Prime Editing Protocol for HEK 293T/293 Cells with Thermo Fisher Neon® Transfection System

Required Materials

1. Neon® transfection system (Thermo Fisher, MPK5000), consisting of the following parts:
   - Neon® device
   - Neon® pipette
   - Neon® pipette station.
2. Neon® transfection system 10 µL kit (MPK1096) containing:
   - 3 × 1 mL Resuspension Buffer R
   - 2 × 150 mL Electrolytic Buffer E
   - 96 × 10 µl Neon® tips
   - 20 Neon® electroporation tubes

   Store at room temperature. After first use, store buffers at 4°C.
3. Cell culture and electroporation reagents:
   - DMEM medium (Gibco, 10569010)
   - FBS (Gibco, 10091148)
   - Trypsin-EDTA (0.25%), phenol red (Gibco, 25200072)
   - DPBS (LIFE, 10099141)
4. PE protein: Catalog PE (GenScript, RC00003-RC00009)
5. pegRNA: desalt or HPLC purified grade (GenScript, SC1518-CRISPR)
6. Cell culture plasticware:
   - 10 cm cell culture dish (Corning, 430167)
   - 12-well cell culture plate (Corning, 3513)

Preparations Before Electroporation

Cell culture recommendations

1. Replace media every 2-3 days.
2. Passage cells at 80-90% confluency.
3. Seed cells at 2 x 10^5 cells/mL.
4. Subculture 1 day before electroporation.

Note: As a general rule, it's recommended to use cells at the lowest passage number possible. HEK293/293T cells should not be used for electroporation after passage number 20. The optimal confluency for electroporation is 60-80%; higher cell densities may cause lower electroporation efficiencies.

Trypsinization

1. Remove media from the cultured cells and wash cells once with DPBS.

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2. Add 1-2 mL Trypsin-EDTA into the culture dish and incubate the cells for ~1-2 minutes at 37 ℃.

Note: Trypsin-EDTA should be incubated at room temperature before use. Otherwise, digestion time will need to be extended.

3. Neutralize trypsinization reaction with supplemented culture medium once most cells (>90%) have been detached.

Note: Insufficient or excessive trypsinization will affect cell viability and ultimately affect the electroporation.

Reagent solution preparation

1. pegRNA preparation
    - Rehydrate lyophilized pegRNA in RNase-/DNase-free, non-pyrogenic water or TE buffer at a final concentration of 100 μM (100 pmol/μL).
    - After dissolution, aliquot into 5-10 μL per tube and store at -20℃ (for short-term storage) or -80℃ (for long-term storage).

Note: Dissolving pegRNA with water is recommended over dissolving with TE buffer to avoid any uncertain effects of electroporation conversion.

2. PE nuclease preparation
    - PE protein is dissolved in 1mM Sodium citrate by default.
    - Store at -20 ℃ in the short-term (use within 3 months) or -80 ℃ for long-term storage.

Electroporation

Set up the Neon® pipette station

1. Ensure the Neon® pipette station is connected to the Neon® device.
2. Fill the Neon® tube with 3 mL of Electrolytic Buffer (use Buffer E for 10 μL Neon® Tip).

Note: Make sure that the electrode on the side of the tube is completely immersed in buffer. The 10 μL Neon® tip is paired with buffer E, and the 100 μL Neon® tip is paired with buffer E2.

3. Insert the Neon® tube into the Neon® pipette station until you hear a click sound.

Note: Make sure that the side electrode of the Neon® tube is well connected to the side ball plunger of the Neon® pipette station.

Prepare HEK293T cells

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1. Grow the required number of cells.
2. Prepare 12-well plates by filing the wells with 1 mL of culture medium containing 10% FBS (supplements without antibiotics). Pre-incubate plates in a humidified 37°C, 5% CO_2 incubator.
3. Pre-warm an aliquot of culture medium containing serum, DPBS, and Trypsin-EDTA solution to room temperature.
4. One day before electroporation, transfer cells into a new 10 cm cell culture dish with fresh growth medium. Cells should be 60–80% confluency on the day of the electroporation experiment.

Note: 2 x 10^5 cells per sample with 10μL Neon® Tip for our most optimized protocols.

5. Aspirate the media from the dish and rinse the cells using DPBS.
6. Trypsinize the cells using room temperature Trypsin-EDTA.
7. Transfer the trypsinized cell suspension to a 15 mL centrifuge tube and centrifuge the cells at 1000 rpm for 5 minutes at room temperature.

Note: Avoid high-speed centrifugation and pipette cells gently to ensure cell viability.

8. Discard the supernatant and suspend cells in DPBS (without Ca^{2+} and Mg^{2+}).
9. Take out some resuspension solution and count cells with Trypan blue to determine the cell density.
10. Transfer the required number of cells (2 x 10^5 cells per sample) to a 15 mL centrifuge tube and centrifuge the cells at 1000 rpm for 5 minutes at room temperature.
11. Aspirate the DPBS and resuspend the cell pellet in Resuspension Buffer R to a final density of 4 × 10^7 cells/mL (Tube 1).
    - At this density, 5 μL of cell suspension per sample will result in 2 x 10^5 cells per sample.
    - Gently pipette the cells to obtain a single-cell suspension.

Note: Ensure that the supernatant is removed as much as possible while avoiding cell loss. When pipetting for electroporation, the cell suspension needs frequent/gentle agitation to prevent the cells from settling. Work quickly but carefully, and avoid storing the cell suspension for more than 15-20 minutes at room temperature, as prolonged exposure reduces cell viability and transfection efficiency. The resuspension cell density may be adjusted to accommodate the recommended cell numbers for electroporation or optimization protocols. The cell preparation steps can be interspersed and reasonably arranged during the incubation of CRISPR reagents.

Electroporation

1. Incubate Resuspension buffer R at room temperature (stored at 4 °C after opening).
2. According to the table below, mix pegRNA, PE protein, and Resuspension buffer R in a sterile, DNase-/RNase-free 1.5 mL centrifuge tube (Tube 2). Mix well and incubate the RNP mixture in Resuspension buffer R at room temperature for 5-10 minutes

Tube 2	Reagent	pegRNA	PE nuclease	Buffer R
	Amount / Rxn	150 pmol	50 pmol	to 7 μL

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Note: The ratio provided in the table above is a general recommendation which can serve as a starting point for experimentation. Experimental optimization is recommended to determine the optimal amount of pegRNA and PE protein. For the first experiment, it is recommended to set up a negative control group (cells only) and a positive control group (validated efficient gene editing system) separately.

3. Add 5 μL of the cell suspension (from Tube 1, containing 2×10^5 cells) to the reagent mixture (Tube 2) carefully and mix gently by pipetting. The total volume should be ~12 μL.
4. Prepare the Neon® pipette:
   - Press the push-button to the second stop to open the clamp.
   - Insert the top head of the pipette into the Neon® tip until the clamp fully grips the mounting stem of the piston.
5. Slowly release the push-button while applying downward pressure on the pipette, ensuring that the tip is sealed onto the pipette without any groups.
6. Press the push-button on the Neon® pipette to the first stop and immerse the Neon® tip into the cell/reagent mixture, and slowly release the push-button to aspirate the mixture into the Neon® tip.

Note:

- Avoid air bubbles during pipetting, as they may cause arcing during electroporation.
- If air bubbles are present, discard the sample and aspirate a fresh one into the trip again carefully without any air bubbles.

7. Insert the Neon® pipette (containing the sample) vertically into the Neon® tube placed in the Neon® pipette station until you hear a click sound.
8. Start the Neon® transfection system and set the appropriate electroporation parameters (for HEK293T cells- **Voltage: 1200 V, Width: 10 ms, Pulse: 3 pulses**).

Note: If necessary, you can further optimize the electroporation parameters according to the equipment's manual to improve efficiency of electroporation.

9. Ensure that the correct electroporation protocol is selected, then press 'Start' on the touchscreen.
10. The Neon® device automatically checks for the proper insertion of the Neon® tube and Neon® pipette before delivering the electric pulse. The touchscreen displays 'Complete' to indicate that electroporation is complete.
11. Slowly remove the Neon® pipette from the Neon® pipette station and gently transfer the samples from the Neon® Tip by pressing the push-button on the pipette to the first stop into the prepared 12-well culture plate. Each sample should be placed into a separate well.

Note: Avoid repeated pipetting and mixing.

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12. Discard the Neon® tip into an appropriate biological hazardous waste container.
13. Gently rock the culture plate to evenly distribute the cells, then incubate the cells at 37°C in a humidified CO_2 incubator.
14. When you are finished using the Neon® device, turn the power switch on the rear to **off**.

Post-Electroporation

1. Incubate the cells in a humidified 37°C, 5% CO_2 incubator. The analysis may proceed once cells have reached 70-100% confluence.
2. Prime editing efficiency can be assessed after 3 days of culture.

Troubleshooting Guide

Problem	Possible Cause(s)	Recommended Solutions
Arcing (sparks)	Air bubbles in the Neon® Tip	Avoid any air bubbles in the Neon® tip while aspirating the sample.
	High voltage or pulse length settings	Reduce the voltage or pulse length settings.
No cells on plate	Loss of cells during pelleting or supernatant removal before nucleofection	Be cautious when handling the supernatant in cell samples.
Dramatic differences in distribution of cells between reactions	Non-homogeneous distribution of the cell suspension	Mix the cell suspension thoroughly and gently before adding it to the cell/reagent mixture. Continue to gently agitate the suspension to prevent settling. Use cells treated at the same time for all reactions in a given experiment.
	Uneven distribution of cells in the cell culture plate	Gently tap the side wall of the culture plate to dislodge cells and promote even distribution.
Low cell survival rate	Cell culture conditions are not optimal	Use cells at the lowest passage number possible (<20) for electroporation. Split cells at a density of 2-4 $\times 10^5$ cells/mL one day before electroporation. Avoid using cells at high densities, which may affect cell survival post-electroporation.
	Cells are stressed or damaged	Avoid severe conditions during cell harvesting, especially high-speed centrifugation, and pipette cells gently. Also, avoid storing cells in the Resuspension Buffer R for more than 20 minutes. After electroporation, immediately plate

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		the cells into pre-warmed culture medium.
	Incorrect cell number	Ensure 2 $\times 10^5$ cells per sample. Cell numbers >3 $\times 10^5$ or <1$\times 10^5$ cells drastically reduce cell viability post-electroporation.
	Multiple uses of the same Neon® tip	Do not use the same Neon® tip for electroporation more than twice, as repeated application of electric pulses reduces the tip quality and impairs its physical integrity.
Low editing efficiency or non-reproducible transfection efficiency	Protocol issues	Perform protocol optimization and test the dosage range of key reagents to determine optimal conditions.
	Cell culture conditions are not optimal	Avoid using cells highly confluent (>90%).
	pegRNA degradation	Aliquot pegRNA into 5-10 µl per tube and store at -20 °C (for up to 6 months) or -80 °C (for up to 1 year). Avoid excessive freeze-thaw cycles (i.e., more than 10 times).
	PE nuclease degradation or inactivation	Do not use the protein past its expiration and avoid prolonged storage (i.e., over 6 months). Avoid repeated exposure of PE nuclease to temperature fluctuations. Aliquot PE nuclease into 20 µl per tube and store at -80°C. Maintain a storage volume of at least 10 µL to avoid a reduction in enzyme activity.
	Incorrect cell number	Cell numbers >3 $\times 10^5$ or <5 $\times 10^4$ per sample drastically reduce transfection efficiency. Use 2 $\times 10^5$ cells per 10 µl per sample.
	Sparks during electroporation	Avoid air bubbles in the Neon® tip and use an appropriate electroporation program.
	Mycoplasma-contaminated cells	Test cells for Mycoplasma contamination. Start a new culture from a fresh Stock.
	Inappropriate analysis method	Check the rationality of the detection and analysis methods and rigorous operation. If necessary, adjust the analysis method.

Special Note: This protocol was designed for use with the Thermo Fisher Neon® electroporation platform only. If other electroporation or transfection platform are used, we highly recommend using this protocol for reference purposes only and making adjustments according to individual experimental systems.

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Appendix 1: Case Studies

1. Gene editing efficiency in HEK293 T cells using different PE nucleases

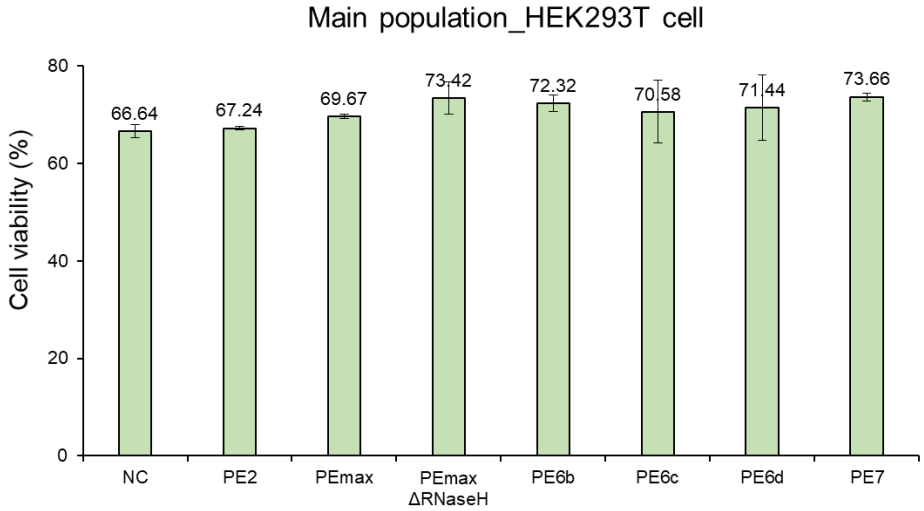

Figure 1: Cell viability of HEK293T cells after 3 days of electroporation.

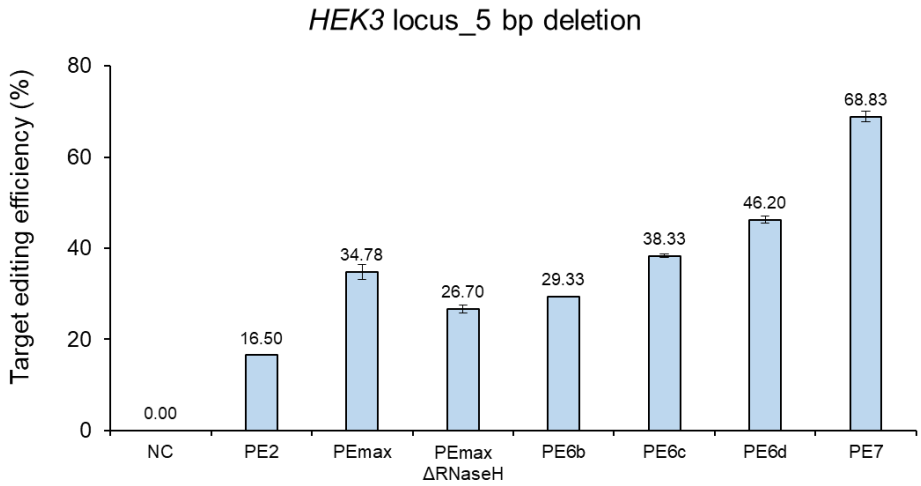

Figure 2: Gene editing efficiency in HEK293T cells using PE protein with Thermo Fisher Neon® electroporation. After 3 days of electroporation.

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RNP preparation:

No.	PE protein		pegRNA		nicking sgRNA	HEK293T cell	Molar ratio
1	PE2	50pmol, 10mg/mL	*HEK3* pegRNA-141nt 5 bp deletion	150 pmol	50 pmol	0.2M*7*2	1：3：1
2	PEmax						
3	PEmax ΔRNaseH						
4	PE6b						
5	PE6c						
6	PE6d						
7	PE7						

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