Enhancing CRISPR knock-in and knock-out with the 4D-Nucleofector® LV Unit PRO

Introduction

The CRISPR/Cas9 system has revolutionized genome engineering by enabling precise, efficient and programmable modification of DNA, which makes it of particular interest for clinical applications. Derived from a prokaryotic adaptive immune mechanism, CRISPR/Cas9 utilizes a single guide RNA (sgRNA) to direct the Cas9 endonuclease to specific genomic loci, where it introduces double-strand breaks (DSBs)[1]. DSBs are repaired by endogenous pathways like non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is a random repair pathway usually leading to gene disruption, whereas HDR facilitates correct gene repair if an HDR template (HDRT) is provided[2]. Due to its simplicity, specificity, and versatility, CRISPR/Cas9 has become a foundational tool for functional genomics, disease modelling and, with high impact for patients suffering from untreatable diseases, in the development of gene-based therapies.

A major challenge for *ex-vivo* gene therapies from bench to bedside lies in a safe and efficient delivery of its components into target cells. In recent years, non-viral methods for cell engineering emerged as promising alternatives to viral transduction as they are safer, have no risk of insertional mutagenesis and provide a greater cargo flexibility. Amongst the virus-free technologies, electroporation is considered the gold standard, with an increasing presence in clinical trials[3]. By applying a brief electrical pulse, electroporation transiently permeabilizes the cell membrane enabling the uptake of CRISPR/Cas9 components in the form of plasmid DNA, mRNA or ribonucleoproteins (RNPs). Our electroporation-based Nucleofector® Technology enables the direct transport of nucleic acids into the nucleus, thus achieving high transfection efficiencies of traditionally challenging cell types like primary T cells or hematopoietic stem cells. The different Nucleofector® Platforms spanning from small scale and higher throughput to large scale have been successfully used in combination with various genome editing tools for cell therapy research and clinical trials.

Our next generation large-volume Nucleofector® Platform, the 4D-Nucleofector® LV Unit PRO, comes with an improved cartridge for the electroporation of complex cargos into large volumes of T cells. The optimal cell handling parameters were developed in small scale using 100 µL reactions and then adapted for the new large-scale cartridge to process up to 1×10^9 cells in 10–20 mL. In addition to optimized scale-up performance, this second generation also allows for more flexibility in medium scale enabling electroporation volumes between 0.5 and 2 mL in the fixed-volume cartridge. Furthermore, it comes with increased robustness for filling and clearing of the fixed-volume cartridge and for sample processing in the flow-through cartridge. In this application note, we show the efficiency and the robustness of the scale up with the 4D-Nucleofector® LV Unit PRO.

Abbreviation	Long form
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
Cas	CRISPR-associated
DSB	Double-strand break
dsDNA	Double-stranded DNA
FT	Flow-through
FV	Fixed volume
(E)GFP	(Enhanced) Green Fluorescent Protein
KI	Knock-in
KO	Knock-out
LV FT	Flow-through cartridge for first generation LV Unit
LV PRO FT	Flow-through cartridge for the LV Unit PRO
LV FV	Fixed-volume cartridge for first generation LV Unit
LV PRO FV	Fixed-volume cartridge for the LV Unit PRO
LV Unit PRO	New large-scale 4D-Nucleofector® Unit
LV Unit	First generation large-scale 4D-Nucleofector® Unit
HDR	Homology-directed repair
HDRT	HDR template
NHEJ	Non-homologous end joining
RAB11A	Ras-associated protein; member of RAS oncogene family
RNP	Ribonucleoprotein
sgRNA	single guide RNA
TRAC	Gene encoding for TCRα constant region
TCRα	T cell receptor alpha

Materials and methods

Rationale and experimental design

The new Nucleocuvette® Cartridges were developed in order to improve the scale up from small (100 µL) to large scale (up to 20 mL) when using our 4D-Nucleofector® System. To achieve that, besides optimizing the design of the reaction room, we optimized our electrical parameters so that the performances match and an easy scale up is possible.

Briefly, activated or resting T cells were transfected with SpCas9, sgRNA and – for knock-in – with dsDNA HDR templates (HDRT) using the 4D-Nucleofector® LV Unit PRO, LV Unit or X Unit. Four days post-transfection, T cells were analyzed for successful gene editing using FACS staining. Based on the data, the performance match between 4D-Nucleofector® X Unit, LV Unit and LV Unit PRO was determined.

Cell culture

Cryopreserved human PBMCs (Lonza) were activated with TransAct™ beads (Miltenyi) for 72 h or $CD3^+$ Pan T Cells (Lonza) were activated with Dynabeads™ human T-Activator CD3/CD28 (Thermo Fisher Scientific) for 48 h prior to transfection. For experiments with resting T cells, cryopreserved human PBMCs (Lonza) were transfected and activated 24 h later. For activation, TransAct™ beads (Miltenyi) were used and resting T cells were activated for 72 h. All experiments were done under standard cell culture conditions for T cells. For this application note, we used 5 x 10^7 cells/mL per electroporation.

RNP + HDRT formation

RNP complexes were assembled by incubating chemically synthesized, HPLC-purified SafeEdit sgRNA (GenScript) with GenCrispr Cas9 nuclease (GenScript) at a 2:1 molar ratio in nuclease-free water. Complex formation was carried out at 37 °C for 15 minutes. RNPs were then incubated with the HDR template (HDRT) for 10 minutes at room temperature. RNP and HDR template concentrations are provided in Table 1.

Experimental setup		
T cells	Cell concentration per electroporation	5 x 10^7 cells/mL
RNP complex	GenCrispr Cas9 nuclease	27.7 µM
	SafeEdit sgRNA	55.5 µM
HDR template	linear dsDNA; L-*TRAC* (3.5 kB)	50 nM
	linear dsDNA; L-*RAB11A* (3.5 kB)	8.4 nM
	linear dsDNA; S-*RAB11A* (1.5 kB)	14.6 nM

Table 1. Experimental setup for CRISPR

A

B

Figure 1.
4D-Nucleofector® System with Core Unit, X-Unit and LV Unit PRO (A). 4D-Nucleofector® Core Unit and LV Unit PRO with full assembly of TheraPEAK® LV Nucleocuvette® Cartridge PRO and TheraPEAK® LV Reservoir with two sample input lines (B).

Non-viral gene editing

On the day of transfection, cells were harvested by centrifugation. T cells were then resuspended in P3 Nucleofector® Solution, combined with cargo, and gently mixed by pipetting.

Next, the cell-cargo mix was transferred to the respective Nucleofector® Vessel for transfection:

- 100 µL Nucleocuvette® Vessel for the 4D-Nucleofector® X Unit (Figure 1A)
- 1 mL Nucleocuvette® Cartridge for the 4D-Nucleofector® LV Unit
- 2 mL Nucleocuvette® Cartridge PRO (Figure 1A) or LV Nucleocuvette® Cartridge PRO (Figure 1B) for the 4D-Nucleofector® LV Unit PRO, both with various volumes

Pulse code	Cell Type code	Used for
Small scale (X Unit; 100 µL)		
CM-125	–	KO or KI
Medium scale, fixed volume (LV Unit; 1 mL)		
CM-125	–	KO or KI
EO-115	T cell, human, stim. T cell, human, unstim. HF	KO or KI
Medium scale, fixed volume (LV Unit PRO; up to 2 mL)		
MUH-2959	T cells, human, activated, option 5	KO or KI
MXW-0943*	T cells, human, activated, option 13	KO or KI
KTU-0192*	T cells, human, activated, option 11	KI
TGL-1308	T cells, human, resting, option 1	KO or KI
Large scale (LV Unit, LV Unit PRO; up to 20 mL)		
Best program determined in fixed volume		

Table 2.
Used Nucleofector® Programs for human T cells. Pulses with * were used in addition to the programs recommended in our T cell protocol for initial pulse selection.

After electroporation, T cells are resuspended in cell culture medium and transferred to the appropriate culture vessel. Electroporated cells are collected in culture medium and subsequently transferred to the appropriate culture vessel.

Analysis

Four days post-transfection, gene editing efficiencies were evaluated by flow cytometry (NovoCyte, Agilent). Cells count and cell viability were assessed by either flow cytometry (DAPI staining) or NucleoCounter® NC-202 (Chemometec).

Results

Comparable performance between low and medium scale for CRISPR knock-out of *TRAC*

To establish a scalable approach for comparable results between small scale, medium scale and large scale, we edited the TCRα receptor gene in activated primary T cells.[4] As TCRα undergoes many recombination events, we decided to target the constant region of the receptor (*TRAC* gene). We designed sgRNAs and transfected them together with SpCas9 as RNP using either a small-scale 100 µL Nucleocuvette® Vessel or a medium-scale 2 mL Nucleocuvette® Cartridge PRO (LV PRO FV) or 1 mL Nucleocuvette® Cartridge (LV FV). We got comparable TCR knock-out (KO) efficiencies independent of the Nucleocuvette® Vessel used (Figure 2A). For all vessels, the KO efficiency was > 90 %, with LV PRO FV matching the performance of the 100 µL slightly better compared to the first generation LV FV.

Equal performance between low and medium scale for CRISPR knock-in of GFP into TRAC

Since CAR-T development is a promising application in CGT, we mimicked the insertion of a CAR into *TRAC* by integrating a 3.5 kb long HDR template expressing GFP in order to simplify the analysis. Using our small-scale 100 µL Nucleocuvette® Vessel (100 µL) and fixed-volume 2 mL Nucleocuvette® Cartridge PRO (LV PRO FV), we achieved high knock-in (KI) efficiencies of GFP with over 40 % (Figure 2B). The results are comparable between small and medium scale suggesting an easy scale up for clinical applications. In order to analyze the cell survival after transfection, we seeded transfected cells after a *TRAC* knock-in experiment with LV PRO FV into G-Rex® 10M and achieved expansion rates of 50 – 70 fold (Figure 2C). This shows that T cells are not only successfully gene edited, but also survive the electroporation process well, so that they can be expanded efficiently for downstream applications.

Furthermore, the new LV PRO FV cartridge has the advantage that it is suited for different filling volumes between 0.5 and 2 mL of cell suspension. The editing efficiency for *TRAC* KI and KO, the mean fluorescence intensity (MFI)

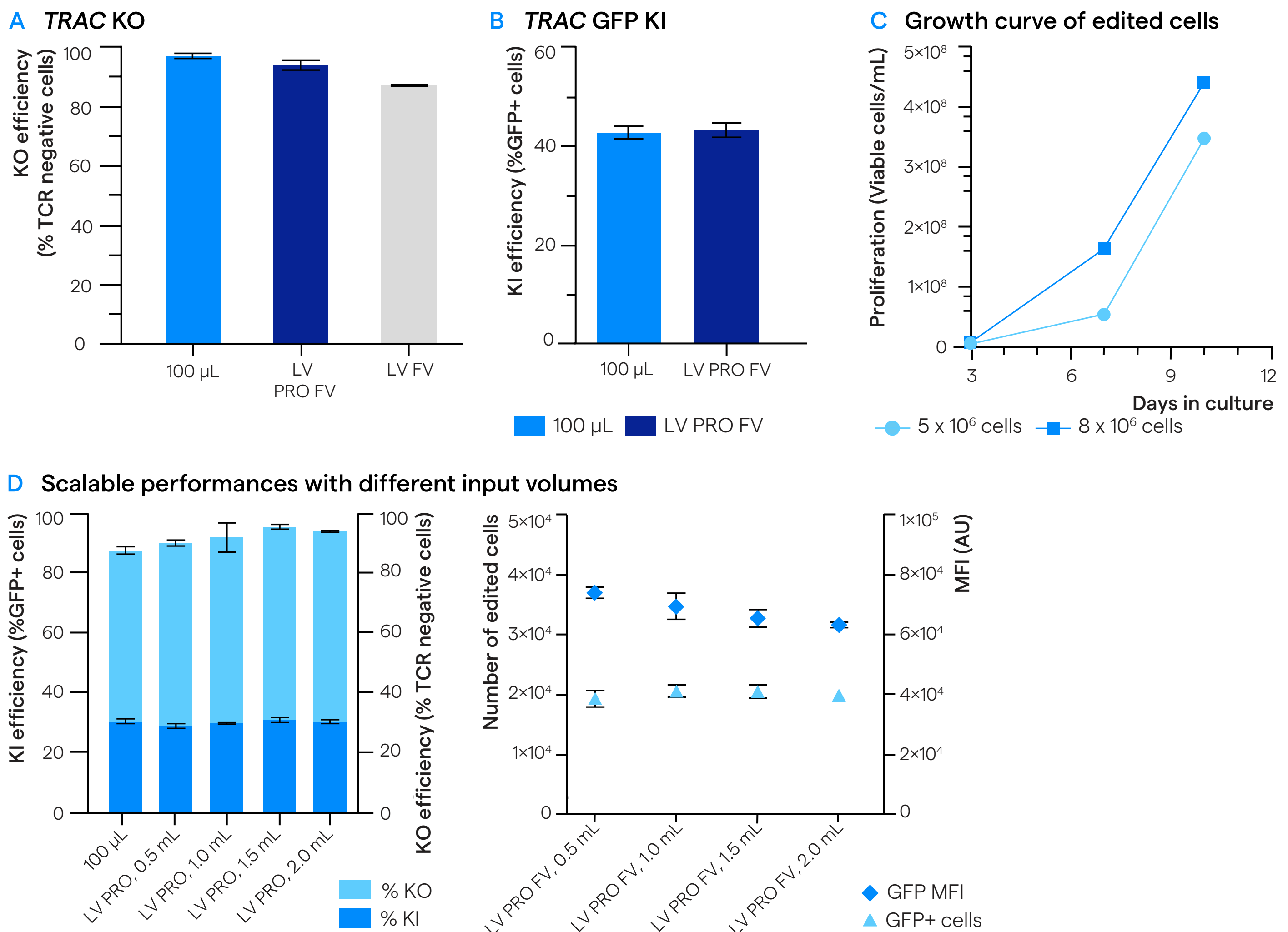


Figure 2.
Scalable performances for 100 µL cuvette and new fixed-volume cartridge. (A) *TRAC* KO efficiency for the LV PRO FV vs small-scale 100 µL reference and first-generation LV FV (2 donor, 4 technical replicates). (B) Representative KI efficiency on day 7 for the LV PRO FV vs small scale 100 µL reference (1 donor, 8 technical replicates). The cargo system delivered was Cas9 RNP and dsDNA HDR template (*TRAC*-GFP; 3.5 kb). (C) Representative growth profile in G-Rex® bioreactors. Two different amounts of cells were seeded after electroporation. (D) The LV PRO FV enables transfection of fixed volumes of cells namely, 0.5, 1.0, 1.5 or 2.0 mL. Different input volumes result in equivalent performances. Representative data are shown for (right) *TRAC* KO and KI (*TRAC*-GFP 3.5 kb), (left) number of edited cells and MFI (median fluorescence intensity). For abbreviations see abbreviations list.

and the number of viable GFP-positive cells are comparable if cells are electroporated at different filling volumes (Figure 2D). Additionally, this experiment again confirms the equal Nucleofection® Performance between 100 µL and LV PRO FV.

Robustness across multiple donors, experiments and starting material

To evaluate the robustness of the 4D-Nucleofector® LV Unit PRO, we knocked a GFP reporter construct of 1.5 kb into the *RAB11A* locus. The fixed-volume 2 mL Nucleocuvette® Cartridge PRO (LV PRO FV) matched the performance of small-scale 100 µL Nucleocuvette® Vessel (100 µL) with two distinct cell type codes for activated T cells (option 11 and 13) across seven different donors in 13 experiments (Figure 3A, 3B). On the contrary, the first-generation fixed-volume 1 mL Nucleocuvette® Cartridge (LV FV) does not perform as well with only 50 % match to the 100 µL.

Next, we aimed to explore whether different starting materials could be used for gene editing approaches with the 4D-Nucleofector® LV Unit PRO. We tested activated T cells derived from PBMCs – our standard choice – alongside with activated T cells from $CD3^+$ Pan T cells and resting T cells. Across all cell sources LV PRO FV consistently demonstrated strong performance, similar to the results obtained with the 100 µL format (Figure 3C).

Large-scale gene editing of up to 1 billion cells enables manufacturing for clinical applications

Clinical applications often require large amounts of edited cells. To scale-up 1 x 10^9 cells in 20 mL were electroporated in the flow-through LV Nucleocuvette® Cartridge PRO (LV PRO FT) by applying pulses identified for medium-scale electroporation. Cell suspension and cargo are processed in consecutive fractions. The sample is pumped from an input vessel into the LV Nucleocuvette® Cartridge, then the transfected fractions are automatically transferred into a collection vessel. The input and collection vessel can either be the 4D-Nucleofector® LV Reservoir developed for use with the flow-through cartridge or cell culture bags. The Nucleofection® Performance in a flow-through experiment matches very well to the small- and medium-scale formats with similar knock-in efficiencies for *RAB11A* in the 100 µL Nucleocuvette® Vessel (100 µL), the 2 mL Nucleocuvette® Cartridge PRO (LV FV PRO) and the LV Nucleocuvette® Cartridge PRO with 20 mL (LV FT PRO) (Figure 4A). As HDRT we used a 3.5 kb GFP reporter construct.

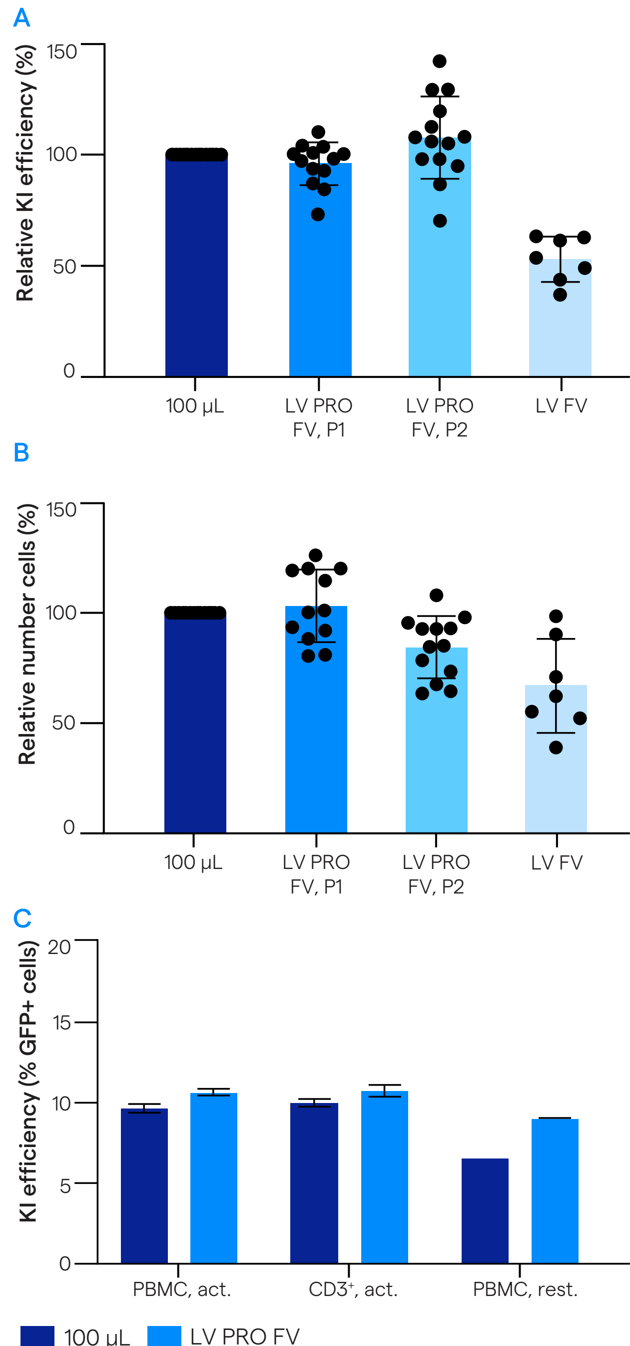
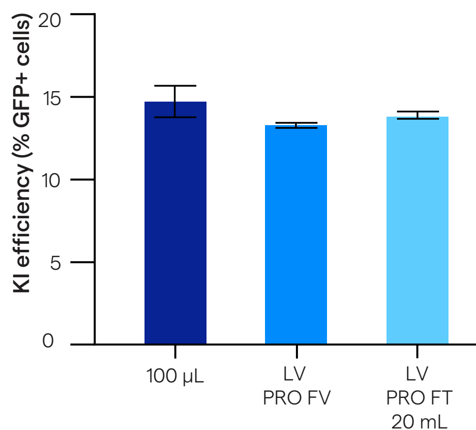
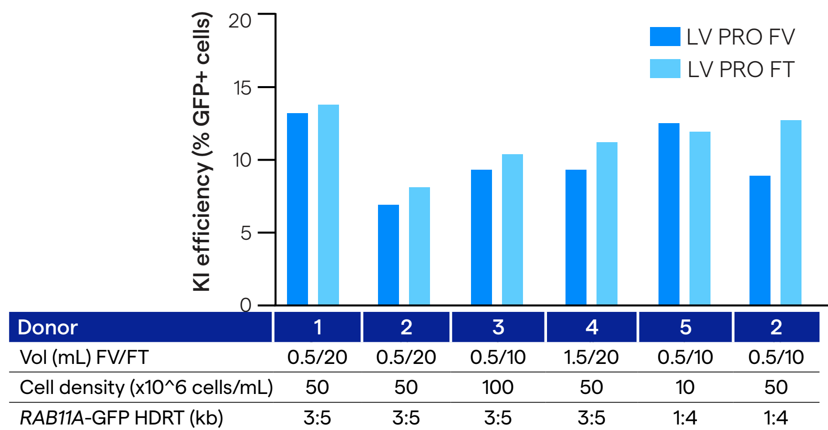


Figure 3.
Robustness across multiple donors, experiments and starting material. (A and B) KI data for *RAB11A* from 7 healthy donors and 13 different experiments were normalized versus the 100 µL reference (set at 100%) to account for donor variability. The graph shows KI efficiency (A) and number of viable edited cells (B) with two electric programs. The performances of the first-generation LV FV cartridge are included for comparison. The cargo system delivered was Cas9 RNP and dsDNA HDR template (*RAB11A*-GFP, 1.4 kb). (C) KI efficiency with dsDNA-*RAB11A*-GFP 1.4 kb for different starting material. For abbreviations see abbreviations list.

Furthermore, scalability between LV FT PRO and LV FV PRO is maintained when different volumes, cell densities or HDR templates are used. Figure 4B shows transfection of 0.5 and 1.5 mL (LV FV PRO) or 10 and 20 mL (LV FT PRO) activated T cells, with cell densities of 5 x 10^7 /mL or 1 x 10^8/mL and 3.5 or 1.4 kb HDRT. The efficiency between LV FT PRO and LV FV PRO are comparable, with LV FT PRO giving slightly better results (Figure 4B). For flow-through experiments, we used a filling volume of 1.5 mL per cycle. This yields 13 fractions for electroporation of a 20 mL sample. For the fractions, we see constant KI efficiency, number of edited cells and fraction volume suggesting a stable performance throughout the filling cycles (Figure 4C). This further confirms the robust performance of the 4D-Nucleofector® LV Unit PRO, making it a valuable tool for manufacturing gene edited cells in large volume.





Donor	1	2	3	4	5	2
Vol (mL) FV/FT	0.5/20	0.5/20	0.5/10	1.5/20	0.5/10	0.5/10
Cell density (x10^6 cells/mL)	50	50	100	50	10	50
RAB11A-GFP HDRT (kb)	3:5	3:5	3:5	3:5	1:4	1:4

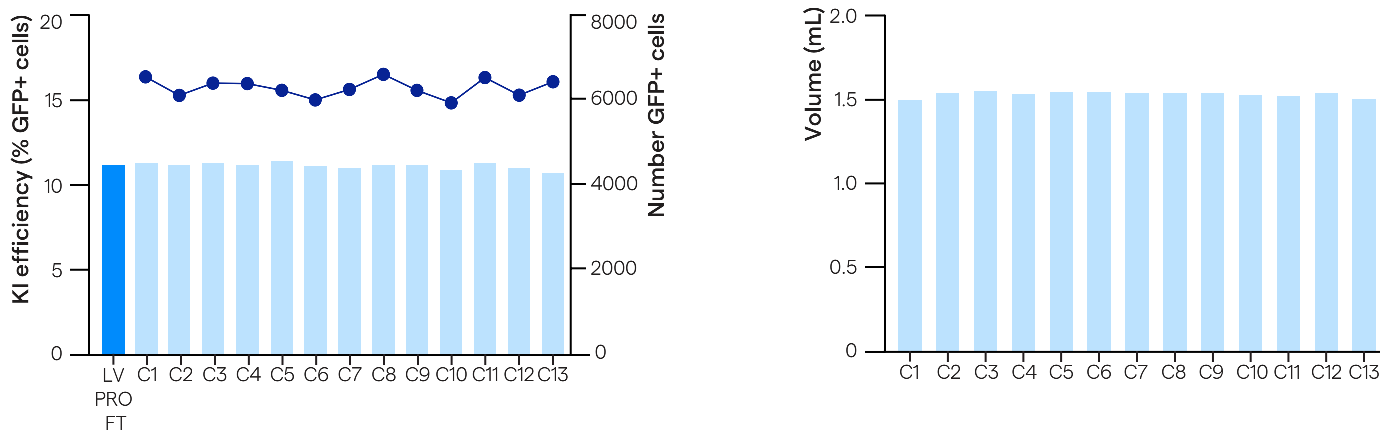


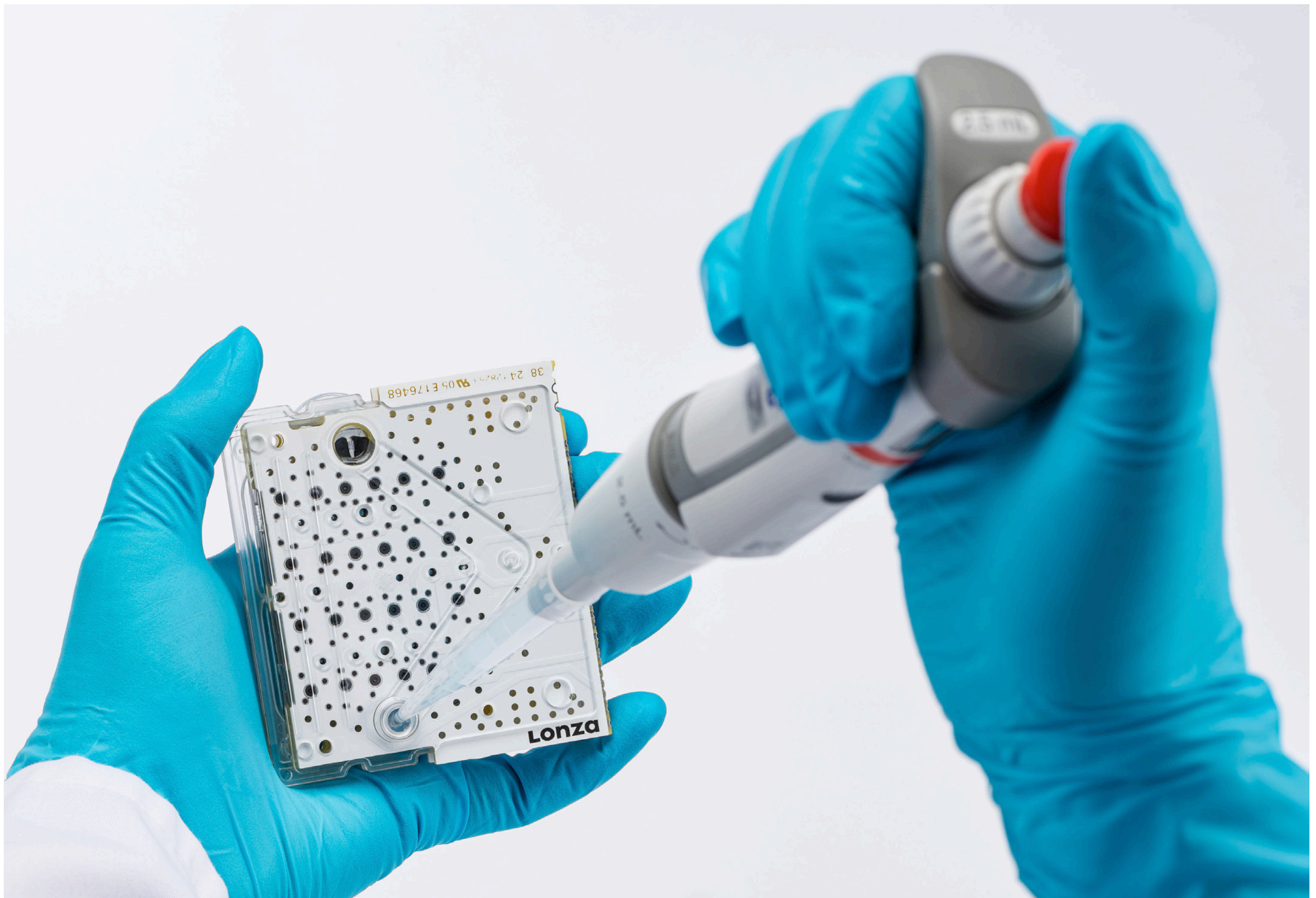
Figure 4.
Scalability from 0.5 to 20 mL maintained across wide range of process parameters. (A) Representative KI efficiency for the LV PRO FV and LV PRO FT cartridges compared to the small scale 100 µL reference (1 donor, 4 technical replicates). (B) Data on 5 different healthy donors whereby Cas9 RNP was co-delivered with a dsDNA HDR template for *RAB11A*-GFP with 3.5 kb or 1.4 kb. Cells were electroporated at 5.0 x 10^7 cells/mL or at 1.0 x 10^8 cells/mL. (C) The LV PRO FT cartridge processes 20 mL volume through subsequent filling and emptying cycles, each addressing, in this case, 1.5 mL cell suspension. The figure shows how stable KI Efficiency (*RAB11A*-GFP, 3.5 kb HDRT) is, from the first aliquot (C1) to the last cycle (C13). Equally stable are the volumes recovered (1.54 mL ± 0.02) and the number of edited cells, suggesting no accumulation of debris or deterioration of the process over time. The total volume recovered is >95% of the input volume. For abbreviations see abbreviations list.

Conclusion

Gene editing with CRISPR Cas9 is shown to be a versatile tool for therapeutic applications. In combination with electroporation, it expands the possibilities of cell and gene therapy by providing an efficient, non-viral way to deliver gene editing tools directly to their target site.

Our new 4D-Nucleofector® LV Unit PRO with Nucleocuvette® Cartridges PRO enables a reliable, robust and efficient delivery of such complex, clinically relevant cargo. We demonstrate an easy scale up from small-scale to large-scale vessels without performance loss. The 2 mL Nucleocuvette® Cartridge PRO can be used with variable volumes between 0.5 and 2 mL, achieving consistent performance. This enables a flexible loading of the cartridge independent of the cell material available. For larger applications, a further scale up to 20 mL and 1 billion cells is possible with the LV Nucleocuvette® Cartridge PRO. Data show good performance at large volumes and the scalability is maintained across a wide range of process parameters.

With this, Lonza's 4D-Nucleofector® LV Unit PRO can reliably support non-viral manufacturing of gene and cell therapy products.



References:
1. **Jinek, M.,** *et al.* A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337, 816 – 821 (2012).
2. **Nami, F.,** *et al.* Strategies for *In Vivo* Genome Editing in Nondividing Cells. *Trends in Biotechnology* Volume 36, Issue 8, 770 – 786 (2018).
3. **H. Balke-Want,** *et al.* Non-viral chimeric antigen receptor (CAR) T cells going viral. *Immuno-Oncology and Technology* Volume 18, 100375 (2023).
4. **Roth, T.L.,** *et al.* Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature* 559, 405 – 409 (2018).

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