Echelon Biosciences Inc.

Protocol for LNP Preparation and Analysis

This protocol is designed with predetermined molar percentages of lipids and RNA:lipid ratio. Other percentages and ratios may be prepared but calculations for alternative formulations should be adjusted accordingly by the end user.

Preparation of the lipid mixture

This procedure is for a 25 mM lipid stock of 50:10:38.5:1.5 % ionizable lipid:DSPC:cholesterol:PEG.

1. Bring all lipids to room temperature.
2. Bring ionizable lipids to 100 mM in EtOH and allow sufficient time for solubilization.
3. Bring DSPC and DMG-PEG-2000 to 10 mM in EtOH and cholesterol to 100 mM in EtOH.
   a. These may require heating to 60-65°C for complete solubilization in EtOH.
   b. Cholesterol in solution should be kept warm (>37°C) to maintain solubility.
4. For each mL of lipid stock add the following:
   - 125 µL of 100 mM ionizable lipid, 250 µL 10 mM DSPC, 96.25 µL 100 mM cholesterol*, 37.5 µL DMG-PEG-2000, and 491.25 µL EtOH.
   - *Note: cholesterol solution should be transferred as quickly as possible to avoid cooling within any tubing or pipette tips used for preparation.

This should result in 25 mM total lipid mix with 50% ionizable lipid (12.5 mM), 10% DSPC (2.5 mM), 38.5% cholesterol (9.625 mM), 1.5% DMG-PEG-2000 (0.375 mM).

All lipid solutions and lipid mixes should be sealed and stored at –20°C.

Preparation of RNA-LNPs

This procedure is for a 5:1 RNA:lipid mix (v:v). In our optimization experiments, the GFP mRNA used was diluted to 0.4 µM in sodium acetate buffer prior to mixing with lipids. LNPs may be prepared by hand mixing or by automated microfluidic devices. The procedure below describes small scale preparation using hand mixing, which can be scaled as needed.

1. Bring lipid mixes to room temperature prior to use and vortex as needed to mix.
2. Thaw RNA stocks on ice.
3. Once thawed, bring the RNA to the desired concentration using sodium acetate buffer.
4. Calculate the amount of lipid required to achieve a 5:1 RNA:lipid ratio. Ex: for every 100 µL of RNA add 20 µL of lipid mix.
5. Add the lipid mix to the RNA solution and mix rapidly. The solution should become cloudy and opaque. We do not recommend mixing by vortex as this may shear free RNA.
6. Once the LNPs have formed, they should be diluted (~20x) or dialyzed into a neutral pH buffer such as PBS.

For small scale preparations a 20x dilution is sufficient to neutralize pH and dilute out the EtOH from the lipid mix. Ex: LNPs formed in a 5:1 RNA:lipid ratio with a 100 µg/mL concentration can be diluted 20x to 5 µg/mL RNA and then diluted further in buffer, vehicle, or media for in vitro or in vivo testing. Dialysis should be used if higher RNA concentrations are desired or if the volume of LNPs prepared is sufficiently large, i.e. >5-10 mL. All LNPs should be 0.2 micron filter sterilized before use if not prepared under sterile conditions.

Once LNPs are in a neutral pH buffer they are stable for at least one week at 4°C. In our experiments 5 µg/mL RNA LNPs were stable at 4°C up to 30 days. Stability may vary depending on individual RNAs and ionizable lipids and should be monitored by the user.

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Encapsulation Efficiency

Quantitative – fluorescence-based quantification using reagents such as QuantiFluor®, Quant-it RiboGreen™, or similar products.

1. Prepare detection reagents as described by manufacturer in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).
2. Prepare a 1% v/v solution of Triton X-100 in TE buffer.
3. Add the 1% Triton X-100 solution to an aliquot of the LNPs for a 0.2% v/v final concentration and incubate on ice for 10-15 minutes. Add an equivalent volume of TE alone to separate aliquots of the LNPs.
4. Add the prepared detection reagent to a 96-well plate (white or black, not clear) and add the required amount of LNP samples (+Triton or +TE). Mix briefly and incubate at room temperature for 5 minutes. Protect from light.
5. Read the plate according to the excitation/emission spectra for the detection reagent.
6. Encapsulation efficiency (EE) is calculated as follows:

   EE = 100 * (T-U)/T

   where 'T' is the total RNA in the sample (+Triton) and 'U' is the free, unencapsulated RNA (+TE).

For absolute quantification of total encapsulated RNA, an RNA standard with known concentration should be run in parallel with the samples.

Qualitative – agarose gel-based assessment of encapsulated/protected RNA.

1. Prepare a 1% agarose (w/v) TAE gel with sufficient lanes for all samples and a gel ladder. Add nucleic acid dye such as SYBR Safe at a 1:10,000 dilution.
2. Prepare a 1% v/v solution of Triton X-100 in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).
3. Add the 1% Triton X-100 solution to an aliquot of the LNPs for a 0.2% v/v final concentration and incubate on ice for 10-15 minutes. Add an equivalent volume of TE alone to separate aliquots of the LNPs.
4. Add gel loading buffer to LNP samples (+Triton or +TE) and load all samples and the gel ladder to the prepared gel.
5. Run the gel for approximately 45 minutes at 100V and image under UV light.

Successful LNP preparation and encapsulation should result in little to no visible bands for the lanes of the gel with +TE (untreated) samples as the RNA is protected from the dye in the gel by the lipids. Conversely, +Triton (treated) samples should have readily apparent bands on the gel due to release of the RNA from the LNPs due to Triton treatment.

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