

Introduction

Current gene delivery technologies for cell therapy applications rely on harsh mechanisms which can negatively impact cell quality. Retaining cell health and function following the delivery of cargo represents a potential game changer for the manufacture of next generation gene-modified cell therapies.

Solupore® by Avectas offers an automated, reproducible transfection system for the delivery of a wide variety of cargo (RNA, DNA, protein) to primary immune cells used in clinical cell therapy applications. Solupore delivers cargoes by physiochemical means using physiological cell concentrations ($\sim 10^6$ cells/mL), yielding cells with superior cell quality compared with electroporation. As a result of the superior cell health, cells transfected using Solupore possess the potential for enhanced *in vitro* and *in vivo* function.

Key features and benefits of Solupore®:

- **Superior cell health & phenotype** – improved proliferative capacity, maintained stem cell memory population and reduced apoptosis compared to electroporation, leading to enhanced *in vitro* and *in vivo* cell function
- **Versatility** – non-viral delivery of RNA, DNA, or protein cargo (Figure 1A) to a range of cell types including T cells, NK cells, and iPSCs (Figure 1)
- **Flexible, complex editing** – allows for multiplexing as well as rapid, sequential gene edits of primary cells while retaining superior cell quality compared with electroporation
- **Ease of adoption/use** – automated, closed system available now with full GMP support by the 2H of 2023

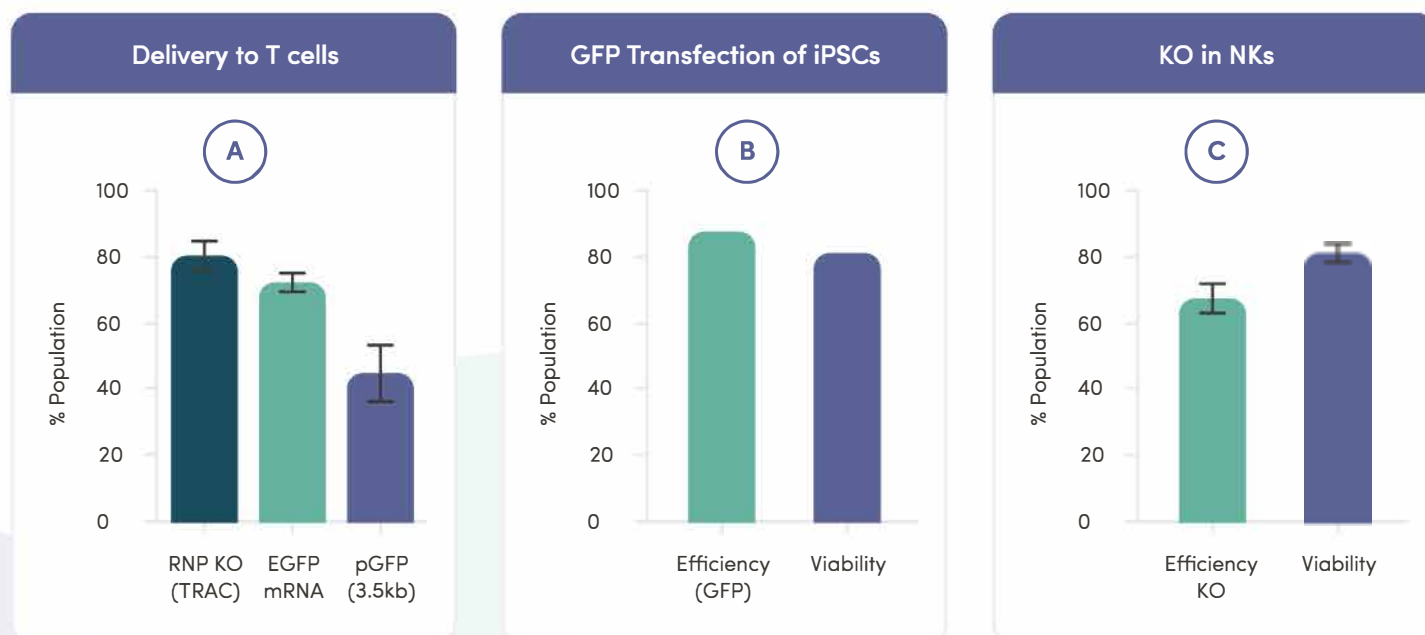


Figure 1: A) Demonstrating diversity of cargo types delivered using Solupore (protein, mRNA, DNA) to T cells, B) Demonstrates ability to deliver to iPSCs, C) demonstrates the ability to deliver to NKs

Solupore delivery of CD19 CAR to primary human T cells led to improved *in vivo* engraftment and reduction of tumor growth compared to electroporation¹ (Figure 2C).

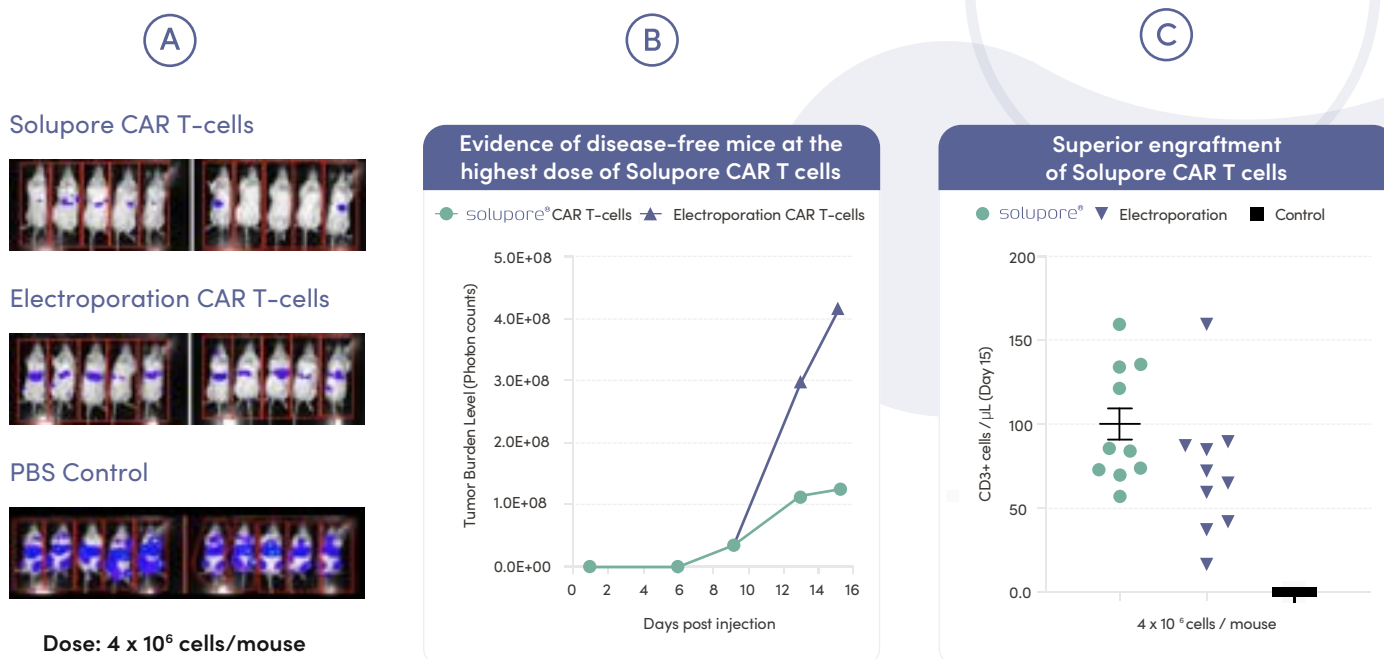


Figure 2: A) Bioluminescent imaging 15-days post dosing for the Solupore CAR T cells, electroporated CAR T cells and PBS control. B) Solupore cell engraftment in blood was significantly higher in mice that received Solupore CAR T cells. C) Average radiance across each group demonstrating reduction in tumor growth for mice dosed with CAR T cells.

Here, we build on this work to demonstrate Solupore efficiently delivers cargo for multiplexed as well as sequential editing of primary T cells. We use CRISPR-Cas RNP based knockout of TCR, B2M, and CD7. These genes are targeted to reduce the risk of alloimmunization and graft vs. host disease for allogeneic cell therapies. Cells edited using the Solupore system display superior cell health and function compared to electroporation.

1: Kavanagh, H., Dunne, S., Martin, D. S., McFadden, E., Gallagher, L., Schwaber, J., Leonard, S., & O'Dea, S. (2021). A novel non-viral delivery method that enables efficient engineering of primary human T cells for ex vivo cell therapy applications. *Cytherapy* 23(9) 852-860 <https://doi.org/10.1016/j.jcyt.2021.03.002>

Results

Solupore Enables Multiplexed and Sequential Editing

Primary T cells were edited using either Solupore or electroporation using 3 different donor sources. At 7 days post editing, Solupore edited cells demonstrated similar knockout cell yield compared to electroporation (Figure 3A).

Release of IFN γ is a standard measure of T cell function responses. At 4 days post editing, Solupore triple-edited cells demonstrated superior cell function (Figure 3B) based on higher levels of interferon gamma (IFN γ) response relative to electroporated cells (1159 pg/mL for SOLUPORE and 598 pg/mL for electroporation).

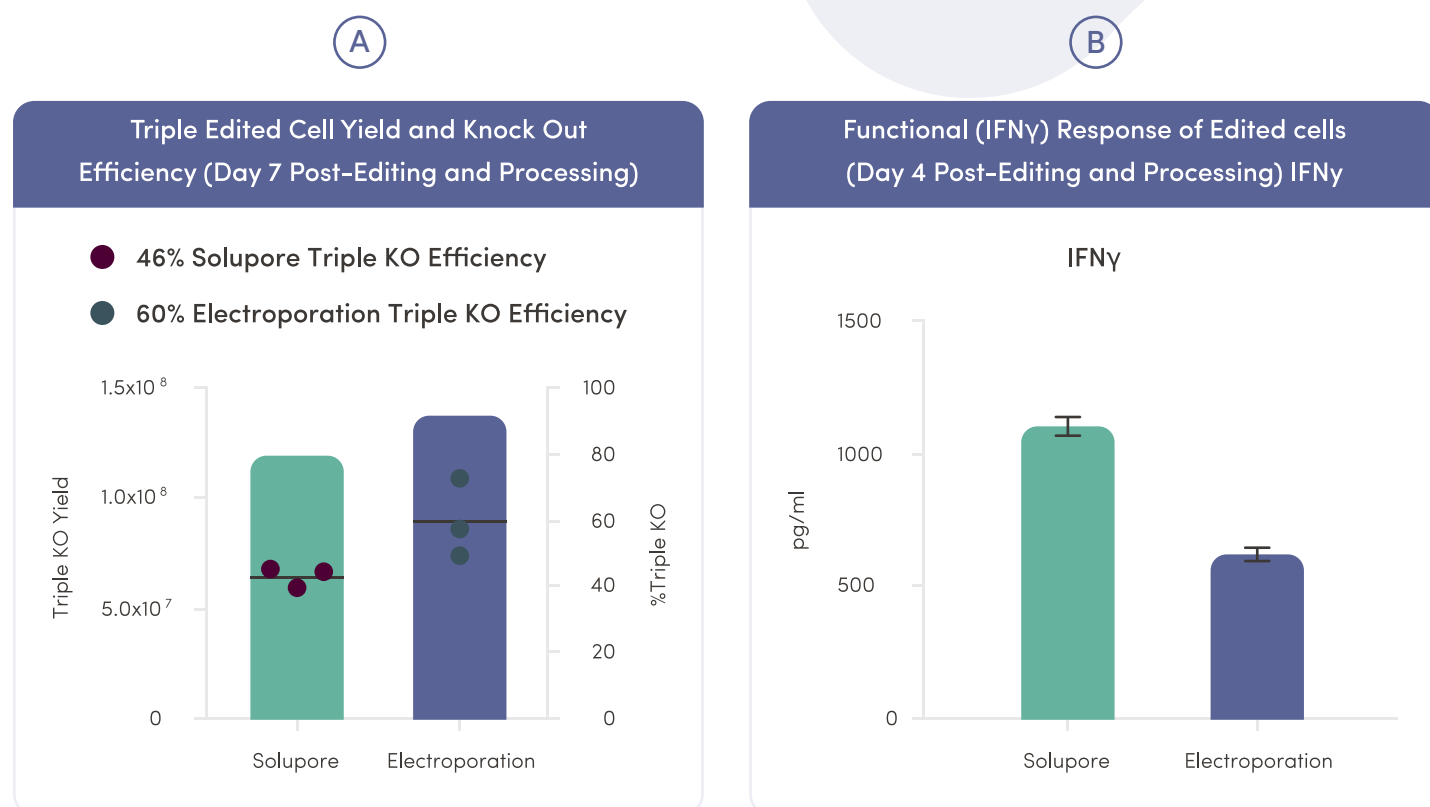
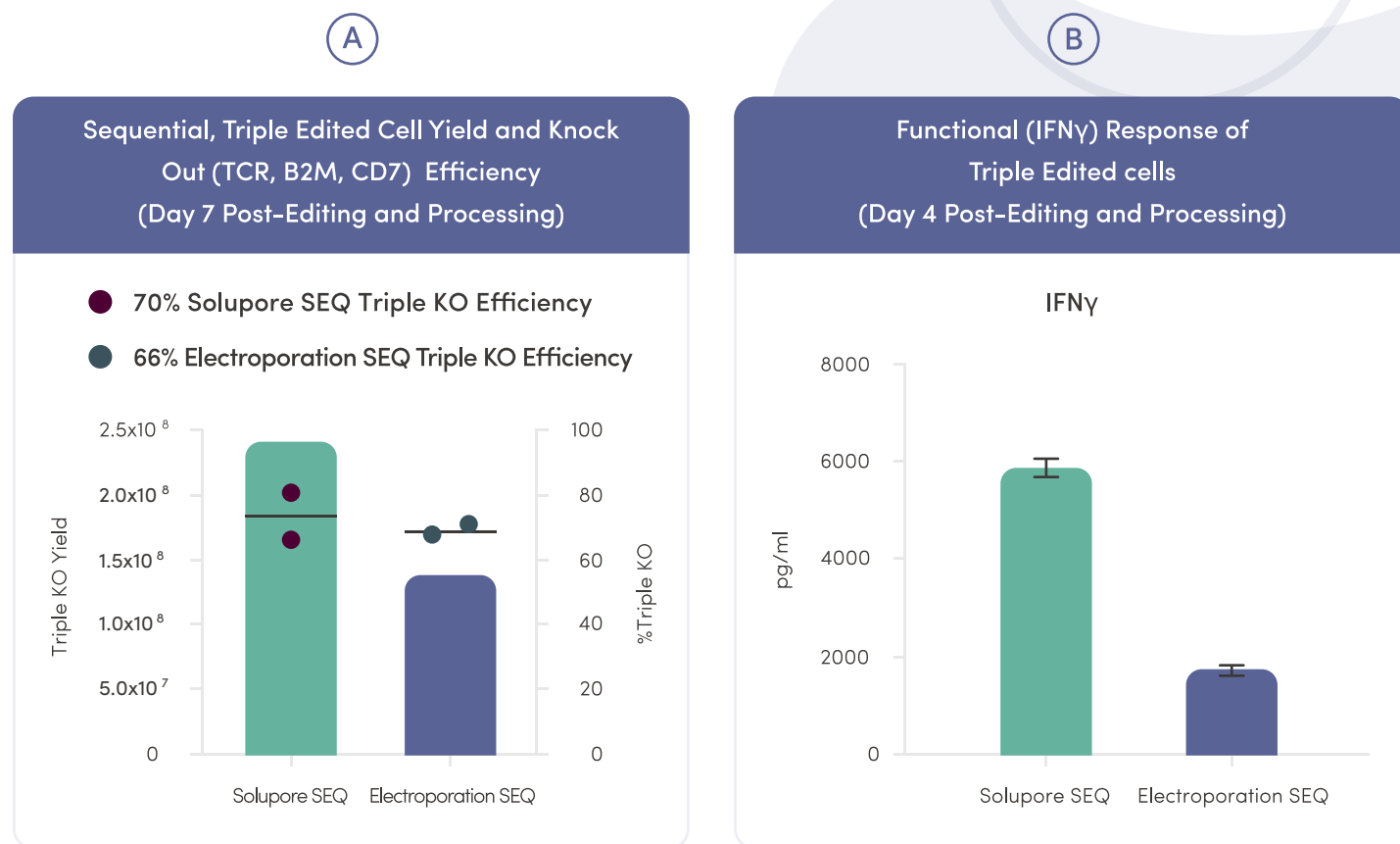


Figure 3: A) Triple knockout efficiency and knockout cell yield following RNP delivery using Solupore or electroporation. B) IFN γ response. (n = 3 donors)

A second round of editing was performed twenty-four hours after the initial round, demonstrating the flexibility of Solupore. The overall number of viable triple knockout cells was higher in the Solupore system while comparable levels of triple knockout efficiency were retained (70% for Solupore, 66% for electroporation) (Figure 4A). The superior cell health led to improved cell function as shown by IFN γ responses of 5922 pg/mL for Solupore compared to 1916 pg/mL for electroporated cells (Figure 4B).



2 donors Used, CD3 isolated, Cargo 3 μ g Per M cells

Figure 4: A) Triple knockout efficiency and knockout cell yield following two rounds of RNP delivery using Solupore or electroporation as well as the resulting B) IFN γ response. (n = 2 donors) SEQ = Sequential

Superior Cell Health and Phenotype

Gene editing can trigger apoptosis, a form of programmed cell death, due to DNA damage from double-stranded breaks required for gene knockout. Furthermore, apoptosis can be exacerbated during gene editing tool delivery due to harshness of the delivery method to further reduce the overall health and yield of the edited cell population. Solupore multiplex edited cells showed reduced signs of apoptosis compared to electroporated cells (Figure 5A), indicative of improved cell health.

Stem cell memory T cells (T_{SCM}) are a desirable sub-population for T cell therapies as they promote sustained proliferation and persistence following treatment. The population of stem cell memory T cells was maintained with the Solupore process (Figure 5B). This observation was maintained after two rounds of sequential editing (not shown).

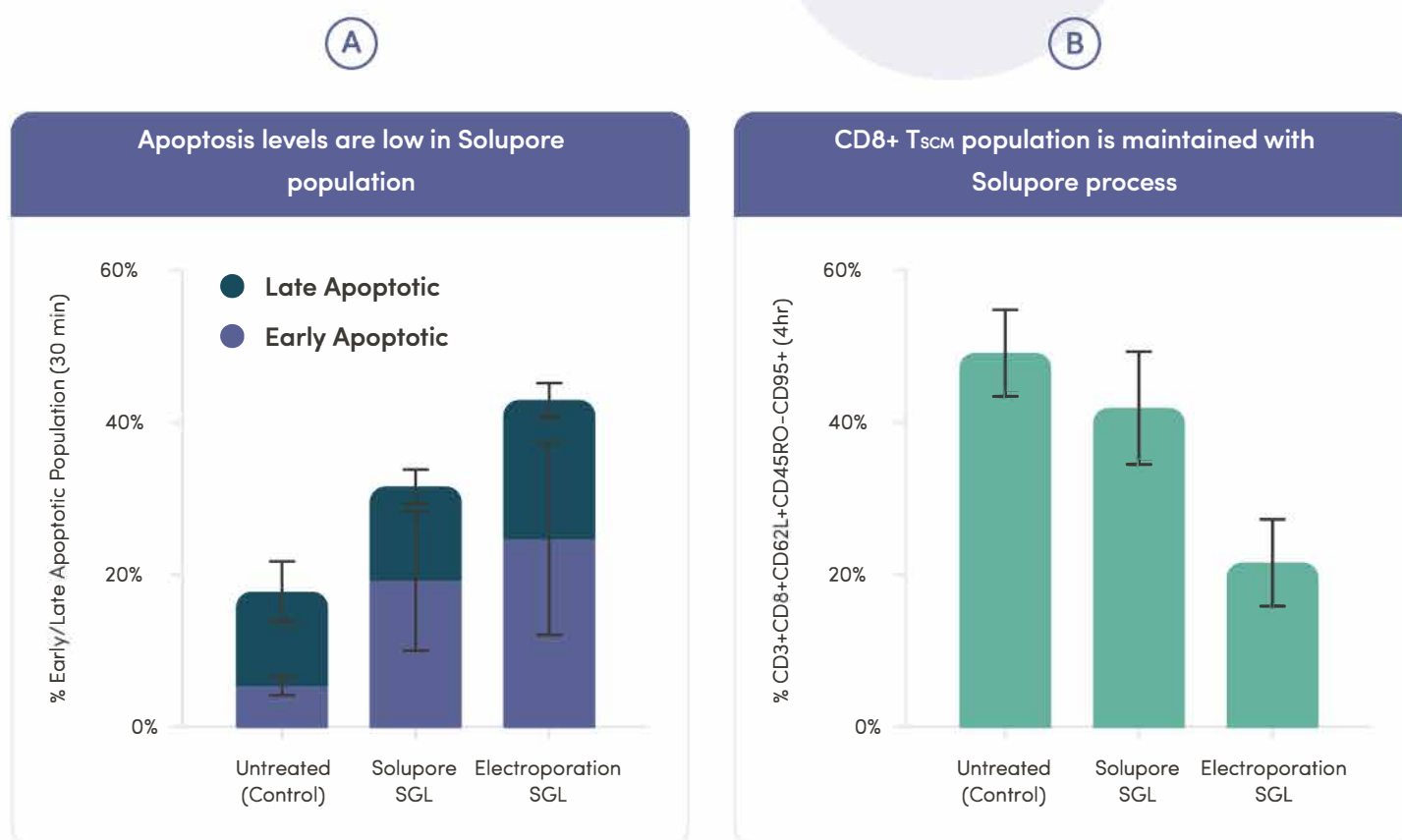
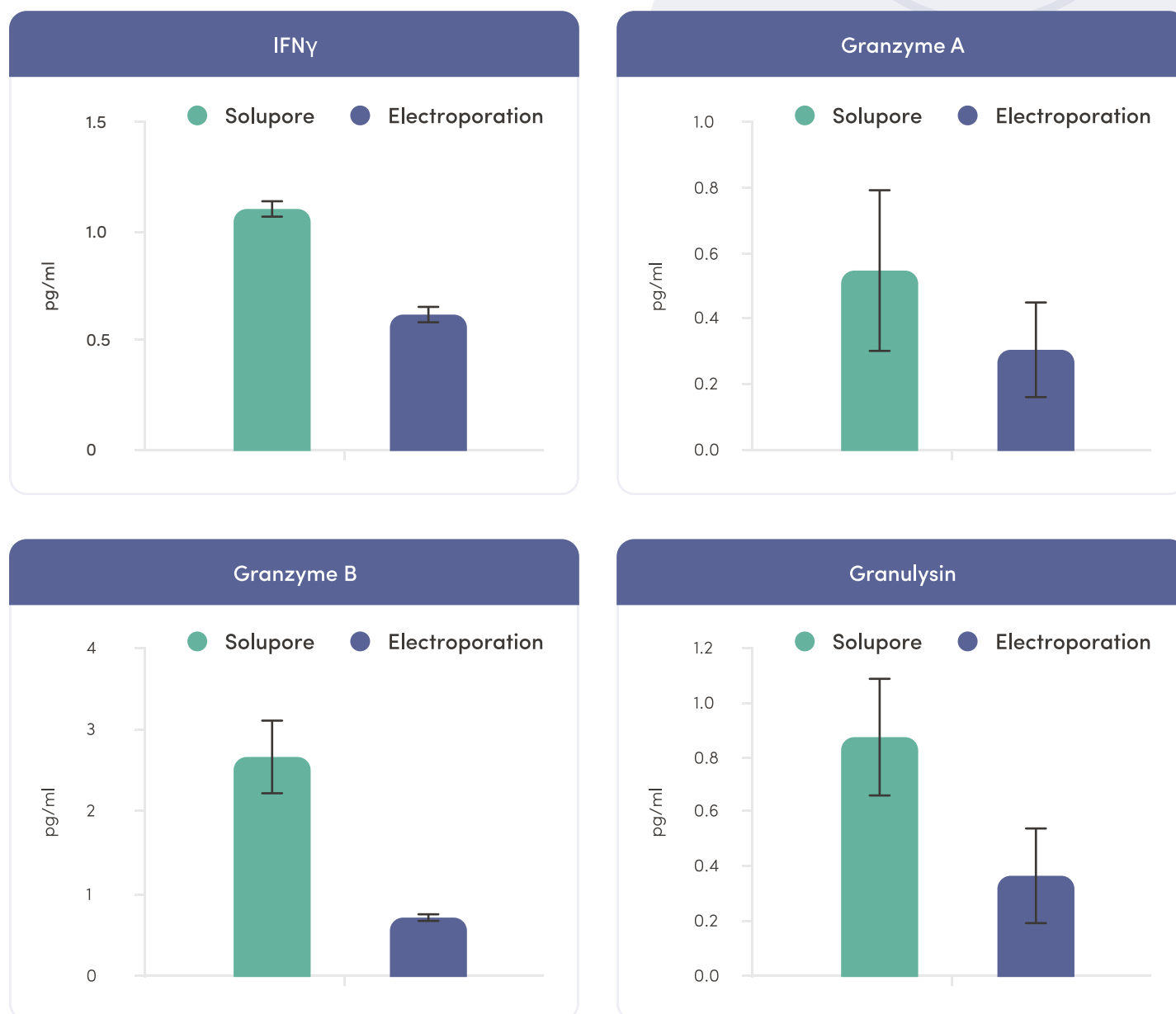


Figure 5: A) percentage of early and late apoptotic markers across untreated and test cells B) Percentage of T_{SCM} cells for each group. T_{SCM} populations defined as CD3(+), CD8(+), CD62L(+), CD45RO(-), CD95(+). Primary T cells were multiplex-edited using 3 KO RNP (TRAC, B2M, CD7) with Solupore or electroporation (n = 3 donors)

Enhanced Cell Function

Several *in vitro* assays were leveraged to characterize T cell function. Three days post triple knockout editing, edited cells were stimulated with TransACT™(CD3/CD28 stimulation), and then tested for levels of secreted IFN γ , Granzyme A, Granzyme B, and Granulysin. Solupore edited CD8+ T cells were highly functional, as exhibited by the higher levels of secreted factors compared to electroporated cells.



Post process CD8+ T cell Activation

Figure 6: Factor secretion (IFN γ , Granzyme A, Granzyme B, and Granulysin) for CD8+ T cells edited with Solupore and electroporation systems (n = 2 donors).

Conclusion

The Solupore platform can deliver complex cargo while maintaining cell health, phenotype, and activity (*in vitro* and *in vivo*). This most practical delivery approach, in which normal cell health and function can be retained while delivering complex cargoes, could be a game-changer for the industry.

Method Summary

Cell Isolation

Primary human CD3⁺ T cells from healthy donors were isolated from LeukoPaks (BioIVT, US) using positive selection for CD4 and CD8 T cells (Miltenyi Straight from Leukopak CD4 or CD8 MicroBead Kit). CD3⁺ T cells were cultured in TexMACS™ media and TransAct™ (Miltenyi, Germany) supplemented with 120 IU/mL IL-2 (CellGenix) and 5% Human AB (Valley Biomedical, US) for 3 days prior to Solupore transfection.

A split stream strategy was employed to account for donor-to-donor variability when comparing the Solupore and electroporation system.

Cell Editing

Briefly, CRISPR-Cas ribonucleoproteins (RNPs) were prepared for gene knockout of TCR, B2M, and CD7. Cas9 RNPs were prepared by adding each sgRNA (100 μ M; TRAC – AGAGTCTCTCAGCTGGTACA; CD7 – GGAGCAGGTGATGTTGACGG; B2M – GGCCGAGATGTCTCGCTCCG) to Cas9 nuclease (Alt-R S.p. HiFi Cas9; IDT) at a 1:2 molar ratio and incubating at room temperature for 10 min.

Closed, single-use transfection chamber

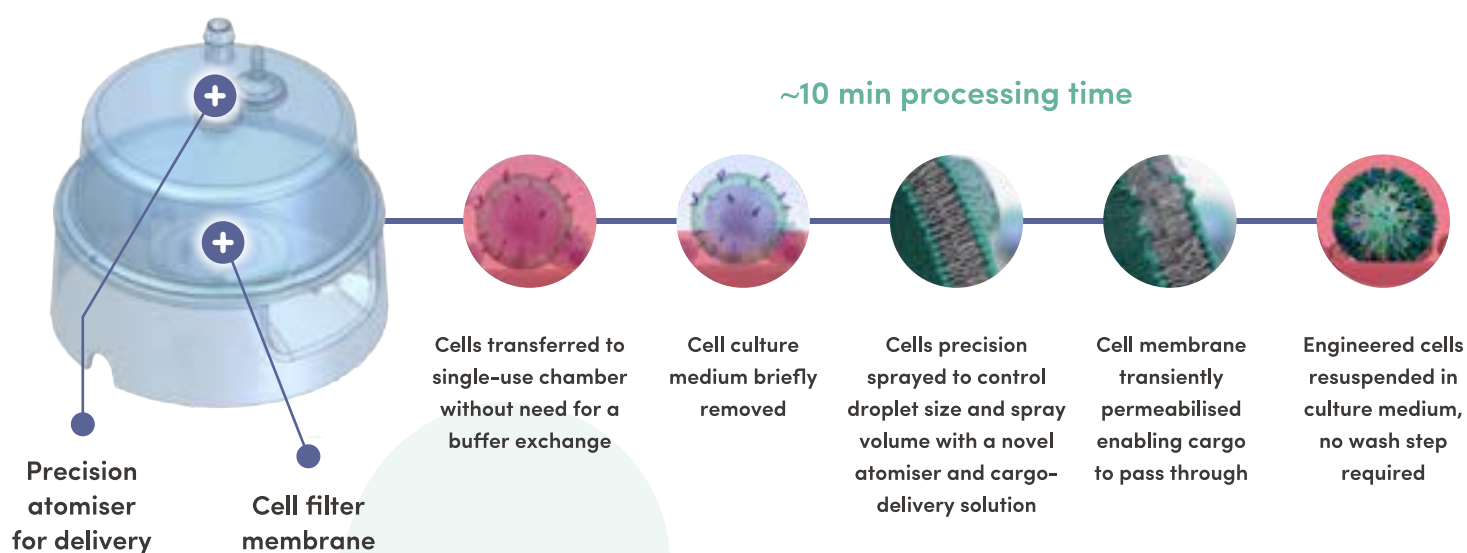


Figure 7: Solupore offers a physiochemical transfection process that is rapid and gentle on cells.

A summary workflow of the Solupore transfection process is provided in Figure 7. For each Solupore transfection, 20×10^6 CD3+ T cells were prepared in basal media, transferred to a cell transfer bag and sterile welded to the Solupore single-use assembly (SUA). Solupore Delivery Solution (DS) was prepared with addition of 9.07 μL of each RNP per 50 μL final spray volume (3 μg of each RNP cargo / 1×10^6 cells) per Solupore transfection. The SUA is loaded onto the Solupore multi-use controller (MUC), all required sterile connections are made, and the Solupore process recipe selected and initiated. Using an atomiser designed by Avectas, the target CD3+ cells are precision sprayed with the suspension of DS containing the cargo. The cargo is transferred across the cell membrane and into the target cells. After transfection, fresh cell culture medium (room temperature) is then pumped into the SUA to resuspend and recover the cells. The engineered cells were automatically harvested from the SUA chamber into a transfer bag for further downstream processing and cultured in T75 and/or G-Rex® (24 well) at concentration 0.5×10^6 /mL depending on the downstream assay requirements.

The Nucleofector® 4D (Lonza) was used to electroporate cells. For each sample, 15×10^6 cells were centrifuged at $350 \times g$ for 5 min and the supernatant removed. The cells were resuspended in 600 μL of PBS and 200 μL was added to 3 x 1.5 mL tubes. The cells were centrifuged at $200 \times g$ for 5 min. The PBS was removed, and the cells resuspended in 90 μL P3 Buffer (Lonza) and added to a 1.5 mL tube containing 3.36 μL of each RNP (10.08 μL total). The cells were mixed gently with the cargo and transferred to a 100 μL cuvette and electroporated using code EO115. For every 1 million cells, 3 μg of each RNP cargo was delivered. Immediately post-transfection ~200 μL of pre-warmed media was added to each cuvette and the cells from the 3 cuvettes were transferred to a single T25 flask containing 15 mL pre-warmed media and/or G-Rex® (24 well) at concentration 0.5×10^6 /mL depending on the downstream assay requirements.

For sequential editing, cells were transfected or electroporated a second time 24 hours later following the same procedure.

Analysis

Cell number and viability were analysed using a NC-200™ NucleoCounter® (Chemometec). Apoptosis was assessed using Annexin-V assay on a NC-3000™ NucleoCounter® (Chemometec). Cell expansion was conducted in G-Rex® (24 well) platform in TexMACS™ media and TransAct™ (Miltenyi, Germany). Cell media was refreshed with TransAct™ and IL-2 on day 3 and 5 post transfection or electroporation. For cytokine analysis supernatants were harvested day 4 post transfection and analysis conducted using a bead-based immunoassay to quantify multiple soluble analytes (BioLegend's LEGENDplex™). Target protein expression and T cell memory phenotyping (CD3 (TRAC), CD7, HLA (B2M), CD4, CD8, CD45RO, CD62L, CD95, Zombie Aqua for viability), were analyzed by flow cytometry using a NovoCyte 3000.