Lipid Nanoparticle Reagents

GenVoy-ILM™

T Cell Kit for mRNA

on NanoAssemblr® Ignite™

User Guide

1001343

Table of Contents

Product Description

GenVoy-ILM™ T Cell Kit for mRNA, Ignite is an off-the-shelf lipid nanoparticle (LNP) reagent mix optimized for the delivery of messenger RNA (mRNA) or Cas9 mRNA/sgRNA into activated human primary T cells. LNPs are prepared on the NanoAssemblr® Ignite™ instrument with Ignite NxGen™ Cartridges. This non-viral delivery method can be seamlessly integrated into any standard human T cell culture workflows using an established protocol with either freshly isolated or cryopreserved T cells.

GenVoy-ILM T Cell Kit for mRNA, Ignite enables researchers to establish a clinically-relevant and scalable method at the preclinical scale for *ex vivo* gene delivery and editing to accelerate the development of T cell therapies.

Why Use the GenVoy-ILM T Cell Kit for mRNA, Ignite?

- Off-the-shelf LNP reagent kit with a path to the clinic
- Maintain high T cell viability, even after multi-step genetic manipulation
- Successfully engineer T cells with high levels of knockout or protein expression
- Extensively validated with downstream T cell culture workflows
- Seamlessly scalable on the NanoAssemblr instruments from discovery to preclinical

Kit Components

GenVoy-ILM T Cell Kit for mRNA, Ignite, 3 mL (1001144)

Component	Product Code	Size	Storage
Lipid Mix	1001145	3 mL	-80 °C
Formulation Buffer (10X)	1001146	2 mL	2–8 °C
Dilution Buffer (10X)	1001147	40 mL	2–8 °C
Cryopreservation Buffer	1001148	3 mL	-80 °C
Apolipoprotein-E3 (ApoE)	1001149	500 µg	-80 °C

GenVoy-ILM T Cell Kit for mRNA, Ignite, 6 mL (1001161)

Component	Product Code	Size	Storage
Lipid Mix	1001162	6 mL	-80 °C
Formulation Buffer (10X)	1001163	4 mL	2–8 °C
Dilution Buffer (10X)	1001164	80 mL	2–8 °C
Cryopreservation Buffer	1001165	6 mL	-80 °C
Apolipoprotein-E3 (ApoE)	1001149	500 µg x 2	-80 °C

Required Products (Not Included in Kit)

Product	Product Code
NanoAssemblr Ignite Instrument	NIN0001
NanoAssemblr NxGen Ignite Cartridges – 100 Pack	NIN0061
NanoAssemblr NxGen Ignite Cartridges – 200 Pack	NIN0062

For more information on the product, please visit www.precisionnanosystems.com/t-cell-kit-for-mrna-ignite.

For more information on the NanoAssemblr platform with NxGen technology, please visit www.precisionnanosystems.com.

General Product Use Limitations and Warranty

Limitations and Warranty

GenVoy-ILM T Cell Kit for mRNA, Ignite, 3 mL and GenVoy-ILM T Cell Kit for mRNA, Ignite, 6 mL are intended for research use only and not for in-human use. We do not make any claims or representations that intend to provide information for the diagnosis, prevention, or treatment of a disease.

GenVoy-ILM T Cell Kit for mRNA, Ignite, 3 mL and GenVoy-ILM T Cell Kit for mRNA, Ignite, 6 mL are subject to Precision NanoSystems' general terms and conditions which can be found at www.precisionnanosystems.com/terms-and-conditions/.

Expected Characteristics and Performance

The anticipated performance is based on the use of eGFP mRNA (Trilink, L-7601) and the biological workflow recommended in this User Guide. Further optimization is expected for different RNA and biological workflows.

Characteristics	Anticipated Performance Based on eGFP mRNA
mRNA encapsulation efficiency	>80% using the RiboGreen Assay
Transfection efficiency	>80% using flow cytometry
Cell viability	>80% compared to untreated sample using Trypan Blue exclusion cell count

Background and General Considerations

Lipid Nanoparticle Background

RNA-LNPs are prepared by mixing the ethanolic solution of lipids with an aqueous solution of RNA at low pH. Upon mixing, the change in polarity of the environment triggers the self-assembly of LNPs. The low pH causes the ionizable lipids to become cationic, where they interact with anionic RNA through electrostatic complexation to form the particle core. Other lipids assemble around the core. Rapid microfluidic mixing through the NanoAssmblr Ignite ensures homogeneous conditions that promote core formation and lead to monodisperse particles.

Among other factors, mixing parameters may influence particle size and PDI. The total flow rate (TFR) and the flow rate ratio (FRR) are the most influential mixing parameters to tune particles for a given application. In general, higher TFR and FRR result in smaller particle sizes, although LNPs are generally stable in size for a wide range of input parameters. **For the GenVoy-ILM T Cell Kit for mRNA, Ignite, the microfluidic mixing parameters are optimized for transfection efficiency in primary T cell with the TFR set at 12 mL/min and FRR at 2:1.**

An important factor for high RNA encapsulation and biological activity of LNP formulations is the ratio of mRNA to lipids. **The GenVoy-ILM T Cell Kit for mRNA, Ignite utilizes an optimized ratio to give high protein expression in primary T cells.**

General RNA Considerations

The following section contains important considerations for the RNA to ensure a successful outcome with GenVoy-ILM T Cell Kit for mRNA, Ignite. We recommend mRNA with the following characteristics for maximum biological activities:

mRNA modifications
5′ capping and 3′ polyadenylation (polyA) protect against enzymatic degradation and are critical for biological activities. In our experience, Cap 1 modification results in significantly higher in vitro translation efficiency in T cells compared to Cap 0 (e.g., ARCA). Base modifications (e.g., pseudouridine, 5mou) are not recommended, with wild type (WT) bases showing significantly higher expression levels in T cells *ex vivo*.

mRNA and guide RNA purity
It is generally advisable to use the highest purity of RNA available. When using in-house synthesized IVT RNA (both for mRNA and sgRNA), gel electrophoresis should be used to verify integrity. Here, a single, sharp band of the expected size should be visible. The encapsulation of impurities will yield reduced performance as non-functional RNA will displace the positive lipid charges. All RNA stock solutions must have a concentration of at least 0.35 mg/mL for the working solution preparation.

Storage and handling
The mRNA should be stored in a low ionic strength buffer, such as 1 mM sodium citrate at pH 6.4 or in RNAse-free water, as high levels of ions in solution may impact encapsulation efficiencies. For sgRNA, we recommend resuspending the pellet in molecular grade water. For all RNAs, avoid multiple freeze-thaw cycles by storing the solutions in aliquots at -80 °C.

CleanCap™ mRNA products from Trilink Biotechnologies are recommended for use with the GenVoy-ILM T Cell Kit for mRNA, Ignite. CleanCap eGFP mRNA (Trilink L-7601) can be used as a positive control.

GenVoy-ILM T Cell Kit for mRNA on NanoAssemblr Spark and Ignite

The GenVoy-ILM T Cell Kit for mRNA is available on the NanoAssemblr Spark and NanoAssemblr Ignite. **Table 1** outlines the differences between these kits to enable researchers to scaleup easily.

Table 1. GenVoy-ILM T Cell Kit for mRNA at the Spark and Ignite Scales

Kit Names	Suggested number of runs per kit	Approximate RNA input per kit	Approximate RNA yield per kit	Anticipated CAR T Cells, dependent on dose	RNA-LNP storage
GenVoy-ILM T Cell Kit for mRNA, Spark	5 x 96 µL run	50 µg	35 µg	~10 million	4 °C, 1 week
GenVoy-ILM T Cell Kit for mRNA, Ignite, 3 mL	9 x 1 mL run 2 x 4 mL run 1 x 9 mL run	2 mg	1.3 mg	~350 million	4 °C, 1 week -80 °C, 1 month, with included Cryopreservation Buffer
GenVoy-ILM T Cell Kit for mRNA, Ignite, 6 mL	18 x 1 mL run 4 x 4 mL run 2 x 9 mL run	4 mg	2.6 mg	~700 million	

Supplies and Equipment

To prepare RNA-LNPs with the GenVoy-ILM T Cell Kit for mRNA, Ignite, the following third-party supplies and equipment are suggested:

General Laboratory Equipment

Description	Recommended Product/Supplier
Single and multi-channel pipettes	General laboratory supplier
Fluorescence plate reader	BioTek™ Synergy™ H1, or similar
Heating block or oven capable of heating to 55°C	General laboratory supplier, Thermo Scientific™ Digital Dry Baths/Block Heaters or similar
Oven, capable of heating to 37 °C	General laboratory supplier
UV spectrometer	NanoDrop™, Thermo Scientific™, or similar
Vortex mixer	General laboratory supplier
Refrigerated centrifuge, swing bucket	Capable of 4000 RCF speeds, general laboratory supplier
Hemocytometer/cell counter	General laboratory supplier
Flow cytometer	CytoFLEX™, Beckman Coulter or similar

General Consumables

Description	Recommended Product/Supplier
15– and 50–mL conical centrifuge tubes	General laboratory supplier, Falcon™ or similar
0.5–2 mL cryo vials, with screw cap and O-ring seal	General Laboratory Supplier
Syringe filters – 13 mm, 0.2 µm	Acrodisc®, 13mm, Supor® hydrophillic polyethersulfone membrane shows high LNP recovery
Amicon® 30 kDa MWCO centrifugal filters, 15 mL	Amicon® Ultra-15 Centrifugal Filter – Ultracel® Regenerated Cellulose. Alternative manufacturers are not recommended. Flexibility in MWCO
1, 3, 5, or 10 mL disposable syringe	Becton Dickinson (BD) Luer-Lok™ Tip or similar (note the internal diameter of syringe on the Ignite instrument)
Blunt needles	Becton Dickinson 18 guage, or similar
48-well tissue culture and 96-well black bottom plates	Corning® Costar™ Flat Bottom 48-well TC treated PS (3548) and Corning® 96-well Black Flat Bottom PS (3915)
T75/T150 flasks	General laboratory supplier, e.g. Fisher Scientific, 13-680-65, 08-772-48
G-Rex® 6M, 6 well plate	Fisher Scientific, NC1498767

Suggested Reagents

Description	Recommended Product/Supplier
CleanCap® GFP mRNA CleanCap® Cas9 mRNA	Trilink Biotechnologies, L-7601 Trilink Biotechnologies, L-7606
Quant-iT RiboGreen Assay Kit	ThermoFisher (R11490)
Triton X-100	Sigma (cat: X100-100ML)
Cryopreserved T cells	STEMCELL Technologies Inc., 70024
EasySep™ Direct Human T Cell Isolation Kit	STEMCELL Technologies Inc., 19661
Human Whole Peripheral Blood, Anticoagulant ACDA	STEMCELL Technologies Inc., 70504
Easy 50 EasySep™ Magnet	STEMCELL Technologies Inc., 18002
ImmunoCult™ Human anti- CD3/CD28/CD2 T Cell Activator	STEMCELL Technologies Inc., 10970
ImmunoCult™-XF T Cell Expansion Medium	STEMCELL Technologies Inc., 10981
Recombinant human IL-2	STEMCELL Technologies Inc., 78036
0.4% Trypan Blue solution	General laboratory supplier or Sigma-Aldrich, 93595
RNAse-free phosphate buffered saline	Corning Dulbecco's PBS, 1x without calcium and magnesium, 21-031-CV
Deionized RNAse-free water	General laboratory supplier
70% isopropanol or ethanol	General laboratory supplier
Nuclease decontamination spray	RNAseZap™ or similar

Additional Documentation

Description	Document Number
NanoAssemblr Ignite User Guide	Ignite-UG-1019
T Cell Ignite Workbook	tcellkitmrnaignite-WB-0322
RNA Quantification Workbook (For RiboGreen Assay)	PNI-WB-S9-001-INT
Application Note: Genome Editing of Human Primary T Cell with Lipid Nanoparticles	CRISPR-AN-0322

Standard Workflow

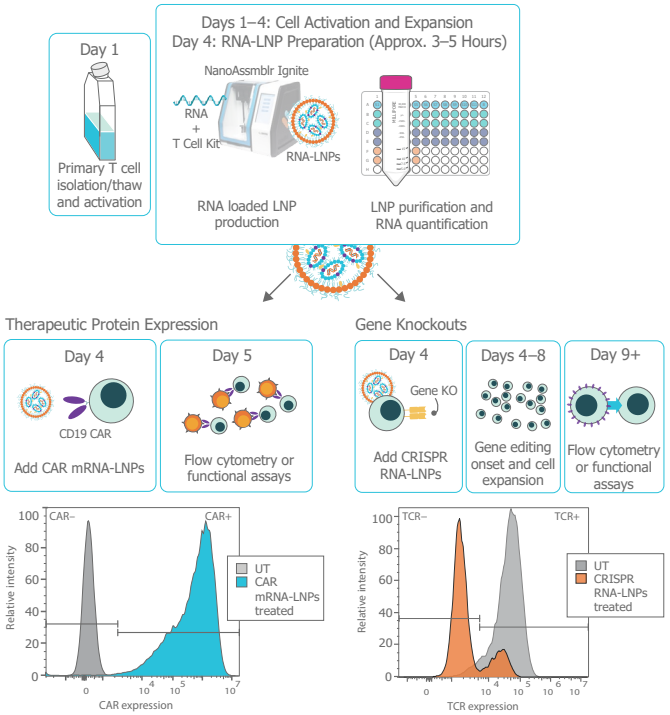


Figure 1. Primary T cell workflow for both mRNA-based gene expression and CRIPSR/Cas gene editing applications using the GenVoy-ILM T Cell Kit for mRNA, Ignite.
Day 1 — Cryopreserved primary human pan T cells are thawed and activated. Days 1–4 – Cells are activated and expanded. Day 4 — RNA-LNPs are produced. RNA encapsulation efficiency is assessed to determine dosing. RNA-LNP production requires 3–5 hours of hands-on time on the NanoAssemblr Ignite. Therapeutic expression: Day 5 — Protein expression or therapeutic effect can be assessed. Gene knockouts: Days 4–8 — Gene editing onset occurs with maximal editing achieved on Day 8. Day 9 and onwards — Gene editing efficiency can be assessed.

The User Guide aims to describe in detail the RNA-LNP production using the NanoAssemblr Ignite and primary T cell culture protocol validated by Precision NanoSystems. For further information on gene editing and multi-step engineering, please refer to our Application Note: Genome Editing of Human Primary T Cell with Lipid Nanoparticles (Document ID: CRISPR-AN-0322).

Step 1. User Input	
Desired LNP Volume (mL)	3
Stock Cas mRNA concentration (mg/mL)	1
Stock sgRNA Concentration (mg/mL)	3.2
Cas9 to sgRNA ratio	1

Step 2. Dilution Buffer Preparation	
Dilution Buffer (10X) (mL)	9
Molecular grade water (mL)	81
Final Volume (mL)	90

Step 3. Aqueous Solution Preparation	
Mol Bio Water (µL)	1833.8
Formulation Buffer (µL)	264.0
Cas9 mRNA (µL)	413.1
sgRNA (µL)	129.1
Final Volume (µL)	2640.0

Step 4. Required Lipid Mix Volume in Syringe	
T Cell Lipid Mix (µL)	1320

Legend
Fillable
Dispense / Important

Step 5. Ignite Parameters	
C (Aq) Syringe **Volume** (mL)	3
R (Lipid) Syringe **Volume** (mL)	3
Flow Rate Ratio C:R	2:1
Total Volume (mL)	3.30
Total Flow Rate (mL/min)	12
Start Waste (mL)	0.30
End Waste (mL)	0

Step 6. Downstreaming Processing	
Number of Amicon Filters	2
Target Concentrated Vol. (mL)	3.0
RNA Theoretical Max (µg)	688

Step 7. Cell Treatment	
Concentration of LNPs (µg/mL)	**112**
T cells (millions)	**50**
Desired RNA Dose (µg/mL)	**3**
Volume of LNPs to Add (mL)	1.34

Figure 2. T Cell Ignite Workbook outlines the preparation, production, and processing steps of RNA-LNP production. Blue cells can be changed to desired input values, such as desired RNA-LNP volume and starting stock concentrations. Yellow cells can be changed and are related to pipetting, dilutions, and instrument parameters.

Production of RNA-LNPs with the NanoAssmblr Ignite

This section describes the workflow for preparing RNA-LNPs on the NanoAssemblr Ignite, including aqueous solution preparation, instrument handling, and particle downstream processing.

A. Equipment Setup and Calculations

Prior to RNA-LNP production, fill out the T Cell Ignite Workbook. An example image of the sheet is shown in **Figure 2**.

NOTE: *The T Cell Ignite Workbook can be obtained by contacting your Field Application Scientist at Precision NanoSystems.*

1. Enter the desired volume of LNPs, with a recommended minimum of 1 mL (**Step 1** in T Cell Ignite Workbook).
2. Clean worktop surface with RNAse Zapp or a similar cleaning agent to reduce chances of sample contamination.
3. Begin thawing mRNA on ice. Always keep the mRNA aliquot on ice.
4. Thaw the Lipid Mix by removing the vial from -80 °C and placing at 55 °C for 5 minutes, e.g., in a bead bath or heat block.
5. Dilute the Dilution Buffer (10X) with nuclease-free water according to **Step 2** in the T Cell Ignite Workbook. The 1X buffer will be used to dilute the RNA-LNPs after production.
6. Pre-cool the centrifuge to 4 °C and prepare centrifugal filters (e.g., Amicon) by spinning ~15 mL of water through the filter at 4000 *x g* for 5 minutes. The water spin removes membrane preservatives and serves to check membrane integrity. If water passes through the filter fully, the membrane may be compromised, and the filter should be discarded.
7. One centrifugal filter per 1.5 mL of undiluted LNPs (45 mL diluted) is recommended. Adding additional filters does not reduce recovery but saves downstream processing time. See **Step 6**. in T Cell Ignite Workbook.

1 **mRNA Expression Calculations**

User Input	
Desired LNP Volume (mL)	1
Stock mRNA Concentration (mg/mL)	1

Aqueous Solution Preparation	
Mol Bio Water (µL)	516.6
Formulation Buffer (µL)	88.0
mRNA (µL)	275.4
Final Volume (µL)	880.0

2 **CRISPR-Cas9 Editing Calculations**

User Input	
Desired LNP Volume (mL)	1
Stock **Cas9 mRNA** concentration (mg/mL)	1
Stock **sgRNA** concentration (mg/mL)	3.2
Cas9 to sgRNA ratio	1

Aqueous Solution Preparation	
Mol Bio Water (µL)	611.3
Formulation Buffer (µL)	88.0
Cas9 mRNA (µL)	137.7
sgRNA (µL)	43.0
Final Volume (µL)	880.0

Figure 3. T Cell Ignite Workbook can be used for RNA aqueous phase calculations for both gene delivery and knockouts. Values are given based on desired RNA-LNP volumes and stock solution concentrations.

B. Aqueous Solution Preparation

Please find recommendations for both mRNA-only and mRNA/sgRNA encapsulation in the T Cell Ignite Workbook. The Workbook has a tab for each type of RNA-LNP production, with an example image shown in **Figure 3**. The RNA weight to Lipid Mix has been optimized and is restricted in the calculations such that increasing the RNA input will reduce product performance.

Recommended: Measure the concentration of the RNA solution(s) with UV-Vis (e.g., NanoDrop™) to ensure the concentration is as expected. If deviation is observed, update the concentration in the T Cell Ignite Workbook field accordingly.

mRNA Delivery

Important: We recommend to complete steps 1–3 in the next section **(C)** prior to aqueous phase preparation. This limits RNA degradation in the acidic buffer environment.

1. Input the concentration of the mRNA stock solution in mg/mL in the T Cell Ignite Workbook (**Step 1**). In this order, pipette the Formulation Buffer, molecular grade water, and mRNA into one aqueous solution according to the volumes indicated in the T Cell Ignite Workbook (**Step 3**). Make sure to mix Formulation Buffer with molecular grade water well before adding mRNA.
2. Mark this tube as "**RNA Aq**" and keep on ice until ready to place in a syringe and Ignite instrument.

CRISPR-Cas RNA Delivery

Important: We recommend to complete steps 1–3 in the next section **(C)** prior to aqueous phase preparation. This limits RNA degradation in the acidic buffer environment.

1. Input the concentration of the Cas mRNA sgRNA stock solutions in mg/mL in the T Cell Ignite Workbook (**Step 1**). A commonly used sgRNA stock concentration is 100 mM or roughly 3.2 mg/mL for synthetic constructs.
2. Input the desired Cas9 mRNA to sgRNA weight ratio. Recommend starting with 1:1 weight ratio.
3. In this order, pipette the Formulation Buffer, molecular grade water, sgRNA and Cas9 mRNA into one aqueous solution in the T Cell Ignite Workbook (**Step 3**). Make sure to mix Formulation Buffer with molecular grade water well before adding sgRNA and Cas9 mRNA.
4. Mark this tube as "**RNA Aq**" and keep on ice until ready to place in a syringe and Ignite instrument.

C. NanoAssemblr Ignite Production of RNA-LNPs

1. Turn on the NanoAssemblr Ignite. From the main menu, select "Quick Run" – an example screen is shown in **Figure 4**.
2. Enter the parameters by selecting syringe brand and size, flow rate ratio, total volume, total flow rate, and start waste. The parameters are shown in **Step 5** in T Cell Ignite Workbook.
3. Open the lid of the NanoAssemblr Ignite, remove a NxGen Cartridge from the package, and insert the cartridge in the cartridge slot until a soft click is felt. Raise the rotating block until the cartridge luers are visible to connect the syringes. Please refer to NanoAssemblr Ignite User Guide (Document ID: Ignite-UG-1019) for further instrument usage details.
4. Draw the required amount of **Lipid Mix** into the specified lipid (R) syringe size (see **Steps 4** and **5** in T Cell Ignite Workbook) using a clean blunt needle. Remove the needle, and clear air bubbles from the syringe by tapping it, and use the plunger to advance the liquid, avoiding drips from the syringe tip. Insert the syringe into the "R" inlet of the Ignite Cartridge.
5. After preparation, draw the entire solution of the tube labelled "**RNA Aq**" prepared in the previous section into the specified aqueous (C) syringe size (see **Step 5** in Workbook) using a clean blunt needle. Ensure appropriate volume is present in the syringe and remove air bubbles as before. Insert the syringe into the (C) inlet of the Ignite Cartridge and twist to engage the Luer-Lok™.

   Tip to help remove the air bubbles: draw 0.2–0.3 mL aqueous phase first and clear the air bubble near the rubber gasket by tapping it. Make sure there is no air bubble inside the syringe before drawing the leftover solution.
6. Return the rotating block to the downwards position. Ensure the sample switch arm for two 15 mL conical tubes is installed on the right of the cartridge.
7. Mark a 15 mL conical collection tube as "**Sample**" and push the tube into the tube clip labeled "**Sample**". Mark another with "**Waste**" and push the tube into the tube clip labeled "**Waste**".
8. Close the Ignite lid and tap "Next" on the screen. Read and confirm the information in the dialog box and press "Start". The motors of the Ignite will start manufacturing the LNPs. The formulation is collected in the sample tube.
9. After the motors have positioned themselves back in the home position, open the lid and remove the sample collection tube and set aside for further processing.
10. Raise the rotating block and remove the syringes from Ignite and discard them. Return the rotating block to the downward position and remove and discard the cartridge.

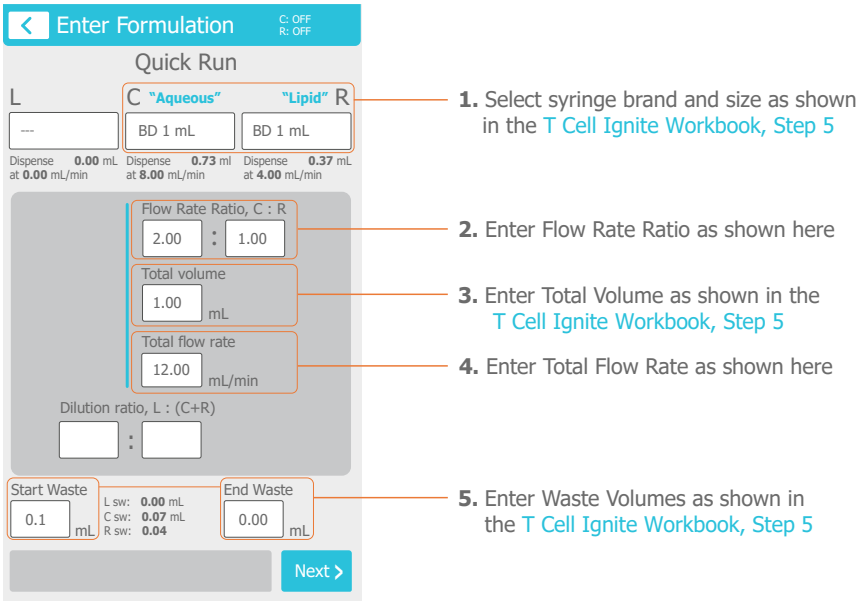


Figure 4. (1) Select the available syringe brand and sizes recommended in Step 5 of the T Cell Ignite Workbook. (2) Enter 2:1 flow rate ratio, a constant parameter for all runs. (3) Enter the total volume recommended in Step 5 of the T Cell Ignite Workbook. (4) Enter 12.00 mL/min total flow rate, a constant parameter for all runs. (5) Enter the start waste, shown in Step 5 of the T Cell Ignite Workbook, or 10% of the total volume. Enter "0.00 mL" for the end waste.

D. Downstream Processing of LNPs

1. After formulation, perform a 30X dilution on the LNPs with **1X Dilution Buffer** (previously diluted from 10X Dilution Buffer provided in the kit in section A5 of the User Guide). This may need to be done in a separate 50 mL conical vial or a media bottle for larger runs.
2. Fill the preconditioned Amicon filters (See **step 6 in section A**) with the diluted LNPs. Spin at 4000 *x g* for 10 minutes at 4 °C (in a swing bucket rotor). Lower speeds are acceptable but increase the processing time.
3. Discard the solution below the filter unit and repeat this step as necessary until the entire sample is re-concentrated to the starting LNP volume.
4. Recover the sample from the filter using a micropipette (wash the membrane with ~100–200 µL **1X Dilution Buffer** to ensure full recovery) and pipette into an RNAse-free tube.
5. In a biosafety cabinet, filter the concentrated sample using a sterile 0.2 µm filter into a new sterile vial. Suggest to use small 13 mm hydrophilic polyethersulfone syringer filters to allow for higher RNA recovery. The use of multiple 0.2 µm filters may be needed for larger sample volumes and should not impact RNA yield.

   Tip to maximize LNP recovery: push the air then small amounts of **1X Dilution Buffer** through the filter after most of the sample has been collected. Repeat the air push for 2–3 times to make sure all residues are collected.
6. Aliquot out 25 µL of the final sample into a new RNAse-free tube in the biosafety cabinet for determining RNA concentration and particle size (optional).
7. Measure the encapsulated RNA concentration by the RiboGreen Assay using Precision NanoSystems Encapsulation Efficiency Protocol (See **Appendix A**).

   Optional: Measure the size of the particles by dynamic light scattering (DLS). Generally, we would expect the particle size to be within 60–100 nm range (measured on Malvern Zetasizer Ultra). Please see **Appendix B** for further details.

E. Long-term Storage of LNPs

1. Dilute aliquots of the LNPs 1:1 with the **Cryopreservation Buffer** included with the kit. Mix the LNPs with the buffer thoroughly by pipetting at least 5 times up-and-down.
2. Formulated RNA-LNPs can be stored in the **Cryopreservation Buffer** at -80 °C for up to one month.

Suggested T Cell Culture Protocol

This section describes the cell culture protocol for RNA delivery into human primary T cells using the GenVoy-ILM T Cell Kit for mRNA, Ignite. The protocol provides guidelines for the preparation of human primary T cells, activation and maintenance, and treatment with RNA-LNPs.

Primary human T cells were cultured using STEMCELL Technologies' ImmunoCult™ products. The GenVoy-ILM T Cell Kit for mRNA, Ignite is expected to be compatible with alternative T cell culture media systems. However, optimization of treatment day may be required if using alternative cell culture reagents. For additional information on appropriate use and storage of the cell isolation, activation, or culture products, please refer to the supplier's instructions.

Preparing Complete T Cell Media

1. Re-constitute lyophilized recombinant human IL-2 to a concentration of 0.1 mg/mL in sterile 1X PBS without calcium or magnesium under sterile conditions.
2. Divide re-constituted IL-2 into small aliquots and store at -80 °C for up to 2–3 months. Do not reuse aliquots after 2 freeze thaw cycles.
3. Prepare the **complete T cell media** by adding 100 µL of 0.1 mg/mL IL-2 to 100 mL of ImmunoCult™-XF T Cell Expansion Medium.

NOTE: *Once IL-2 has been added the media is stable for 1 week at 4* °C*. Pre-warm the complete T cell media, to 37* °C *prior to use.*

Preparing Human Primary T Cells

Human primary T cells can be isolated from peripheral blood using the EasySep™ Direct T Cell Isolation Kit. Alternatively, frozen human primary T cells can be used by following this procedure:

1. **Day 1:** Warm ImmunoCult-XF T Cell Expansion Media to 37 °C prior to use.
2. Quickly thaw the frozen vial of T cells (2 x 10^7 cells/vial) in a warm (37–40 °C) water bath for approximately 2–3 minutes (no more than 3 minutes). Wipe the outside of the vial with 70% ethanol. Transfer the cell suspension into a 15 mL tube containing 9 mL of warm ImmunoCult-XF T Cell Expansion Media.
3. Centrifuge the tube at 300 *x g* for 10 minutes at room temperature (21–25 °C) then aspirate to remove the supernatant (First wash). Resuspend the cell pellet with 10 mL of of ImmunoCult-XF T Cell Expansion Media.
4. Repeat centrifugation at 300 *x g* for 10 minutes at room temperature and remove the supernatant (Second wash). Resuspend the pellet in 5 to 10 mL of complete T cell media.
5. Aliquot out 10 µL of the cell suspension and mix with 10 µL trypan blue to perform cell count using a hemocytometer or an automatic cell counter.
6. Bring T cells to a final concentration of 1 x 10^6 cells/mL in the complete T cell media in a T-75 flask. We recommend limiting total cell volume to 25 mL in the T-75 flask.

T Cell Activation

We suggest activating T cells immediately after isolation or thawing.

7. To activate T cells, add 25 µL of ImmunoCult Human CD3/CD28/CD2 T Cell Activator per million T cells; that is 25 µL per 1 mL of cells at 1 x 10^6 cell/mL.
8. Incubate the flask in an upright position at 37 °C in a humidified atmosphere with 5% CO_2 for 72 hours (3 days).
9. On Day 4 (3 days post-activation), count the cells using trypan blue exclusion.
    a. If there are enough cells for RNA-LNP treatment, proceed to the RNA-LNP addition to activated primary T cells section.
    b. If more cells are required for LNP treatment, activated T cells may be expanded further prior to RNA-LNP treatment (only applicable for therapeutic protein expression). Dilute the cells in complete T cell media and maintain in the incubator for additional 4–5 days to facilitate expansion. If required, activated T cells may be re-activated for further expansion. We do not recommend multiple rounds of re-activation cycles. Refer to the cell expansion protocol described in **Appendix D** for a detailed example.

NOTE: *CD25 and LDLR surface expression levels of activated T cells can be evaluated by flow cytometry prior to treatment. This will provide insight on the activation state and the ability of the cells to uptake LNPs. On Day 4 (3 days post-activation), the viable T cell population is expected to show at least 80% CD25 expression or 60% CD25 & LDLR co-expression to ensure successful delivery of RNA-LNPs into T cells.*

RNA-LNP Addition to Activated Primary T Cells

1. Thaw an aliquot of ApoE stock (100 µg/mL) on ice. See **Appendix C** for instructions on preparation of 100 µg/mL ApoE stocks.
2. Dilute activated T cells to a concentration of 0.5 x 10^6 cells/mL in complete T cell media.
3. Add ApoE to the diluted T cell suspension to achieve a final concentration of 1 µg/mL.
4. Based on the RiboGreen assay results, calculate the volume of RNA-LNPs to be added to the cells. *In vitro* titration is recommended to identify the optimal treatment based on payload dose and/or to modify levels of protein expression as desired.
    a. Suggested starting dose:
        ii. For therapeutic protein expression, start with a treatment dosage between 1–3 µg RNA-LNP per 1 x 10^6 T cells.
        iii. For gene knockouts, we recommend a 1:1 ratio of Cas9 mRNA and sgRNA at a dose between 2–4 µg total RNA per 1 x 10^6 T cells.
    d. The T Cell Ignite Workbook **Step 7** includes a calculation aid for determining the volume of RNA-LNPs to add to the cells.
5. RNA-LNP treatment may be done in flasks or in 48-well TC treated plates at a T cell concentration of 0.5 x 10^6 cells/mL.

a. 48-well Plate Treatment: Seed each well with 250 µL of cells at 0.5 x 10^6 cells/mL. Add the calculated volume of RNA-LNP formulation directly into the cell suspension in the wells using a micropipette. Gently pipette up and down with the same micropipette twice to mix. We do not recommend any further perturbation of the cells beyond this point.

b. Flask Treatment: Add RNA-LNPs into the cell suspension at 0.5 x 10^6 cells/mL in the flask. Gently rock the flask in a side-to-side motion twice.

NOTE: *Avoid vigorous mixing after addition of RNA-LNPs.*

6. Incubate the treated cells at 37 °C in a humidified atmosphere with 5% CO_2 for 24 or 48 hours. Treatment duration may differ depending on the kinetics of protein expression for the mRNA of interest.
7. For gene editing, we recommend further cell expansion after RNA-LNP addition to achieve maximal knockouts by Day 8. Follow the optional cell wash step below to obtain higher cell yields after expansion. Incubate at 37 °C/5% CO_2 and maintain the cell density below 2 x 10^6 cells/mL for the duration of expansion. Monitor the cell growth using trypan blue cell counts and dilute the cells if they reach 2 x 10^6 cells/mL with complete T cell media.

Optional for Gene Editing Applications: Cell Washing After LNP Treatment for Increased Cell Proliferation

1. Wash the cells 24 hours after the initial RNA-LNP treatment.
2. Centrifuge cells at 300 *x g* for 10 minutes at room temperature and discard the supernatant.
3. Resuspend the cell pellet with complete T cell media supplemented with triple activator (12.5 µL activator/mL).

Downstream Analysis of T Cells

Transfection efficiency can be assessed by quantifying the levels of protein expression or gene knockout using flow cytometry or other methods. It is critical to consider the biological implications of the RNA-LNP treatment and the culture conditions on the target detection in selecting the appropriate bioanalytical method.

In vitro cell-based assays can be used to assess function through T cell-mediated cytotoxicity. Please refer to the application note: Genome Editing of Human Primary T Cell with Lipid Nanoparticles (CRISPR-AN-0322), for a detailed protocol.

Appendix A: RiboGreen Assay for Determination of mRNA Encapsulation Efficiency

Determining the mRNA encapsulation efficiency is necessary for accurate dosing of RNA-LNPs with T cell experiments. If the RNA-LNP preparation protocol is followed as outlined in this guide, the RNA-LNPs are expected to be loaded with 100–120 µg/mL of RNA, with variation typically arising from changes in the final sample volume.

The recommended encapsulation efficiency protocol is as follows:

RiboGreen Assay for Determine of mRNA Encapsulation Efficiency

Additional Reagents/Disposables

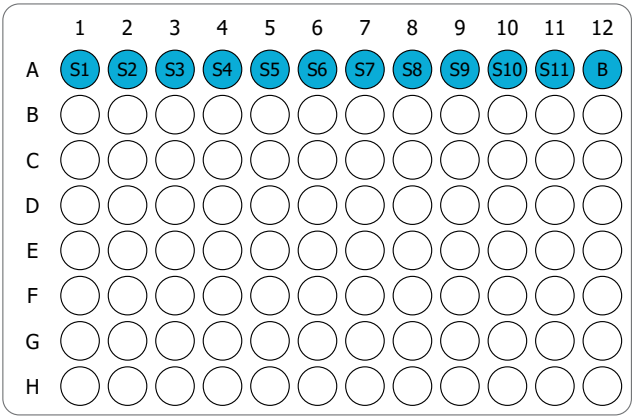
Description	Recommended Product/Supplier
Invitrogen™ TE Buffer (20X), RNase-free	Thermo Fisher Scientific, T11493
Quant-iT™ Ribogreen® Assay Kit	Thermo Fisher Scientific, R11490
Triton™ X-100	Sigma Aldrich, X100-100ML
RNase-free water	General Laboratory Supplier
RNase-Free Filter Pipette Tips (10, 20, 200, and 1000 µL)	General Laboratory Supplier
Pipette basins	General Laboratory Supplier
96-well black plate	Corning® 96-well Black Flat Bottom PS (3915), or similar
Mg^{2+} / Ca^{2+} free PBS 1X	General Laboratory Supplier
18 Gauge Needles	General Laboratory Supplier

Additional Equipment Required

Description	Recommended Product/Supplier
Plate Reader	Synergy™ H1 Biotek® Plate Reader
Multichannel Pipette (10–300 µL)	General Laboratory Supplier
Micropipettes (10, 20, 200, and 1000 µL)	General Laboratory Supplier

Preparation of Sample Stock Solutions

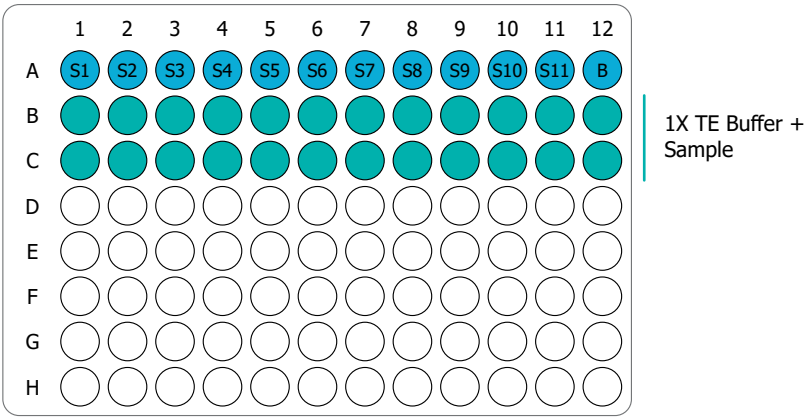
1. Prepare 1X TE buffer from 20X TE buffer by adding 10 mL of 20X TE buffer to 190 mL RNase-free water in a clear glass bottle. Shake the bottle to mix.
2. To 100 mL of prepared 1X TE buffer, add 2 mL of Triton X-100. Stir using a magnetic stirrer for 15 min. This solution is the Triton Buffer.
3. Pour the 1X TE buffer and Triton buffer in separate pipette basins.
4. In the top row of the 96-well plate (Row A), add 15 µL of mRNA-LNP sample to these wells (S1-S11). Add 15 µL of PBS to the blank well (B).
5. Using a multi-channel pipette, add 1X TE buffer to Row A to make up the volume to 250 µL. Pipette to mix.



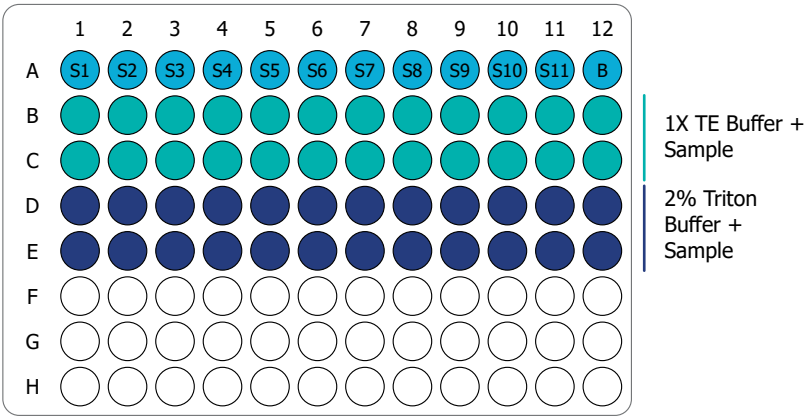
mRNA-LNP Sample Setup

This assay is run in duplicate. It is recommended that liquid handling be done using a multi-channel pipette.

1. Add 50 µL of 1X TE buffer to the two wells directly below each mRNA-LNP sample (Rows B and C).
2. Add 50 µL of mRNA-LNP sample stock solution from Row A into the wells in Row B and C.
3. Add 50 µL of Triton buffer to the wells in Rows D and E below each sample.



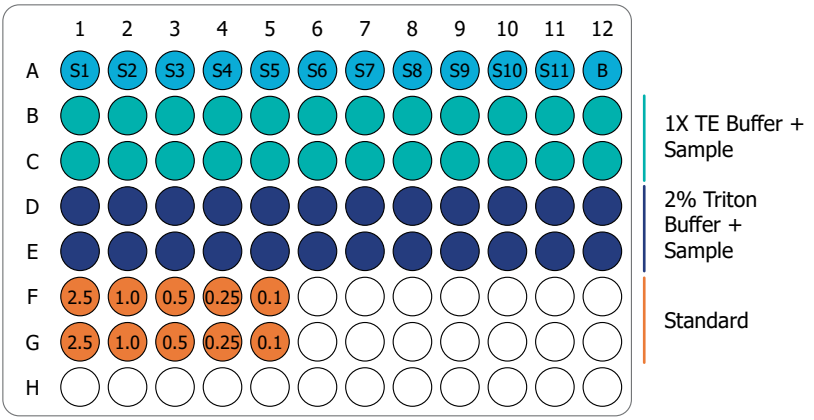
4. Add 50 µL of sample stock solution from Row A into the wells in Rows D and E.



RNA Standard Curve Setup

1. Setup a standard curve (in duplicate in rows F and G) as shown in the table below using the RNA Stock (20 µg/mL RNA), 1X TE Buffer, and Triton Buffer.

Final RNA (µg/mL)	**RNA Stock Required** (µL)	**TE Buffer Required** (µL)	**Triton Buffer Required** (µL)	**Total Volume per Well** (µL)
2.5	25	25	50	100
1	10	40	50	100
0.5	5	45	50	100
0.25	2.5	47.5	50	100
0.1	1	49	50	100



2. Once the samples and standard curve are plated, incubate the plate at 37 °C for 10 minutes to lyse the mRNA-LNPs in the presence of Triton buffer.

Preparation of Ribogreen Solution

1. Sum the total number of sample wells and standard curve wells. Add 4 to this number, and multiply the total by 100. This is the total volume, in µL, of Ribogreen Solution needed for this assay.
2. In a 15 mL RNase-Free Falcon Tube, dilute the Ribogreen Reagent 1:100 into 1X TE buffer to the total volume calculated in the previous step.

NOTE: *For example, if 3000 µL of Ribogreen Solution is needed, add 30 µL of Ribogreen Reagent to 2970 µL of 1X TE buffer.*

3. Vortex the Ribogreen Solution for 10 seconds to mix.

Addition of Ribogreen Solution and Sample Reading

1. Remove 96-well plate from 37 °C incubator.
2. Add 100 µL of Ribogreen Solution to each well.
3. Pop any bubbles with a needle.
4. Read using fluorescent plate reader with the following settings:

Plate Reader Parameters	
Excitation	485 nm
Emission	528 nm
Optics	Top Read
Gain	55
Read Height	8mm

NOTE: *The Gain and Read height will change depending on the instrument.*

Sample Analysis

1. Enter each mRNA-LNP sample and each Standard Curve sample into the RNA Quantification Workbook (PNI-WB-S9-001-INT). This sheet will calculate the encapsulation efficiency and mRNA concentration of each sample.

NOTE: *The RNA Quantification Workbook can be obtained by contacting your Field Application Scientist at Precision NanoSystems.*

2. The second sheet on this workbook (Name: Plate Setup) gives the well numbers from which the O.D. values would be fed into the first sheet (Name: RNA Quantification).
3. The third sheet (Name: Dilution factor-calculation) in the workbook gives the calculation to input the dilution factor values in column 'O' of the first sheet (Name: RNA Quantification).

Appendix B: Lipid Nanoparticle Sizing and PDI

Evaluation of the LNP size is optional but can be considered when using in-house produced RNA that may yield more heterogenous particles. Extensive testing using commercially available mRNA and/or sgRNA has shown consistent sizes in the range of 60–100 nm with PDIs well below 0.2. Therefore, with commercial reagents, size measurement is not recommended. For primary T cells transfection, all sizes below 120 nm work equally well, with PDI having no impact on the resulting biological performance.

To confirm uniform size characteristics, mix **5 µL** of the LNPs with **345 µL** of **1X PBS** in a semi-micro cuvette and measure on a DLS instrument. The recommended Malvern Zetasizer Ultra instrument parameters are listed below.

Instrument Parameter

Parameter	Value/Selection
Cell	DTS0012 (Disposable 10x10 Plastic Cell)
Material	Polystyrene Latex Refractive Index: 1.59 Absorption: 0.01
Dispersant	1X PBS Refractive Index: 1.34 Viscosity (mPa.s): 1.02
Temperature (°C)	25
Return to default temperature	yes
Equilibration time (s)	0
Analysis Model	General Purpose
Angle of detection	Back Scatter
Positioning method	Measure at optimal position
Attenuation	Automatic
Measurement Process	Automatic
Use pause after sub runs	No
Optical Filter	No Filter
Pause between repeats (s)	0

NOTE: *Attenuation is considered good if within the range of 7–8. At attenuations below 6, dilute your measurement sample with the same dispersant; and attenuations above 9, depending on the concentration of the nanoparticle formulation, add an additional 5–20 µL of your nanoparticle formulation to the measurement sample to achieve a higher concentration for the measurement.*

Appendix C: Preparation of 100 µg/mL ApoE Stock Solution

ApoE stock solution should be prepared in a sterile environment.

1. Thaw lyophilized vial of ApoE (500 µg) at room temperature for 10 to 15 minutes.
2. Add 1 mL of 1X PBS and vortex for 30 seconds. Let it stand at room temperature for 10 to 20 minutes.
3. Vortex again for 30 seconds and transfer the 1 mL solution to a tube containing 4 mL of 1X PBS for a final volume of 5 mL (500 µg powder dissolved in 5 mL 1X PBS) in the tube, vortex again for 30 seconds.
4. Vortex intermittently and visually confirm ApoE has completely dissolved completely before aliquoting into sterile screw cap vials.
5. Label the tubes and store at -80 °C, avoid more than 2 freeze thaws of the aliquots.

Appendix D: Optional Expansion of Activated Human T Cells

This optional protocol is to allow the production of sufficient activated human T cells from a small number of cells. Expansion may be achieved using multiple T-150 flasks or specialized vessels, such as the G-Rex® systems. Expansion may be performed before mRNA-LNP treatment of cells, but not recommended for gene knockouts, as high cell division reduces non-homologous end joining (NHEJ) repair rates with increasing HDR rates instead.

1. Count the cells and determine CD25 and LDLR expression levels by flow cytometry to ensure that T cells are activated and are expressing LDLR. The recommended timepoint is Day 4 (3 days post cell activation) when using the cell activation and culture reagents suggested in this User Guide.
2. Dilute the activated T cells to 4 x 10^4 cells/mL in complete T cell media, in a large cell culture vessel (T-150 flask or G-Rex 6M well plate) and incubate at 37 °C in a humidified atmosphere with 5% CO_2. Depending on the incubator setup, T-150 flask may be placed inside in a horizontal position (flat) or in a vertical position (upright). The maximum recommended volume for flat and upright orientations are 30 mL and 60 mL respectively. See **Table 1**. for detailed examples.

NOTE: *Do not remove the anti-CD3/CD28/CD2 T Cell activator (or alternative activator).*

Table 1. Suggested cell seeding densities and volumes in different vessels on day 4. Cells are significantly diluted to promote cell expansion.

Concentration	# of Cells	Volume (mL)	# of T-150 flasks (flat)	# of T-150 flasks (upright)	# of G-Rex 6M wells
4 X 10^4 cells/mL	1.2 X 10^6	30	1	1	1
	4.8 X 10^6	120	4	2	2

3. Allow the cells to expand until Day 8 or until desired cell numbers are achieved. Cell growth should be monitored, and the concentration should not exceed 2 x 10^6 cells/mL for the duration of expansion.

Table 2. Suggested cell seeding densities and volumes in different vessels on day 8.

Concentration	# of Cells	Volume (mL)	Amount of activator	# of T-150 flasks (flat)	# of T-150 flasks (upright)	# of G-Rex 6M wells
5 X10^5 cells/mL	30 X 10^6	60	0.75 mL	2	1	1
	144 X 10^6	288	3.6 mL	10	5	4

4. On Day 8, count the expanded cells and determine if further expansion is required. Refer to **Table 2**. for detailed examples.
5. Transfer the calculated volume of cells into a sterile 50 mL tube then centrifuge at 300 *x g* for 10 minutes at room temperature.
6. Remove the supernatant and resuspend the pellet to 0.5 x 10^6 cells/mL in complete T cell media supplemented with triple activator (12.5 µL/mL of 0.5 x 10^6 cells) as shown in Table B.
7. Incubate the cells at 37 °C in a humidified atmosphere with 5% CO_2 and allow them to expand until Day 11.

   Optional: At the end of expansion, count the cells and determine CD25 and LDLR expression levels by flow cytometry.
8. On Day 11, count the cells then prepare them for RNA-LNP addition as follows: dilute the required number of cells to 0.5 x 10^6 cells/mL in complete T cell media. Do not remove the CD3/CD28/CD2 T Cell activator (or alternative activator). Refer to the protocol described in section RNA-LNP addition to activated primary T cells.

Notes

Notes

Notes

Notes

Ordering Information

GenVoy-ILM T Cell Kit for mRNA, Ignite

	Name	Includes	Product Code
	GenVoy-ILM™ T Cell Kit for mRNA, Ignite, 3 mL	1 Kit	1001144
	GenVoy-ILM™ T Cell Kit for mRNA, Ignite, 6 mL	1 Kit	1001161

Instruments, Cartridges and Accessories

	Name	Includes	Product Number
	NanoAssemblr® Ignite™	1 Instrument 2 Sample Switch Arms 2 Cartridge Adapters	NIN0001
	NxGen™ Cartridges	100 pack 200 pack	NIN0061 NIN0062

Related Products

	Name	Includes	Product Code
	NanoAssemblr® Spark™	1 NanoAssemblr® Spark™ Instrument 1 Power Supply (Worldwide) 1 One-year Warranty	NIS0001
	Spark™ Cartridge	20 Pack 80 Pack	NIS0009 NIS0013
	GenVoy-ILM™ T Cell Kit for mRNA, Spark	1 Kit	1000701
	GenVoy-ILM™ T Cell Kit for mRNA, Spark with Cartridges	1 Kit 5 Cartridges	1000683

End-to-end Solutions Enabling the Creation of Transformative Medicines

Precision NanoSystems is a global leader ushering in the next wave of genetic medicines of infectious diseases, cancer and rare diseases. We work with the world's leading drug developers to understand disease and create the therapeutics and vaccines that will define the future of medicine. Our proprietary NxGen technology platforms and comprehensive expertise enable researchers to translate disease biology insights into non-viral genomic medicines.

For more information on Precision NanoSystems' NanoAssemblr manufacturing platform, see the webpage: https://www.precisionnanosystems.com/platform-technologies/product-comparison

PRECISION NANOSYSTEMS

Create Transformative Medicines™

For technical assistance and related documents:

- Contact your Field Application Scientist
- Or email us at info@precision-nano.com
- Or call 1-888-618-0031

Contact
info@precision-nano.com
1-888-618-0031

Headquarters:
50 - 655 West Kent Ave. N.,
Vancouver, BC, V6P 6T7
Canada

Precision Nanosystems USA:
395 Oyster Point Boulevard, Suite 145
South San Francisco, CA 94080
USA

Precision Nanosystems Europe:
Two Snowhill Road
Birmingham, UK. B4 6WR

Precision Nanosystems APAC:
10 Anson Road #12-14
International Plaza
Singapore 079903

www.precisionnanosystems.com

Product Number: 1001343

Document ID: tcellkitmrnaignite-UG-0322