



PROTOCOL

RNA-LNP Formulation Protocol (ALC-0315)

A step-by-step workflow for producing ALC-0315-based RNA-LNPs using TAMARA.

September 2026

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Introduction

Lipid nanoparticles (LNPs) have become the platform of choice for delivering messenger RNA (mRNA), small interfering RNA (siRNA) and other nucleic acid payloads into cells while shielding them from degradation [1,2]. The rapid development of mRNA vaccines during the COVID-19 pandemic gave the clearest public demonstration of what LNPs can achieve at scale, and the same system is now being extended toward a broad range of applications including protein replacement, gene editing, and cancer immunotherapy.

What makes an RNA-LNP therapeutic effective has as much to do with the particle built around the payload as with the RNA itself: LNPs protect nucleic acids from enzymatic degradation, promote cellular uptake, and facilitate endosomal escape of the payload into the cytoplasm [3]. Their performance can be adjusted through formulation parameters, such as lipid type and composition, as well as process parameters. This versatility makes LNPs adaptable to different nucleic acid payloads, therapeutic applications, and delivery requirements.

A conventional RNA-LNP consists of four lipid classes [4,5]:

- **Ionizable lipids** (40–50 mol%) represent the central functional component of RNA-LNPs. At acidic pH, below their acid dissociation constant (pKa, typically ~6.0–6.5), these lipids become positively charged and interact electrostatically with negatively charged RNA, promoting its encapsulation during LNP formation. At physiological pH, they remain largely neutral, helping to minimize cytotoxicity. Following cellular uptake, the acidic environment of the endosome promotes their protonation and interaction with anionic endosomal membrane lipids, facilitating membrane destabilization and release of the RNA payload into the cytoplasm [6–8]. ALC-0315, used throughout this protocol, is the ionizable lipid employed in the Pfizer-BioNTech COVID-19 mRNA vaccine. Other clinically established examples include SM-102 (Moderna) and DLin-MC3-DMA (Patisiran).
- **Helper phospholipids** (10–15 mol%) provide structural support to particle and can influence intracellular RNA release and *in vivo* circulation time [7,9]. DSPC and DOPE are among the most commonly used ones, with DSPC notably present in FDA-approved RNA-LNP formulations.
- **Sterols** (38–50 mol%) contribute to nanoparticle stability, modulate lipid packing and fluidity, facilitate membrane fusion, and decrease the adsorption of serum proteins to the LNP surface [7,9]. Cholesterol is the most widely used sterol and is the one used in clinically approved RNA-LNP formulations.

- **PEG-lipids** (1.5–2 mol%) form a hydrophilic steric layer at the nanoparticle surface that helps limit aggregation, enhance colloidal stability, extend circulation time, and help adjust particle size [7]. Representative PEG-lipids include ALC-0159, used together with ALC-0315 in the Pfizer-BioNTech formulation, as well as PEG-DMG (Moderna) and PEG-c-DMG (Patisiran).

This protocol uses the lipid composition of the clinically approved Pfizer-BioNTech COVID-19 mRNA vaccine, comprising **ALC-0315:DSPC:cholesterol:ALC-0159** at a molar ratio of 46.3:9.4:42.7:1.6, which has become a widely used research reference.

Beyond lipid composition, the **formulation process** itself is a key determinant of LNP quality. The formulation technique and associated process parameters can directly influence critical quality attributes (CQAs), including particle size, size distribution, RNA encapsulation, and ultimately biological performance. A controlled and reproducible manufacturing process is therefore essential for formulation development.

Microfluidic mixing enables rapid and reproducible RNA-LNP formation while providing precise control over key process parameters. **TAMARA**, Inside Therapeutics' plug-and-play microfluidic formulation platform, provides controlled mixing conditions in a user-friendly format and supports workflows ranging from early-stage formulation screening through *in vitro* and *in vivo* studies (Fig. 1).

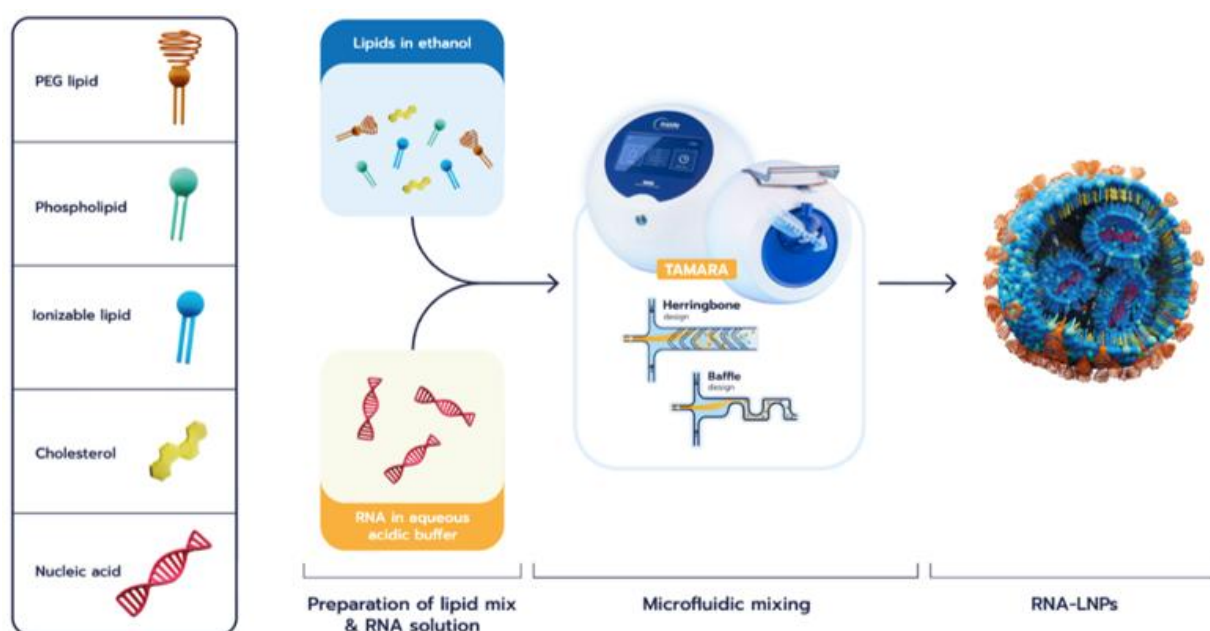


Figure 1. RNA-LNP formulation workflow using the TAMARA microfluidic system.

Materials

Table 1. Equipment, reagents and consumables required for RNA-LNP formulation.

Equipment	• TAMARA formulation system • Magnetic stirrer (if dialysis) or centrifuge (if centrifugal filter units) for buffer exchange • Dynamic Light Scattering (DLS) for colloidal analysis • Fluorescence microplate reader for RNA quantification (e.g., RiboGreen assay)
Reagents	• RNA stock solution (e.g., eGFP siRNA) • Lipid stock solutions (ALC-0315, DSPC, cholesterol, ALC-0159) • Ultra-pure ethanol for dissolving lipids • Aqueous buffer for RNA (e.g., 10 mM citrate buffer, pH 4.0) • Dilution buffer (e.g., 1X phosphate-buffered saline (PBS), pH 7.4)
Consumables	• Eppendorf and Falcon tubes • Dialysis membrane (e.g., Pur-A-Lyzer Midi kit, 3.5 kDa MWCO) if using dialysis • Centrifugal filters (e.g., Amicon Ultra, 100 kDa MWCO) if using centrifugal ultrafiltration • 0.22 µm filter (e.g., PVDF) if sterile filtration

Protocol for RNA-LNP formulation using TAMARA



RNase-free handling: Perform the protocol under RNase-free conditions. Clean work surfaces and pipettes with an RNase-decontamination solution and use RNase-free tubes and pipette tips throughout.

Step 1: Prepare the lipid phase

- Prepare a stock solution of each lipid in ethanol (e.g., 10 mg/mL) and store as single-use aliquots at -20°C to minimize repeated freeze-thaw cycles.
- Bring lipid stocks to room temperature (≥ 20 min) before use, and check they are fully dissolved – warm gently, vortex, or sonicate if needed.
- Prepare the **organic lipid phase** by combining the lipid components in a single tube in the molar ratio ALC-0315:DSPC:Cholesterol:ALC-0159 = 46.3:9.4:42.7:1.6, then add ultra-pure ethanol to reach the target concentration and volume. Mix by pipetting or gently vortexing until homogeneous.
 - **Calculation support:** Use our [RNA-LNP Formulation Calculator](#) to determine the lipid and RNA quantities required for your formulation and experimental scale.
 - **Formulation support:** Our [LNP Starter Kits](#), offered in collaboration with CordenPharma, provide pre-validated lipid compositions to simplify formulation preparation and accelerate development, including ALC-0315-based formulation.

Practical example: For a 1 mL formulation run at an aqueous-to-organic flow rate ratio (FRR) of 3:1, combine 750 μ L of aqueous RNA solution with 250 μ L of lipid mixture. Three replicate runs therefore require a total of 750 μ L of lipid mixture (3×250 μ L). It is recommended to prepare an excess volume to account for potential handling losses. Table 2 provides an example for the preparation of 1 mL of ALC-0315-based lipid mix, providing sufficient material for three replicate runs while allowing for these losses.

Table 2. Example lipid mixture for ALC-0315-based LNP formulation. The lipid mixture comprises ALC-0315:DSPC:cholesterol:ALC-0159 at a molar ratio of 46.3:9.4:42.7:1.6. Quantities are provided for the preparation of 1 mL of lipid mixture at a total lipid concentration of 5 mg/mL, using individual lipid stock solutions at 10 mg/mL. Combine 500 μ L of the prepared lipid mix with 500 μ L of ultra-pure ethanol to obtain a final volume of 1 mL.

Lipid	MW (g/mol)	Molar ratio (%)	Mass required (mg)	Volume from 10 mg/mL stock (μ L)
ALC-0315	766.3	46.3	2.80	280
DSPC	790.0	9.4	0.59	59
Cholesterol	386.6	42.7	1.30	130
ALC-0159	2464.2	1.6	0.31	31

Step 2: Prepare the RNA phase

- Prepare the **aqueous RNA phase** by calculating the RNA quantity required for the target N/P ratio. For ALC-0315-based RNA-LNPs, an N/P ratio of 6 can be used as a starting condition; optimization may be required depending on the RNA payload and experimental objective.
-  **N/P ratio** represents the molar ratio of positively charged amine groups (N) on the ionizable lipid to negatively charged phosphate groups (P) on the RNA.
- Thaw the RNA stock solution, preferably on ice to limit degradation.
- Dissolve the RNA in an acidic aqueous buffer (e.g., 10 mM citrate buffer, pH 4.0), adjusting concentration and volume to match your calculation.

Practical example (continued): For the 1 mL ALC-0315 formulation described above, using an N/P ratio of 6 and an FRR of 3:1, each run requires 750 µL of aqueous RNA phase. For the 22 base pair eGFP siRNA (44 nucleotides in total) used in this example, the calculated siRNA concentration in the aqueous phase is approximately 67 µg/mL in 10 mM citrate buffer (pH 4.0), corresponding to approximately 50 µg of siRNA per 1 mL formulation. Three replicate formulations therefore require 2250 µL of RNA phase (3 × 750 µL per run) and approximately 150 µg of siRNA. To account for handling losses, prepare approximately 2500 µL of RNA phase at 67 µg/mL, corresponding to approximately 167.5 µg of siRNA in total.

Step 3: Perform microfluidic mixing

3.1 Prepare the TAMARA formulation system

- Power on TAMARA and allow the instrument to complete initialization.
- Check that the microfluidic chip, reservoirs, and fluid-contact surfaces are clean and fully dry before starting.

3.2 Run a pressure test

Before the first run of the day, or when necessary, perform a pressure test as described in the [TAMARA user guide](#) (pp. 24-26).

3.3 Define chip, reservoir, solvent, and temperature settings

From the TAMARA home page, open the menu in the top-left corner and confirm the system settings for the run:

- Select the **micromixer geometry** from the “Chips” page. The staggered herringbone mixer (SHM) generally produces smaller particles at the same total flow rate (TFR), while the baffle mixer offers greater flexibility across formulation conditions.

- Select the appropriate **reservoir size** (small or large) from the “Reservoirs” page according to the volume required for the run.
 - Select the **organic solvent** from the “Solvent” page. Ethanol, isopropyl alcohol, acetone, and methanol are pre-configured in the system; other solvents can be added by entering their viscosity parameters.
-  Non-polar solvents, such as dichloromethane or chloroform, are not compatible with the cyclic olefin copolymer (COC) microfluidic chips.
- Enter the **laboratory temperature** on the “Temperature” page and confirm that the setting reflects the current room temperature before starting each run.

Practical example (continued): Select the staggered herringbone mixer (SHM) and small reservoir, set ethanol as the organic solvent, and enter the current laboratory temperature in the system.

3.4 Set microfluidic parameters

Click “New Run” to enter the microfluidic parameters for the formulation:

- **Flow Rate Ratio (FRR):** Ratio of the aqueous to organic phase flow rates.
- **Total Flow Rate (TFR):** Combined flow rate of the aqueous and organic phases.
- **Total volume:** Total formulation volume to be processed during the run.

TAMARA operating ranges: FRR 1:1–10:1, TFR 0.8–15 mL/min, and total formulation volume 0.2–30 mL.

 FRR and TFR both shape LNP characteristics and should be chosen to fit your experimental goal. A higher aqueous-to-organic FRR or a higher TFR generally yields smaller particles.

Practical example (continued): Set the FRR to 3:1 (aqueous:organic), the TFR to 1 or 5 mL/min, and the total volume to 1 mL.

3.5 Load the reservoirs

Load the aqueous RNA phase first, followed by the organic lipid phase, into their respective reservoirs.

 Dispense gently toward the center of the reservoir bottom, keeping the pipette tip near the liquid level as it rises to minimize bubble formation. Always load the aqueous phase before the organic phase ([TAMARA user guide](#), p. 31).

Practical example (continued): For the 1 mL formulation at an FRR of 3:1, load 750 µL of aqueous RNA phase into the aqueous reservoir, followed by 250 µL of organic lipid phase into the organic reservoir.

3.6 Install the chip and prepare collection

- Position the silicone gasket and microfluidic chip in the instrument, ensuring that the chip is correctly oriented.
- Close the lid securely and place a suitable collection tube in the designated holder.

3.7 Run the formulation

- Start the run and collect the resulting LNP sample in the collection tube.
- Follow the progress of the run in real time through the TAMARA interface. You can verify the selected parameters at the top of the screen during the run.

3.8 Clean the system

- Place a 50 mL falcon tube in the holder to collect waste.
- Run at least one full cleaning cycle: a purge followed by a wash with the recommended cleaning solutions.
 - **For consecutive runs using the same lipid mixture and RNA solution**, perform at least one wash using ultra-pure water in the aqueous reservoir and ethanol in the organic reservoir.
 - **When changing lipid composition or RNA solution**, perform a more extensive washing sequence, for example: (1) water/ethanol, (2) water/water, followed by (3) ethanol/ethanol.



The cleaning routine can be adapted to your experimental needs.

- After cleaning, confirm the system and the chip are dry and in good condition before the next run.

Step 4: Downstream processing

4.1 Buffer exchange

Following microfluidic mixing, the LNP suspension contains ethanol and acidic buffer. Buffer exchange removes residual solvent and transfers the particles into a physiologically compatible buffer such as PBS at pH 7.4. For small formulation volumes, dialysis and centrifugal ultrafiltration are practical options.

- **Dialysis** is a simple and cost-effective method for buffer exchange and removal of residual ethanol. In this process, rehydrate the dialysis membrane (e.g., a Pur-A-Lyzer 3500 Da Midi kit) with the desired buffer according to the manufacturer's instructions. Carefully load the LNP sample (typically 50–800 μ L for Midi kits) into the membrane and ensure proper sealing. Place the membrane in a sufficient volume of PBS and dialyze with gentle stirring at room temperature or 4°C overnight. The dialysis buffer may be replaced as needed to improve solvent removal. Although simple and economical, dialysis requires a relatively long processing time.
- **Centrifugal ultrafiltration** provides a faster alternative for buffer exchange and can simultaneously concentrate the LNP suspension. In this method, transfer the LNP sample to a suitable centrifugal filter device (e.g., Amicon Ultra centrifugal filter, 100 kDa MWCO) and perform repeated centrifugation and buffer-refill cycles with PBS to remove residual ethanol and exchange the formulation into the desired buffer. For example, three centrifugation cycles at 3600 $\times$ g for 30 min at 4°C may be used. Although faster than dialysis, centrifugal ultrafiltration is generally more costly.

4.2 Sterile filtration & storage

- **Sterile filtration:** Filtration of the LNP suspension through a 0.22 μ m filter (e.g., PVDF) is recommended to minimize the risk of microbial contamination, particularly for experiments involving sensitive cellular models or *in vivo* applications. However, some particle loss or aggregation may occur during filtration.
- **Storage:** Store the LNP formulation in PBS at 4°C for short-term use, ideally under an inert atmosphere (nitrogen or argon), for up to one week. For longer-term storage, freezing or freeze-drying (lyophilization) may be considered. Cryoprotectants, such as sucrose or trehalose, are commonly used to help preserve LNP stability during freezing and lyophilization.

Step 5: Characterization of RNA-LNPs

Characterization is crucial for assessing the quality, consistency, and performance of RNA-LNP formulations for their intended application. **Critical quality attributes (CQAs)**, including particle size, polydispersity, RNA encapsulation efficiency, and morphology, influence LNP stability, biodistribution, delivery performance, and safety. Table 3 summarizes commonly used analytical methods for evaluating these and other relevant attributes.

For routine physicochemical characterization in academic research laboratories, **dynamic light scattering (DLS)** is widely used to determine particle size and polydispersity, while fluorescence-based assays such as **RiboGreen** are commonly used to assess RNA encapsulation efficiency. For evaluation of LNP morphology and internal structure, **cryo-TEM** is widely regarded as the gold-standard technique.

Table 3. Common analytical assays for LNP characterization.

Quality attribute	Analytical methods
Particle size & size distribution	DLS; NTA; AF4-MALS
Zeta potential	ELS
Particle concentration	NTA
Morphology & structure	Cryo-TEM; SAXS; SANS
Lipid composition	HPLC-CAD; HPLC-ELSD; HPLC-MS
RNA/DNA encapsulation	RiboGreen/PicoGreen assay; QuantiFluor RNA/DNA kits; AF4-MALS-UV; AEX-HPLC-UV; IP-RP HPLC-UV
RNA integrity & purity	Capillary electrophoresis; AEX-HPLC-UV; IP-RP HPLC-UV
Transfection efficiency	RT-qPCR; Western blot; ELISA; flow cytometry; confocal microscopy
Cytotoxicity	Flow cytometry; MTT assay; WST-1 assay
Endosomal escape	Confocal microscopy; STED; FRET-based assays

(*) **Abbreviations:** **AEX-HPLC**, anion-exchange high-performance liquid chromatography; **AF4-MALS**, asymmetric flow field-flow fractionation coupled with multi-angle light scattering; **CAD**, charged aerosol detection; **cryo-TEM**, cryogenic transmission electron microscopy; **DLS**, dynamic light scattering; **ELISA**, enzyme-linked immunosorbent assay; **ELSD**, evaporative light scattering detection; **ELS**, electrophoretic light scattering; **FRET**, Fluorescence resonance energy transfer; **HPLC**, high-performance liquid chromatography; **IP-RP HPLC**, ion-pair reversed-phase high-performance liquid chromatography; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NTA**, nanoparticle tracking analysis; **RT-qPCR**, reverse transcription quantitative polymerase chain reaction; **SAXS**, small-angle X-ray scattering; **SANS**, small-angle neutron scattering; **STED**, stimulated emission depletion microscopy; **UPLC**, ultra-performance liquid chromatography; **UV**, ultraviolet; **WST-1**, water-soluble tetrazolium salt-1.

Example formulation data

ALC-0315-based LNPs were prepared using CordenPharma's LNP Starter Kit (Kit #1) on the **TAMARA microfluidic platform** equipped with a staggered herringbone mixer (SHM). eGFP siRNA was encapsulated at an N/P ratio of 6, with 1 mL formulations produced at TFRs of 5 and 10 mL/min while maintaining an FRR of 3. Following formulation, the LNPs were purified by dialysis and evaluated for particle size and polydispersity by DLS and for siRNA encapsulation using the RiboGreen assay.

At a TFR of 5 mL/min, the ALC-0315 LNPs displayed particle sizes of 76–78 nm, with PDIs around 0.13–0.22. Increasing the TFR to 10 mL/min resulted in slightly smaller particles of approximately 69–73 nm, while maintaining low PDIs around 0.19–0.24 (Fig. 2a). Encapsulation efficiency remained consistently high, at approximately 89–95% across the tested conditions (Fig. 2b).

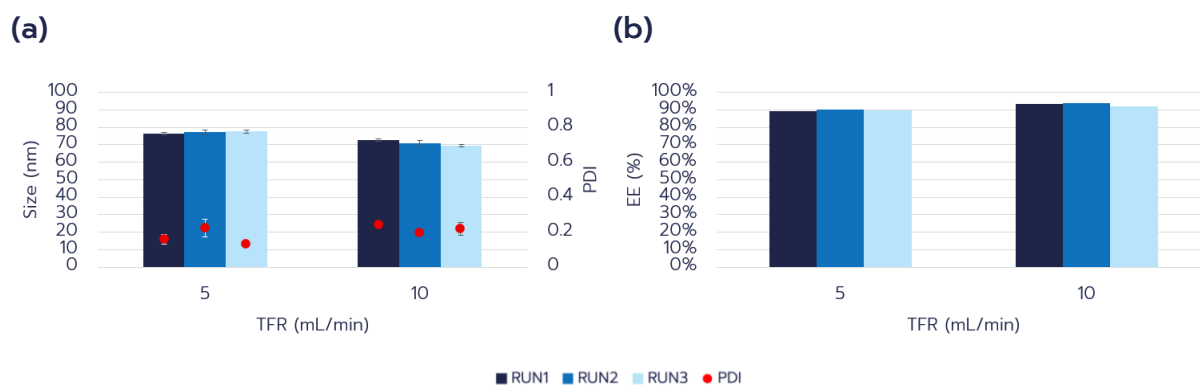


Figure 2. Physicochemical characterization of eGFP siRNA-loaded ALC-0315 LNPs formulated using TAMARA at TFRs of 5 and 10 mL/min following dialysis. (a) Particle size and polydispersity index (PDI). (b) Encapsulation efficiency (EE%).

Acknowledgments

We thank CordenPharma for providing the LNP Starter Kits used to generate the experimental data presented in this protocol.

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