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Protocol

NanoFabTx™ DOTAP Lipid Mix- for synthesis of cationic (DOTAP) liposomes

Protocol for Catalog No. 926027

Introduction

NanoFabTx™ nanoformulation kits and lipid mixes enable users to encapsulate a wide variety of therapeutic drug molecules for targeted or extended drug delivery without the need for lengthy trial-and-error optimization. ***NanoFabTx™*** kits provide an easy-to-use toolkit for encapsulating a variety of therapeutics in nanoparticles, microparticles, or liposomes. The resulting particles are biocompatible and biodegradable and can be further modified to target specific tissues or to ensure slow and sustained drug release. Drug encapsulated particles synthesized with the ***NanoFabTx™*** kits are suitable for biomedical research applications such as oncology, immuno-oncology, gene delivery, and vaccine delivery.

The kits and mixes minimize laboratory setup with optimized protocols and step-by-step instructions for synthesizing drug-encapsulated liposome-based formulations. Protocols for two different particle synthesis methods are included: one for lipid film hydration/extrusion and one for microfluidics. The lipid film hydration/extrusion method uses standard laboratory equipment and glassware. The microfluidics method uses our ***NanoFabTx™*** device kit, complete with the microfluidics chip, fittings and tubing, and compatible pressurized pump systems to support your microfluidics-based liposome preparation (compatible microfluidics system or syringe pump required).

NanoFabTx™ **DOTAP Lipid Mix- for synthesis of cationic (DOTAP) liposomes** is designed for the synthesis of specifically sized cationic liposomes for gene delivery applications. The lipid mix contains rationally selected lipid in precise ratios that have been optimized to achieve a specific size range and positive charge for efficient complexation with negatively charged nucleic acids. The ***NanoFabTx™*** **DOTAP Lipid Mix, for synthesis of cationic (DOTAP) liposomes** includes a curated ready-to-use lipid mix and step-by-step protocols for conventional lipid film hydration/extrusion and microfluidics methods to synthesize approximately 100nm liposomes.

Disclaimer

NanoFabTx™ **DOTAP Lipid Mix- for synthesis of cationic (DOTAP) liposomes** is for research use only; not suitable for human use. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Specifications

Storage	Store *NanoFabTx™ DOTAP Lipid Mix- for synthesis of cationic (DOTAP) Liposomes* at -20°C. Protect from light.
Stability	Refer to the expiration date on the batch-specific Certificate of Analysis.

Materials

Materials required for use with both methods, but not supplied

Catalog Number	Quantity
459836	Ethanol, 200 proof
276855	Dimethyl Sulfoxide (DMSO)
GE17-0851-01	Disposable PD 10 Desalting Columns
27024	Glass vials, clear glass (4 ml capacity)
V7130	Glass vial, clear glass (20 ml capacity)
09978	Ammonium sulfate, BioUltra, ≥99.0% (T)
	RNase free water
SLFHX13	Syringe filters 0.45µm (for filtering non-aqueous solutions like ethanol)
SLHAR33SS	Syringe filters 0.45µm (for filtering aqueous solutions like buffer)
PURX12050	Pur-A-Lyzer™ Maxi Dialysis Kit
D8537	Dulbecco's phosphate buffered saline (DPBS), no calcium, no magnesium (pH 7.4)
	Deionized water

Materials required for use with the Microfluidics system, but not supplied

Catalog Number	Description
911593	NanoFabTx™ microfluidic – nano device kit
	Pressurized pump system or syringe pumps

Materials required for use with Extrusion, but not supplied

Catalog Number	Description
610000 ***or*** Z373400	Extruder Set with Holder Heating Block (Avanti Polar lipids) ***Or*** LiposoFast Liposome Factory (Avestin)
Z373419	LiposoFast Liposome Factory-pore size 100 nm, polycarbonate membrane
Z373427	LiposoFast Liposome Factory-pore size 200 nm, polycarbonate membrane

Before you start: Important tips for optimal results

Filter solutions. For best results, filter the lipid solution through a 0.45 µm syringe filter (Cat. No. SLFHX13NL) before use. In addition, filter the buffer or water through a 0.45-µm syringe filter (Cat. No. SLHAR33SS) before use.

Lipid film hydration/extrusion method – The 50mg lipid mix (Cat No. 926027-50mg) will result in approximately 2 ml of liposomes solution upon hydration and extrusion whereas the 100mg lipid mix (Cat. No. 926027-100mg) will make approximately 4 ml of liposome solution.

Microfluidics method – Volume of collected nanoparticles. The volume of liposomes suspension can be controlled by adjusting the running/collection time. If only a small volume (1-2 ml suspension) of liposomes is required, run the microfluidics system for 2-3 mins. Similarly, for larger volume, collect the liposome suspension for approximately 5 minutes.

Microfluidics method – Reduce blockages with proper cleaning. Clean the microfluidics system after synthesis of each batch of liposomes. Improper cleaning can result in blockages in the micromixing microfluidics chip and tubing. A well-maintained microfluidics chip can be used multiple times through cleaning and proper storage.

Microfluidics method – Prime the tubing and chip. Prime the tubing and the micromixing microfluidics chip before starting liposomes synthesis. Priming purges gases from the fluid pathways, conditions the chip surface and serves as a check of chemical compatibility for all wetted parts of the system. In addition, priming reduces or prevents precipitation of lipids inside the system in the case of backflow, jetting, or chaotic mixing. Precipitation of reagents can irreversibly block the microfluidics chip.

Procedure

Two procedures to synthesize cationic DOTAP liposomes are provided below: **Procedure 1 –Lipid film hydration and extrusion method** and **Procedure 2 –microfluidics-based method**. **Procedure 1** is a widely used method to prepare liposomes because of its scalability, simplicity, and lack of requirements for expensive laboratory instruments. **Procedure 2** is a microfluidics or continuous flow-based technique that results in narrow size distribution, enhanced control over each stage of particle fabrication, greater particle yields, ease of scalability, and excellent reproducibility. This microfluidics-based protocol uses the rapid micromixing method to synthesize liposomes.

The procedure below for siRNA encapsulation was developed and optimized to achieve a desired size range using a specific siRNA sequence as a model. The *NanoFabTx™ DOTAP Lipid Mix, for synthesis of cationic (DOTAP) liposomes* protocol can be modified for other therapeutics/genes of interest. The procedure below is suggested as a guide for your own optimization. The table below can be used to help you determine the lipid concentration.

Table 1: Total lipids and DOTAP (moles)

	TOTAL LIPIDS (µMOLE)	DOTAP (µMOLE)
CAT NO. 926027-50mg	84.67	38.7
CAT. NO. 926027-100mg	169.34	77.4

Prepare reagents

- 1. Prepare Ammonium sulfate (AS) buffer (240 mM, pH 5.4)
- Dissolve 31.71g of ammonium sulfate salt in 1 liter of deionized water
- The pH of the solution is natively 5.4, additional pH adjustment is not required
- Filter the AS buffer through a 0.45µm filter before use (Cat. No. CLS430625)

Procedure 1 – Prepare liposomes by hydration/extrusion method

- Lipid extrusion is a technique in which a lipid suspension is forced through a polycarbonate filter with a defined pore size. Liposome size is determined by the pore size of the filter (i.e. 100nm pore size filter results in approximately 100nm diameter liposomes). The extrusion process can be carried out with the suggested list of extruders in the materials table. The following protocol was optimized for liposome preparation using the Extruder Set with Holder Heating Block (Cat. No. 610000). See Figure 1 for more details.

- 1. Prepare lipid mix solution
- Remove the crimp seal/septum from the lipid mix vial and add RNAse free water to a final concentration of 25mg/ml (2ml for 50mg, 4ml for 100mg)

  Note: The lipid mix concentration is suggested as a guide for your own optimization. Lipid concentration will vary depending on desired N/P ratio. Please refer to Table 1 for molar concentrations.

- Vortex the solution for 5 mins to completely dissolve the lipid mix into the buffer.

  Note: Mild sonication can be used for dissolving the lipid mix completely.

2. Prepare multi-layer vesicles (MLVs)

- Place the vial containing the lipid mix solution in a water bath or incubator set at 65 °C.
- Gently shake for 1 hour at 65 °C.
- Complete five freeze-thaw cycles: 3 minutes on ice and 3 minutes in a 65 °C water bath to reduce MLV size.

- 3. Assemble the extruder
- Assemble the extruder as per manufacturer recommendation.

  Note: The extrusion process can be carried out with the suggested list of extruders in the materials table. The following protocol was optimized for liposome preparation using the Extruder Set with Holder Heating Block (Cat. No. 610000)

- Assemble the extruder with a 200 nm polycarbonate membrane (Cat No. Z373427)
- Keep the assembled extruder on a hot plate to maintain 65 °C.

Note: Use caution, do not touch hotplate during the extrusion process.

4. Extrude liposomes

- Load 1 ml of MLV solution in an extruder syringe (Syringe 1). Attach the syringe to one side of the extruder assembly.
- Attach an empty syringe (Syringe 2) on the other side of the extruder.

Figure 1: *The lipid extrusion process. The MLV solution in Syringe 1 is transferred to Syringe 2 by gently pushing on the plunger of Syringe 1. The solution is then transferred back to Syringe 1 by gently pushing on the plunger of Syringe 2. This process is repeated for a total of 11 cycles.*

- Gently push the plunger of Syringe 1 to completely transfer the MLV solution from Syringe 1 to Syringe 2 (Figure 1).
- Gently push the plunger of Syringe 2 to completely transfer the solution back to the original syringe (Syringe 1). This completes one cycle.
- Repeat the above steps 10 more times (for 11 total cycles). The solution should be completely transferred to Syringe 2 after the final pass-through.
- Remove the extruder from the block.
- Replace the 200nm polycarbonate membrane (Cat No. Z373427) with the 100nm polycarbonate membrane (Cat No. Z373419). The MLV solution should remain in Syringe 2.
- Gently push the plunger of Syringe 2 to completely transfer the MLV solution to Syringe 1.
- Gently push the plunger of Syringe 1 to completely transfer the MLV solution to Syringe 2. This completes one cycle.
- Repeat the above steps 10 more times (for 11 total cycles). The solution should be completely transferred to Syringe 1 after the final pass-through.
- Remove the extruder from the block.
- Remove the syringes from the extruder.
- Inject the lipid solution into a clean vial (approximately 1 mL) and store at 4°C until further use.
- For immediate gene loading, please proceed to the “siRNA complexation with cationic liposomes” section.
- For empty liposomes, centrifuge at 30,000-60,000 x g for 30 minutes to 1 hour to collect liposome pellets. If desired, liposomes can be lyophilized for long-term storage.

5. Clean the extruder

- Wipe the extruder apparatus with isopropyl alcohol and thoroughly dry it before storing.
- Rinse the syringes with isopropyl alcohol followed by DI water and thoroughly dry before storing.

Note: Membranes are intended to be used for a single liposome preparation and should not be reused.

6. Measure liposome size

- Measure the size of the liposomes with a dynamic light scattering instrument (DLS) and transmission electron microscopy (TEM).

Procedure 2 – Prepare liposomes by microfluidics-based method

A. Set up the microfluidics system

This microfluidics protocol is designed for use with the corresponding *NanoFabTx™ Microfluidic - Nano* Device kit (Cat. No. 911593) which contains all required device components for synthesizing liposomes and a detailed protocol for use with a syringe pump or pressurized pump system. Please follow the protocol with *NanoFabTx™ Microfluidic - Nano* Device kit (Cat. No. 911593) to set upthe microfluidics system. **Figure 2** shows a schematic of the microfluidics setup and tubing connections for a syringe pump system.

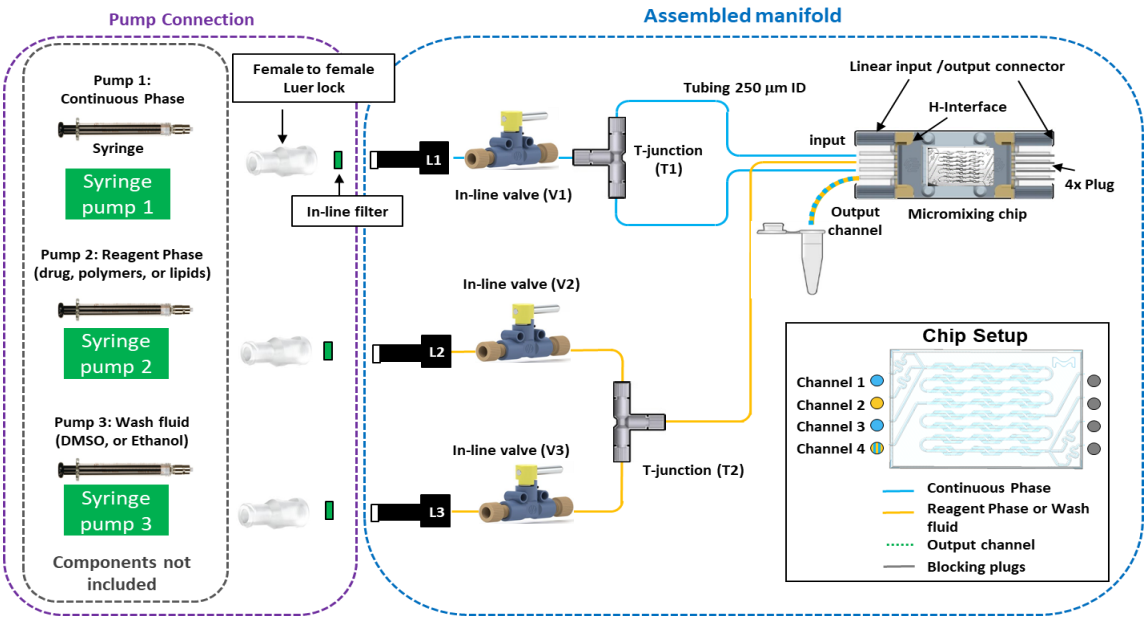

Figure 2: *Schematic of the microfluidics setup for a syringe pump. The manifold is supplied preassembled. The microfluidics chip is packaged separately. In-line filters and leur locks are supplied for connection to syringe pumps.*

B. Prepare liposomes by microfluidics

1. Filter RNAse free water

- Filter 10 ml of RNAse free water using syringe filter (Cat. No. SLHAR33SS) and add to a new glass vial (Cat. No. V7130)

2. Prepare lipid mix solution

- Remove the crimp seal/septum from the lipid mix vial and add ethanol (200 proof) for a final concentration of 22.4mM (3.78ml for 50mg, 7.56ml for 100 mg)

Note: The lipid mix concentration is suggested as a guide for your own optimization. Lipid concentration will vary depending on desired N/P ratio. Please refer to Table 1 for molar concentrations.

- Vortex the solution to completely dissolve the lipid mix
- Filter the lipid solution using a syringe filter (Cat. No. SLHAR33SS) into a 4 ml glass vial (Cat No 27024)

3. Assemble microfluidics system

- Assemble the microfluidics system as described in protocol with *NanoFabTx™ Microfluidic - Nano* Device kit (Cat. No. 911593)

Note: The synthesis of liposomes can be carried out in either a three-pumps (as shown in **Figure 2**) or two-pumps configuration (pump 1 and 2 only). In the three-pumps configuration, a vial of priming solution is kept in pump 3 throughout the process. Pump 3 can be used for the washing step without the need for swapping the vials in pump 2 when using a two-pumps configuration. For washing, simply close valve V1 and V2 and open valve V3 and start the flow of DMSO using the pump software.

4. Insert priming solvent DMSO into microfluidics system

- As shown in the setup illustrated in **Figure 2,** place a vial (Cat. No. V7130) of DMSO inside pump 1. Pump 1 is connected to channels 1 and 3 of the micromixing microfluidics chip.
- Place another vial (Cat. No. V7130) of DMSO inside pump 2 or pump 3. Pump 2 and pump 3 are connected to the channel 2 of the micromixing microfluidics chip via T-junction (T2).
- Keep valves V1, V2, and V3 closed. They connect to pump 1, pump 2, and pump 3 respectively.

5. Prime the system

- Place a waste collection vial at the output channel to collect waste generated during setup and priming.
- Open valve V2 to flush the assembled manifold with the DMSO (no lipid solution) by setting a flow rate of 100 µl/min for pump 2. Keep valve V3 closed if using the two-pumps configuration.
- Close valve V2 and open valve V1 to flush the assembled manifold with DMSO by setting a flow rate of 100 µl/min for pump 1. For further details on priming the system refer to the device kit protocol (Cat. No. 911593)

6. Prepare liposomes

- Place the vial (Cat. No. V7130) containing 10 ml of filtered RNAse-free water inside pump 1 and the vial (Cat No 27024) containing 2 ml of lipid mix in ethanol inside pump 2.
- Check that both valves V1, V2, and V3 are closed. Always keep valve V3 closed when using the two-pump configuration.
- Select the flow rates for pump 1 and 2 based on desired liposome size. An initial flow rate of 100ul/min for reagent phase and 250 ul/min for RNAse-free water is suggested. Increasing the flow rate ratio (reagent phase: water phase) decreases liposome size. A flow rate between 1:2 to 1:20 is suggested. **Table 2** provides liposome sizes achieved with various lipid concentrations using the same flow rate settings.

Table 2: Flow rate settings for synthesizing liposomes

Lipid concentration (mM)	Desired liposomes size (nm)	Flow rate ratio	Flow rate – Pump 2 Reagent phase Lipid mix solution (µl/min)	Flow rate – Pump 1 Water phase RNAse free water (µl/min)
11.2	113	**2.5**	**100**	250
22.4	118	**2.5**	**100**	250

- Open valve 1 and set the flow rate of pump 1 (RNAse free water) to 250µl/min.

  Note: This is a suggested flow rate for lipid concentrations of 11.2mM and 22.4mM and a desired liposome of approximately 115nm. Flow rate optimization may be required to achieve your desired liposome size and lipid concentration.

- Set the flow rate for pump 2 (lipid mix solution) to 100 µl/min and open valve 2.

  Note: This is a suggested flow rate for lipid concentrations of 11.2mM and 22.4mM and a desired liposome size of approximately 115nm. Flow rate optimization may be required to achieve your desired liposome size and lipid concentration.

- The flow rates of both solutions will stabilize within a few seconds.

  Optional: fluid flow of the two solutions can be visualized by a high-speed microscope.

- After the flow rates of the two solutions have stabilized, replace the waste collection vial with a sample collection vial at the output channel and collect the liposomes suspension.
- When you have collected the desired volume of the liposome suspension, transfer the output channel tubing to the waste collection vial, close valves V1 and V2, use the Flow Control Center software or syringe pump interface to stop fluid flow, and remove the solution vials from pump 1 and pump 2.

7. Remove excess ethanol from liposome suspension

- Transfer 1-2 ml of liposomes suspension into a dialysis cassette (Cat. No. PURX12050) and dialyze the samples against 1 L of 240 mM ammonium sulfate (AS) buffer for at least 4 hrs. Collect the purified samples and store at 4 °C for further use.
- Measure the size of the liposomes with a dynamic light scattering instrument (DLS) and transmission electron microscopy (TEM).
- For immediate loading, proceed to the “siRNA complexation of cationic liposomes” section.
- For empty liposomes, centrifuge at 30,000-60,000 x g for 30 minutes to 1 hour to collect liposome pellets. If desired, liposomes can be lyophilized for long-term storage.

8. Clean the microfluids system

Note: Clean the microfluidics system after each use using the method below. Improper cleaning can result in chip and tubing blockage.

- Follow this cleaning procedure after each run to remove any remaining lipid precipitates or deposited buffer.
- Use DMSO to clean the tubing and micromixing microfluidics chip. DMSO is the preferred cleaning solvent, because both the lipids and buffer have high solubility in DMSO.
- Filter 10 ml DMSO through a 0.45 µm syringe filter into each of two vials (Cat. No. V7130).
- Close valves V1, V2, and V3 and place a waste collection vial at the output channel tubing.
- Place the vials of filtered DMSO in pumps 1 and 2.
- Open valve V1 and set the flow rate of pump 1 to 100 µl/min.
- Set the flow rate of pump 2 to 100 µl/min and immediately open valve V2.

Note: If using a three-pump configuration, washing is not required for pump 3.

- Gradually increase the flow rate on both pumps to 300 µl/min. Run the system for 3 minutes to completely remove any lipids, or buffer precipitated inside tubing or micromixing microfluidics chip.
- When the cleaning process is complete, close valves V1 and V2 and use the software or pump interface to immediately stop the flow of the liquids through pumps 1 and 2.
- Remove the DMSO vials.
- Disconnect the linear input/output connectors and remove the micromixing microfluidics chip from the H-interface.
- Store the micromixing microfluidics chip in its box, or place it in another clean, dust-free environment.

siRNA complexation with cationic liposomes

Materials: siRNA (10 µM in stock)
Liposomes: NanoFabTx™ DOTAP liposomes, prepared from the NanoFabTx™-DOTAP lipid mix
Buffer: 100mM HEPES buffer, pH 7.4 (Cat No. H4034), sterile filtered or serum-free media

Guidance for Optimal Transfection Conditions

The protocol below was designed for the transfection of an siRNA sequence of 21 bp long and an N/P ratio of 3.5. The volumes and concentrations of siRNA and liposomes listed are specific to these experimental conditions and will need to be optimized for your desired application.

It is recommended to optimize the following conditions: cell lines and seeding densities, siRNA-targeted sequences and treatment concentrations, N/P ratio (quaternary amines (N) of DOTAP/Phosphate (P) of siRNA), liposome-siRNA (lipoplexes) complexation time and cell treatment/incubation time.

Note: Complexation of siRNA-NanoFabTx™ DOTAP liposomes must be prepared in serum-free media or buffer.

1. Prepare siRNA stock
    - Dilute siRNA to 1 µM with HEPES buffer or serum-free media

2. Prepare DOTAP liposome stock
    - Dilute preformed liposomes to 0.25mg/ml (193.5µM DOTAP) with HEPES buffer or serum-free media

3. Liposome-siRNA complexation

Note: The volumes listed are specific to the following experimental parameters:

Total lipoplexes volume: 15 µl (follow recommendations in Table 3 for cells cultured in one well of a 24-well plate)
N/P ratio: 3.5
siRNA sequence: 21 bp long
The N/P ratio is calculated as the ratio of quaternary amines (N) of DOTAP/ the phosphate (P) of the siRNA. For this example protocol, N/P =3.5 = 1.1 nmol amines (from DOTAP)/ 315 pmol phosphate (from siRNA)

- Add 5.7 µl of the liposome stock (193.5 µM DOTAP) to an empty tube

  Note: 5.7 µl is equivalent to 1.1 nmol DOTAP (1.1 nmol amines)

- Add 1.8 µl of HEPES buffer or serum-free media

- Add 7.5 µl of the siRNA stock to the liposome stock

  Note: 7.5 µl is equivalent to 7.5 pmol siRNA (315 pmol phosphate)

- Incubate for 15 minutes at room temperature

Optional Step: Remove uncomplexed siRNA by ultracentrifugation prior to adding the liposomes to cells.

4. Add liposome-siRNA (lipoplexes) to cells following recommended volumes and concentrations in Table 3.
5. Treat cells for 24-72 hours at 37°C
6. Analyze transfected cells according to experimental design (ie. fluorescence, qPCR, etc.)

Table 3. Suggested experimental conditions for siRNA transfection

Cell Culture Conditions

Cell culture plate	**96-well**	**24-well**	**6-well**
Adherent Cells	~0.26×10^5	~0.13×10^6	~0.78×10^6
Cell Culture Medium (with Serum)	0.15 mL	0.75 mL	4 mL

Formulation Conditions

Cell culture plate	**96-well**	**24-well**	**6-well**
siRNA per well (1 µM)	1.5 µL (Final: 10nM, 1.5 pmol)	7.5 µL (Final: 10nM, 7.5 pmol)	40 µL (Final: 10nM, 40 pmol)
NanoFabTx DOTAP liposomes per well (0.25mg/ml)	1.1 µL (N/P ratio 3.5)	5.7 µL (N/P ratio 3.5)	30.4 µL (N/P ratio 3.5)
HEPES buffer per well	0.4 µL	1.8 µL	9.6 µL
Lipid-siRNA complex per well	3.0 µL	15.0 µL	80.0 µL

Tips for siRNA transfection via cationic liposomes:

- When optimizing transfection efficiency, use a constant siRNA concentration and vary the amount of liposomes solution to adjust the N/P ratio and fully entrap the nude siRNA. To start, a suggested treatment concentration of 10nM is recommended.
- siRNA entrapment at various N/P ratios can be tested using gel electrophoresis. Nude siRNA and incomplete entrapped siRNA will present as a dim to bright band on an agarose/ethidium bromide gel. It is suggested to choose an N/P ratio where the band is no longer present, and the siRNA cannot be detected (see Figure 3 for details). Use this or higher N/P ratio to prepare lipoplexes.
- Once the N/P ratio has been determined, siRNA concentrations can be adjusted to determine the best transfection efficiency conditions.

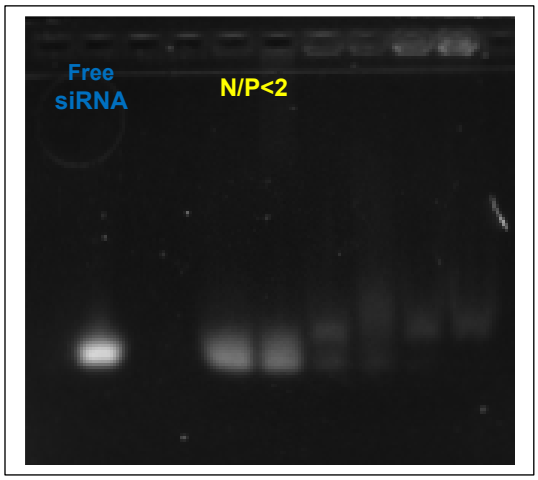

Figure 3. liposome-siRNA (lipoplexes) complexation at different N/P ratios. Model siRNAs were incubated with NanoFabTx DOTAP Lipid Mix at different N/P ratios in HEPES buffer for 15 minutes at room temperature and run on a 1% agarose/ethidium bromide gel. Nude model siRNA showed the brightest band on left. Lipoplexes with incomplete entrapped siRNA showed dim siRNA band at N/P < 2

Troubleshooting

Detailed troubleshooting on the microfluidics setup is provided in the troubleshooting guide included in the *NanoFabTx™ Microfluidic - Nano, device kit* (Cat. No. 911593). Due to the numerous connections between microfluidics components, and the narrow flow paths for the fluids, you may encounter leaks or blockages. This section presents information on and potential solutions for commonly encountered problems.

1. Liposomes are not in the desired size range

Possible cause – This protocol is optimized for synthesis of liposomes in the 80 nm to 120 nm using the model drug, doxorubicin. If you encapsulate a different drug in liposomes, the size of your liposomes may vary from the size range reported here.

Possible cause – Procedure 2 is optimized for synthesis of liposomes in the size ranges of 80 nm to 100 nm using a pressurized pump system or the recommended syringe pumps. The flow rates listed in the protocol are optimized using pressurized pumps with flow sensors attached. If you use different flow rates, or alternative syringe pumps, the size of your liposomes may vary from the size range reported here.

2. Polydisperse liposomes

PDI is the standard deviation of the particle diameter distribution divided by the mean particle diameter. PDI is used to estimate the average uniformity of a particle solution; higher PDI values correspond to a greater size distribution in the nanoparticle sample. A sample is considered monodisperse when the PDI value is less than 0.1

Possible cause – Polydisperse samples can result as a result of improper extrusion process.

Solution – Follow the instructions properly using the right membrane filters with every batch to minimize polydispersity.

Possible cause – In the microfluidics method, polydisperse samples can occur if the flow in the tubing or micromixing microfluidics chip is uneven or blocked.

Solution – The next sections provide tips to minimize uneven flow or remove blockages.

3. Uneven flow in the microfluidics-based method

Possible cause – Uneven flow can be caused by bubbles of air in the system.

Solution – Fluid flowing through the system will clear bubbles within 1–2 min. You can usually see the bubbles passing through the micromixing microfluidics chip. If this approach does not remove the bubbles, sonicate the solutions for 30 min and vent the pressure chamber.

Possible cause – If the flow becomes unstable when the microfluidics system has been in operation for a while, one of the solution supplies may have run dry or the pick-up tubing might not reach to the bottom of a vial.

Solution – Check that the vials contain enough reagent and that the 250-µm pick-up tubing is long enough to collect from the bottom of each vial.

Possible cause – If none of the above solutions leads to even flow, the software may need to be rebooted.

Solution – Stop all flow, close and reopen the Flow Control Centre software, and restart flow. If this method does not solve the problem, the system may have a blockage. Check for blockages as detailed in the next section.

Possible cause – If the system has no blockages, the flow sensor may not function correctly.

Solution – Replace the flow sensor.

4. Leak in system

Possible cause – Changes/fluctuations in system pressure or flow rate can arise from a leak in the system.

Solution – Before troubleshooting a possible blockage, make sure that all connectors are properly fitted and that the system has no apparent leaks.

5. Blockage of tubing or micromixing microfluidics chip

Possible cause – During the synthesis of liposomes using the microfluidics setup, the introduction of dust fibers, deposition of precipitated buffer/lipids, or drying of buffer/lipids inside the micromixing microfluidics

chip or tubing, or improper cleaning procedures can cause blockage in the micromixing microfluidics chip or tubing.

Several indications suggest that a partial or complete blockage has occurred:

a. Consistent flow rate is maintained when a pump is in flow control mode, but the pressure increases.
b. Consistent pressure is maintained when a pump is in pressure control mode, but the flow rate decreases.
c. The instrument software has set changes to the flow rate, but apparent flow rate does not change.
d. The flow is significantly slower than expected.
e. The flow rate fluctuates unexpectedly and affects droplet stability.

Possible cause – If a partial or transitory blockage is present, the pressure may increase gradually, then suddenly drop as the blockage moves along the flow path, and then increase again when the obstruction becomes lodged.

Solution – Blockages can occur anywhere in the flow path of the system; identifying the location of a blockage is a process of elimination.

Start with the micromixing microfluidics chip, because sometimes blockages (dust or hair) are visible under a microscope. If you find a blockage on the chip, monitor it while you vary the pump pressure to try to dislodge it. If a blockage on the chip cannot be cleared, the chip will need to be replaced.

If you see no physical blockage in the micromixing microfluidics chip, disconnect the chip interface and check whether liquid flows from the tubing. If liquid now flows from the disconnected tubing the blockage is likely either in the chip or the connector was improperly seated against the chip. If the system has a T-connector that splits the flow of a solution into two inputs, check that the flow rates through each input are identical. If the flow is asymmetric, a blockage could be somewhere between the T-connector and the chip. First replace the tubing and see if this fixes the problem; if not, replace the T-connector.

If it is not already apparent which line is blocked, vary the flow rate of the solutions one at a time while observing the ends of the tubing. This step will help to identify which line is blocked.

Work your way back through the system, from the chip to the pump, one component at a time, and check for stable flow at each stage. When you find the section that contains the blockage, simply replace it.

The blockage may have occurred because of particulate contamination in your solution(s). Refilter solutions through a 0.45µm syringe filter before use.