Lipid Nanoparticle Reagents

GenVoy-ILM™

T Cell Kit for mRNA

on NanoAssemblr® Spark™

User Guide

1000684

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Product Description

GenVoy-ILM T Cell Kit for mRNA is a lipid nanoparticle (LNP) reagent mix optimized for the delivery of messenger RNA (mRNA) into activated human primary T cells using lipid nanoparticles (LNPs) prepared on the NanoAssemblr® Spark™ instrument with Spark Cartridges. This non-viral delivery method can be seamlessly integrated into 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 enables researchers to establish a clinically relevant method for *ex vivo* gene delivery to accelerate the development of T cell therapies. The NanoAssemblr Spark instrument and cartridges allow rapid and easy preparations of ultra-low volumes of mRNA-LNP with this reagent at the discovery scale.

Why Use LNPs for Human T Cell Gene Delivery?

- LNPs are a potent mRNA delivery technology.
- LNPs engineer T cells with uniform cell surface protein expression.
- LNPs are gentle on T cells.
- LNPs can be easily integrated into any standard T cell culture workflow with minimal manipulation of cells.

Kit Components

GenVoy-ILM T Cell Kit for mRNA with Spark Cartridges (1000683)

Component	Component #	Size	Storage
Lipid Mix	1000680	90 µL	-80 °C
Formulation Buffer 1 (10X)	1000681	30 µL	2–8 °C
Formulation Buffer 2	1000682	900 µL	2–8 °C
Apolipoprotein-E4 (ApoE)	NWW0007	50 µg	-80 °C
5 x NanoAssemblr Spark Cartridges	NIS0002	1	15–25 °C

GenVoy-ILM T Cell Kit for mRNA (1000701)
- Spark Cartridges purchased separately

Required Components (Not Included in Kit)

Component	Component #
NanoAssemblr Spark Instrument	NIS0001

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

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

General Product Use Limitations and Warranty

GenVoy-ILM T Cell Kit for mRNA is intended for research use only and not for in-human use. No claim or representation is intended to provide information for the diagnosis, prevention or treatment of a disease.

GenVoy-ILM T Cell Kit for mRNA is 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

General Considerations

The following section contains important considerations for the mRNA to ensure a successful outcome with GenVoy-ILM T Cell Kit for mRNA.

General mRNA Considerations

We recommend mRNA with the following characteristics for maximum biological activity:

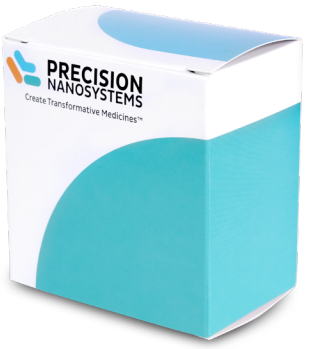
Modifications: 5′ capping and 3′ polyadenylation (polyA) protect against enzymatic degradation and exhibit increased biological activity compared to uncapped naked mRNA. Cap 1 modification results in significantly higher *in vitro* translation efficiency in T cells compared to Cap 0 (e.g., ARCA) with this kit. Additional recommendations include using uridine-depleted mRNA by substituting uridine-free synonymous codons or adding base substitutions. For example, 5-methoxyuridine and pseudouridine substitution have been shown to decrease innate immune responses *in vivo* and increase translation efficiency *in vitro*.

Purity: It is generally advised to use the highest purity mRNA available.

Storage and handling: The mRNA concentration should be above 0.5 mg/mL for the working mRNA solution preparation. This protocol uses a starting mRNA concentration of 1 mg/ml. 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. mRNA stock solutions should be divided into 50-100 µL aliquots to avoid multiple freeze-thaw cycles and should be stored at -80 °C for maximum shelf-life.

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

GenVoy-ILM T Cell Kit for mRNA



Related Precision Nanosystems Products

Product Name	PNI Catalog Number
GenVoy-ILM T Cell Kit for mRNA	1000701
GenVoy-ILM T Cell Kit for mRNA with Spark Cartridges (5 Spark Cartridges)	1000683
NanoAssemblr Spark Instrument	NIS0001
Spark Cartridges (20 pack)	NIS0009
Spark Cartridges (80 pack)	NIS0013

Other Required Kits

Description	Recommended Product/Supplier
Quant-iT™ RiboGreen® RNA Assay Kit, incl. 20X TE Buffer, RNase-free	Thermo Fisher Scientific, R11490

Suggested Supplies and Equipment

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

Description	Recommended Product/Supplier
Fluorescence plate reader	BioTek™ Synergy™ H1, or similar
Micropipettes and RNAse free pipette tips (10, 20, 200 and 100 µL)	General Laboratory Supplier
Multichannel pipette and tips (100 and 300 µL)	General Laboratory Supplier
Heating block, capable of heating to 55 °C	General Laboratory Supplier
Oven, capable of heating to 37 °C	General Laboratory Supplier
0.5 ml cryo vials, with screw cap and O-ring seal	General Laboratory Supplier
48-well plates	Corning, or similar
Benchtop centrifuge	General Laboratory Supplier
Hemocytometer/cell counter	General Laboratory Supplier
T75 flask	General Laboratory Supplier

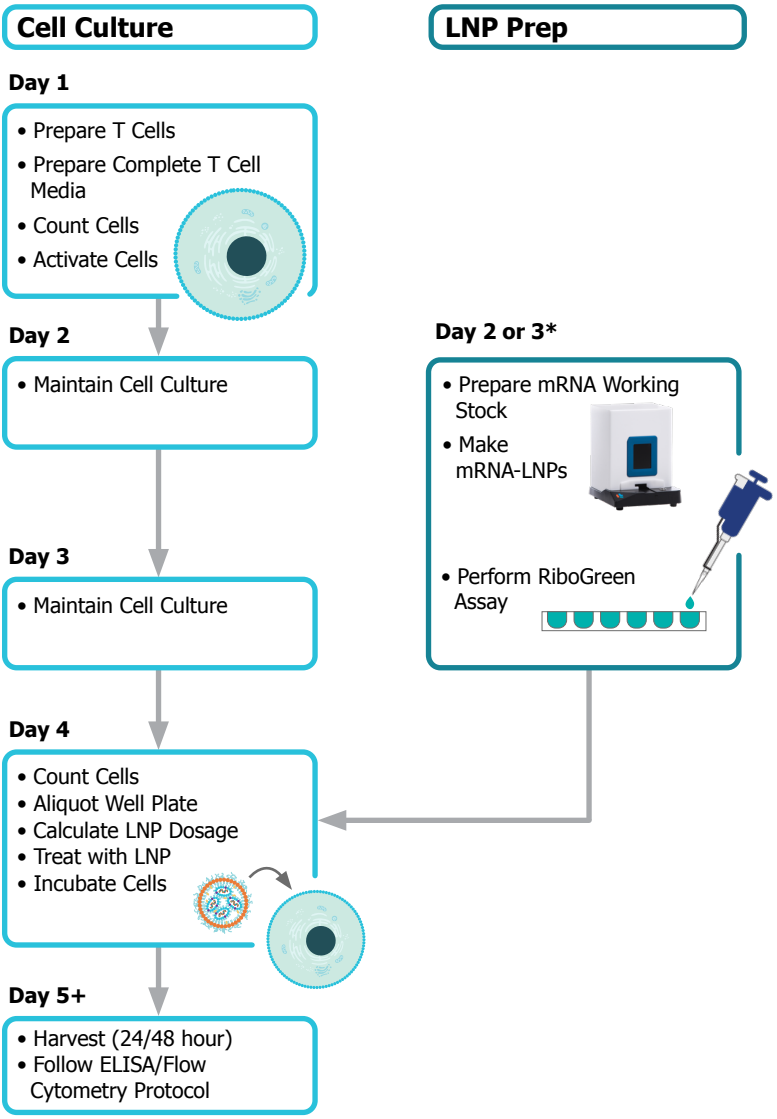
Suggested Reagents

Description	Recommended Product/Supplier
CleanCap® eGFP mRNA (5moU)	Trilink Biotechnologies, L-7601
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 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
Trypan Blue	General Laboratory Supplier
RNAse-free phosphate buffered saline	Corning Dulbecco's PBS, 1x without calcium and magnesium 21-031-CV
Deionized water, RNAse-free water	General Laboratory Supplier
70% isopropanol or ethanol, sterile	General Laboratory Supplier
Nuclease decontamination spray	RNAseZap™ or similar

Additional Documentation

Description	Document Number
Spark User Guide	sparksystem-UG-0119
RNA Quantification Workbook (For RiboGreen Assay)	PNI-WB-S9-001-INT

Standard Workflow



***NOTE:** Protocol takes approximately 1 hour with no manipulation of cells.*

Formulation of mRNA-LNPs

Equipment Setup

1. Turn on and clean the biosafety cabinet (BSC) by wiping down with 70% alcohol and RNAse decontamination spray.
2. Set up the NanoAssemblr Spark within the BSC by wiping down with 70% alcohol, connecting to a grounded outlet and turning on the instrument. More information on installation and operation of the Spark can be found in the NanoAssemblr Spark User Guide.
3. Retrieve a frozen mRNA aliquot and thaw on ice. Keep the aliquot on ice at all times.
4. Retrieve the GenVoy-ILM T Cell Kit for mRNA and thaw the Lipid Mix vial at 55 °C for 5 minutes then keep at room temperature. Both the mRNA aliquot and the kit should be kept sterile at all times.
5. In the BSC, place the following items:
    - On ice:
        - mRNA aliquot (1 mg/mL)
    - At room temperature:
        - Spark cartridges
        - T Cell Kit components:
            - Lipid Mix
            - Formulation Buffer 1 (10X)
            - Formulation Buffer 2
            - ApoE
        - Two 0.5 mL cryo vials for each sample, labeled (technical replicates recommended):
            - mRNA working solution
            - Formulated mRNA-LNP for cell culture
        - 20 and 200 µL micropipettes and sterile pipette tips
6. Prior to formulation, "purge" the Spark instrument
7. From the *Menu,* select **Formulation Mode**, and then set the instrument to **Setting 3**.

mRNA Working Solution Preparation

Preparation of the mRNA working solution should be done immediately prior to formulation to minimize degradation caused by the acidic buffer environment. This step should be completed after the Spark instrument has been set up, and all the consumables and equipment are ready in the BSC.

1. Assuming a starting mRNA concentration of 1 mg/mL, prepare the following components for one sample:
    a. 10 µL mRNA (1 mg/mL)
    b. 3.2 µL Formulation Buffer 1 (10X)
    c. 18.8 µL RNAse-free water

The total volume of the mRNA working solution for a single sample should be 32 µL, and the mRNA concentration should be 1 mg/mL.

It is recommended to pipette in 10% more of each component of the mRNA working solution to account for any potential pipetting losses. Use reference **Table 1** below for the preparation of one (1) to five (5) samples.

2. Combine the components to form the working mRNA solution.

   Once the components are combined, the working mRNA solution is ready to be used for mRNA-LNP formulation on the Spark.

Table 1. mRNA working solution preparation for 1 mg/mL mRNA. Volumes listed below include an extra 10% to account for pipetting errors.

No. Samples	Aqueous Vol (µL)	**Water** (µL)	**Formulation Buffer 1 (10X)** (µL)	**mRNA** (µL)
1	35.2	20.7	3.52	11.0
2	70.4	41.4	7.04	22.0
3	105.6	62.0	10.56	33.0
4	140.8	82.7	14.08	44.0
5	176.0	103.4	17.60	55.0

Preparation of mRNA-LNPs Using Spark

Spark Cartridge Loading and Formulation

1. Open the package containing a Spark Cartridge and the accompanying Cartridge Cap.

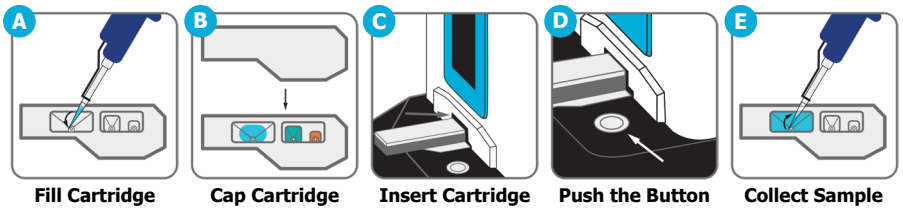


Figure 1. Overview of formulation steps with the Spark.

2. Using a micropipette, fill the largest well (Well 1) of the cartridge with 48 µL of Formulation Buffer 2 (refer to Figure 2).

NOTE: *Slowly pipette into each well of the Spark Cartridge while following the pipette positioning shown in Figure 2 to avoid spilling and bubble formation.*

3. Fill the middle well (Well 2) of the cartridge with 32 µL of the mRNA working solution prepared previously.
4. Fill the smallest well (Well 3) of the cartridge with 16 µL of the Lipid Mix.

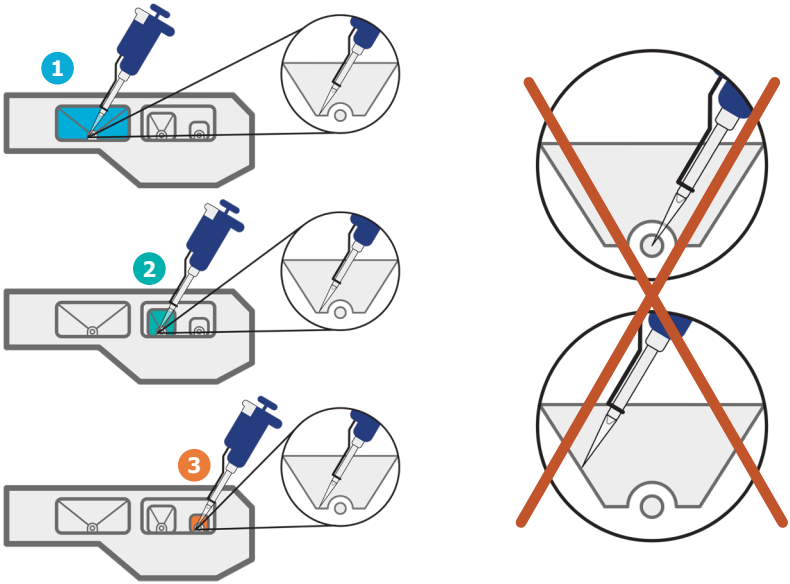


Figure 2. Spark cartridge well numbering scheme with insets showing how to pipette into the wells of a Spark Cartridge without spilling or forming bubbles.

5. Assemble the cartridge and insert into the Spark, as shown in Figure 1 panel B and C.
6. From the *Menu,* select **Formulation Mode**, and then select **Setting 3** if not already set.
7. Press the button on the Spark to formulate the mRNA-LNP sample.

Sample Collection

Once the formulation step is complete and as instructed by the instrument:

1. Add 96 µL of Formulation Buffer 2 to the vial labeled Formulated mRNA-LNPs.
2. Remove the Spark Cartridge from the instrument and lift the cap off carefully.

3. Using a micropipette, remove the mRNA-LNP sample from the largest well (Well 1) of the cartridge, and add it to the microcentrifuge tube containing 96 µL of Formulation Buffer 2. This tube contains the formulated mRNA-LNPs at an estimated concentration of 30-40 µg/mL.
4. Reserve 25 µL of the mRNA-LNPs for the RiboGreen Assay to determine encapsulation efficiency.

The sample of mRNA-LNPs is now ready for use. The sample should be stored under sterile conditions at 4 ºC and used for cell treatment within 1 week.

T Cell Culture Protocol

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

Human T cell cultures were performed using STEMCELL Technologies' ImmunoCult™ products. The GenVoy-ILM T Cell Kit for mRNA is expected to be compatible with alternative T cell culture media systems.

NOTE: *For additional information for appropriate use and storage of the ImmunoCult products, please refer to the supplier's instructions.*

Media and T Cell Prep

Preparing Complete T Cell Media

1. Re-constitute the 100 µg of 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 IL-2 into 4x250 µL aliquots, and store at -80 °C for up to 2-3 months.
3. Prepare the complete T cell media by adding 50 µL of 0.1 mg/mL IL-2 to 50 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. Warm media to 37 °C prior to use.*

Preparing Human Primary T Cells

1. Human primary T cells can be isolated from peripheral blood using the EasySep Direct T Cell Isolation Kit.
2. Alternatively, frozen human primary T cells can also be used as a starting point using the following procedure:
    a. Quickly thaw the frozen vial of T cells in a warm water bath.
    b. Wipe the outside of the vial with 70% ethanol.
    c. Transfer the cell suspension into a 15 mL tube containing complete T cell media.
    d. Perform centrifugation at 300 g for 10 minutes followed by resuspension of the cell pellet with 10 mL of complete T cell media.
    e. Repeat the previous step once.
    f. After the last centrifugation, resuspend the cells in 2 mL of complete T cell media.
    g. With a 10 µL aliquot of the cell suspension, perform Trypan Blue exclusion cell count using a hemocytometer or an automatic cell counter.
3. Bring T cells to a final concentration of $1x10^6$ cells/mL in the complete T cell media in a T75 flask. These are suspension cells, and the flask should be at 37 °C and 5% CO_2.

Cell Activation and Maintenance

1. To activate T cells, add 20 µL of ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator per $1x10^6$ cells/mL of complete T cell media. It is suggested to activate T cells directly after isolation or thawing.
2. Monitor cell growth through a daily cell count.
3. As the cells continue to expand, keep the cells at $1x10^6$ cells/mL as necessary by topping up with complete T cell media.

NOTE: *Day 4 of the workflow (3 days post-activation), CD25 expression should be greater than 80% as assessed by flow cytometry. CD25 expression of over 80% is important for successful delivery of mRNA-LNPs into T cells.*

Treatment of Activated Human T cells

mRNA-LNP treatment works best before the cells have entered the exponential growth phase (around 3 days post-activation). As such, mRNA-LNPs for treatment should be prepared on Day 2 or 3 of the workflow allowing for sufficient time to conduct the RiboGreen Assay to determine encapsulation efficiency.

1. Re-constitute the lyophilized recombinant human ApoE to a concentration of 0.1 mg/mL in sterile 1X PBS without calcium or magnesium under sterile conditions.
2. Divide diluted ApoE into aliquots and store at -80 °C for up to 2 months.
3. On Day 4, count T cells using Trypan Blue exclusion dye.
4. Bring cells to a concentration of 5×10^5 cells/mL in complete T cell media containing 1 µg/mL of ApoE.

NOTE: *It is important for the CD3/CD28/CD2 T Cell Activator to remain in solution for the duration of treatment.*

5. From the cell suspension, add 1 mL/well to a 12 well plate or 250 µL/well to a 48 well plate.
6. Based upon RiboGreen assay results calculate the volume of mRNA-LNPs to be added to the cells.

NOTE: *The recommended starting treatment dosage is 2 µg of mRNA-LNPs per 1 x 10^6 T cells. The dose can be titrated to modify levels of protein expression.*

7. Treat the T cells with the calculated volume of mRNA-LNP formulation.

NOTE: *It is important to not pipette up and down extensively or vigorously when mRNA-LNPs are introduced into the cell culture. Use a p10 or p20 to pipette mRNA-LNP in and gently pipette up and down 2–3 times. Do not use a p1000 to resuspend the cells.*

8. Incubate the plate of cells for 24 or 48 hours, or the appropriate time point. Timing may differ based on the kinetics of protein expression for the mRNA of interest.

Downstream Analysis of T Cells

1. At the end of incubation period or appropriate time point, follow the appropriate protocol for detection of protein expression by flow cytometry, ELISA, or Western blot.

Appendix A: RiboGreen Assay for Determination of mRNA Encapsulation Efficiency

Determining the mRNA encapsulation efficiency is highly recommended for accurate dosing of mRNA-LNPs with T cell experiments. If the mRNA-LNP preparation protocol is followed as outlined in this guide, the mRNA-LNPs are expected be loaded with 40 ± 8 µg/mL of mRNA, with variation typically arising from non-specific adsorption of the mRNA to the consumables used.

The recommended encapsulation efficiency protocol is as follows:

RiboGreen Assay for Determination of mRNA Encapsulation Efficiency

Additional Reagents/Disposables

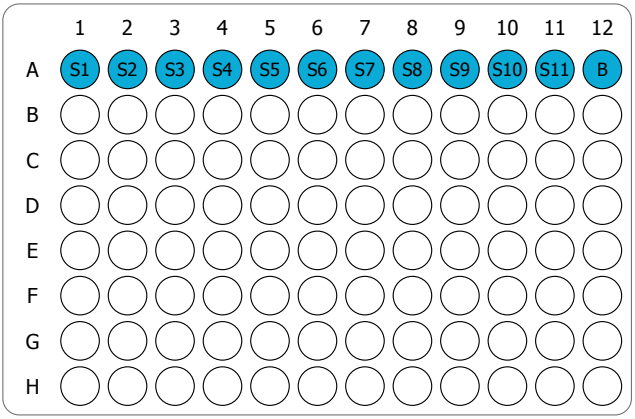
Description	Recommended Product/Supplier
Quant-iT™ Ribogreen® Assay Kit	Thermo Fisher Scientific, R11490
Triton™ X-100	Sigma Aldrich, X100-100ML
RNase-free water	General Laboratory Supplier
RNase-Free Filter Pipette Tips (10, 20, 200, and 1000 µL)	General Laboratory Supplier
Pipette basins	General Laboratory Supplier
96-well clear plate	General Laboratory Supplier
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

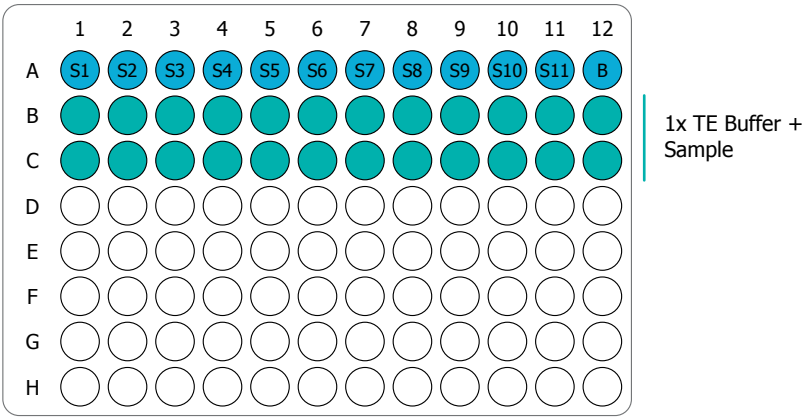
1. Prepare 1X TE buffer from 20X TE buffer by adding 10 mL of 20X TE buffer to 190 mL RNase-free water in a clear glass bottle. Shake the bottle to mix.
2. To make the Triton Buffer, add 2 mL of Triton X-100 to 100 mL of prepared 1X TE buffer and stir using a magnetic stirrer for 15 min.
3. Pour the 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.



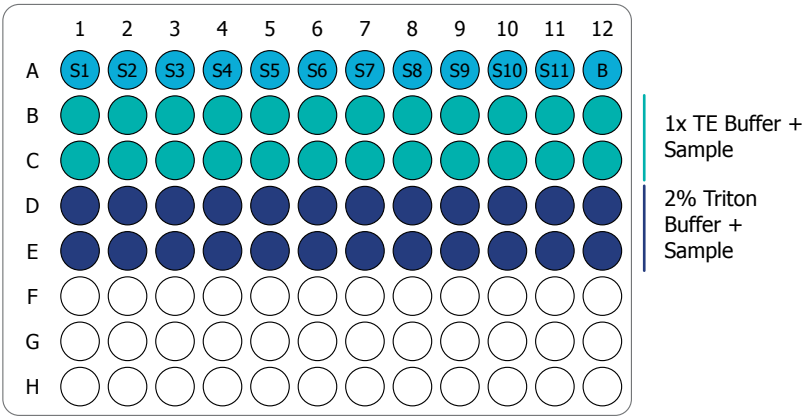
mRNA-LNP Sample Setup

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.



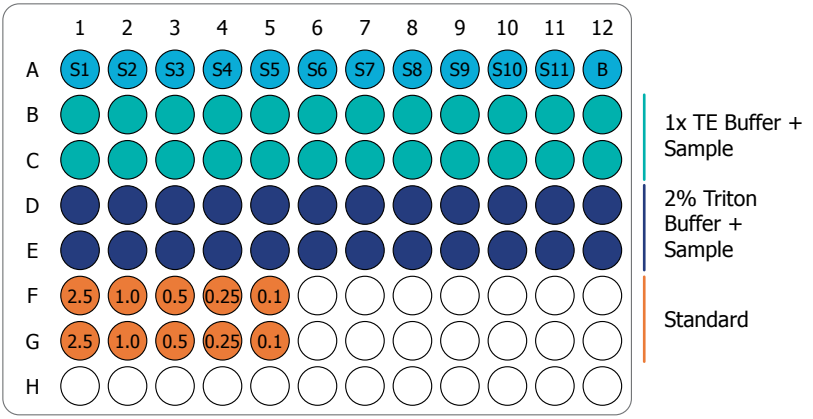
4. Add 50 µL of sample stock solution from Row A into the wells in Rows D and E.



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	RNA Stock Required	TE Buffer Required	Triton Buffer Required	Total Volume per Well
µg/mL	µL	µL	µL	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.
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).

Notes

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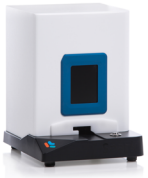
Ordering Information

T Cell Kit for mRNA

Image	Name	Product Number
	GenVoy-ILM T Cell Kit for mRNA	1000701
	GenVoy-ILM T Cell Kit for mRNA with Spark Cartridges	1000683

Instruments, Cartridges and Accessories

Image	Name	Product Number
	NanoAssemblr Spark	NIS0001
	NanoAssemblr Spark Cartridges (20 pack, 80 pack)	NIS0009, NIS0013

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

About Precision NanoSystems Inc. (PNI)

PNI 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. PNI offers proprietary technology platforms and comprehensive expertise to enable researchers to translate disease biology insights into non-viral genetic medicines.

PRECISION NANOSYSTEMS

Create Transformative Medicines™

For technical assistance and related documents:

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Product Number: 1000684

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