Electroporation

Systematic nonviral CAR-iPSC engineering

Optimizing nonviral genome editing workflows for iPSCs with advanced electroporation technology

Introduction

Efficient and precise genome engineering of human induced pluripotent stem cells (iPSCs) is essential for applications spanning functional genomics, disease modeling, and the generation of genetically defined cell lines for therapeutic research [1]. iPSCs offer unlimited self-renewal and developmental plasticity, but their sensitivity to physical and molecular perturbations presents unique challenges for genome editing. Genetic modifications introduced at the pluripotent stage are ideally achieved with high efficiency while preserving cell viability, pluripotency, and genomic integrity.

CRISPR/Cas9 technology has become a standard approach for targeted genome modification in iPSCs, enabling gene knockout through nonhomologous end joining (NHEJ) and precise transgene insertion via homology-directed repair (HDR). Despite its versatility, implementation of CRISPR/Cas9 editing in human iPSCs remains technically demanding [2]. HDR-mediated knock-in is often inefficient, and delivery-associated stress can compromise post-editing recovery and pluripotency maintenance. As a result, systematic optimization of genome-engineering workflows is required to balance editing performance with maintenance of iPSC quality.

Nonviral delivery of synthetic guide RNA (sgRNA) and Cas9 nuclease as ribonucleoprotein (RNP) complexes has emerged as a preferred strategy for iPSC genome editing with the CRISPR/Cas9 system. This approach offers transient nuclease activity and reduced risk of off-target effects compared with DNA-based methods [3]. Electroporation is particularly well suited for RNP delivery into iPSCs; however, editing outcomes are highly sensitive to electroporation parameters, buffer composition, and Cas9:sgRNA stoichiometry. Small changes in these variables can result in substantial differences in knockout or knock-in efficiency and cell survival [4].

The Invitrogen™ Neon™ NxT Electroporation System provides a programmable platform for genome editing in sensitive cell types, including human pluripotent stem cells. By enabling precise control over voltage, pulse width, and pulse number, the system supports the systematic optimization of delivery conditions for both NHEJ-mediated gene disruption and HDR-mediated targeted integration [5]. Such optimization is critical for generating reproducible and genetically uniform iPSC lines.

Here, we describe a nonviral-mediated cell engineering workflow to generate CAR-iPSC lines using Neon NxT system–mediated delivery of CRISPR/Cas9 RNPs combined with linear double-stranded DNA donor template. We systematically optimize electroporation parameters, RNP stoichiometry, donor template concentration, buffer conditions, and post-electroporation recovery to maximize gene editing efficiency while preserving cell viability and pluripotency. This study establishes a robust nonviral framework for precise and reproducible genome engineering and CAR-iPSC recovery.

Materials and methods

Human fibroblast-derived iPSCs were cultured in Gibco™ StemFlex™ Medium on 6-well plates coated with Gibco™ rhLaminin-521 (Figure 1). At 70–80% confluency, cells were washed with DPBS without calcium or magnesium, dissociated using Gibco™ CTS™ Versene™ Solution (10–15 min, 37°C), and resuspended as single cells. Cell viability (>80%) and counts were determined using a NucleoCounter™ NC-3000™ cytometer (ChemoMetec). Cells were washed twice and resuspended in Invitrogen™ Neon™ NxT Resuspension R Buffer at 2 x 10^7 cells/mL (unless a different cell density or electroporation buffer condition is mentioned in results section).

Cas9 RNP complexes were prepared by combining Gibco™ CTS™ HiFi Cas9 Protein (240 µg/mL) and Invitrogen™ TrueGuide™ sgRNA (64 µg/mL) at a 1:1 molar ratio and incubated for 10–15 min at room temperature. A ~5 kb linear dsDNA donor (anti–mesothelin 3 CAR sequence with flanking regions of homology to the *CD38* locus) was added at 100 µg/mL (2X). For each electroporation, 10 µL of cells were mixed with 10 µL of payload prepared in the same electroporation buffer (final: 1 x 10^7 cells/mL, 120 µg/mL Cas9, 32 µg/mL sgRNA, 50 µg/mL donor DNA).

Electroporation was performed using the Neon NxT system to test multiple different electroporation conditions and parameters (10 µL tips). Cells were plated on rhLaminin-521–coated plates in StemFlex Medium with Gibco™ CTS™ RevitaCell™ Supplement (contains ROCK inhibitor) or Gibco™ CultureCEPT™ Supplement for recovery. Media was replaced every other day without supplements.

After 4 days, cells were analyzed for viability, recovery, pluripotency markers (TRA-1-81, SSEA4), and CAR expression (V5 tag) by using the Invitrogen™ Attune™ CytPix™ Flow Cytometer. Editing efficiency was confirmed by amplicon sequencing (Ion GeneStudio™ S5 System). Statistical analysis was performed using GraphPad Prism™ Software (*t*-test or ANOVA; mean ± SEM, n >3). Experimental parameters are summarized in Table 1.

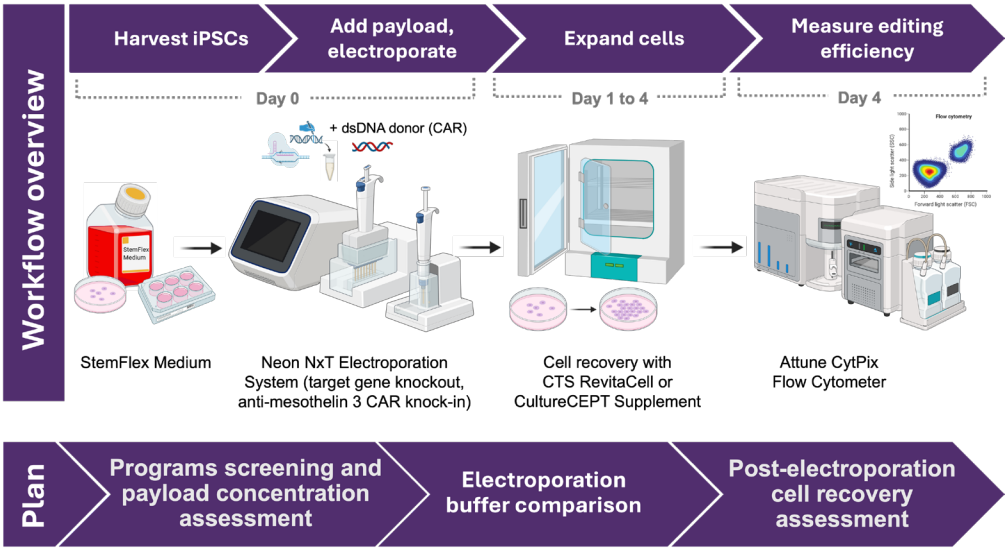

Figure 1. Workflow overview and experimental plan. The top panel describes gene editing in human iPSCs via electroporation, followed by cell recovery, expansion, and editing efficiency analysis. The bottom panel describes the experimental plan to compare and optimize the following key variables: electroporation program, payload concentration, electroporation buffer (R vs. GE buffer), and post-electroporation cell recovery supplement (CTS RevitaCell vs. CultureCEPT Supplement).

Table 1. Summary of experimental parameters used in the study.

Step	Description
Electroporation buffer	R, GE
Programs	Variable (indicated in results figures)
Payload	120 µg/mL CTS HiFi Cas9 Protein 32 µg/mL TrueGuide sgRNA targeting the *CD38* locus 25–100 µg/mL linear dsDNA donor encoding anti–mesothelin 3 CAR (V5-tagged)
Post-electroporation recovery supplement	CTS RevitaCell vs. CultureCEPT Supplement
Target	*CD38* locus (exon 1 start codon)
Cells per electroporation	1 x 10^7 cells/mL
Analysis	Total CAR$^+$ viable cells analyzed by flow cytometry *CD38* knockout levels analyzed by next-generation sequencing

Results

Optimization of electroporation parameters and Cas9:sgRNA ratio enables efficient genome editing with improved cell recovery

To establish electroporation (EP) conditions that balance high genome editing efficiency with robust cell recovery, we initially screened 25 EP programs differing in pulse voltage, width, and number using the Neon NxT Electroporation System to deliver RNP complexes (Figure 2A). Across programs, *CD38* knockout (KO) efficiency varied widely and was inversely related to cell recovery, highlighting a trade-off between editing performance and cell viability (Figure 2B). Several EP programs (red points) achieved intermediate to high KO efficiencies while maintaining acceptable viable cell yields, and these conditions were prioritized for further studies.

Quantification of total viable edited cells on day 4 post-EP revealed that a subset of EP programs generated substantially higher numbers of edited cells compared with other conditions, despite comparable KO percentages (Figure 2C). These findings underscore the importance of considering absolute edited cell output, rather than editing efficiency alone, when optimizing EP workflows.

In parallel, Cas9:sgRNA molar ratios were systematically evaluated using EP parameters of 1,200 V, 10 ms, and 3 pulses. Increasing sgRNA relative to Cas9 improved editing efficiency; however, excessive sgRNA reduced overall cell recovery (Figure 2D, 2E). A 1:2 Cas9:sgRNA ratio provided an optimal balance, yielding high editing efficiency while maximizing total edited cell recovery per EP (Figure 2E).

A

Program ID	Pulse voltage (V)	Pulse width (ms)	Pulse No.
P1	1,400	20	1
P2	1,500	20	1
P3	1,600	20	1
P4	1,700	20	1
P5	1,100	30	1
P6	1,200	30	1
P7	1,300	30	1
P8	1,400	30	1
P9	1,000	40	1
P10	1,100	40	1
P11	1,200	40	1
P12	1,500	20	2
P13	1,200	20	2
P14	1,300	20	2
P15	1,400	20	2
P16	850	30	2
P17	950	30	2
P18	1,050	30	2
P19	1,150	30	2
P20	1,300	10	3
P21	1,400	10	3
P22	1,500	10	3
P23	1,600	10	3
P24	1,300	10	2
P25	1,200	10	3

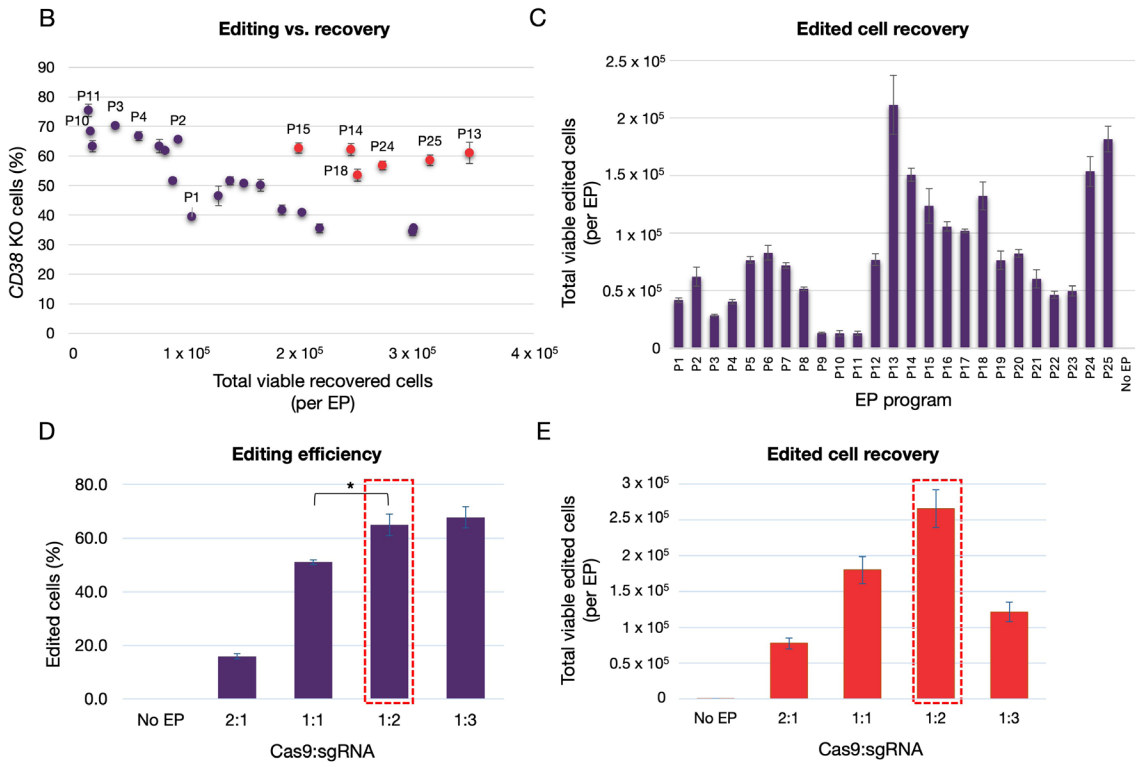

Figure 2. Optimization of EP parameters and Cas9:sgRNA ratio for efficient genome editing and cell recovery. (A) Table summarizing the EP programs (different in pulse voltage, pulse width, and pulse number) tested using the Neon NxT Electroporation System. **(B)** Relationship between editing efficiency and cell recovery across tested EP programs. Percentage of *CD38* gene KO cells is plotted against total viable cells recovered per EP. Each point represents an individual EP program. Red points highlight selected EP conditions with balanced editing efficiency and cell recovery. *CD38* KO levels in iPSCs were analyzed by amplicon-based next-generation sequencing (Ion Torrent™ semiconductor sequencing technology and Ion Plus Fragment Library Kit). **(C)** Total number of viable edited cells recovered on day 4 following each EP program. "No EP" indicates the non-electroporated control. **(D)** Editing efficiency and **(E)** total viable edited cell recovery per EP as a function of Cas9:sgRNA molar ratios. The dashed red box highlights the optimal Cas9:sgRNA ratio balancing editing efficiency and cell recovery. Across all graphs: bars represent mean values with error bars indicating variability across replicates. Statistical significance between conditions is indicated (*p <0.05).

CAR knock-in efficiency and recovery are strongly influenced by donor DNA concentration and EP conditions

Using optimized EP conditions (1,200 V, 10 ms, 3 pulses) and a 1:1 Cas9:sgRNA ratio, we titrated dsDNA donor concentrations, and CAR knock-in efficiency was assessed by flow cytometry on day 4 post-EP (Figure 3A). Increasing dsDNA donor concentration from 25 µg/mL to 100 µg/mL resulted in a dose-dependent increase in CAR knock-in frequency (Figure 3B). However, higher donor concentrations were associated with reduced cell recovery (Figure 3C), consistent with donor-associated cytotoxicity. When both parameters were considered together, 50 µg/mL dsDNA donor concentration maximized the total number of CAR$^+$ cells recovered per EP (Figure 3D), indicating that maximal knock-in frequency does not necessarily translate into maximal CAR$^+$ cell yield. Evaluation of selected EP programs further demonstrated that EP conditions significantly impacted CAR knock-in efficiency, cell recovery, and total CAR$^+$ output (Figures 3E–3G). While some programs yielded higher knock-in percentages, others produced substantially greater numbers of CAR$^+$ cells due to improved survival. EP programs that balanced efficient knock-in with higher recovery generated the greatest absolute CAR$^+$ cell numbers and were identified as optimal conditions.

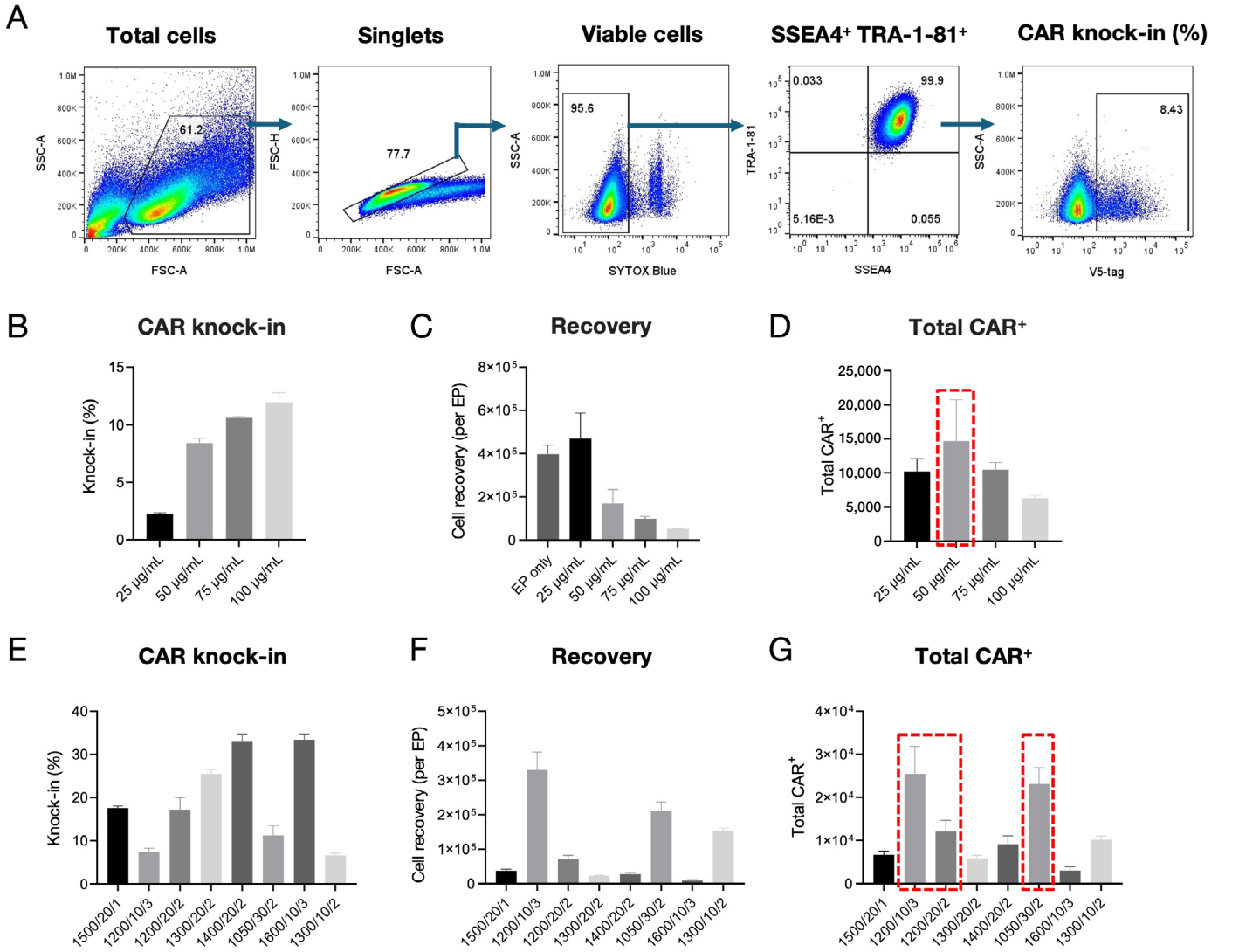

Figure 3. Transgene integration and CAR expression in iPSCs following payload concentration and EP program optimization.
(A) Representative flow cytometry gating strategy used to quantify CAR knock-in efficiency on day 4 post-EP. Cells were sequentially gated on total cells (FSC-A vs. SSC-A), singlets (FSC-H vs. FSC-A), viable cells (negative for Invitrogen™ SYTOX™ Blue Dead Cell Stain), and CAR knock-in cells identified by co-expression of SSEA4 and TRA-1-81. The percentage of CAR knock-in cells within the viable population is shown. **(B)** Percentage of CAR knock-in cells, **(C)** cell recovery, calculated by count of total viable cell number on day 4 post-EP, and **(D)** total number of CAR$^+$ cells per EP at different dsDNA donor concentrations. "EP only" indicates electroporation without payload. **(E)** Percentage of CAR knock-in cells, **(F)** cell recovery, and **(G)** total CAR$^+$ cells recovered per EP across selected EP programs. Parameters are given in the format of voltage/pulse width/pulse number. Across all graphs: bars represent mean values with error bars indicating variability across replicates (n = 3). Dashed red boxes highlight EP conditions that maximize total CAR$^+$ cell number.

EP buffer composition and post-EP culture conditions further modulate CAR⁺ cell yield

To further improve CAR knock-in (KI) outcomes, the impact of EP buffer composition was assessed under fixed EP parameters of 1,200 V, 20 ms, and 2 pulses. While GE buffer supported the highest proportion of KI events within the edited population, as a percentage of CAR⁺ cells (Figure 4A), a drop in cell recovery was observed (compared to EP buffer R), affecting total CAR⁺ cell output (Figures 4B, 4C).

Finally, post-EP culture supplementation was evaluated as an additional lever to enhance total CAR⁺ cell recovery. CultureCEPT Supplement is a chemically defined xeno-free supplement formulated with small-molecule components that support cell survival, stress recovery, and post-manipulation viability. Short-term supplementation with CultureCEPT Supplement immediately after electroporation using GE buffer significantly increased total CAR⁺ cell recovery compared with CTS RevitaCell Supplement (Figure 4D), indicating that post-EP culture conditions play a critical role in maximizing total edited cell yield.

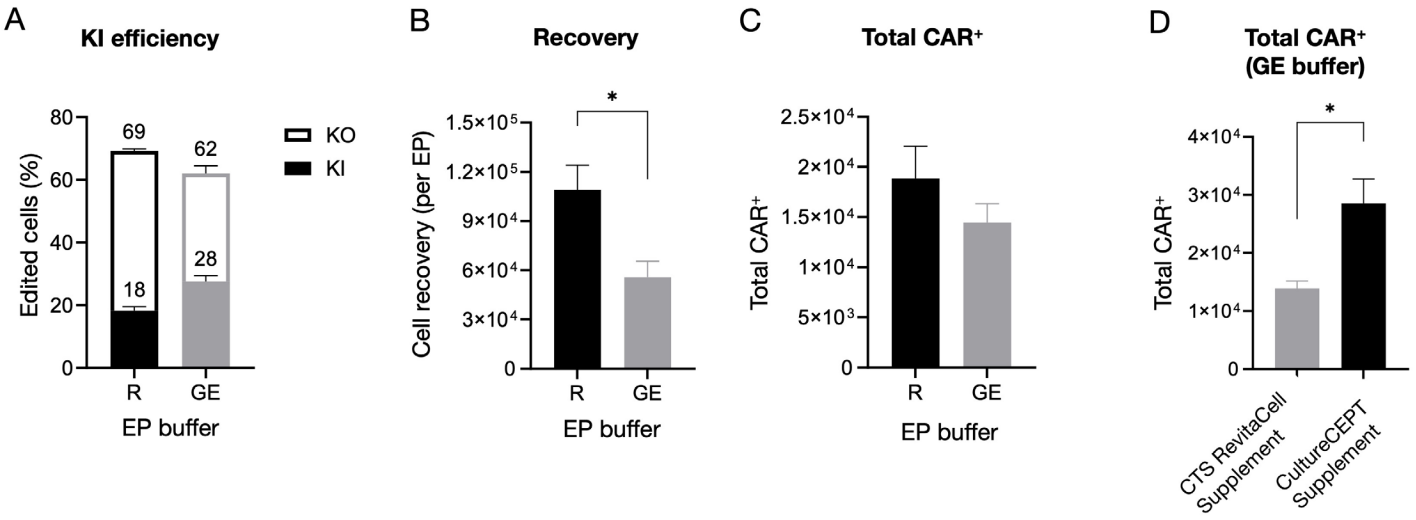

Figure 4. Effect of EP buffer composition and post-EP culture conditions on CAR knock-in efficiency and cell recovery. (A) Editing outcomes following EP in different EP buffers under parameters of 1,200 V, 20 ms, and 2 pulses. The percentage of edited cells is shown, with knock-in (KI) and knockout (KO) fractions indicated for each buffer condition (R, GE). Numbers within bars denote the proportion of KI cells within the edited population. **(B)** Cell recovery and **(C)** total number of CAR⁺ cells recovered per EP across EP buffer conditions. **(D)** Effect of post-EP culture supplementation on total CAR⁺ cell recovery. Total CAR⁺ cells recovered per EP on day 4 are shown for cells recovered with CTS RevitaCell or CultureCEPT Supplement in the culture medium for 24 hr immediately post-EP. Across all graphs: bars represent mean values with error bars indicating variability across replicates (n = 3). Statistical significance between conditions is indicated (*p <0.05).

Characterization of CAR-iPSC clones demonstrates maintenance of pluripotency and genomic integrity

Following enrichment of V5⁺ cells by the Invitrogen™ Bigfoot™ Cell Sorter and establishment of monoclonal CAR-iPSC lines (Figure 5A), three independent CAR-iPSC clones were selected for downstream characterization.

To evaluate preservation of stem cell identity after editing, pluripotency status was assessed using PSC 4-marker immunocytochemistry (ICC) staining and the PluriTest™ assay (Figure 5B, 5C). All engineered clones clustered within the pluripotent reference population and remained clearly distinct from non-iPSC controls, confirming maintenance of pluripotency following CAR knock-in (Figure 5C). Genomic integrity was further evaluated using the Applied Biosystems™ KaryoStat™ HD assay, which demonstrated normal chromosomal profiles without detectable large-scale chromosomal abnormalities after electroporation and clonal expansion (Figure 5D). Furthermore, qPCR-based differentiation analysis with the Applied Biosystems™ TaqMan™ hPSC Scorecard™ Panel demonstrated preservation of trilineage differentiation potential across all selected CAR-iPSC clones (Figure 5E).

Collectively, these findings demonstrate that Neon NxT system–mediated CAR engineering supports generation of stable CAR-iPSC clones that retain pluripotency, differentiation capacity, genomic integrity, and sustained CAR expression following clonal expansion.

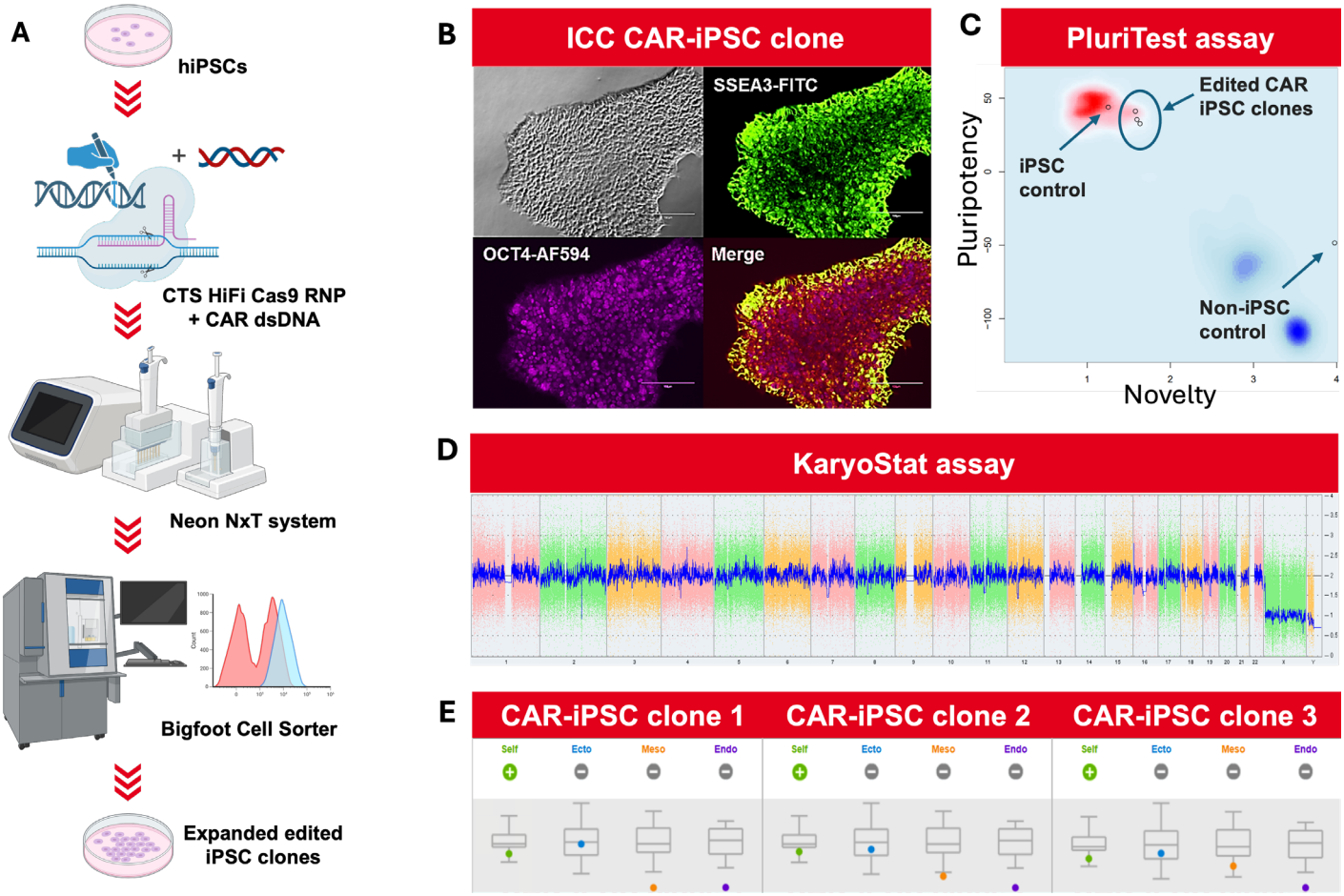

Figure 5. Characterization of engineered CAR-iPSC clones following Neon NxT system–mediated genome engineering. (A) Workflow for CAR knock-in, electroporation, cell sorting, and clonal expansion. **(B)** ICC staining demonstrating maintenance of pluripotency marker expression (OCT4 and SSEA3) in edited CAR-iPSC clones. **(C)** PluriTest assay confirming pluripotent transcriptional profiles of engineered CAR-iPSC clones relative to iPSC and non-iPSC controls. **(D)** KaryoStat assay analysis showing normal chromosomal integrity after genome engineering and clonal expansion. **(E)** qPCR-based differentiation analysis with the TaqMan hPSC Scorecard Panel demonstrating preservation of trilineage differentiation potential across selected CAR-iPSC clones.

Conclusion

All together, these results establish a systematically optimized EP workflow in iPSCs in which electroporation parameters, Cas9:sgRNA ratio, donor DNA concentration, buffer composition, and post-EP cell recovery culturing conditions collectively determine genome editing efficiency and, critically, the total yield of engineered CAR-iPSCs. Furthermore, engineered CAR-iPSC clones retained pluripotency, differentiation capacity, and genomic integrity following clonal expansion.

References

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5. Xu X et al. (2018) Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation. *Sci Rep* 8(1):11649.

Ordering information

Product	Quantity	Cat. No.
Neon NxT Electroporation System with 1-Channel and 8-Channel Pipettes	1 system	NEON18S
Neon NxT Electroporation System Starter Kit with 1-Channel and 8-Channel Pipettes	1 kit	NEON18SK
Neon NxT Electroporation System 10 µL Kit with 1-Channel Tubes	25 x 2 reactions	N1096
Neon NxT Electroporation System 10 µL Kit with 8-Channel Tubes	96 x 2 reactions	N1096-8
CTS StemFlex Medium	500 mL	A5465001
StemFlex Medium	500 mL	A3349401
CTS Versene Solution	100 mL	A4239101
rhLaminin-521	100 µg	A29248
CTS RevitaCell Supplement	5 mL	A4238401
CultureCEPT Supplement	500 µL	A56799
SYTOX Blue Dead Cell Stain	1 mL	S34857
TRA-1-81 (Podocalyxin) Monoclonal Antibody, APC, eBioscience	100 tests	17-8883-42
SSEA4 Monoclonal Antibody (eBioMC-813-70 (MC-813-70)), Alexa Fluor 488, eBioscience	100 tests	53-8843-42
V5 Tag Monoclonal Antibody (TCM5), PE, eBioscience	100 tests	12-6796-42
Attune CytPix Flow Cytometer	1 system	A51848
eBioscience Flow Cytometry Staining Buffer	600 mL	00-4222-26
CTS PSC Cryomedium	50 mL	A4238801
CTS HiFi Cas9 Protein	2.5 mg	A54223
TrueCut HiFi Cas9 Protein	100 µg	A50576
TrueGuide sgRNA *CD38* start codon locus	CTGAACTCGCAGTTGGCCAT PAM (AGG)	

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