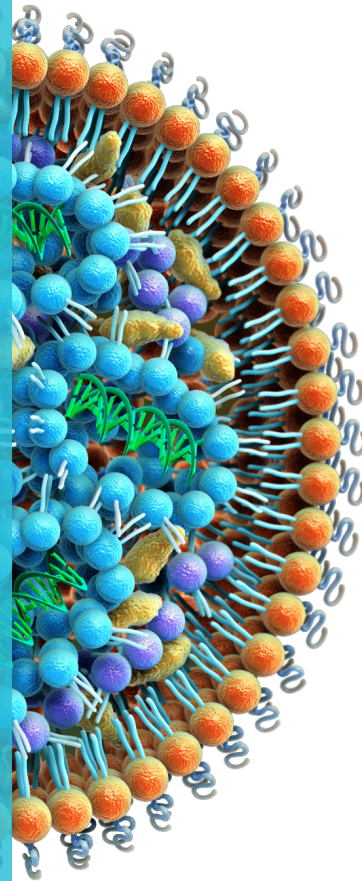




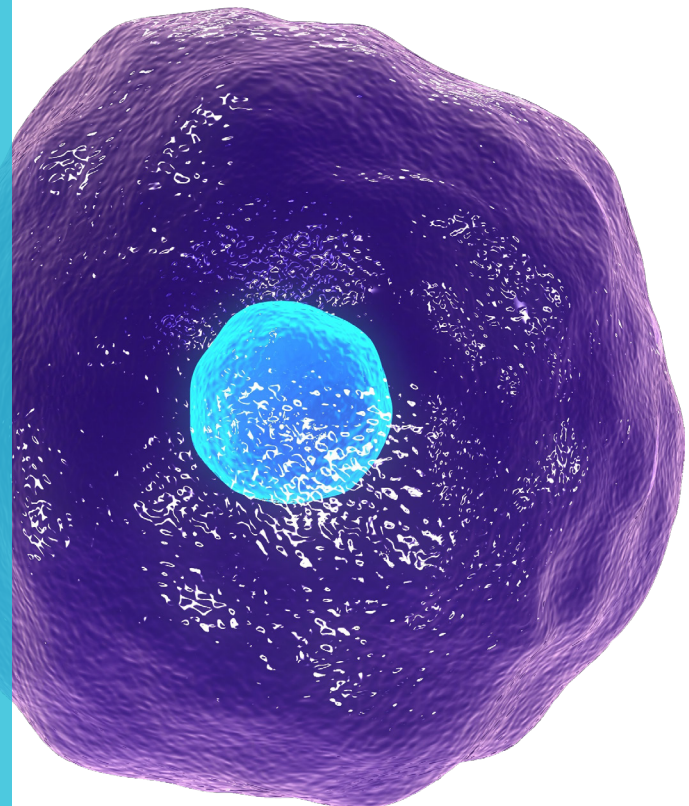
**PRECISION
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Genome Editing of Human Primary T Cells with Lipid Nanoparticles



Gene-edited CAR T cells
for next-generation cell
therapies



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Introduction

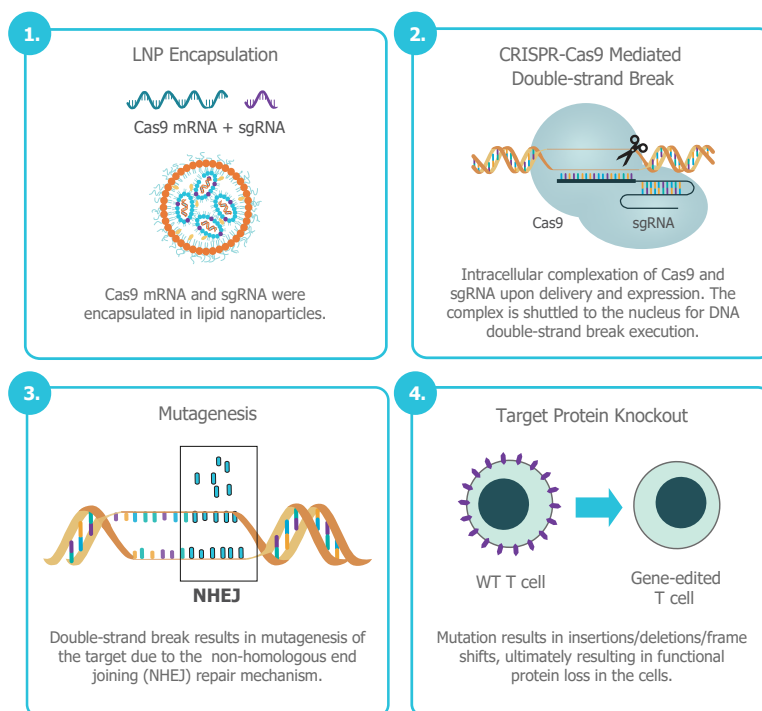
The expression of the chimeric antigen receptor (CAR) on T cells turns a patient's cells into cell-based cancer therapies and has revolutionized cancer treatment today¹. Despite its successes and high response rates, evidence suggests an increasing need for more complex genetic engineering enabled by CRISPR/Cas-mediated genome editing technologies. Such examples include the disruption of inhibitory pathways exploited by the tumor microenvironment^{2, 3}, improvement of CAR T cell efficiency^{4, 5}, and manufacturing of universal CAR T cells from allogeneic donors^{6, 7}. The desire to achieve both gene editing and transgene expression in next-generation T cell therapies emphasizes the significance of the genetic material delivery method, which plays a critical role in cell function, cell yield, ease of production, and scale-up.

A promising new approach for T cell engineering is the use of RNA to express therapeutic proteins and gene editing nucleases. RNA is typically delivered to cells using electroporation; however, the sequential electrical pulses for multi-step gene engineering leads to a dramatic trade-off between efficiency and cell viability. This type of trade-off is not observed with lipid nanoparticles (LNPs), making it an attractive alternative for RNA delivery. LNPs are entirely synthetic lipid formulations designed to encapsulate and protect RNA before delivering it into cells. The production of LNPs is well-established and is scalable for large-scale gene delivery and gene editing, which are key to meeting clinical demand now and in the future. The RNA-LNP complex structurally resembles low density lipoproteins (LDL) and can co-opt the endogenous uptake pathway of LDL to enter cells using receptor-mediated endocytosis. This gentle uptake mechanism enables successful genome engineering of T cells while maintaining high cell viability.

Herein, we report a novel method for sequential genetic engineering of T cells using the GenVoy-ILM™ T Cell Kit for mRNA. We utilized a manufacturing workflow optimized to deliver various RNA cargoes (**Figure 1**). In this case study, we show CRISPR/Cas9-mediated knockouts (KO) of the T cell receptor (TCR $\alpha\beta$) and explore multi-step LNP engineering to produce TCR $\alpha\beta$ KO CAR T cells, a promising approach towards allogeneic CAR T cell therapy^{6, 8, 9}. We describe in detail LNP production and cell culture treatment protocols, as well as optimization strategies for T cell gene editing to ensure success with the GenVoy-ILM T Cell Kit for mRNA.

Figure 1. Gene editing with lipid nanoparticles in human primary T cells.

Gene knockouts are achieved through the delivery of Cas9 mRNA and synthetic single guide RNA (sgRNA). Following delivery and translation, the Cas9 protein and sgRNA complex intracellularly and are shuttled to the nucleus to execute a double-strand break of the target gene. Without a DNA donor template, the vast majority of the breaks are combined through the mutation-prone non-homologous end joining (NHEJ), yielding permanent target protein knockout.



Background

Gene editing in primary T cells can be achieved through a number of enzymes, including transcription activator–like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) Cas9. CRISPR/Cas9 is becoming increasingly preferred due to the ease of target selection and optimization^{10, 11}. In CRISPR/Cas9-mediated gene editing, the Cas9 protein is directed to the target DNA by the guide RNA, where it induces double strand breaks that are mostly resolved through non-homologous end joining (NHEJ). The RNA-guided nature of CRISPR/Cas9 system enables flexibility of target selection and multiplexed gene editing, creating tremendous potential in CAR T cell therapy.

Traditionally, viral vectors are used for T cell engineering, but they pose several limitations, including limited cargo capacity, adverse immunological reactions, and high manufacturing costs¹². To circumvent the downsides of viral vectors, electroporation has been increasingly utilized^{10, 13-15}; however, as mentioned above the sequential electric pulses required to achieve both gene editing and protein expression can be detrimental to cells. This can cause low cell viabilities and yield, create dysregulation of genes¹⁶, and increased cell exhaustion marker expression¹⁷.

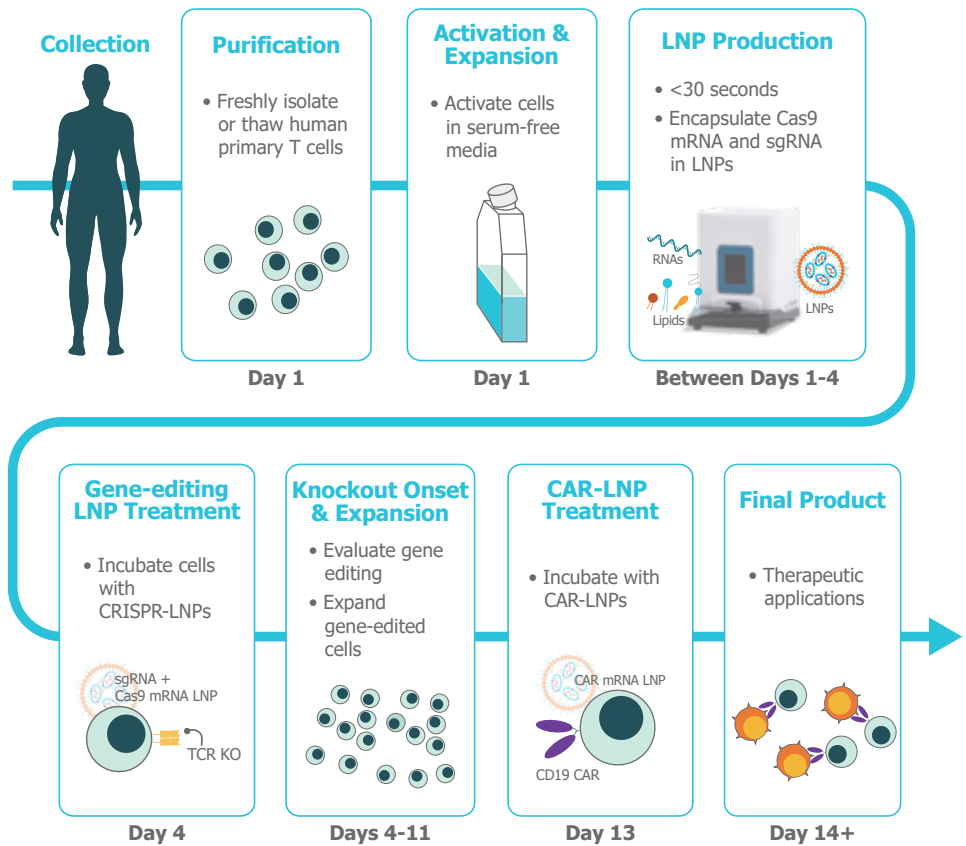
Although the number of works utilizing LNPs is limited, two recent studies by Billingsley et al. show promising results with LNP-mediated expression of CD19 CAR mRNA in primary T cells, which demonstrated equivalent tumor killing potency compared to electroporation and/or lentiviral transfection^{12, 18}.

The [GenVoy-ILM™ T Cell Kit for mRNA](#) offers a clinically-relevant and scalable method^{21, 22} for genome editing using LNPs to advance the field of cell therapies, while leveraging the powerful technology that has revolutionized COVID-19 mRNA vaccine development efforts. This work aims to exemplify the power of LNPs, for both therapeutic protein expression and advanced T cell engineering. LNPs encapsulating commercially-available Cas9 mRNA and single-guide RNA (sgRNA) were optimized for packaging, cargo ratios, and the combination of multiple guides. In addition, we show the seamless integration of LNPs into a standard primary T cell culture workflow and demonstrate how multiple LNP additions enable production of gene-edited CAR T cells. These discovery-scale RNA-LNPs were produced in less than 5 minutes using the [NanoAssmblr® Spark™](#) microfluidic platform and added directly to the cells.

This work highlights that LNPs produced with GenVoy-ILM T Cell Kit for mRNA are highly efficient at knocking out targets ($80 \pm 8\%$ with two sgRNAs) and expressing CAR protein ($91 \pm 5\%$), all the while maintaining high cell viability ($>90\%$). The resulting gene-edited CAR T cells were co-cultured with leukemia cells and showed highly efficient target-specific killing, whereas gene editing itself had no negative impact on the therapeutic potential.

Figure 2. Primary T cell gene editing and multi-step engineering workflow.

Day 1 – Cryopreserved primary human pan T cells were thawed and activated. Days 1-4 – LNPs were produced with RNA encapsulation efficiency assessed to determine dosing. Day 4 – Activated T cells were incubated with the gene editing RNA-LNPs. Days 4-11 – Cells were expanded to increase cell numbers for subsequent experiments. Day 13 – Gene-edited cells were treated with CAR mRNA-LNPs to express a therapeutic anti-CD19 CAR protein. Day 14 – Approximately 24 hours post-CAR treatment, cytotoxicity assays against the tumour target were conducted.



Materials & Methods

GenVoy-ILM T Cell Kit for mRNA with Spark Cartridges	Precision NanoSystems, 1000683
mRNA, Cas9 and CD19-CAR	CleanCap® Cas9 mRNA (wt), TriLink Biotechnologies, L-7606, in 1 mM sodium citrate, pH 6.4 CleanCap® CD19 CAR mRNA (wt), TriLink Biotechnologies, custom product, in 1 mM sodium citrate, pH 6.4
sgRNAs	Alt-R® CRISPR-Cas9 sgRNA, Integrated DNA Technologies, custom targets, in RNase free water
RNA quantification	Quant-iT™ RiboGreen® RNA Assay Kit, incl. 20X TE Buffer, RNase-free, Thermo Fisher Scientific, R11490
Primary T cells	Human Peripheral Blood Pan T Cells, Frozen, STEMCELL Technologies Inc., 70024
T cell activator	ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator, STEMCELL Technologies Inc., 10970
T cell media	ImmunoCult™-XF T Cell Expansion Medium, STEMCELL Technologies Inc., 10981
T cell media supplement	Recombinant IL-2, STEMCELL Technologies Inc., 78036
CD19+ Target Cells	SUP-B15 CRL-1929™, ATCC
B cell and co-culture media	Gibco™ RPMI 1640, ThermoFisher Scientific, 11875093
B cell and co-culture media supplements	Gibco™ 100X GlutaMax™ Supplement, 35050061 100 mM Gibco™ Sodium Pyruvate 11360070 Gibco™ MEM Non-Essential Amino Acids Solution 11140050 Fetal bovine serum, A3840302 (all ThermoFisher Scientific)

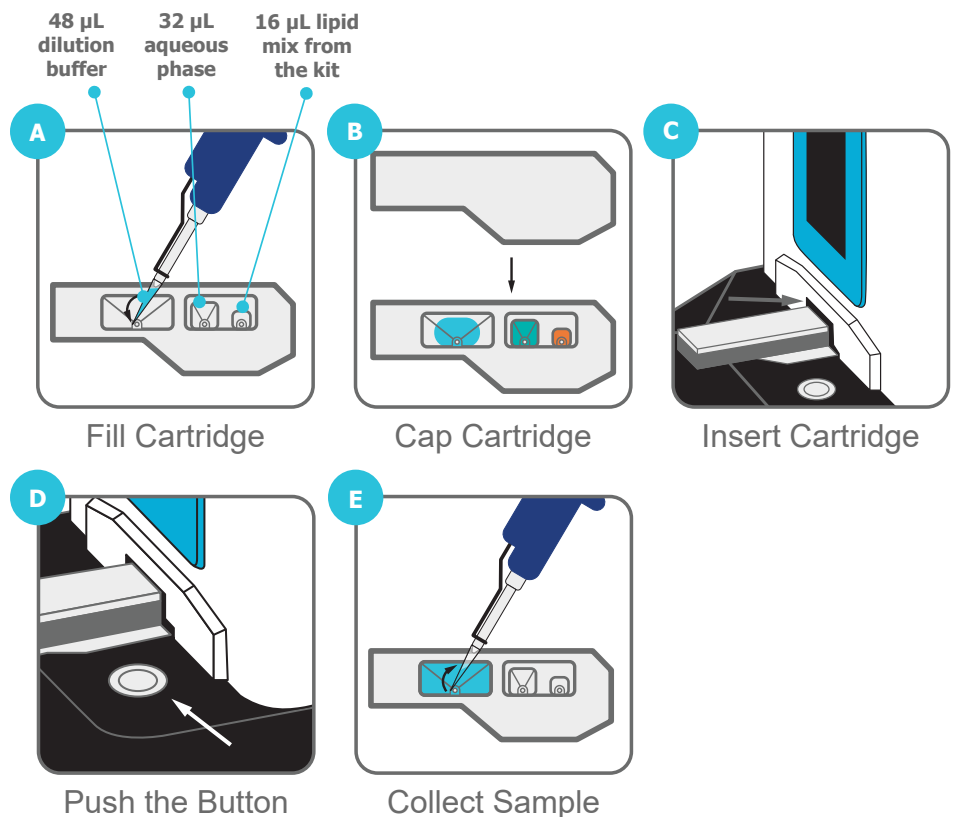
TCR negative selection	EasySep™ Human TCR Alpha/Beta Depletion Kit, STEMCELL Technologies Inc., 17847
Flow cytometry stains/antibodies	Anti-TCR α/β, clone IP26, BioLegend Inc., 306712 Anti-CD3, clone HIT3a BioLegend, Inc. 300308 Biotinylated CD19 CAR Detection Reagent, Miltenyi Biotec, 130-115-965 Anti-Biotin-APC Miltenyi Biotec, 130-110-952 Anti-CD8, BioLegend Inc., 300936 FVS660, BD Bioscience, 564405 Violet Proliferation Dye 450, BD Biosciences, 562158 FVS575V, BD Biosciences, 565694
Stain Buffer	BSA, BD Biosciences, 554657
T cells treatment plates	Costar® 48-well Clear TC-treated Multiple Well Plates, Corning Inc., 3548
Co-culture treatment plates	Falcon™ 96-Well, Non-Treated, U-Shaped-Bottom Microplate, Fisher scientific, 0877254

Equipment

NanoAssemblr Spark	Precision NanoSystems, NIS0003
Fluorescence plate reader	BioTek™ Synergy™ H1, or similar
Flow cytometer	CytoFLEX™ V4-B2-Y4-R3, Beckman Coulter, C09766

Figure 3. Workflow for producing lipid nanoparticles using the NanoAssemblr Spark.

A) Buffer, aqueous phase and lipid solutions are pipetted into the wells of the Spark microfluidic cartridge as follows: (1): 48 μL Formulation Buffer 2, (2): 32 μL RNA-aqueous phase, (3): 16 μL lipid mix. B) The cartridge cap is fitted. C) The cartridge is inserted into the Spark instrument. Setting 3 is selected via the touch screen interface. D) The "Start" button is pressed to begin mixing. Mixing takes ~3 seconds. E) The resulting RNA-LNP suspension is pipetted out of the collection well and mixed with 96 μL of Formulation Buffer 2 in a microcentrifuge tube.



Gene Targets

Target	DNA target sequence (5' to 3')
TCR sgRNA 1	CTCTCAGCTGGTACACGGCA ²³
TCR sgRNA 2	GAGAATCAAAATCGGTGAAT ²⁴
Neg. sgRNA	GTTCCGCGTTACATAACTTA ²⁵

A. LNP Preparation

1. LNPs were generated through microfluidic nanoprecipitation, using the NanoAssemblr Spark instrument, according to the instructions in the User Guide (NIS1024). The organic phase consisted of GenVoy-ILM T Cell Kit for mRNA and the aqueous phase encapsulating a total of 10 µg RNA per LNP formulation. Overview of LNP production is shown in **Figure 3** and is referenced throughout this section. The LNPs were prepared within a biosafety cabinet to maintain sterility.
2. The aqueous phases were prepared by mixing the RNAs with the provided Formulation Buffer 1 (10X) and molecular grade water. The aqueous phase should be kept on ice at all times.
 - For gene editing applications, the aqueous phase consisted of 5 µg Cas9 mRNA and 5 µg sgRNA. An example calculation of the aqueous phase is shown in **Table 1** where an extra 10% was added to account for pipetting errors.

Table 1. Gene editing aqueous phase preparation from 1 mg/mL mRNA and 100 mM sgRNA stocks*.

# of sgRNAs	Water (µL)	Formulation Buffer 1 (10X) (µL), 10X stock	sgRNA 1 (µL), 100 µM stock	sgRNA 2 (µL), 100 µM stock	Cas9 mRNA (µL), 1 mg/mL stock
1	24.5	3.52	1.72	---	5.5
2	24.5	3.52	0.86	0.86	5.5

*Note: Volumes listed below include an extra 10% to account for pipetting errors.

- For gene delivery applications, the aqueous phase consisted of 10 µg CAR mRNA. Example calculation of the aqueous phase is shown in **Table 2** where an extra 10% was added to account for pipetting errors.

Table 2. CAR mRNA aqueous solution preparation from a 1 mg/mL mRNA stock*.

Number of samples	Water (µL)	Formulation Buffer 1 (10X) (µL), 10X stock	CAR mRNA (µL), 1 mg/mL stock
1	20.68	3.52	11.00
2	41.36	7.04	22.00

*Note: Volumes listed below include an extra 10% to account for pipetting errors.

3. The particles were prepared by pipetting 48 µL of Formulation Buffer 2, 32 µL (of the above prepared) RNA-aqueous phase, and 16 µL GenVoy-ILM T Cell for mRNA kit lipid mix into the microfluidic cartridge (**Figure 3A**), in this order.
4. The cartridge was capped (**Figure 3B**), inserted into the NanoAssemblr Spark instrument (**Figure 3C**) and button pressed to produce the LNPs (**Figure 3D**).
5. Once the production is complete (in a few seconds), the LNPs were collected by pipetting out from the largest reservoir (**Figure 3E**). Formulated RNA-LNPs were refrigerated until further use.

B. LNPs characterization

1. RNA concentration was measured using the RiboGreen® RNA reagent. This allows for the calculation of the applied RNA dose on the T cells.
2. For RNA quantification, detailed steps and accompanying calculation sheet are provided in the GenVoy-ILM T Cell Kit for mRNA User Guide (1000684).

Optional step: LNPs may be characterized through dynamic light scattering (DLS), with an expected size of 60-90 nm.

C. T cell culture

1. Cryopreserved primary human pan T cells ($\sim 2 \times 10^7$) were thawed and washed twice with ImmunoCult™ XF T-cell Expansion medium by centrifugation at $300 \times g$ for 10 minutes.
2. Cells were diluted to 1×10^6 cells/mL with $0.1 \mu\text{g/mL}$ IL-2 supplemented ImmunoCult-XF T Cell Expansion Medium (complete T cell expansion medium) then immediately activated with $25 \mu\text{L}/10^6$ cells of ImmunoCult Human CD3/CD28/CD2 T Cell Activator.
3. Cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for three days prior to treatment.

D. LNP treatment

1. The recommended timepoint for RNA-LNP treatment is around 72 hours post activation (Day 4).
To ensure success, it is recommended to perform flow cytometric detection of either CD25+ (expected 60 to 80%) or CD25+ LDLR+ (>60%) cell populations prior to RNA-LNP treatment.
2. Activated T cells were diluted to 0.5×10^6 cells/mL in complete T cell expansion medium supplemented with $1 \mu\text{g/mL}$ ApoE.
3. $250 \mu\text{L}$ of the diluted T cells were seeded on 48-well tissue culture plates (0.125×10^6 cells per well).
4. In these experiments (both for gene knockout and subsequent CAR expression), RNA-LNPs were dosed at $3.2 \mu\text{g RNA}/10^6$ cells ($\sim 10 \mu\text{L RNA-LNPs}$). An optimal treatment dose is likely to fall between $1\text{-}4 \mu\text{g}/10^6$ cells, but will depend on the design, size and quality of the RNA. Titration is recommended for optimal dose identification.
5. The treatment plates were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for up to 96 hours.

Optional step: for increased cell yield, centrifuge the plate at $300 \times g$ for 10 minutes at room temperature after 24 hours of LNP treatment then discard the supernatant. Resuspend the pellet in $250 \mu\text{L}$ complete T cell media with triple activator ($25 \mu\text{L}/10^6$ cells).

E. TCR knock out analysis by flow cytometry

The biological implications of the LNP treatment and the culture conditions on the selected target must be considered for proper detection by surface staining. For optimal cell surface TCR/CD3 detection, soluble activator was removed at least 24 hours prior to flow cytometry to reduce antibody blocking. Activator removal is not a pre-requisite for targets whose surface expression levels are unaffected by the presence or absence of the soluble activator.

1. Soluble activator was removed 96 hours post Cas9/sgRNA-LNP addition (or day 7 after thaw) by centrifugation. Cells were diluted to 0.5×10^6 cells/mL maintained at $37^\circ\text{C}/5\% \text{CO}_2$ for 3 days.

2. TCR knockout was assessed on day 7 post-treatment (day 11 after thaw).
3. Cells were first stained for live/dead discrimination, then subsequently co-stained for TCR α/β and CD3 surface expression.
4. Data was acquired on CytoFLEX then analyzed using FlowJo™ V10.7.

F. Multi-step engineering with CAR and expression analysis by flow cytometry

1. Upon knockout determination cells were purified using TCR negative selection by following manufacturer's instructions and re-activated to prime for CAR-LNP treatment. For this, cells were diluted to 0.5×10^6 cells/mL in complete T cell expansion medium with triple activator ($25 \mu\text{L}/10^6$ cells) and cultured at $37^\circ\text{C}/5\% \text{CO}_2$ for 2 days.
2. After 2 days of re-activation (day 9 post treatment), cells were prepared for secondary LNP treatment as follows: diluted to 0.5×10^6 cells/mL in complete T cell expansion medium containing $1 \mu\text{g}/\text{mL}$ of ApoE.
3. CAR mRNA-LNP treatment was carried out in T-25 flasks where the LNPs were added at $3.2 \mu\text{g RNA}/10^6$ cells. Upon addition, flasks were gently tilted to evenly disperse the particles, then incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 24 hours.
4. CAR transfection efficiency was assessed after the incubation period using flow cytometry. Cells were stained for live/dead discrimination then subsequently stained for the surface expression of anti-CD19 CAR. Data was acquired on CytoFLEX and then analyzed using FlowJo V10.7.

G. Maintenance of target cells for the cytotoxic T cell assay

1. Cryopreserved target cells SUP-B15 were thawed and maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1 mM Sodium Pyruvate, 1x MEM and 1x GlutaMax (R+FBS media) for at least 7 days after thaw and used for up to 12 passages.
2. On the day of T cell cytotoxicity evaluation, target cells were metabolically labelled with violet proliferation dye 450 (VPD450) and the dye uptake was confirmed by flow cytometry.
3. Target cells were maintained at $37^\circ\text{C}/5\% \text{CO}_2$ until co-culture treatment.

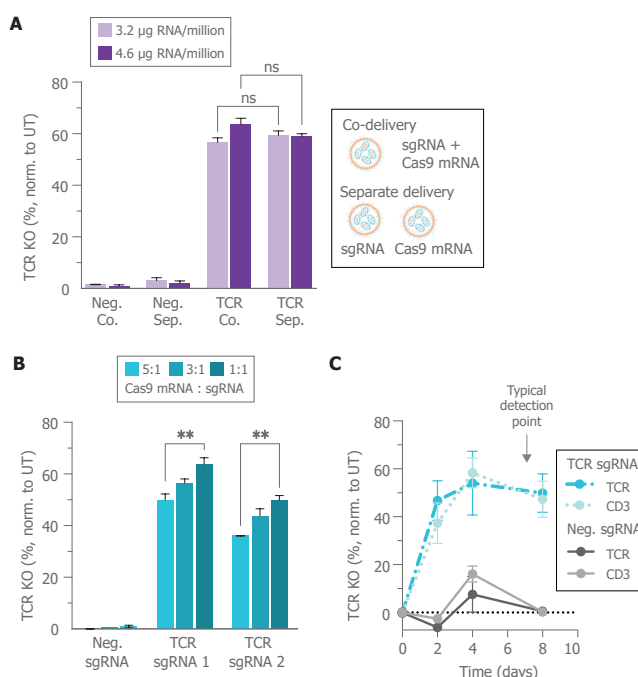
H. Tumor-specific T cell cytotoxicity assay

1. CAR⁺ T cells (effector) and untreated (UT) controls were washed and suspended in R+FBS media as described above.
2. The co-culture experiment was performed in triplicates in 96-well round bottom plates at the following effector to target ratios: 0:1, 1:1, and 2:1. Target cell number in each well was fixed at 1×10^4 cells. Final volume per well was 200 μL .
3. Cells were co-cultured overnight (16 to 18 hours) at $37^\circ\text{C}/5\% \text{CO}_2$.
4. Following incubation, cells were stained to assess viability (on all cells) and CD8 surface expression (on effector cells). Data was acquired on CytoFLEX then analyzed using FlowJo V10.7.
5. Cytotoxic potential for each group was quantified calculating specific lysis as the metric.
6. Specific lysis was determined by normalizing cell viability in anti-CD19 CAR T cell co-culture (CAR) to either untreated T cell-target cell co-culture control (UT) or target cell single culture control (Mock) according to the following formula:
$$\% \text{ SL relative to UT T cell co-culture} = 1 - (\% \text{ Viable CAR} / \% \text{ Viable UT or Mock})$$

Results & Discussion

Figure 4. Packaging strategies, weight ratios, and kinetics for optimal performance.

A) Co- vs. separate delivery of Cas9 mRNA and negative sgRNA or TCR sgRNA 1 at 3.2 or 4.6 μg total RNA per million cells. In co-delivery both the Cas9 mRNA and sgRNA were mixed in the aqueous phase in a 1:1 ratio and LNPs formed. For separate delivery, each component was encapsulated in separate LNPs, then added to the T cells in a 1:1 weight ratio. B) Cas9 mRNA and sgRNA weight ratios were varied during microfluidic encapsulation, 1:1, 3:1, and 5:1 mRNA to sgRNA. Primary T cells were treated with LNPs at an encapsulated mRNA dose of 3.2 μg /million cells. C) Kinetics of TCR knockout onset was performed with detection over the course of 8 days post RNA-LNP treatment using TCR sgRNA 1. One-way ANOVA was conducted on shown pairs, with $n\text{s}$ $P > 0.05$ and $**$ being $p \leq 0.01$.



CRISR/Cas9 Gene Editing Optimization

Gene editing was achieved using Cas9 mRNA and sgRNA, and delivered to human primary T cells with LNPs using the GenVoy-ILM T Cell Kit for mRNA. TCR was selected as a model target due to its clinical relevance. LNP formulation parameters were investigated in depth, including the method of encapsulation, Cas9 mRNA:sgRNA ratio optimization, onset of gene editing, and the use of multiple guides for a single target. The resulting LNPs were subjected to particle characterization and evaluated using in vitro cell-based assays to assess knock out efficiency.

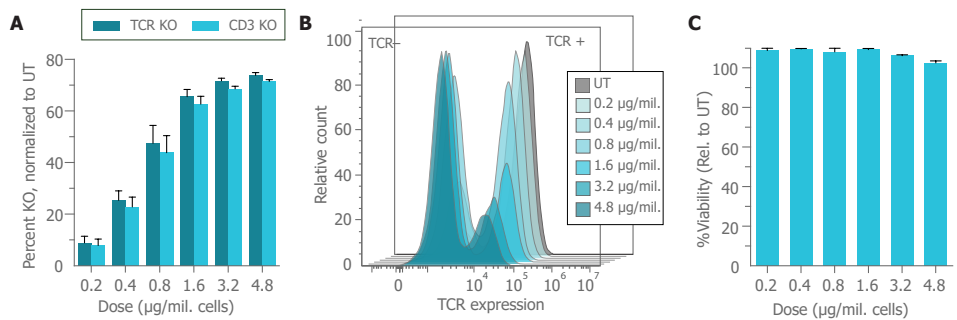
RNA encapsulation methods for Cas9 mRNA and sgRNA were investigated to determine the optimal method. We compared formulations that packaged both payloads in a single LNP (referred to as co-encapsulation or co-delivery) with formulations that separated the payloads in two different LNPs. Shown in **Figure 4A**, there were no differences observed in the knockout efficiency between the two encapsulation methods. To simplify the workflow, co-encapsulation was used in all subsequent experiments.

Next, we determined the optimal Cas9 mRNA to sgRNA weight ratio during encapsulation. Previously, LNP-mediated delivery of weight ratios 1:1 to 5:1 Cas9 mRNA to sgRNA have been successful²⁶⁻²⁹; therefore, we investigated around this range. As shown in **Figure 4B**, 1:1 Cas9 mRNA to sgRNA weight ratio was the ideal formulation parameter and used henceforth in all experiments.

TCR knockout was monitored over the course of 8 days post-LNP addition to study the kinetics of gene editing and knockout onset. We observed rapid onset of gene editing: There was a high level of knockout within 48 hours and reached highest levels at 96 hours (**Figure 4C**). The small decline in relative target knockout efficiency is attributed to the difference in the proliferative propensity of TCR⁻ and TCR⁺ cells in presence of an anti-CD3 based activator. We have not observed a similar effect when studying other targets, such as CD52 (data not shown), as this effect stems from a lower activation status of the TCR/CD3 negative cells.

Figure 5. Dose-dependent knockout efficiency and cell viabilities in a single knockout.

A) Percent TCR knockout (and subsequent CD3 surface loss) of TCR sgRNA 1+2 treated samples at the indicated 0.2–4.8 µg RNA/million cell dose. B) Surface expression of TCR as detected by flow cytometry at the indicated RNA dose. C) Percent cell viability normalized to untreated for samples treated at the indicated RNA doses.

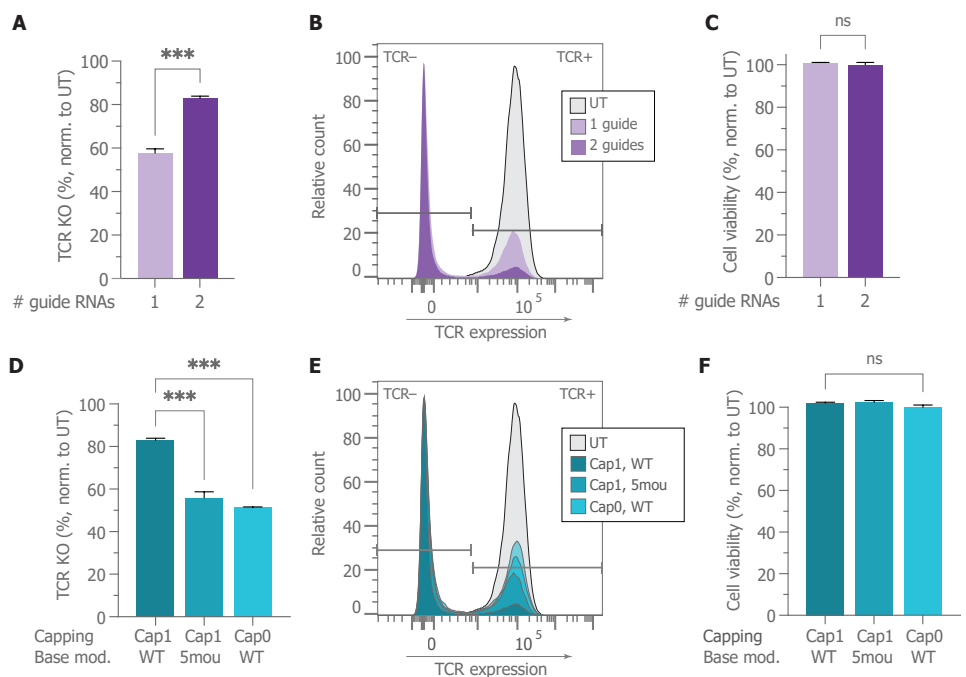


Precise dose modulation is a unique advantage of RNA delivery and translation³⁰ that allows researchers to fine-tune protein expression for optimal performance and avoid adverse effects such as off target editing or cell viability reduction. **Figures 5A and 5B** show distinct LNP dose-dependent loss of TCR surface expression. The dose-dependence of knockout efficiency was particularly notable in the 0.2 to 1.6 µg/million range, indicative of the capacity to fine-tune the knockout performance for a given target. Furthermore, we observed loss of CD3 surface expression in response to TCR knockout, at levels corresponding to the respective TCR KO efficiencies (**Figure 5A**). Cell viabilities remained high at all doses tested and was comparable to the untreated counterpart (**Figure 5C**).

Overall, delivery optimizations yielded a recommendation of encapsulating at a 1:1 weight ratio of Cas9 to sgRNA in a single LNP. Additionally, we recommend a starting treatment dose between 2-4 µg/million T cells, with subsequent detection 4 days post RNA-LNP treatment.

Figure 6. Synthetic guide RNA and mRNA capping/base modification selection.

A) TCR knockout efficiency (%) when delivering a single sgRNA (TCR 1) or two sgRNAs (TCR 1+2) at half relative mol% each. B) Corresponding flow histograms to knockout levels in A. C) Corresponding cell viabilities as determined through flow cytometry. D) TCR knockout efficiency (%) of various capping and base modifications of the Cas9 mRNA, as indicated, TCR 1+2 sgRNA was utilized. E) Corresponding flow histograms to knockout levels in D. F) Corresponding cell viabilities as determined through flow cytometry. For all, a dose of 3.2 µg total RNA / million T cells was used. Significance evaluated using t-tests among selected groups, with ns $p > 0.05$, * $p \leq 0.05$, and *** $p \leq 0.001$.



Guide RNA and mRNA Selection

It is important to recognize that the efficiency of gene editing is dependent on several factors and not solely on the delivery system. One such factor is the selection of gene editing reagent or payload, for example, the source of mRNA/sgRNA and the target sequences^{31, 32}. To maximize gene editing efficiency, further investigation of the gene editing materials was conducted.

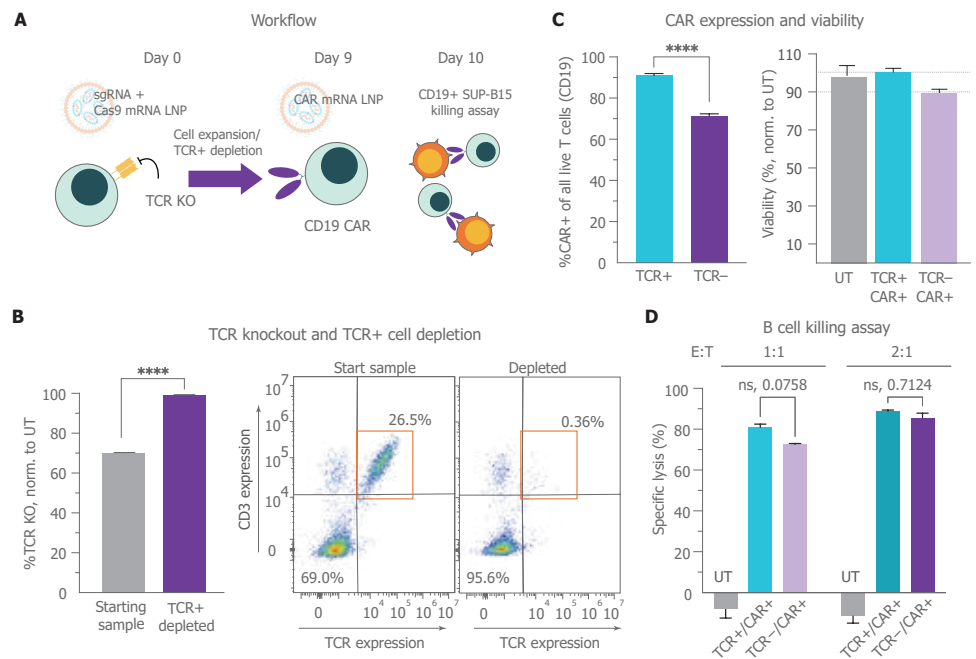
First, we studied the role of guide RNA construct. Previously, single TCR guides were utilized for delivery optimization (**Figure 4**). These selected sgRNA target sequences were tested as a single guide or as a combination of two different guides (each guide used at half mol% concentration of the single guide condition). **Figures 6A and 6B** show that TCR KO ranged from 57-85% in knockout efficiency such that utilizing both TCR 1 and TCR 2 sgRNA significantly increased knockout efficiency compared to using a single sgRNA (**Table 1** in the Material and Methods provides additional detail when using multiple sgRNAs). Cell viabilities upon LNP treatment remained at 98-100% relative to the UT controls (**Figure 6C**), with the UT cells having 87% absolute cell viability.

It is well understood that RNA capping and base modification make a significant impact for RNA-mediated in vitro gene editing³³. We tested Cap1 and Cap0 Cas9 mRNA with either wild-type (WT) or 5-methoxyuridine (5mou) bases. Shown in **Figures 6D and 6E**, both Cap0 and 5mou base modifications yielded lower TCR knockout efficiency, corroborating existing works showing that Cap1 and WT bases yield higher biological translational efficiencies^{30, 34}. The cell viabilities remained high for all constructs delivered (**Figure 6F**).

Overall, for efficient gene editing, high quality sgRNAs, the combination of multiple target sequences, and Cap1 WT-base mRNA are recommended. With this method, using TCR 1 and 2 sgRNAs we achieved on average $80 \pm 8\%$ knockout (range 66-91%, n=12 experiments).

Figure 7. Multi-step T cell engineering for off-the-shelf cell therapy.

A) Schematic illustration of experimental approach. LNPs containing TCR sgRNA 1+2 and Cas9 mRNA were added to primary T cells. Cells were expanded for 9 additional days and treated with CD19 CAR mRNA LNPs. 24-h post CAR RNA treatment, B cell (SUP-B15) killing assay was conducted. B) TCR knockout on Day 7 post-LNP treatment when treated with TCR sgRNA 1+2 and Cas9 mRNA at a dose of 3.2 $\mu\text{g}/\text{million}$ cells. Starting sample was TCR negative selected to purify the knockout sample. C) Left: Percent CD19 CAR expression 24h after treatment with CAR mRNA LNPs at 3.2 μg RNA/million cells, when population is TCR+, or TCR-. Right: Corresponding cell viabilities, normalized to the untreated population. UT, TCR+, TCR-. D) Specific lysis of SUP-B15 cells by UT, TCR+/CAR19+, or gene-edited TCR-/CAR19+ at the indicated effector to target ratios (E:T). One-way ANOVA analysis was conducted with ns $P > 0.05$, **** $p \leq 0.0001$.



Multi-Step Engineering: Knockout and Gene Delivery

To further demonstrate the versatility of RNA-LNPs, we generated gene-edited CAR T cells through multi-step LNP additions. For this, LNPs allow for multiple gene delivery treatments without compromising cell viability, an important consideration for next generation cell therapies.

Our workflow resulted in high gene editing efficiencies in combination with strong CAR expression (**Figure 7**). Following initial LNP treatment, the gene-edited T cells were expanded to facilitate low-density lipoprotein receptor (LDLR) recycling and LNP uptake in the subsequent treatment step³⁵. In the first treatment step, we utilized this optimized workflow, achieving ~70% TCR knockout on Day 7 (post-LNP addition). After TCR detection, the sample was purified to yield > 99% TCR⁻ population (**Figure 7B**).

After achieving a pure TCR⁻ population, LNPs encapsulating CD19-CAR mRNA were added to the cells. More than 70% of the TCR⁻ cells treated expressed the CAR surface protein compared to 90% in the unedited cells (TCR⁺) (**Figure 7C**). We attributed the lower transfection efficiency to the reduced activation state of the TCR⁻ cells, owing to the loss of both TCR and CD3 receptors. Both LNP treated samples, whether TCR⁺ or TCR⁻, maintained >90% relative viability to the untreated controls.

The resulting TCR⁺ and TCR⁻ CD19 CAR T cells were functionally assessed in a co-culture assay where CD19-specific killing was determined. CAR T cells were compared at two different T cell to SUP-B15 cancer cell ratios (E:T ratio) and the specific lysis was calculated to quantify cytotoxic potential. In **Figure 7D** we show statistically indistinguishable performance between the TCR⁺/CAR⁺ and the LNP-mediated TCR⁻/CAR⁺ cells. Both populations lysed 70-80% of CD19⁺ cancer cells at 1:1 E:T ratio.

Overall, this multi-step CAR T cell engineering workflow exemplifies the performance of LNPs for achieving high level of gene editing and CAR expression, while maintaining high cell viabilities.

Conclusion

The interest and demand for novel cell and gene therapies continues to grow. As the field turns its focus on addressing new diseases and therapeutic targets, the complexities and scope of genetic engineering will only increase. As a clinically-validated technology in vaccines (i.e., COVID-19 mRNA vaccines) and gene therapies (i.e., siRNA-based Onpattro(R)), LNPs are well-positioned to address the limitations of electroporation and viral vectors in cell therapies to successfully address a rapidly evolving clinical landscape. LNPs are an effective, gentle, and scalable gene delivery and editing technology that can help accelerate T cell therapy research and drug development.

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