Chapter 5

Prime Editing of Mouse Primary Neurons

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Abstract

Prime editing is a hybrid genome editing technology that introduces small edits on the genome with high precision. It combines nickase Cas9 with reverse transcriptase to prime and synthesizes edited DNA from RNA, reducing unintended insertions and deletions on the genome. This protocol describes the design of prime editing guide RNAs (pegRNAs), cloning of plasmids, nucleofection of mouse primary neurons, and preparation for next-generation sequencing. Directions are given for pegRNA and PE3b gRNA design and construction using PegAssist, a publicly available webtool and plasmid set. Prime editing in neurons allows genome manipulation while maintaining endogenous gene expression, making it ideal for studying protein structure/function relationships and pathogenic variants in a native neuronal context.

Key words Prime editing, CRISPR, Neurons, Genome editing, pegRNA, Nucleofection, PegAssist

1 Introduction

The advent of genome editing technologies has been a boon for biomedical research. CRISPR/Cas9-based editing in particular has provided unmatched control over genomic sequences both in vitro and in vivo. Cas9-based techniques broadly operate through the association of the Cas9 nuclease with a guide RNA which directs the complex to a specific genomic locus [1, 2]. Once bound to the target sequence on genomic DNA (**gDNA**), Cas9 induces a double-strand break (**DSB**) at a defined position. The DSB activates the non-homologous end joining (**NHEJ**) machinery for repair, but with repeated cleavage and ligation the error rate in this process causes insertions and deletions (**indels**) on nucleotides resulting in frameshift mutations and a functional knock-out (**KO**) of the gene of interest [1, 3–6]. This strategy is highly effective for frameshift and loss-of-function mutations. However, when wanting

Tolga Soykan and Alexandros Poulopoulos (eds.), *Synapse Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2910, https://doi.org/10.1007/978-1-0716-4446-1_5,

to study specific mutations for structure-function analyses, or to study the effects of specific variants associated with disease, more refined editing techniques are required [7].

Earlier advances in specific genome editing approaches included homology-directed repair (**HDR**)-mediated genomic knock-in (**KI**) and base editing. While each improves on the specificity of genomic outcomes, they are still limited in application. HDR methods rely on DSB, which increases the propensity of indels [8]. Base editors do not cause indels, but do have specificity issues when it comes to nearby bases, and are currently limited to four transition mutations (A > G, G > A, T > C, and C > T). Additionally, both strategies are prone to widespread off-target effects.

An alternative genome editing strategy, termed Prime Editing (**PE**), uses Cas9 targeting of specific sequences on the genome without causing DSBs, limiting indels and off-target editing [9]. To prevent DSBs, PE uses the H840A nickase variant of Cas9 (**nCas9**) [1], which cuts only the non-complementary strand of DNA. nCas9 is fused to an engineered reverse transcriptase (**RT**) which synthesizes DNA with the desired edit based on an RNA template (Fig. 1). Both the guide RNA (**gRNA**) that targets the nCas9 and the template RNA for the RT are encoded in a single prime editing guide RNA (**pegRNA**) containing four sequence modules: spacer (a standard guide sequence designed for all CRISPR/Cas9 applications), scaffold (required for Cas9 binding),

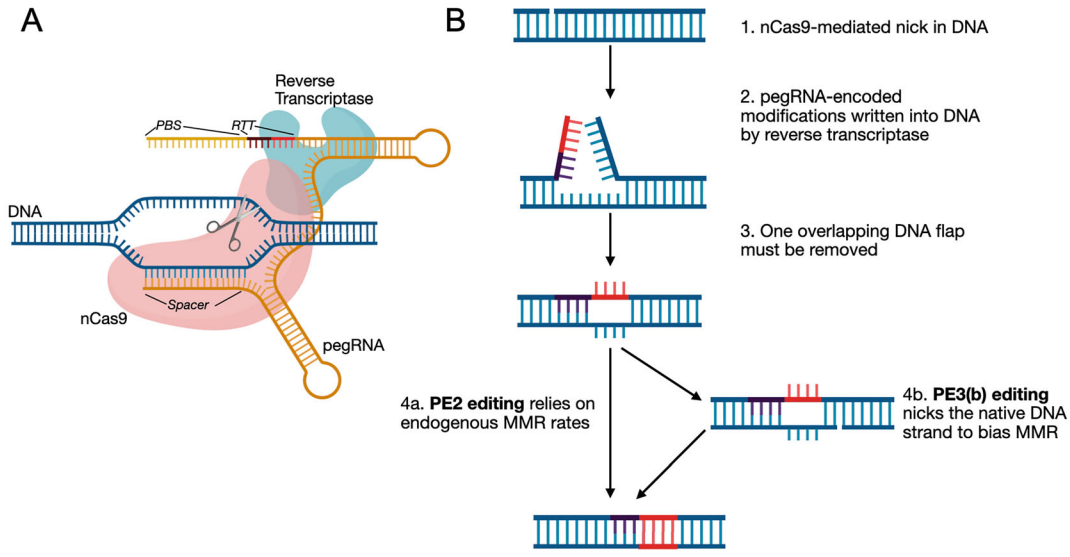

Fig. 1 Mechanism of prime editing. A. Schema representing prime editor (pink nCas9 and blue reverse transcriptase) bound to target DNA (dark blue) with relevant regions labeled. B. The target DNA progresses through four stages for successful incorporation of the written strand (red and purple). PE3 strategy is depicted on the left, and PE3b strategy is shown on the right. (Created with BioRender.com)

primer-binding sequence (PBS; will hybridize to the cleaved 3′ strand of DNA to prime the RT), and reverse transcriptase template (RTT; encodes the desired edit). Successful incorporation of the desired edit is dependent on two molecular coin flips: first the incorporation of transcribed product, and second the correction of the newly formed mismatch. The mismatch repair can be biased by the inclusion of a second nicking gRNA in an approach termed PE3 (there is also PE3b which binds and nicks after RT activity due to proximity to the spacer PAM sequence) [9]. The PE3 approach increases efficiency at the cost of also increasing indel rates (these still tend to be minority products unlike DSB approaches), so specific experimental considerations should be weighed when selecting an approach [9].

All told, PE approaches allow for highly specific genome editing while maintaining design flexibility, reducing indels, and nearly eliminating off-target effects. The major sacrifice of this approach is its low efficiency for larger edits, though adaptor technologies are being developed to mitigate that.

Prime editing excels at precisely introducing point variants or small and specific insertions and deletions. Although limited in applications, this aligns well with the needs of neuroscience research. In addition to aiding in studies of protein structure and function, almost 70% of the 6000 known monogenic diseases affect the nervous system [10]. The high precision of this editing approach enables genome editing without stringent requirements for selection and expansion of correct edits. This could expand the utility of powerful in vitro models like primary neuronal cultures for structure/function studies, analysis of single-nucleotide variants [11–13], and even correction of pathogenic variants. This protocol will focus on nucleofecting prime editing plasmids in developing neurons in culture: pegRNA design, cloning, nucleofection, primary neuron culture, and preparation for next-generation sequencing of the gene target.

2 Materials

2.1 Cloning and Preparation of Plasmid Containing pegRNA and PE3b gRNA Plasmids

1. Synthesized pegRNA fragment (sequence given by PegAssist.app).
2. Forward and reverse oligonucleotides for PE3b (sequence given by PegAssist.app).
3. Plasmids:

   P457 (pJ2.2.U6 < pegRNA scaffold), Addgene plasmid #226288.

   P396 (pJ2.U6 < gRNA scaffold), Addgene plasmid #226289.

   P387 (pJ2.17v2), Addgene plasmid #226290.

4. Primers:
   prRR940 (pegRNA_1.0_F): GGTCTCAggagTTTCCCC-GAAAAGTGCCAC.
   prRR918 (pegRNA_2.0_R): GGTCTCAagtaGCCGATTCA TTAATGCAGCG.
   prRR941 (PE3b_2.0_F): GGTCTCAtactTTTCCCCGAAAA GTGCCAC.
   prRR921 (PE3b_7.0_R): GGTCTCAagcgGCCGATTCA TTAATGCAGCG.
5. BbsI and BsaI restriction enzymes.
6. T4 DNA ligase and 10x ligase buffer.
7. Chemically competent *E. coli*.
8. SOCS medium.
9. LB agar plates supplemented with ampicillin.
10. LB supplemented with ampicillin.
11. Plasmid miniprep kit.
12. Polymerase master mix and PCR buffer (*see* **Note 1**).
13. DNase-free water.
14. Gel DNA recovery/extraction kit.
15. PCR tubes.
16. Agarose.
17. Glycerol.
18. Cryogenic Tubes.
19. Sterile cotton swab.
20. Plasmid miniprep kit.

2.2 Neuron Culture and Nucleofection

1. P0 mouse pups (*see* **Note 2**) (Approximately 2 pups per 9.5 cm^2 culture plate).
2. poly-L-lysine.
3. Glass coverslips (*see* **Note 3**).
4. Borosilicate Pasteur pipettes.
5. Sterile, autoclaved MilliQ water.
6. Culture Medium—Neurobasal Plus with 1x B-27 Plus and Penicillin-Streptomycin (*see* **Note 4**).
7. Dissection medium—1x HBSS (Mg- and Cl-), 10 mM HEPES, 30 mM glucose, and 12 mM magnesium sulfate in MilliQ water; filter sterilize through 0.22-micron filter.
8. Papain.

9. 10x Papain activation buffer—68 mM Cysteine-HCL, 0.01% β-mercaptoethanol, and 12 mM EDTA pH 8.0 in MilliQ water; filter sterilize through 0.22-micron filter.
10. Activated papain solution—1:1 papain with 2x papain activation buffer (*see* **Note 5**).
11. Electroporation medium—Combine 120 mM KCl, 10 mM KH_2PO_4, 2 mM EGTA, 25 mM HEPES, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ adjusted pH 7.5–7.6. Add 5 mM reduced glutathione GSSG and 2 mM ATP immediately before use or freezing. This can be stored at −80 °C (*see* **Note 6**).
12. Plasmid expressing pegRNA/PE3(b) (constructed and prepared in **part 2**).
13. Plasmid TU516 pJ2.CAG < EGFP-2A-PE2.
14. 15 mL screwcap tube.
15. Latex pipette bulb.
16. 15 mL pipettes.
17. 50 mL conical tubes.
18. Trypan blue.
19. Hemocytometer.
20. Microcentrifuge tubes.
21. 0.4 cm electroporation cuvettes.

2.3 Instrumentation

1. Thermocycler.
2. Shaking heat block.
3. Nanodrop or spectrophotometer.
4. Autoclave.
5. Laminar flow hood.
6. Amaxa Nucleofector II.
7. CO_2 incubator.

2.4 Software

1. Genome browser genome.ucsc.edu or benchling.com.
2. Pegassist.app or other pegRNA design tool.

3 Methods

3.1 Design of pegRNA and PE3(b) Guides

1. Import the desired gene of interest using the Benchling.com gene importer. If not using benchling, the following steps can also be completed using UCSD Genome Browser, NCBI gene viewer and Ensembl to identify specific gene regions.
2. Identify and annotate specific locus intended for editing (*see* **Note 7**).

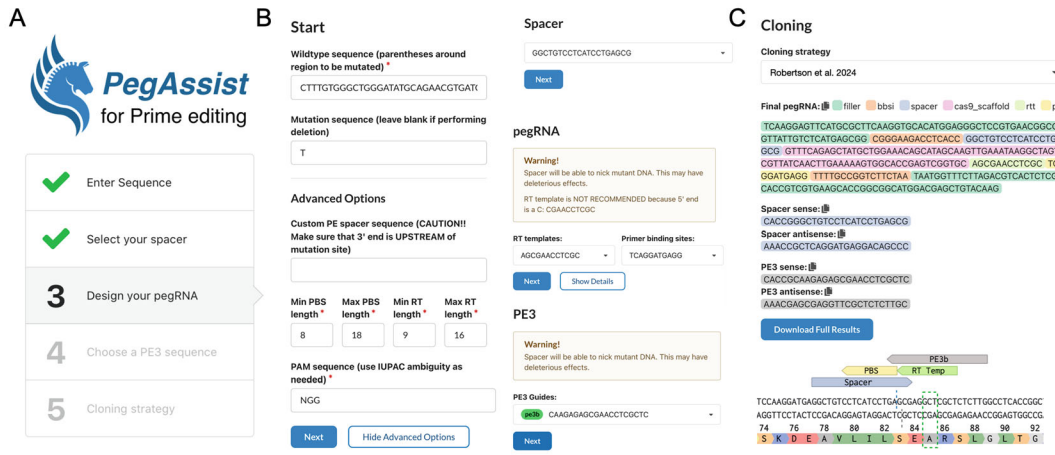

Fig. 2 Using PegAssist.app to design prime editing guides. (**a**) The PegAssist.app workflow. (**b**) An example wildtype sequence and mutation sequence allows PegAssist.app to generate options for Spacer, RT Template, Primer binding site (PBS), and optional PE3(b) guides. PegAssist will display relevant warning messages dependent on the sequence and provide final options to facilitate cloning. (**c**). Alignment of the chosen pegRNA and PE3b guide regions on the target DNA. The direction and orientation of spacer, PBS, and RT template in relation to one another is crucial for successful prime editing. Nicking sites for the pegRNA (blue dashed line) and PE3b guide (gray dashed line), and the region to be modified (green dashed box) are shown on the DNA sequence. (Partially created with Benchling.com)

3. Copy the nucleotide sequence with 150 bases upstream and downstream of the intended edit.
4. Paste the sequence in a separate document labeled "Wild-type sequence."
5. Copy and paste ~300 nucleotide sequence in the "Wildtype sequence" box on pegassist.app. PegAssist provides a user-friendly interface to design and select optimal pegRNAs (Fig. 2), though other pegRNA design tools can be used equivalently (*see* **Note 8**).
6. In the "Mutation sequence" box, input the sequence desired within the parentheses. Example inputs:

   Substitution G > T

   Wildtype: GATC(G)TGTCC;

   Mutation sequence: T

   Deletion

   Wildtype: GATC(G)TGTCC;

   Mutation sequence: *leave blank*

   Insertion

   Wildtype: GATC()GTGTCC;

   Mutation sequence: A

7. Prime editing parameters can be left at the default settings for initial designs.

   PBS length: 8–18 nucleotides

   RTT length: 9–16 nucleotides

   PAM sequence: NGG

8. PegAssist will display available spacer sequences. Once a spacer sequence is selected (*see* **Note 9**), the options for compatible PBS and RTT are listed (*see* **Note 10**). Select the desired spacer, PBS, RTT, and optional PE3(b) (*see* **Note 11**) sequences.
9. Double check the compatibility of these parts by annotating the known genomic sequence. Copy these individual selections and paste into a separate file. Annotate the Wild-type sequence to confirm the sequences will bind in the desired locations and orientation (*see* **Note 12**).
10. Order the oligonucleotides and double-stranded DNA fragment required for cloning (*see* **Note 13**). PegAssist will display the parts with necessary cloning elements in the "Cloning" section.

3.2 Cloning of pegRNA

The following steps will produce a single vector with the pegRNA and PE3(b) guides present in duplex and under the control of independent U6 promoters. This is beneficial for downstream applications as it limits the total number of plasmids required for prime editing.

3.2.1 Preparing the pegRNA

1. Bring the synthetic pegRNA to a concentration of 100 ng/μL using DNAse/RNAse-free water (*see* **Note 14**).
2. Perform a Golden Gate Assembly (GGA) to subclone the pegRNA into the first vector backbone. In a PCR tube, mix 100 ng of the pegRNA, 100 ng P457, 1 μL BbsI, 1 μL T4 DNA ligase, and 2 μL 10x T4 ligase buffer. Bring the final volume of 20 μL using DNAse/RNAse-free water.
3. Place the PCR tube in a thermocycler and run a standard GGA protocol for 15–50 cycles (*see* **Note 15**).
4. Transform the GGA mixture using the following method: Add 4 μL of the resulting mixture to TOP 10 chemically competent *E. coli* or an equivalent strain. Rest on ice 30 min. Heat-shock the mixture at 42 °C for 30 s in a heating block (*see* **Note 16**). Return to ice for 2 min. Add SOCS medium using sterile technique and incubate at 37 °C with shaking for 30 min. Plate this mixture on LB agar plates supplemented with ampicillin and incubate overnight at 37 °C.
5. Using a sterile pipette tip, pick individual colonies and inoculate small volumes (~4 mL) of LB supplemented with ampicillin. Grow these liquid cultures overnight at 37 °C with shaking.

6. Pellet the cultures by centrifugation. Extract plasmid DNA using a plasmid miniprep kit according to the manufacturer's protocol and sequence the plasmid using a U6 universal primer (*see* **Note 17**) to confirm proper insert of the pegRNA. Select one clone with the correct sequence and proceed to the next step.
7. Perform a PCR on the pegRNA plasmid using primers prRR940 and prRR918. Combine 100 ng of the pegRNA plasmid, 10 ng each primer, 12.5 μL KAPA HiFi HotStart ReadyMix, and DNAse-free water in a final volume of 25 μL. A standard hot start PCR protocol can be used with a 60 °C annealing temperature and 1 min extension time.
8. Run the PCR product on a 1% agarose gel and purify the 740 bp band using a Gel DNA Recovery/Extraction Kit according to the manufacturer's instructions. Save this purified PCR band at 4 °C as "Part I" for a future GGA.

3.2.2 Preparing the PE3 (b) Guide

1. Resuspend the forward and reverse oligonucleotides for the PE3(b) guide in DNase/RNase-free water to a final concentration of 100 μM. Add 1 μL of each oligo to a PCR tube with 1 μL 10x T4 ligase buffer and 7 μL DNase/RNase-free water. Place the sample in a thermocycler and denature at 95 °C for 5 min followed by a descending temperature ramp of 0.1 °C per second to 25 °C to anneal the oligos.
2. Perform a GGA using 1 μL annealed oligo mixture, 100 ng P396, 1 μL BbsI, 1 μL T4 DNA ligase, 2 μL 10x T4 ligase buffer, and DNase/RNase-free water to a final volume of 20 μL.
3. Repeat the steps from the pegRNA cloning section to perform a GGA, transform in *E. coli*, culture, miniprep plasmids, and sequence with a U6 universal primer.
4. After confirming proper plasmid sequence perform a PCR as above using primers prRR941 and prRR921. Run the product on a 1% agarose gel and purify the 1000 bp band using a Gel DNA Recovery/Extraction Kit according to the manufacturer's instructions. Save this purified PCR band at 4 °C as "Part II" for a future GGA.

3.2.3 Constructing the pegRNA/PE3(b) Plasmid

1. Perform a GGA using 100 ng Part I, 100 ng Part II, 100 ng P387, 1 μL BsaI, 1 μL T4 DNA ligase, 2 uL 10x T4 ligase buffer, and DNase/RNase-free water to a final volume of 20 μL. Place this reaction tube in a thermocycler and run a GGA protocol with 50 cycles.
2. Refer to the steps above to transform the GGA and culture the bacteria to isolate plasmid clones. Confirm the sequence by Sanger sequencing using primer prRR945.

3. Make a glycerol stock of the bacteria containing the proper sequence by combining 800 μL of bacteria in suspension in LB broth with 500 μL sterile 80% glycerol in a cryogenic tube. Store the glycerol stock at −80 °C. This stock can be used to prepare plasmid DNA for experimental applications.
4. Using the wooden end of a sterile cotton swab, scrape the top layer of the glycerol stock and use this to inoculate 220 mL LB broth supplemented with ampicillin in a 1 L flask (*see* **Note 18**). Incubate the flask at 37 °C with shaking overnight up to 18 h. Use a Plasmid Midiprep Kit to purify the plasmid DNA. Quantify the total DNA concentration using a NanoDrop Spectrophotometer by applying 1.5 μL plasmid to the NanoDrop (*see* **Note 19**).
5. For short-term storage and repeated use, keep plasmid DNA at 4 °C. For long-term storage, keep the DNA at −20 °C. Aliquot as needed to avoid repeated cycles of freeze/thaw.

3.3 Primary Culture of Neonatal Mouse Cortical Neurons and Nucleofection of Prime Editing Plasmids

1. Two days prior to culturing neurons, prepare culture dishes and pipettes. Coat dishes with poly-L-lysine (P4832) by adding a 1/12 solution of poly-L-lysine in 1x PBS equal to the culturing volume of the dish/plate. Incubate this dish in a cell culture incubator until the day of culture. Borosilicate Pasteur pipettes should be flame-polished by rotating the tip over the flame of a Bunsen burner to decrease the size of the opening and round the glass edges (*see* **Note 20**). Autoclave the flame-polished pipettes and place in a sterile laminar flow hood in preparation for culturing.
2. At least one hour prior to culturing cells, while working in a sterile laminar flow hood, aspirate the poly-L-lysine from the culture dish and wash 3x with equal volume sterile water. Add culture medium to the dish and return to cell culture hood.
3. On the day of cell culture prepare dissection medium and activated papain buffer (*see* **Note 21**). Warm electroporation medium to room temperature. Measure and mix DNA in microcentrifuge tubes (*see* **Note 22**).
4. Fill a 50 mm petri dish with ice-cold dissection media. Under a dissecting microscope, dissect the brain of a p0 mouse neonate by first euthanizing via rapid decapitation using sterilized surgical scissors. Grasp the head using a Kimwipes tissue and make an incision in the scalp along the midline of the skull. Pull pack the skin layer to expose the underlying skull and cut the skull along the midline. Peel the skull away from the incision site using sterile tweezers to expose the brain. Remove the brain to the dish with cold dissection media.
5. Under a dissecting microscope, remove the cerebellum. Remove the pia from the cortical surface using sharp-tipped tweezers and separate the hemispheres (*see* **Note 23**). Cut and

discard the subcortical structures from the overlying cortex for both hemispheres. Transfer the cortical tissue to a 15 mL screwcap tube containing 1 mL dissection media on ice.

6. While working in a sterile laminar flow hood, aspirate the dissection media until around 0.5 mL remains with the cortical tissue. Add 30 μL activated papain solution and incubate in a 37 °C water bath for 30 min.
7. Return the tube containing cortical tissue to the laminar flow hood. Inactivate papain by washing tissue 3x with 10 mL dissection media (*see* **Note 24**). Add 5 mL growth media to tissue pieces. Using a flame-polished Pasteur pipette with a latex pipette bulb, gently triturate the tissue 4–8 times. Use a 15 mL pipette to transfer the cell suspension. Pass the suspension through a 70 μm cell strainer into a 50 mL conical tube. Bring the total volume of the suspension to 10 mL. Take a 10 μL sample of the suspension and transfer to a tube containing 10 μL trypan blue. Pipette this mixture into a hemocytometer and count viable cells in each quadrant to estimate the density of viable cells.
8. For a 6-well plate aliquot the suspension to microcentrifuge tubes so that at least 720,000 cells are present in each aliquot (*see* **Note 25**). For other plate sizes a density of 75,000 cells/cm^2 should be retained.
9. Spin down the cells at 1000 RCF for 2 min.
10. In the laminar flow hood, aspirate and discard the media. Resuspend the pelleted cells in 300 μL of electroporation buffer. Add 1–3 μg of DNA mixture. Gently transfer this suspension to a 0.4 cm electroporation cuvette. Place the cuvette in an Amaxa Nucleofector II and select program O-005. Immediately after electroporation transfer the cell suspension to the coated culture dish containing pre-warmed culture media (*see* **Note 26**). Incubate the cells in a cell culture incubator. After four hours, replace the medium with fresh, prewarmed growth medium to remove non-adherent cell debris.
11. Change medium again 24 h after plating.
12. Results of the nucleofection should be detectable by fluorescence within 24–48 h of nucleofection (*see* **Note 27**). The efficiency of co-electroporation can be appreciated by the fluorescence imaging of neurons and glia at DIV 2 (Fig. 3).
13. Media should be changed every other day by removing 50% of the total culture media and replacing with fresh, prewarmed culture media. Transfected cells can be identified by green fluorescence encoded on the TU516 plasmid.

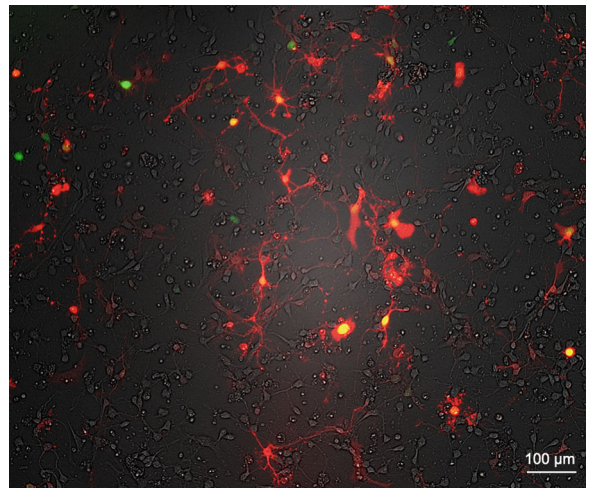

Fig. 3 Primary cortical culture following AMAXA nucleofection. A. Cells include neurons and glia shown at DIV 2 following plasmid nucleofection. RFP (red) and nuclear-GFP (green) on separate plasmids are co-electroporated to appreciate efficiency of co-expression. Observed overall efficiency is typically between 10–30%. Scale bar 100 µm

3.4 Preparation for Next-Generation Sequencing

While these cells can be used for a multitude of downstream assays, it is critical to first validate the editing approach. While deconvolution of Sanger sequencing is possible, it is unlikely to provide proper fidelity to detect and assay editing precision. Instead, next-generation sequencing (NGS) should be employed. The following steps will provide the necessary guidance to begin a NGS preparation. After these steps are followed, further library preparation can be done either by the user or passed on to a third-party company or core facility.

1. Design PCR oligos to amplify the region surrounding the desired edit. The resulting amplicon should be 250–500 base pairs in length, with at least 40% of the total length downstream of the PAM sequences recognized by the pegRNA and PE3 (b) guides. To make these oligos compatible with NGS applications, attach the following Illumina universal adapter sequences: Forward ACACTCTTTCCCTACACGA CGCTCTTCCGATCT—[Oligo Sequence] and Reverse GA CTGGAGTTCAGACGTGTGCTCTTCCGATCT—[Oligo Sequence] (*see* **Note 28**).
2. Genomic DNA can be isolated using the salting-out method [14] (*see* **Note 29**). Cells can be collected by the addition of minimal 0.25% trypsin/EDTA. Incubate cells at 37 °C for 5 min, then gently pipette to remove remaining adherent cells.

3. Transfer the suspended cells into a microcentrifuge tube containing 3-4x volume lysis buffer containing 0.2 mg/mL proteinase K, 150 mM NaCl, 10 mM Tris, 10 mM EDTA, and 0.1% sodium dodecyl sulfate. Incubate this tube at 55 °C overnight.
4. Add 0.5 total volume saturated NaCl to precipitate proteins. Mix by inversion and spin at max speed in a benchtop centrifuge for 2 min.
5. Retain the supernatant and ethanol precipitate by adding 0.1 total volume 3 M sodium acetate and 3 total volumes ice cold 100% isopropanol.
6. Chill at −80 °C for 20 min. Spin down at max speed at 4 °C for 10 min.
7. Remove the supernatant. Add ice-cold 100% ethanol to the pelleted DNA and mix by flicking the tube. Centrifuge at maximum speed at 4 °C for 10 min.
8. Remove the supernatant and dry the tube at room temperature. Resuspend the pellet in DNA resuspension buffer with 10 mM Tris pH 8.5 and 0.1 mM EDTA.
9. Quantify DNA concentration using a NanoDrop.
10. Use 10–50 ng of genomic DNA for a PCR using the oligos designed for NGS. Combine genomic DNA with 10 ng each primer, 12.5 μL KAPA HiFi HotStart ReadyMix, and DNase-free water in a final volume of 25 μL (*see* **Note 30**).
11. The resulting PCR product can be purified and used to generate an NGS library.

4 Notes

1. A high-fidelity polymerase should be used to decrease error rate when cloning. Commonly used polymerases are available with error rates lower than 0.5 in 10^5 nucleotides.
2. Approximately two mouse neonates will provide enough cells for a single 9.5 cm^2 culture plate.
3. Consider the size of the culture wells when selecting coverslips. If the coverslips are too large, removing them for fixation and mounting will be difficult. If mounted coverslips for imaging is not required, glass bottom plates can be used instead.
4. Culture medium should be aliquoted and kept frozen for long-term storage. An aliquot in active use can be stored at 4 °C for seven days.
5. Both the papain and the activation buffer will be suspensions requiring thorough mixing before pipetting.

6. Prolonged storage will oxidize glutathione and lower electroporation efficiency. Always note electroporation efficiency, and if substantial decreases are observed prepare fresh buffer.
7. Mismatches between the targeting guide RNA and the genomic locus will reduce editing efficiency. Multiple reference genome assemblies may be listed for any given species. Best practice would include checking the desired target locus across each assembly. Additionally, Sanger sequencing of the target locus using genomic DNA prepared from sample animals can eliminate uncertainty in the nucleotide sequence at the target locus.
8. PegAssist.app will aid in the design of and construction of prime editing guide RNAs, but other webtools exist. It may be beneficial to use multiple webtools to identify strong candidates for high editing efficiency. Other options include (*in alphabetical order*) Easy-Prime [15], PE-Designer [16], peg-Finder [17], PINE-CONE [18], PnB Designer [19], Prime-Design [20], and multicrispr [21].
9. The PBS and RTT sequences are determined by the site of the DNA nick and are therefore determined by the spacer sequence. For highest efficiency editing choose a spacer that has a compatible PE3b guide. If no spacers are displayed, a PAM sequence is not located within the search window. Increase the maximum allowable RTT length in the search parameters to expand the search window.
10. The resulting PBS and RTT sequences differ only in total length. A high-throughput analysis of pegRNAs found that the highest editing efficiency is achieved with a PBS 11- or 12-nt in length, and an RTT 12+/−2- nt in length [22]. There may still be variability in overall efficiency dictated by the genomic locus, so multiple pegRNAs can be designed and compared empirically.
11. Preference should be given for a PE3b guide over a PE3 guide. The PE3b guide will bind closer to the pegRNA cut site resulting in sequential binding and nicking of genomic DNA and decreasing rates of unintended gene knock-out [23].
12. The spacer should cut upstream (5′) of the desired edit. The PBS should bind on the strand opposite of the spacer. The RTT should directly abut the PBS and contain the desired mutation.
13. The “final pegRNA” displays the full pegRNA sequence with the selected spacer, PBS, and RTT as well as BbsI cloning sites and stretches of “filler” sequence. This sequence should be ordered as synthesized double-stranded DNA. The filler is only necessary if the company used to synthesize the pegRNA requires a minimum number of base pairs and will be removed in subsequent cloning steps. The “Spacer sense” and “Spacer

antisense" sections can be ignored if ordering the "final pegRNA" in full. The optional "PE3(b) sense" and "PE3 (b) antisense" sequences contain additional nucleotides for cloning compatibility and should be ordered as displayed from an oligonucleotide synthesis company (like IDT).

14. This pegRNA part can be used directly in a GGA, or it can be subcloned using a TOPO TA Cloning Kit (Thermo Scientific, K4575J10).
15. An example GGA protocol follows: (1) Initial digest at 37 °C for 15 min. (2) Digest at 37 °C for 2 min. (3) Ligation at 16 °C for 5 min. (4) Return to "**step 2**" 15–50 times. (5) Final digest at 37 °C for 15 min. (6) Heat inactivation at 65 °C for 20 min. (7) Hold at 4 °C. The final digest is meant to cleave any remaining backbone vector without the desired insert and should improve cloning success. Heat inactivation will destroy the enzymes and prevent ligation during the "Hold" step. When selecting the cycle number for a GGA, the major consideration is the number of parts being inserted into a backbone. With 1–2 inserts a 15-cycle GGA is typically sufficient. If cloning efficiency is low, the cycle number can be increased to 50.
16. For any steps involving a heating block, water can be added to the well prior to inserting a tube to distribute heat more evenly.
17. If the service provider does not offer commonly used universal primers, the following sequence can be ordered as an oligonucleotide to submit for Sanger sequencing: GACTATCATATGCTTACCGT.
18. The volume of this culture should not exceed ~1/5 total capacity of the culture flask. Larger volumes will not be properly aerated and plasmid quality and yield could be impacted. In this case, an extra 20 mL is initially added to account for evaporation during the long growth period.
19. The expected concentration of plasmid is 2–4 μg/μL. Check that the A260/A280 ratio is around 1.8. Values of 2.0 or above could suggest RNA contamination. Check that the A260/230 ratio is between 2.0 and 2.2. Lower values suggest impurities are present in the sample.
20. Flame polishing the pipettes is essential for cell viability and quality. The process rounds the edges at the opening of the pipette to reduce mechanical stress on the dissociated cells.
21. Dissection medium should be kept cold on ice. Activated papain should be incubated at 37 °C for at least 30 min before adding to tissue.
22. The plasmids should be mixed at an even molar ratio to promote co-electroporation.

23. The pia can be difficult to remove. Gently scrape the surface of the brain with sharp tweezers until the pia is slightly furrowed. Grab the furrowed region with the tweezers and pull parallel to the surface of the brain to peel the pia. If not properly removed dissociation of the tissue may be more difficult.
24. The tissue is very delicate and can easily be aspirated during pipetting. Allow the tissue to fully settle to the bottom of the tube before pipetting.
25. Viability of cells after nucleofection decreases dramatically if fewer cells are used in the suspension during the cuvette electroporation. For high viability, aim to have over 10^6 cells per electroporation.
26. After adding the cell suspension to the culture plate, rock the plate in a figure eight motion to promote even distribution of the cells.
27. Other plasmid transfection methods, including lipofection, polyethylenimine, or calcium phosphate co-precipitation also work with neurons. However, in our hands, the efficiency of plasmid transfection and co-transfection are order-of-magnitudes lower compared to nucleofection.
28. These NGS-compatible primers have a universal adapter sequence that is recognized in a second PCR during library preparation. Primers with unique barcode sequences will prime each sample independently and then samples can be pooled. The barcodes enable sorting of individual samples after sequencing.
29. As an alternative, there are also commercially available kits that make genomic DNA isolation from these samples easy and quick.
30. The annealing temperature for this PCR will be variable based on the design of the primers. Care should also be taken to avoid saturation during PCR. To test for saturation, a sample can be collected at the second to last cycle and compared to a sample from the final sample on an agarose gel. The amplicon should nearly double between these samples.

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