAVHS site, harvest half of the cells for genomic DNA by pelleting. Process the pellet using the Zymo Quick-gDNA Microprep kit, following the manufacturer's instructions.
- 26 PCR amplify genomic DNA sample with primers specific for adjacent PPP12CR1 sequences and sequences specific for inserted sequences. A second set of primers can detect uninserted AAVSH. Run products on gel to confirm predicted size. Uninserted genome, using UninsSH_F and _R will produce a 538bp product. Inserted genome, using InsSH_F and _R will product a 1048 bp product.
- 27 Cut out amplified band from the gel and Sanger sequence if desired.
- 28 To obtain cloned, individual colonies from the mixed cell population, cells may be distributed to 96-well plates by FACS or hand-diluted using existing protocols.² Select wells that produce a single colony for further screening. Expand and screen as above.





Protocol references

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