NanoGenerator Flex Lipid Nanoparticle Synthesis System

Introduction

Nanoparticles are at the leading edge of the rapidly developing field of nanotechnology. Their unique size-dependent properties make these materials superior and indispensable in many areas. They have been used in many industries, such as drug delivery, energy, and electronics. Nanoparticle synthesis is one of the key steps to enable nanoparticle applications.

The microfluidic miniaturized reactors of the PreciGenome NanoGenerator Flex nanoparticle synthesis system enable rapid reagent mixing and precise spatial-temporal manipulation of reactions. Using such microfluidic synthesis methods results in smaller and more uniform nanoparticles than traditional bulk synthesis. The physicochemical properties of nanoparticles can also be precisely controlled in a reproducible manner. Controlling the reaction environment leads to improvements in the quality of nanoparticle size distribution, higher reproducibility, and ultimately increased preparation process yield of nanoparticles.

NanoGenerator Flex provides a wide throughput range of 0.1–12 mL, which meets the needs from early discovery to early pre-clinical study.

NanoGenerator Flex Systems:
Flex S (Left) and M (Right)

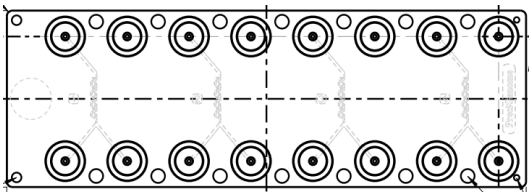
System Benefits

- Tunable and controllable particle size
- Low PDI
- High encapsulation efficiency
- Low payload consumption (<0.2ml output for Flex-S)
- Wide application from early screening to early pre-clinical study
- OEM service and module integration available

Microfluidic Chip

PreciGenome offers a variety of microfluidic chips in different materials to meet most of our customers' application requirements for nanoparticle synthesis. Three types of materials, including polymers, glass, and silicon, are commonly used to fabricate microfluidic chips. Chip material selection depends on the application requirements, including chip design, types of solvent or reagent used for experiments, needs of applications, budget, fabrication time, etc.

CHP-MIX-4 is a polymer based microfluidic mixer chip which is specifically designed for the NanoGenerator System. One CHP-MIX-4 chip contains four individual mixing channels.



Microfluidic Mixer Chip (CHP-MIX-4)

System Components

NanoGenerator Flex Instrument & Chip

- Nanogenerator Flex-S

- Or Flex-M, 1 set

- Microfluidic mixer chips, 3 pcs

Flex-M accessories

- Reservoir kit, 15ml, 2 sets

Flex-S accessories

- Reagent tanks, 10 pc
- Silicon gaskets, 2 pc

Working Mechanism

The following section will describe results of experiments done in-house by PreciGenome to show the performance of our product. The microfluidic passive mixing chip CHP-MIX-4 is used in the experiments.

Working mechanism for Flex-M: In liposome synthesis, the aqueous solution ("aqueous" phase) and lipids in ethanol ("organic" phase) are loaded in 15mL reservoir. During experiments, pressures from the controller were applied to the reservoir kits. Solutions in the reservoir kits were pushed into the PTFE tubings. The pressures were adjusted based on the readings from the flow senors and the preset flow rates were reached and stabilized quickly. Before the stabilization of the flow rates, both solutions would go to the waste bottle to avoid synthesized liposomes with non-uniform sizes. Once the flow rates reached the preset values, the valves would switch to the ports connected to the microfluidic chip. Two phases would be mixed in the microfluidic chip to form liposomes. The mixed solution (liposome solution) was collected from the outlet of the microfluidic chip. Users can optimize the mixing ratio, flow rates, and synthesis effect by changing the flow rate settings using the pressure controller.

Working mechanism for Flex-S: Reagent solutions are loaded in reservoir tanks. Preset pressures are directly applied to the reservoir tanks. The solutions are pushed into channels in the mixing chips. Pressures are set in such a way that a total flow rate and flow ratios are fixed.

Payloads

- DNA/mRNA/siRNA
- Small molecule drugs
- Proteins and peptides
- Other payloads

Applications

- Drug delivery
- Nucleic acid lipid nanoparticles (LNP) synthesis
- Polymeric nanoparticles, such as PLGA, PLGA-PEG
- Gel particle synthesis

Application Notes – Liposome/Lipid Nanoparticle Synthesis

Materials and Experimental

Materials

Reagent list included PG-LipidFlex (PreciGenome), SM102 ionizable lipid (Cayman Chemical), gWiz GFP DNA plasmid (Aldevron), ethanol (99%, Sigma), sodium acetate buffer (3M, pH 5.2, Sigma), PBS 1x (Corning), Triton X-100 (Sigma), DNA Quantification Assay Kit (BioVision), deionized water (MilliQ) and ethanol (>99%, Sigma).

Instrument list included NanoGenerator Flex (PreciGenome), nano particle analyzer (SZ-100V2, HORIBA) and 96-well plate reader (ELx808, Tecan Spectrasfluor Plus).

Lipid solution preparation

For liposome generation, PG-LipidFlex (PreciGenome, 30 mM in ethanol) was directly diluted to 15 mM in pure ethanol. For DNA lipid nanoparticle synthesis, SM102 ionizable lipid was mixed with PG-LipidFlex (SM102/LipidFlex = 40/60 molar ratio) and further diluted in ethanol to give a working solution of 12.5 mM total lipid concentration. Before sample loading, lipid working solution was passed through a 0.45 µm PES filter to remove dust and large particles.

Aqueous solution preparation

For liposome generation, sodium acetate buffer 100 mM pH 5.2 (Sigma) was used directly. For DNA lipid nanoparticle (LNP) synthesis, gWiz GFP (5757 bp, Aldevron) was dissolved in sodium acetate buffer (100 mM, pH 5.2, Sigma) to give a DNA working solution (93.5 µg/mL, N/P ratio 5.5). Before sample loading, DNA working solution was passed through a 0.45µm PES filter to remove dust and large particles.

Small volume synthesis using Flex-S Mode (0.2–2 mL)

Connect the Flex-S flow unit to the iFlow Touch pressure controller. Switch software to Flex-S mode. Load a microfluidic mixing chip on the chip holder. Pre-fill the mixing channel with 5 µL ethanol and insert the reagent tank unit to the of corresponding mixing channel. Load buffer or DNA working solution to the “aqueous tank” and lipid working solution to the “solvent tank”. Optionally pre-load PBS to the sample collection tank for in-line dilution. The total flow rate and flow rate ratio is fixed in Flex-S mode. Select the controller channels in use and input the target product volume. Save the recipe then press the start button; liposome or LNP product will be generated in seconds. For size and PDI analysis, 100 µl liposome or DNA encapsulated LNP product was dissolved in 1 mL PBS. The diluted solution was then analyzed in a nano particle analyzer.

Large volume synthesis using Flex-M Mode (2–12 mL)

Connect the Flex-M mounting fixture to pressure controller. Switch software to Flex-M mode. Load lipid and aqueous solutions into two separated Falcon centrifuge tubes (15 or 50mL). Connect the tubes to NanoGenerator system through compatible adaptors in the starter kit. Connect a clean tube to the product collection adaptor. Load a microfluidics mixing chip on the chip holder. Connect the chip with both aqueous and oil inlets, as well as the product outlet. Set the total flow rate and flow rate ratio (W:O), then press start button. Liposome and DNA LNPs are generated automatically. 100ul Liposome or DNA encapsulated lipid nanoparticle product was dissolved in 1 mL PBS. The diluted solution was used for size and PDI analysis in a nano particle analyzer.

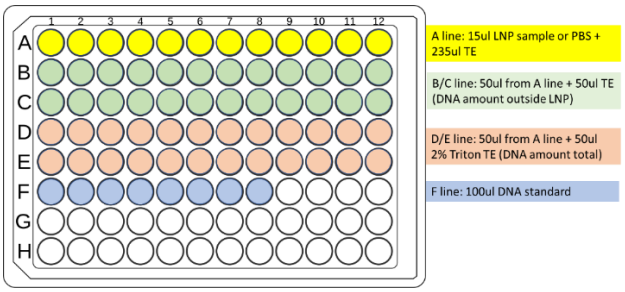
DNA encapsulation efficiency measurement

DNA Quantification Assay Kit (BioVision) was used following manufacturer’s protocol. Briefly, on a 96-well plate, add 15ul Lipid nanoparticle sample a well on line A. Then add 235ul 1x TE buffer to the same well to get 250ul sample stock solution.

Add 50ul 1x TE buffer to wells on line B and C. Transfer 50ul sample stock solution from line A to line B and C, in which lipid nanoparticle structure is still well maintained. In this case, DNA amount outside LNP is detected.

Add 50ul Triton buffer (2% Triton X-100 in 1x TE buffer) to wells on line D and E. Transfer 50ul sample stock solution from line A to line D and E, in which lipid nanoparticle structure is destructed and DNA is released. In this case,

total DNA amount is detected.



Prepare fluorescence dye working solution according to manufacturer's protocol. Add 100ul fluorescence dye working solution to each sample well on the plate (line B to F). Incubate 5 min in dark before fluorescence reading (E_x=492nm, E_m=585nm).

Plot the calibration curve based on the fluorescence signal of DNA standard. Then use the calibration equation to calculate the DNA amount of each sample well. The encapsulation efficiency (E.E.%) is calculated by the following equation:

$$E.E.\% = \frac{Total\ DNA\ amount - outside\ LNP\ DNA\ amount}{Total\ DNA\ amount} \times 100\%$$

In vitro cell transfection

HepG2 (human liver cancer cell line) was cultured in Eagle's Minimum Essential Medium (EMEM) with 10% FBS. K562 (human leukemia cell line) was cultured in RPMI with 10% FBS. HepG2/K562 cells were seeded 24 hours before transfection at the density of 2–4 x 10^4 cells/well. Lipid nanoparticles with GFP DNA plasmid were added into cells at the concentration of 400 ng DNA/well. The cells were incubated at 37 ℃ in 5% CO_2 atmosphere.

Nucleus staining and GFP fluorescence imaging

48 hours after transfection, HepG2/K562 cells were stained with Hoechst 33342 solution (blue fluorescence) for nucleus imaging. Briefly, Hoechst 33342 stock solution (1mg/ml in DI water) was diluted into 10 ug/ml in PBS right before staining. Add 20ul diluted Hoechst solution into cells. Incubated at r.t. for 10 minutes. Then the cells were investigated in a fluorescence microscopy with blue and green fluorescence channels.

Results and Discussion

The NanoGenerator Flex system can achieve a wide throughput range of 0.1–12 mL by using either S or M modules. The Flex-S module is for small volume synthesis (0.1–2 mL) for early screening, while Flex-M module is for large volume synthesis (2–12 mL) for animal studies.

Both Flex-S and M use the same microfluidic mixing chip (CHP-MIX-4). This feature allows convenient data transfer from early screening to large production.

Size Tuning by Total Flow Rate (TFR):

The size of liposomes and LNPs can be tuned by adjusting the total flow rate (TFR). In general, higher TFR results in faster mixing, which generates smaller liposomes and LNPs. For example, synthesized liposomes were 43.1 nm with TFR 5 ml/min, increasing to 64.9 nm with TFR 1 ml/min.

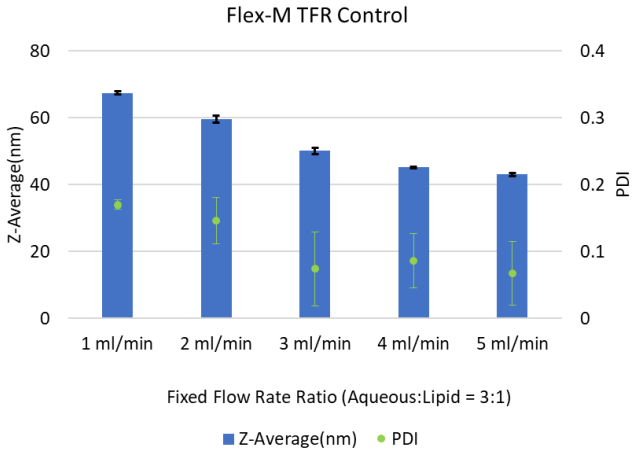


Figure 1. Liposome size tuning by total flow rate with Flex-M module. Sodium acetate buffer (100 mM, pH 5.2) and LipidFlex ethanol solution (15 mM) were used as "aqueous" and "solvent" phases correspondingly. Flow rate ratio (W:O) was fixed at 3:1.

Size Tuning by Flow Rate Ratio (W:O ratio):

Under the same TFR, liposome or LNP size was slightly changed by adjusting the aqueous to solvent flow rate ratio (W:O ratio). Although W:O = 3:1 is the most widely used ratio for liposome or LNP preparation, users of the Flex-M may set it from 2:1 to 5:1 while maintaining a narrow size distribution (**Figure 2**).

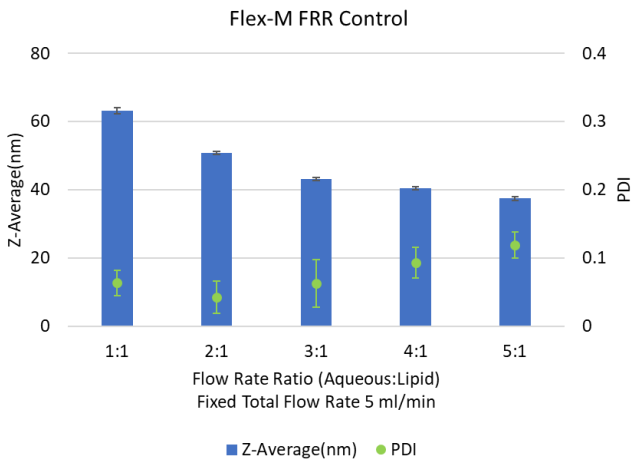


Figure 2. Liposome size tuning by flow rate ratio with Flex-M module. Sodium acetate buffer (100 mM, pH 5.2) and LipidFlex ethanol solution (15 mM) were used as "aqueous" and "solvent" phases correspondingly. Total flow rate was fixed at 3 ml/min.

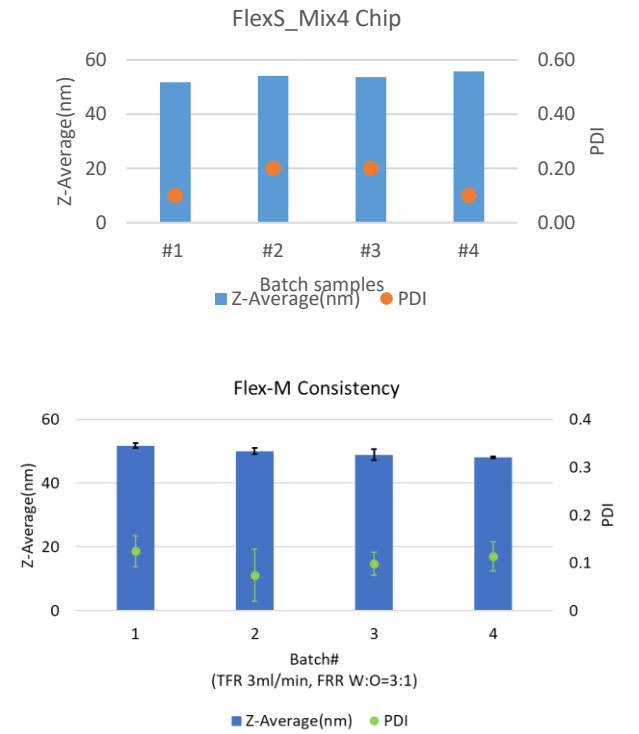


Figure 3. Batch consistency by Flex-S and Flex-M modules. Sodium acetate buffer (100 mM, pH 5.2) and LipidFlex ethanol solution (15 mM) were used as "aqueous" and "solvent" phases correspondingly. Total flow rate and flow rate ratio were preset in Flex-S module.

Batch-to-Batch Consistency

Batch to batch variation is the main problem of conventional bulk preparation methods. But empowered by advanced microfluidics technology, the PreciGenome NanoGenerator Flex system can generate highly consistent and reproducible results and reduce batch to batch variation. **Figure 3** shows the results from four individual samples with the same formulation. Both Flex-S and M modules have excellent repeatability.

Nucleic Acid Encapsulated LNPs

Liposomes have a hollow interior protected by a lipid bilayer. This structure makes them an ideal vehicle for payloads such as DNA, mRNA, protein etc. The size, PDI and encapsulation efficiency are therefore critical parameters for LNPs. The following paragraphs show the capability and flexibility to prepare DNA encapsulated LNPs using PG-LipidFlex and Nanogenerator Flex.

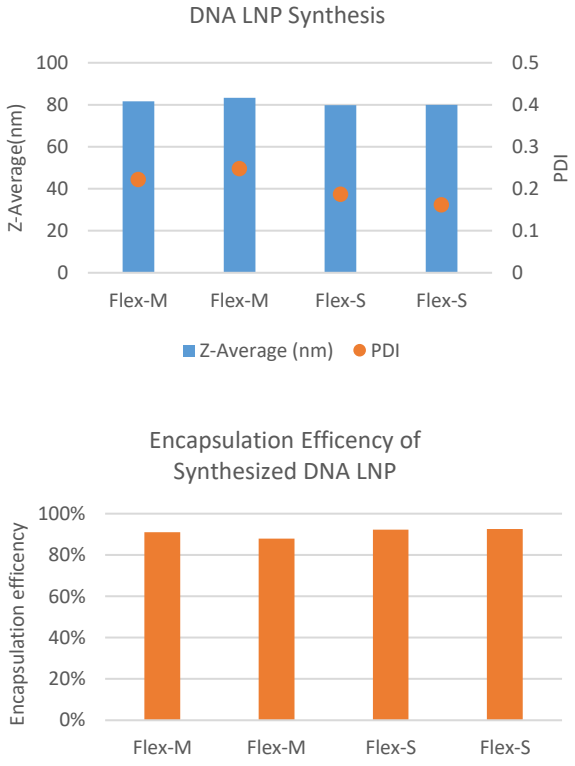


Figure 4. Size, PDI (upper) and encapsulation efficiency (lower) of synthesized DNA LNP. DNA working solution (93.5 µL/mL, sodium acetate buffer 100 mM, pH 5.2) and SM102/LipidFlex working solution (40/60 mol%, 12.5 mM total lipid) were used as "aqueous" and "solvent" phases correspondingly. Flex-M was set to 3 ml/min TFR and 3:1 (W:O) FRR. Total Flow rate and flow rate ratio was preset in Flex-S module.

PG-LipidFlex is a 3-component lipid mixture including

structured lipid, cholesterol, and stabilizer. By mixing PG-LipidFlex with a cationic/ionizable lipid of choice, users can easily generate nucleic acid encapsulated LNPs using NanoGenerator Flex. Here we used gWiz GFP DNA plasmid as a model payload for demonstration.

SM102 was used as the ionizable lipid and mixed with PG-LipidFlex to give a total lipid concentration of 12.5 mM (SM102/LipidFlex = 40/60 mol%). GFP DNA plasmid was dissolved in sodium acetate buffer (100 mM, pH 5.2) to 93.5 µg/mL (N/P = 5.5). PBS was pre-loaded into the sample collection tube/tank to increase product stability. Synthesized DNA LNPs shared similar size (~80nm), PDI (0.15-0.25) and encapsulation efficiency (>90%). (**Figure 4.**) This demonstrates the consistency between Flex-S and Flex-M results, allowing convenient data transfer from early screening to large production.

***In vitro* Cell Transfection using Synthesized DNA LNP**

The *in vitro* cell transfection study was conducted on HepG2 and K562 cell lines. 24 hours before transfection, HepG2 and K562 cells were seeded in a 96-well plate at a concentration 2-4x10^4 cell/well. On the transfection day, GFP DNA plasmid in SM102 LNPs were added to HepG2/K562 cell medium at a concentration of 400 ng/well.

48 hours after transfection, HepG2 cells were treated with Hoechst 33342 (1 µg/ml) for nucleus staining. Both green (GFP expression) and blue (nucleus) fluorescence images were recorded. Selected green field / blue field overlay image and bright field image are shown in **Figure 5 (upper).** Green field / bright field images of K562 cells are shown in **Figure 5 (lower).**

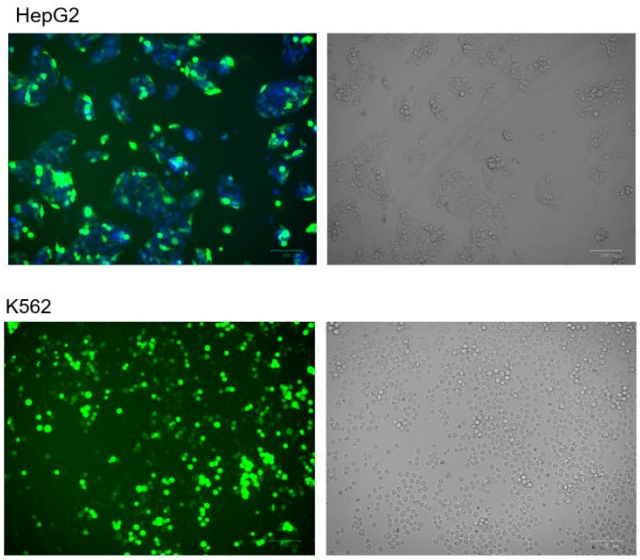


Figure 5. HepG2 (upper) and K562 (lower) fluorescence and bright field images 48 hours after treated with synthesized GFP DNA lipid nanoparticles.

Conclusions

The NanoGenerator Flex system is a robust and convenient tool for liposome and LNP synthesis. The synthesized product shows excellent size control, high uniformity, and narrow size distribution. High cell transfection efficacy of synthesized GFP DNA LNPs was also demonstrated using HepG2 and K562 cell lines.

The NanoGenerator Flex system is available in two different modules, S and M, which provide a wide throughput range between 0.2–12 mL. DNA LNPs synthesized by Flex-S and Flex-M show high consistency in size, PDI and encapsulation efficiency. This indicates the feasibility of data transfer from small to large scale synthesis. The NanoGenerator Flex system therefore provides an economical solution from early discovery to early pre-clinical studies.

References

Chiesa, E, et.al., The Microfluidic Technique and the Manufacturing of Polysaccharide Nanoparticles, *Pharmaeutics,* 2018, 10:267-289