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Title: Formulating and Characterizing Lipid Nanoparticles for Gene Delivery using a Microfluidic Mixing Platform

Authors and Affiliations: Christina M. Bailey-Hytholt[1]*, Paroma Ghosh[1]*, Julia A. Dugas[1], Isidro E. Zarraga[1], and Amey Bandekar[1]
*These authors contributed equally

[1]Biologics Drug Product Development & Manufacturing, Sanofi

Corresponding Authors:
Amey Bandekar
amey.bandekar@sanofi.com

Christina M. Bailey-Hytholt
christina.bailey-hytholt@sanofi.com

Co-authors:
paroma.ghosh@sanofi.com
julia.dugas@sanofi.com
dan.zarraga@sanofi.com

Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees self-record interview statements outside of the filming date. JoVE can provide support for this option.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **48**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Christina Bailey-Hytholt**: This protocol can be used to produce nucleic acid-encapsulated lipid nanoparticles with a high reproducibility and speed and low volumes. The formulation parameters can also be tuned to achieve a desired biodistribution based on the clinical application **[1]**.

1.1.1. LAB MEDIA: Christina Bailey-Hytholt.mp4.

REQUIRED:

1.2. **Paroma Ghosh**: The staggered herringbone design of the microfluidic cartridge allows a rapid, precise, and controlled laminar flow during mixing. The method is amenable to scaling-up and generates uniform, highly encapsulated particles **[1]**.

1.2.1. LAB MEDIA: Paroma Ghosh.MOV

OPTIONAL:

1.3. **Julia Dugas**: With most commercially approved gene therapy products relying on viral methods, this procedure can provide insight into gene delivery, as its nonviral approach allows applications for which repeat dosing is necessary **[1]**.

1.3.1. LAB MEDIA: Julia Dugas 1.MOV.

Protocol

2. Lipid Mix and Nucleic Acid Solution Preparation - Paroma

2.1. Begin by adding the appropriate amount of each lipid stock solution to a glass vial with intermittent vortexing **[1]** as indicated in the Table **[2]** … followed by the addition of 200 proof ethanol to a final volume of 533 microliters **[3]**.

2.1.1. WIDE: Talent adding solution(s) to vial

2.1.2. LAB MEDIA: Table 1

2.1.3. Talent adding ethanol to vial, with ethanol container visible in frame
Videographer: Important step

2.2. Then add the appropriate amount of mRNA/milliliter of citrate buffer to a final volume of 1.5 milliliters **[1]**.

2.2.1. Talent adding mRNA to buffer, with mRNA and buffer containers visible in frame
Videographer: Important step

3. Microfluidic Channel Priming - Christina

3.1. To prime the microfluidic channels, first enter the priming parameters into the instrument software as outlined in the Table **[1]**.

3.1.1. LAB MEDIA: Table 2 *Video Editor: please emphasize Priming column*

3.2. Next, open the instrument lid **[1]** and place a microfluidic cartridge into the rotating block **[2]**.

3.2.1. WIDE: Talent opening lid
3.2.2. Talent placing cartridge into block

3.3. Load at least 500-microliters of ethanol into a 1-milliliter syringe, taking care that there are no bubbles or air gaps at the syringe tip **[1]**, and insert the syringe into the right inlet of the cartridge **[2]**.

3.3.1. Talent loading ethanol into syringe, with ethanol container visible in frame
3.3.2. Talent loading syringe into inlet

3.3.3 Added shot: Instrument running.

3.4. Load a 3-milliliter syringe with 1.5-milliliters of aqueous buffer, taking care that there are no air bubbles or gaps **[1]**, and insert the syringe into the left inlet of the cartridge **[2]**.

3.4.1. Talent loading buffer into syringe, with buffer container visible in frame
3.4.2. Talent loading syringe into inlet

3.5. Place two 15-milliliter conical tubes into the clip holders to serve as waste containers **[1]** and click **Run** to mix the solutions **[2]**.

3.5.1. Talent placing tube(s) into holder(s)
3.5.2. Talent clicking Run

3.6. When the instrument stops priming, as indicated by shutting off of the bottom blue light **[1]**, open the lid and properly dispose of the conical tubes and syringes **[2]**.

3.6.1. Shot of blue light, then light shutting off
3.6.2. Talent opening lid and/or removing material(s)

4. Lipid Nanoparticle (LNP) Formation - Christina

4.1. For lipid nanoparticle formation, set the appropriate formulation parameters as indicated in the Table **[1]** and load a 1-millilter syringe with the previously prepared lipid mix **[2]**.

4.1.1. LAB MEDIA: Table 2 *Video Editor: please emphasize Formulation column*
4.1.2. Talent loading mix into syringe

4.2. Remove any air gaps or bubbles at the syringe tip **[1]** and insert the syringe into the right side of the cartridge **[2]**.

4.2.1. Gaps and/or bubbles being removed *Videographer: Important/difficult step*
4.2.2. Talent loading syringe into inlet

4.3. Load the previously prepared nucleic acid solution into a 3-milliliter syringe, taking care that there are no bubbles or air gaps in the syringe tip **[1]**, and insert the syringe into the left inlet of the cartridge **[2]**.

4.3.1. Talent loading solution into syringe *Videographer: Important/difficult step*
4.3.2. Talent loading syringe into inlet

4.4. Place a 15-milliliter RNase-free conical tube labeled with the sample name into the left tube clip **[1]** and place a 15-milliliter waste conical in the right tube clip **[2]**.

4.4.1. Talent placing tube into tube clip
4.4.2. Talent placing tube into tube clip

NOTE: Shot number 4.4.1. and 4.4.2. were combined and filmed together as one shot.

4.5. Then dilute the lipid nanoparticles with 5 milliliters of PBS **[1]**, close the instrument lid, and click **Run [2]**, retaining the conical tube with the lipid nanoparticle sample at the end of the formulation process **[3]**.

4.5.1. Talent closing lid and/or clicking Run
Added shot: Instrument running.
4.5.2. Talent removing LNP tube OR Shot of LNP sample
4.5.3. Talent at BSC adding PBS to LNP, with PBS container visible in frame

5. Buffer Exchange - Julia

5.1. To perform a buffer exchange, pre-wash a 100-kilodalton-pore size ultracentrifuge filter with 2 milliliters of PBS **[1-TXT]** and empty the PBS from the bottom compartment **[2]**.

5.1.1. WIDE: Talent adding PBS to filter **TEXT: 5 min, 1000 x *g*, RT**
Added shot: Talent centrifuging.
5.1.2. Talent emptying PBS

5.2. Add the diluted lipid nanoparticles to the top compartment of the pre-washed ultra-centriuge filter for three centrifuge washes **[1-TXT]**, discarding the flow through and adding 5 milliliters of PBS to the ultra-centrifuge filter after the first two washes **[2]**.

5.2.1. Talent adding LNP to filter *Videographer: Important step* **TEXT: 12 min, 1000 x *g*, RT, x3**
5.2.2. Talent discarding flow through and/or adding PBS, with PBS container visible in frame *Videographer: Important step*

5.3. After the last wash, pipet the lipid nanoparticle solution against the walls of the ultra-centrifuge filter a few times to minimize the nanoparticle loss **[1]** before transferring the nanoparticle solution to a nuclease free vial **[2]**.

5.3.1. Talent pipetting solution against filter wall

5.3.2. Talent adding solution to vial.

NOTE: Shot number 5.3.1. and 5.3.2. were combined and filmed together as one shot.

5.4. Then add PBS to the bring the nanoparticle suspension to the appropriate experimental concentration as necessary to a final volume of 1 milliliter **[1]**.

5.4.1. Talent adding PBS to tube, with PBS container visible in frame

6. Encapsulation Efficiency Measurement - Christina

6.1. To assess the encapsulation efficiency of the lipid nanoparticles, first prepare 2-fold serial dilutions of working nucleic acid solution in PBS to generate a standard curve, starting from 500 nanograms/milliliter and making at least five dilutions **[1]**.

6.1.1. WIDE: Talent adding nucleic acid to tube(s), with PBS and nucleic acid containers and dilution tubes visible in frame

6.2. Next, prepare the nanoparticle sample dilutions in PBS to achieve an approximate theoretical concentration that lies around the mid-point of the standard curve **[1]**.

6.2.1. Talent adding LNP to tube(s), with PBS and LNP containers visible frame

6.3. Mix 0.5% RNA reagent with 0.4% Triton X-100 and 99.1% PBS to generate RNA quantification reagent **[1]** and mix 0.5% RNA reagent with 99.5% PBS to obtain quantification reagent without Triton X-100 **[2]**.

6.3.1. Talent adding reagent to Triton X-100 and PBS, with reagent, Triton X-100, and PBS containers visible in frame.
6.3.2. Talent adding reagent to PBS, with reagent and PBS containers visible in frame.

NOTE: Shot number 6.3.1. and 6.3.2. were combined and filmed together as one shot.

6.4. Load at least four replicates of each nanoparticle and nucleic acid standard solution into each of four wells of a black, fluorescence-capable, 96-well plate **[1]** and add an equal volume of RNA quantification reagent with and without Triton X-100 to half of the standard and sample replicates **[2]**.

6.4.1. Talent adding LNP and/or standard to well(s), with LNP and standard containers visible in frame *Videographer: Important step*
6.4.2. Talent adding reagent to well(s), with reagent container visible in frame

NOTE: Shots 6.4.2. and 6.5.1. and their corresponding VO text were combined together.

6.5. Add an equal volume of reagent without Triton X-100 to the remaining wells **[1]** and shake the plate in the plate reader for 5 minutes at room temperature protected from light to obtain a thorough mixing of the samples **[2]**.

NOTE: Step number 6.5. was split and shot number 6.5.1. along with its VO text was combined with 6.4.2. while the rest of the VO was added to step 6.6.

6.5.1. Talent adding reagent to well(s), with reagent container visible in frame
Videographer: Important step
6.5.2. Talent placing plate into plate reader.
NOTE: Shot 6.5.2. was dropped and hence not filmed.

6.6. Shake the plate in the plate reader for 5 minutes at room temperature protected from light to obtain a thorough mixing of the samples **[1]**. Then measure the fluorescence on a microplate reader at an excitation wavelength of 480 nanometers and an emission wavelength of 520 nanometers **[2]**.

NOTE: The remaining VO text from step 6.5. was added at the beginning of step 6.6.

6.6.1. Talent placing plate into reader
6.6.2. Added shot: Talent setting instrument parameters and clicking read

7. LNP Hydrodynamic Size and Polydispersity Measurement - Paroma

7.1. To measure the hydrodynamic size and polydisperity of the lipid nanoparticles, first dilute the nanoparticle solution 40 times in PBS **[1]** and add the solution to a semi-micro cuvette **[2]**.

7.1.1. WIDE: Talent adding PBS to LNP, with PBS container visible in frame
7.1.2. Talent adding solution to micro cuvette

7.2. Load the cuvette onto the zetasizer **[1]** and select a standard operating procedure to set the instrument to measure the nanoparticles according to their material, dispersant, temperature, and cell type **[2]**.

7.2.1. Talent loading cuvette into instrument
7.2.2. Talent setting measurement parameters

7.3. Then click **Start** to measure the hydrodynamic size and polydisperity of the lipid nanoparticles **[1]**.

7.3.1. Talent clicking Start

8. LNP Zeta Potential Measurement - Julia

8.1. To measure the zeta potential of the lipid nanoparticles, dilute the nanoparticle solution 40 times in nuclease free water **[1]** and load the solution into a cuvette to the fill line **[2]**.

8.1.1. WIDE: Talent adding LNP to water
8.1.2. Talent add solution to cuvette

8.2. Load the cuvette onto a zeta potential analyzer, taking care that the electrodes make contact with the instrument **[1]** and set the instrument to measure the zeta potential according to the specific makeup of the lipid nanoparticles **[2]**.

8.2.1. Cuvette being loaded with electrodes contacting instrument
8.2.2. Talent setting instrument parameters

8.3. Then click **Start** to measure the zeta lipid nanoparticle potential **[1]**.

8.3.1. Talent clicking Start

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.

2.1., 2.2., 4.2., 4.3., 5.2., 6.4., 6.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.2., 4.3. – NanoAssemblr set up (no air bubbles and correct volume)
Dilution step after NanoAssemblr before the wash (immediately) – this step needs to be added into the protocol.

Results

9. Results: Representative LNP Formulation and Characterization

9.1. In this analysis, multiple batches of lipid nanoparticles with the same lipid formulation and amine-to-phosphate ratio were developed on separate days to demonstrate the reproducibility of the technique **[1]**.

9.1.1. LAB MEDIA: Figure 2

9.2. As observed, Batches 1 and 2 exhibited overlapping size distributions with a similar polydispersity **[1]** with no significant differences observed in the size **[2]** or encapsulation efficiency between the batches **[3]**.

9.2.1. LAB MEDIA: Figure 2 *Video Editor: please individually emphasize data lines*
9.2.2. LAB MEDIA: Figure 2 *Video Editor: please blue data bars*
9.2.3. LAB MEDIA: Figure 2 *Video Editor: please green data bars*

9.3. Typically, changes in the formulation parameters induce some small, yet statistically significant differences **[1]**.

9.3.1. LAB MEDIA: Figure 3

9.4. For example, decreasing the amine-to-phosphate ratio results in a 4% decrease in the encapsulation efficiency **[1]** with a concomitant increase in the hydrodynamic diameter of the nanoparticles **[2]**.

9.4.1. LAB MEDIA: Figure 3 *Video Editor: please add/emphasize green bracket and asterisks*
9.4.2. LAB MEDIA: Figure 3 *Video Editor: please add/emphasize blue bracket and asterisks*

9.5. Lipid nanoparticles formulated with different ionizable lipids but the same amine-to-phosphate ratio exhibit a significant change in the encapsulation efficiency **[1]** as well as slight differences in particle diameter **[2]**.

9.5.1. LAB MEDIA: Figure 3 *Video Editor: please add/emphasize green bracket and asterisks*
9.5.2. LAB MEDIA: Figure 3 *Video Editor: please add/emphasize blue bracket*

9.6. The encapsulation of plasmid DNA results in larger particles **[1]** compared to mRNA encapsulating lipid nanoparticles **[2]**, although both types of nanoparticle demonstrate a similar encapsulation efficiency **[3]**.

9.6.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize blue pDNA data bar*
9.6.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize blue mRNA data bar*
9.6.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize green data bars*

9.7. Changes in the flow rate process parameter, however, do not impact the lipid nanoparticle development **[1]**.

9.7.1. LAB MEDIA: Figure 4 *Video Editor: please add brackets and n.s. texts OR no animation*

Conclusion

10. Conclusion Interview Statements

10.1. **Amey Bandekar**: Lipid nanoparticles have many applications. For example, they are currently being used in COVID-19 vaccine delivery. This technique paves the way for future applications by allowing low volume formulation screening **[1]**.

10.1.1. LAB MEDIA: Amey Bandekar.mov.