PRECISION NANOSYSTEMS

Create Transformative Medicines™

NanoAssemblr® Ignite™ Training Kit with GenVoy-ILM™

1001507

Contents

Description

The NanoAssemblr® Ignite™ is designed specifically for the research and development of nanomedicine formulations at bench scale. Ignite uses microfluidics and computer-controlled fluid injection to rapidly, reproducibly, and controllably tune the conditions governing the precipitation of drug and nanoparticle precursors into nanoparticles. Ignite shares proprietary NxGen microfluidic architectures with the rest of the NanoAssemblr® Platform to allow formulations to be scaled seamlessly from discovery to the clinic.

This Training Kit consists of reagents, consumables, and instructions required to encapsulate RNA into lipid nanoparticles (LNPs). The training goals for this Kit are to:

- Learn to formulate an RNA-LNP on the NanoAssemblr Ignite
- Understand how the Ignite workflow fits into your research programs
- Experience the simplicity and efficiency of formulating with Ignite
- Produce a formulation that acts as a positive control RNA-LNP for the hydrodynamic size, size distribution and encapsulation efficiency using GenVoy-ILM™ (ionizable lipid mix)
- Perform downstream processing and analytical procedures such as solvent removal and nucleic acid encapsulation

Important Notes

1. Please read all instructions thoroughly prior to using this Training Kit.
2. This Kit is designed for training, demonstration of workflow and assessment of particle characteristics only. LNP applications in biological systems require extensive optimization of various parameters. These include payload, formulation, concentration, dosage, media, route of administration, treatment timing, encapsulation efficiency and others. Optimizing research challenges specific to your project needs are beyond the scope of this training.
3. This kit does not include any material or reagents required for RNA quantification (for use in the Encapsulation Efficiency Assay). Please contact your representative at Precision NanoSystems for more information.
4. The instructions provided here are for 1 formulation. This Kit can be used to make 4 additional formulations with different payloads (for example for a control sample containing non-coding NC1 siRNA). Repeat these steps for the subsequent formulations.
5. This kit is not intended for human therapeutic or diagnostic use.

Product Components and Storage

NanoAssemblr® Ignite™

The NanoAssemblr Ignite will be shipped by courier or brought to your site by a Precision NanoSystems representative. The Ignite may arrive separately from the Training Kit components (described below). Store instrument in a cool dry place until a Precision NanoSystems representative can install it.

Training Kit Components

Reagents

Quantity	Component	Catalogue Number	Storage
5	GenVoy-ILM™ (0.7 mL)	NWW0056	-80 °C
2	Formulation Buffer (5 mL)	NWW0011	2–8 °C
5	Dilution Buffer (10 mL)	NWW0001	2–8 °C

Consumables

Quantity	Component	Catalogue Number	Storage
5	NanoAssemblr® Ignite™ NxGen cartridge (Precision NanoSystems)	NIN0002	15–25 °C
10	Syringes 1 mL Slip Tip (Beckton Dickinson)	309659	15–25 °C
10	Syringes 3mL Luer-Lok™ (Beckton Dickinson)	309657	15–25 °C
14	Blunt Needles, 18-Gauge (Beckton Dickinson)	305180	15–25 °C
15	Conical sterile polypropylene centrifuge tubes; 15mL (ThermoFisher Scientific)	339650	15–25 °C
10	Conical sterile polypropylene centrifuge tubes; 50mL (ThermoFisher Scientific)	339652	15–25 °C
3	Acrodisc® Syringe Filters with Supor® Membrane; 13mm, 0.2µm (PALL)	4602	15–25 °C

Consumables continued

Quantity	Component	Catalogue Number	Storage
5	Centrifugal filter concentrator – 10kDa MWCO (Millipore® Amicon® Ultra-15)	UFC-010-24	15–25 °C

Available Upon Request

Quantity	Component	Catalogue Number	Storage
3	siRNA model payload [0.4 mg (23.8 nmol)] (Integrated DNA Technologies)	NWW0013	-80 ºC
5	2000 nt mRNA 0.3 mg at 1 mg/mL in RNAse free water (TriLink)	NWW0010	-80 ºC

Required Equipment and Supplies Not Provided

Description	Example Product/Supplier	Product Catalog Number
UV Spectrometer	Nanodrop, ThermoFisher	ND-Lite
Particle Sizer	Zetasizer Nano-ZS, Malvern Panalytical	ZS-90
Cuvettes for Sizer	1.5–3.0 mL UV Cuvettes, VWR (or equivalent)	97000-586
Vortex Mixer	VWR Analog Vortex Mixer (or equivalent)	10153-838
Benchtop Centrifuge (for 50mL conical tubes)	Sorvall ST 40R Centrifuge	75004524
RNAse free water (200mL)	Hyclone	SH30221.25

Note: *See Appendix for additional requirements for measuring encapsulation efficiency*

Additional Documentation

Name	Document ID
NanoAssemblr® Ignite™ User Guide	ignite-UG-0622

General Product Use Limitations and Warranty

NanoAssemblr Ignite Training Kit with GenVoy-ILM is intended for training purpose 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.

NanoAssemblr Ignite training kit with GenVoy-ILM is subject to Precision NanoSystems' general terms and conditions which can be found at:

www.precisionnanosystems.com/terms-and-conditions/

General Considerations

Lipid Nanoparticles (LNPs) such as those prepared with GenVoy-ILM employ ionizable cationic lipids to avoid toxicity associated with permanently cationic lipoplexes yet are highly effective gene delivery vehicles. Their function is critically dependent on physical properties such as size and size distribution. LNP size is important both for allowing sterile filtration through 0.2 µm filters prior to injection and for determining biodistribution following administration. A homogeneous (narrow) size distribution is critical because deviations from size can lead to fouling of the sterile filters during processing or passive accumulation in off-target tissues following in vivo administration.

LNPs made using NanoAssemblr mixers have narrow size distributions (PDI typically ~0.1 or less) and are at the limit-size: the smallest particle size compatible with the packing of molecules[1]. LNPs made using NanoAssemblr technology also differ from liposomes because they exhibit a homogeneous core structure that is associated with higher potency.

RNA-LNPs are prepared by mixing the ethanolic solution containing GenVoy-ILM™ with RNA dissolved in PNI formulation buffer 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 first interact with anionic RNA through electrostatic complexation to form the particle core. Other lipids assemble around the core. Rapid mixing ensures homogeneous conditions and promotes core-formation over growth, leading to a homogenous population of LNPs.

Though optimizing LNPs for in vivo use is outside the scope of this Training Kit, this Training Kit will demonstrate the advantages of using the NanoAssemblr Ignite microfluidic manufacturing process to make LNPs, specifically to:

- Rapidly load and generate LNPs approaching the limit size[1] in one step
- Achieve high encapsulation efficiency of an RNA payload

1. Zhigaltsev I, Tam Y, Leung A, Cullis P. Production of limit size nanoliposomal systems with potential utility as ultra-small drug delivery agents. Journal of Liposome Research 2015, 1-7.

Expected Performance

When using Precision NanoSystems formulation buffer, and following the provided protocol, with the specified RNA, the expected range of typical characteristics as measured by dynamic light scattering are tabulated below.

Payload	Supplier	Payload Size	Z-Avg Diameter	PDI	Encapsulation Efficiency
DsiRNA	Integrated DNA Technologies	27 bp	45–75 nm	< 0.15	> 90%
CleanCap mRNA	TriLink	~2000nt	60–120 nm	<0.25	> 80%

Protocol

Overview

The following are guidelines for how to prepare working solutions of RNA in Precision NanoSystems Formulation buffer and how to prepare LNPs using GenVoy-ILM with the NanoAssemblr Ignite.

Reagent Preparation

1. To prepare the RNA working solution that will be the aqueous phase for the formulation, follow the steps in Table 1 appropriate for the type of RNA being prepared.
2. To prepare the GenVoy-ILM working solution, remove the GenVoy-ILM lipid mix from -80 °C and thaw it by placing at 55 °C for 5 min and then keep at room temperature.

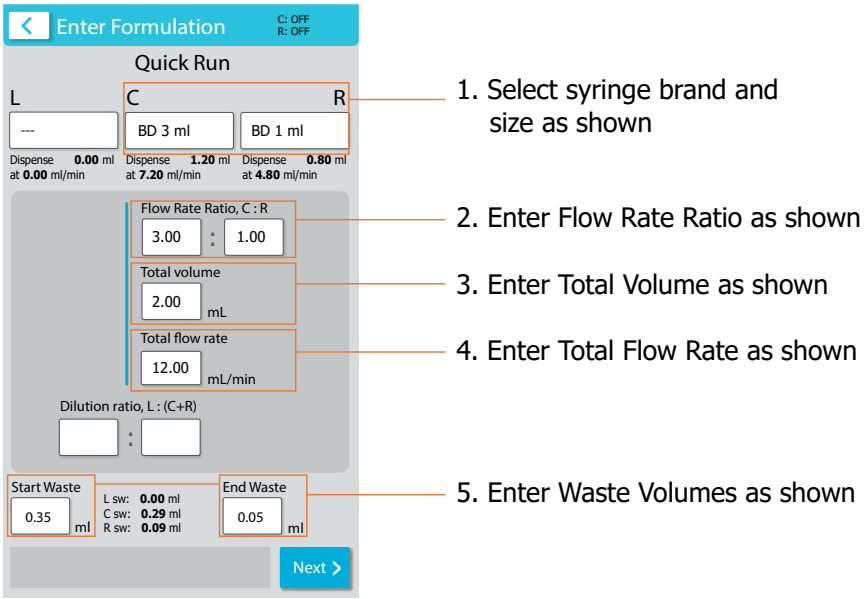
Table 1: Instructions for preparing aqueous phase based on source of RNA

For siRNA (provided by PNI)	For user-provided siRNA	For mRNA
1. Clean the working area with 70% ethanol. 2. Thaw lyophilized siRNA and **Formulation Buffer (FB)** to room temperature. Spin down the siRNA for 20s. 3. Combine 1.5 mL of RNAse-free water with 0.5 mL of **FB**. 4. Add 1.702 mL of diluted **FB** to siRNA and vortex for 10s at medium speed. 5. Using a Nanodrop, confirm that the concentration of the solution is ~235 µg/mL (220 – 240 µg/mL). Readjust with diluted **FB** if the value is >240 µg/mL.	1. Clean the working area with 70% ethanol. 2. Thaw the lyophilized siRNA and Precision NanoSystems **Formulation Buffer (FB)** to room temperature. Spin down the siRNA for 20s. Prepare a stock solution of 10 mg/mL of siRNA. We recommend using 10 mM sodium citrate at pH 6.4 to reconstitute siRNA. 3. Combine 1.5 mL of RNAse-free water with 0.5 mL of **FB** 4. Add 1.662 mL of **FB** to 40 µL of the siRNA stock solution. Vortex solution for 10s at medium speed. 5. Using a Nanodrop, confirm that the concentration of the solution is ~235 µg/mL (220 – 240 µg/mL). diluted Formulation buffer from step 3 or siRNA stock if the value is >240 µg/mL or < 220 µg/mL, respectively.	1. Clean the working area with RNAse Spray (e.g. RNAseZAP) and 70% ethanol. 2. Thaw the mRNA (PNI-provided mRNA or user-provided mRNA at 1 mg/mL in RNAse-free water) and bring **Formulation Buffer (FB)** to room temperature. 3. Add 1.390 mL of **FB** to 300 µL of mRNA and vortex for 10 s at medium speed. 4. Using a Nanodrop, confirm that the concentration of the solution is ~177 µg/mL. Readjust with FB or mRNA stock, if needed.

Formulate Lipid Nanoparticles

1. Turn on the NanoAssemblr Ignite. From the main menu, select "Quick Run".

2. Enter the parameters as shown below by selecting a field, selecting a value from the drop down OR entering the number with the onscreen keyboard then tapping the check mark. Repeat with the next field until your screen looks the same as below:



3. Open the lid of the Ignite and ensure the Cartridge Adaptor is installed over the "L" inlet of the cartridge slot with the silver side up, then remove a NxGen Cartridge from the package and insert it in the cartridge slot until a soft click is felt. Raise the rotating block until the cartridge luers are visible.

4. Draw the entire solution of RNA in Formulation Buffer 1 prepared in the previous section into a 3 mL syringe (volume should be ≥ 1.5 mL). Use a blunt needle if necessary. Remove the needle, clear air bubbles from the syringe and use the plunger to advance the liquid to the tip, but avoid drips from the syringe tip. Insert the syringe into the "C" inlet of the Ignite Cartridge and twist clockwise to engage the Luer Lok.

5. Draw the contents of one entire GenVoy-ILM vial into a 1 mL syringe using a blunt needle if necessary (volume should be ≥ 0.5 mL). Remove the needle, clear air bubbles from the syringe and use the plunger to advance the liquid to the tip, but avoid drips from the syringe tip. Insert the syringe into the "R" inlet of the Ignite Cartridge.

6. Return the rotating block to the downwards position. Ensure the sample switch arm for two 15 mL conical tubes (15–15) is installed.

7. Mark a 15 mL conical collection tube as "RNA-LNP" and push the tube into the clip labeled "Sample". Mark another with "waste" and push the tube into the clip labeled "Waste".

8. Close the Ignite lid and tap "Next" on the screen. Confirm the parameters, read and confirm the information in the dialog box and press "Start". The motors of the Ignite are now injecting the fluids into the microfluidic cartridge. The formulation is collected in the tube labeled "RNA-LNP".

9. After the motors have positioned themselves back in the home position the screen will indicate when it is safe to open the lid. Open the lid and remove the conical collection tube labeled "RNA-LNP" and set aside for characterization and 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 NxGen Cartridge.

11. To make additional samples, tap the back " < " button to return to the Quick Run Screen and repeat steps 3–10.

Downstream Processing: Particle Characterization, and Solvent Removal

Particle Characterization Prior to Dialysis

1. Dilute the 10X Dilution Buffer to 1X by mixing with 90 mL nuclease free water.
2. Pipette 40 µL of each sample into separate cuvettes containing 300 µL of 1X Dilution Buffer and gently mix.
3. Measure the particle size of the sample on the Malvern Zetasizer Nano ZS using the Malvern Zetasizer - Recommended Parameters provided in the Appendix.

Solvent removal

1. Dilute 1.6 mL of the "RNA-LNP" sample with 64 mL of 1X Dilution Buffer (= 40x dilution). Label the tube as "RNA-LNP Dilute".

2. Measure the particle size of "RNA-LNP Dilute" by DLS. For this, use 300µL sample without further dilution.

3. Fill the centrifugal filtration tube with the "RNA LNP Dilute" formulation. Spin the tube at 2000 x g for 30 minutes at 4 °C.

   Note: *refer to the manufacturer's (Millipore®) protocol for use of the Amicon® Centrifugal Filter Tubes*.

4. Discard the solution below the filter unit and add more "RNA-LNP Dilute" to the top of the filter, pipetting over the membranes several times to wash. Then, centrifuge according to step 4. Repeat this as necessary until the entire sample of "RNA-LNP Dilute" is re-concentrated to 1.6 mL.

5. Recover the concentrated sample from the filter using a pipette and gently wash the sample over the filter membrane several times before transferring into an RNAse-free tube. Mark the tube as "RNA-LNP Concentrated".

6. In a biosafety cabinet, using the Acrodisc® sterile filter and a 3 mL syringe, sterile filter the concentrated sample into a new sterile tube. Mark the tube as "RNA-LNP Final".

   Note: *This step is only necessary for downstream biological application and is included here only to demonstrate that workflow.*

Particle Characterization After Dialysis

1. Measure the RNA concentration in the sample "RNA-LNP Final" by RiboGreen Assay using Precision NanoSystems' Encapsulation Efficiency Assay (Appendix).

2. Measure the size of the particles by DLS. For this, mix 30 µL of "RNA-LNP Final" with 300 µL of 1X Dilution Buffer. Measure the sample by DLS on the Malvern ZetaSizer Nano as indicated to confirm that no aggregates are present in the sample.

Note: *The "RNA-LNP final" is now ready for downstream applications. Please consult your local Precision NanoSystems representative for guidance.*

Appendix

A) Malvern Zetasizer - Recommended Parameters

Software Parameters

Material	Protein
Refractive Index	1.45
Absorption	0.001
Dispersant	PBS
Temperature	25 °C
Viscosity	1.0200 cP
Refractive Index	1.335
Dielectric Constant	80.4
General Options	
Mark-Houwink Parameter	
A parameter	0.428
K parameter (cm2/s)	7.67 e-05
Sample Viscosity	Use Dispersant Viscosity
Temperature	25 °C
Equilibration Time	2 min
Cell	Disposable Cuvette 97000-587

Measurement

Measurement angle	173 Back-scatter (NIBS default)
Measurement duration	Manual
Number of Runs	10
Run Duration (Seconds)	10
Number of Measurements	2
Delay Between Measurements	0
Append measurement Number to sample Name	
Mark-Houwink Parameter	
Positioning method	Fixed, 4.65
Attentuation setting	Automatic

Note: *The attenuation is indicated during measurement in the lower right corner of the measurement window of the Malvern Software. Attenuation is considered good within the range of 6–9. 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.

Data Processing

Analysis Model	General Purpose (normal resolution)

B) Encapsulation Efficiency Assay Protocol for RNA-LNPs

Additional Reagents/Disposables

Description	Ordering Information
Quant-iT Ribogreen Assay Kit	ThermoFisher (R11490)
Triton X-100	Sigma (cat: 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 plate	General Laboratory Supplier
Mg^{2+} / Ca^{2+} free PBS 1x	General Laboratory Supplier
18 Gauge Needles	General Laboratory Supplier

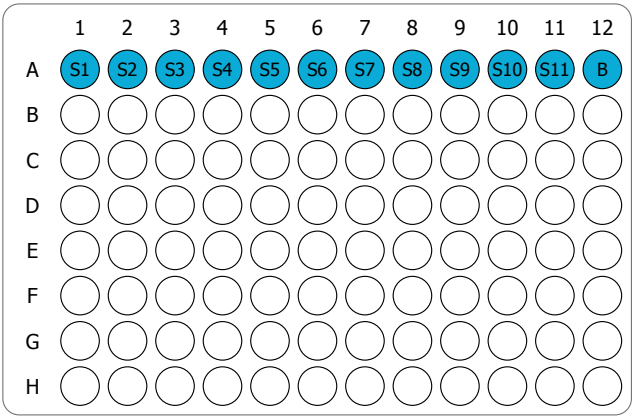
Additional Equipment Required

Description	Ordering Information
Synergy H1 Biotek Plate Reader	11-120-533
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 sample** to these wells **(S1-S11)**. Add **15 µL of PBS** to the blank well **(B)**.

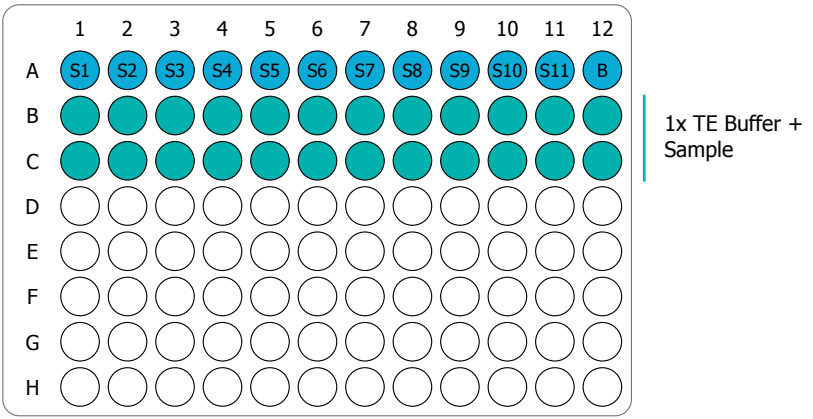
5. Using a multi-channel pipette, add 1X TE buffer to **Row A** to make up the volume to **250 µL**. Pipette to mix.



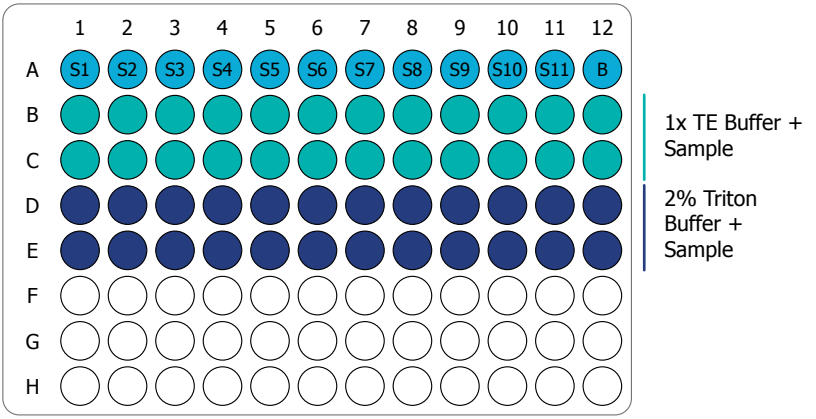
RNA-LNP Sample Setup

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 sample **(Rows B and C).**
2. Add **50 µL** of sample stock solution from Row A into the wells in **Row B and C.**



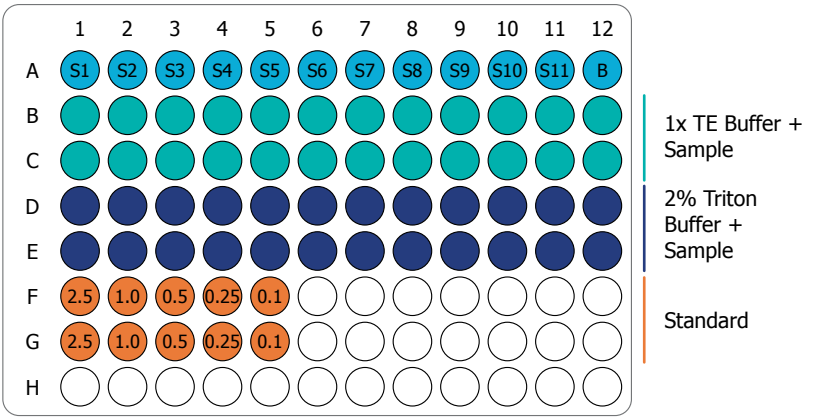
3. Add **50 µL** of Triton buffer to the wells in **Rows D and E** below each sample.
4. Add **50 µL** of sample stock solution from **Row A** into the wells in **Rows D and E.**



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 samples and standard curve are plated, incubate the plate at 37°C for 10 min to lyse the RNA-LNP in the presence of Triton buffer.

Preparation of Ribogreen Solution

1. Sum the total number of sample wells and standard curve wells. Add four 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 **10s** 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:

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 RNA-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 siRNA 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

GenVoy-ILM Reagents

Image	Name	Product Number
	GenVoy-ILM (2 mL, 5 mL)	NWW0041, NWW0042
	GenVoy-ILM with Dye (2 mL, 5 mL)	NWW0039, NWW0040

Instrument

Image	Name	Product Number
	NanoAssemblr Ignite Instrument	NIN0001

Cartridges

Name	Product Number
NanoAssemblr Ignite NxGen Cartridges (100, 200 pack)	NIN0061, NIN0062
NanoAssemblr Ignite NxGen Dilution Cartridges (50, 100 pack)	NIN0063, NIN0064

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For technical assistance and related documents:

- Contact your Field Application Scientist
- NanoMedU Hands-on Training Course: www.precisionnanosystems.com/resources-and-community/training-education/nanomedu
- Or email us at info@precision-nano.com
- Or call 1-888-618-0031.

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