ELVEFLOW
PLUG & PLAY MICROFLUIDICS
an ELVESYS brand

Elveflow User Guide

LIPID NANOPARTICLE SYNTHESIS PACK

DOCUMENT REF: UGLNP22-0204

Introduction

Figure 1: Lipid Nanoparticles collection

The lipid nanoparticle synthesis pack offered by Elveflow is designed for researchers with no prior knowledge in microfluidics and/or lipid nanoparticle generation who are aiming at easily synthesizing lipid nanoparticles (LNP) using microfluidic techniques.

Simple and intuitive instructions are provided to quickly and smoothly make lipid nanoparticles with fine control over nanoparticle generation parameters. When starting, the user can follow the provided step-by-step protocol to obtain LNPs of the desired size and composition, while optimizing the polydispersity index, using the numerous tips provided throughout the document. Finally, this guide provide numerous information to master the LNP fabrication technique, including how to automate the cleaning process and adapt the fluidic system to fully benefit from the versatility offered by this lipid nanoparticle generation system.

This pack has been tested for the fabrication of liposomes and solid lipid nanoparticles (SLNs), see figure 2. However, this Pack can be used to generate other types of nanoparticles, depending on your needs. LNPs are now widely used in biomedical applications as nanocarriers, as they are biocompatible, have high encapsulation efficiencies and can be optimized for a variety of different targets. The principal fields of applications, among others, are in drug delivery, gene therapy and vaccines. LNPs have attracted a lot of attention in light of the Covid-19 pandemic for their use as mRNA nanocapsules in Pfizer/BioNTech and Moderna vaccines.

As most LNP applications involve the encapsulation of molecules (cancer drugs, mRNA, siRNA...), control over their size is very important.

The size of the nanoparticles will determine the number of molecules that can be encapsulated, influence LNP interaction with cells and biological tissues and the kinetics of the release phase. Even a small difference in particle diameter can lead to very different results in terms of drug delivery efficiency.

Moreover, heterogeneous size distribution will result in variability in encapsulation and release efficiencies.[1]

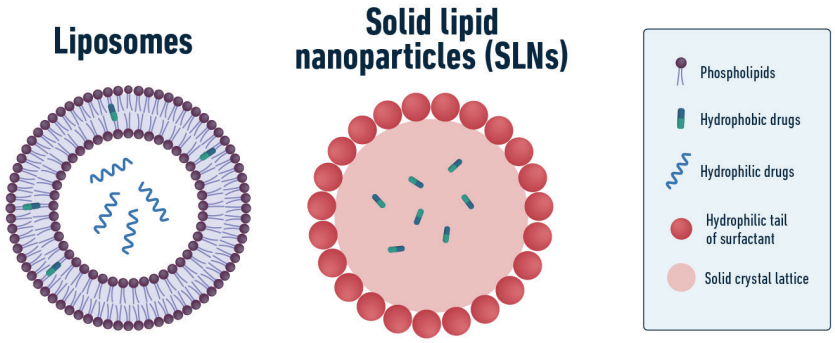


Figure 2: Illustration and composition of liposomes and solid lipid nanoparticles

Historically, the generation of LNPs was carried out using standard bulk processes (precipitation, emulsion, solvent evaporation and sonication). However, these techniques suffer from broad size distribution and poor batch-to-batch reproducibility. This is highly problematic in clinical trials and production stages of drug development. Microfluidics is a highly promising alternative that has attracted a lot of attention for its advantages as a LNP fabrication method. Some of these advantages include:

- Reduced mixing time
- Increased homogeneity
- High monodispersity (polydispersity index (PDI) lower than 0.2)
- Continuous production and high throughput
- Nanoparticle production integration and automatisation
- Wide range of monodisperse nanoparticle sizes possible due to precise flow rate control
- Excellent repeatability from one run to another
- Possibility of working with both small (µL) and large (L) volumes using the same system

[1] *Danaei, M et al. "Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems." Pharmaceutics vol. 10,2 57. 18 May. 2018, doi:10.3390/pharmaceutics10020057*

How does lipid nanoparticle generation work?

There are a few ways to generate lipid nanoparticles in microfluidics. In any case, the most important part relies on the fast mixing of two phases (organic and aqueous). The more efficient and homogeneous the mixing is, the better the control over the size and its distribution.

Figure 3 illustrates how the mixing speed influences the LNPs size; a slow dilution of the ethanol phase (a) leads to larger particles in comparison with a faster dilution (b). This pack will offer you different mixing strategies depending on your needs.

Lipid nanoparticles are typically made using one of these two microfluidic chip designs:

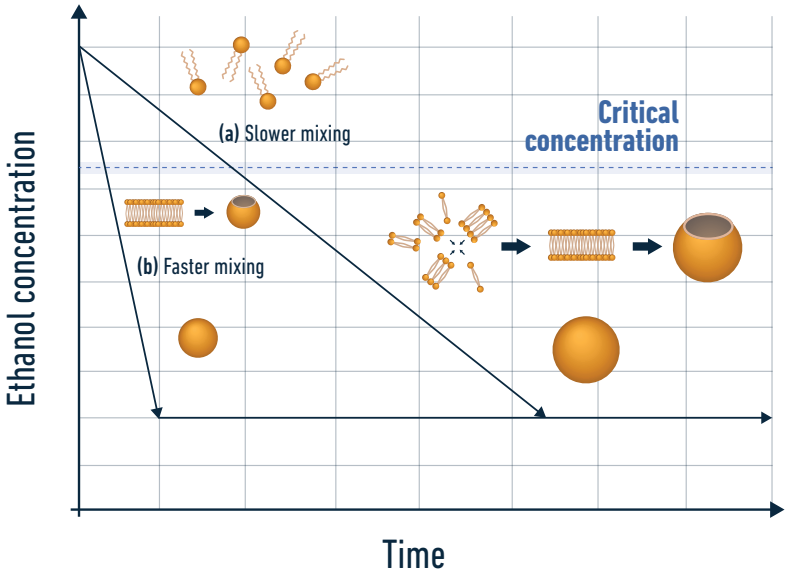


Figure 3: Illustration of the mixing time influence over the lipid nanoparticle size

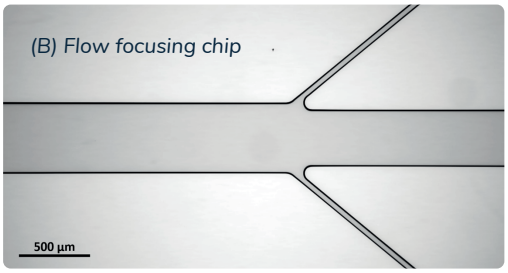


Figure 4: Picture of the (A) herringbone mixer and of the (B) flow focusing microfluidic chip

The first micromixer chip is based on a herringbone mixer where the two streams of reagents are mixed by micro-vortices created in a ridged microchannel.

The second chip relies on a “flow focusing” method. The flow of the organic phase (here, the lipid solution) is squeezed between two streams of the aqueous phase (water or buffer), which leads to the formation of nanoparticles by nanoprecipitation.

Both these chips have a common goal, even though they do not use the same strategy to obtain it, that is ensuring the fastest mixing and control of the organic phase and the aqueous phase. Indeed, a fast dilution of the ethanol phase is correlated with the formation of small LNPs.

The next section will describe the different parameters that can be adjusted to finely tune LNP size. However, it is important to keep in mind that the impact of these parameters will be different if you use the herringbone (chaotic) or flow focusing (diffusion) mixing techniques.

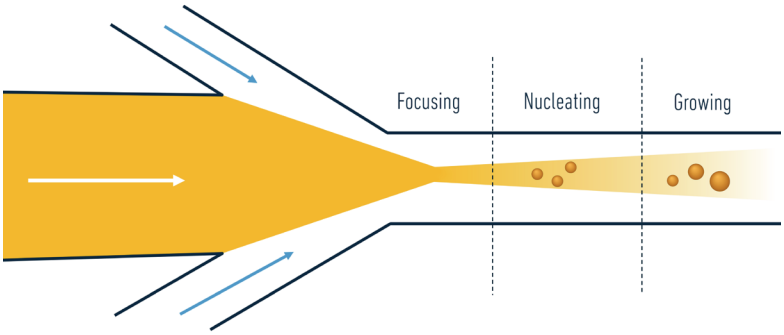


Figure 5: Fluid circulation inside the flow focusing microchip

What are the most important parameters influencing the size of your LNPs?

The size of the generated NPs depends on the chip geometry (shape and dimensions of the channels), the formulation of the lipid solution (type, molecular weight, molar ratios and concentration) and the composition of the aqueous solution (pH, salt content, presence of surfactants).

We will now give an overview of these important parameters and how they influence LNP size.

The flow rate ratio (FRR) is an important variable to play with when generating LNPs using microfluidics, especially with diffusion-based mixing techniques.

This is defined as the ratio between the flow rate of the aqueous phase and the flow rate of the organic phase. For example, a FRR of 10:1 corresponds to the flow rate of the aqueous buffer being 10 times higher than the flow rate of the lipids in ethanol. As a rule of thumb, higher flow rate ratios will lead to smaller LNPs to an extent. The FRR is especially important when using the flow focusing microfluidic chip, while it has a lower impact on LNP size when using the Herringbone chip.

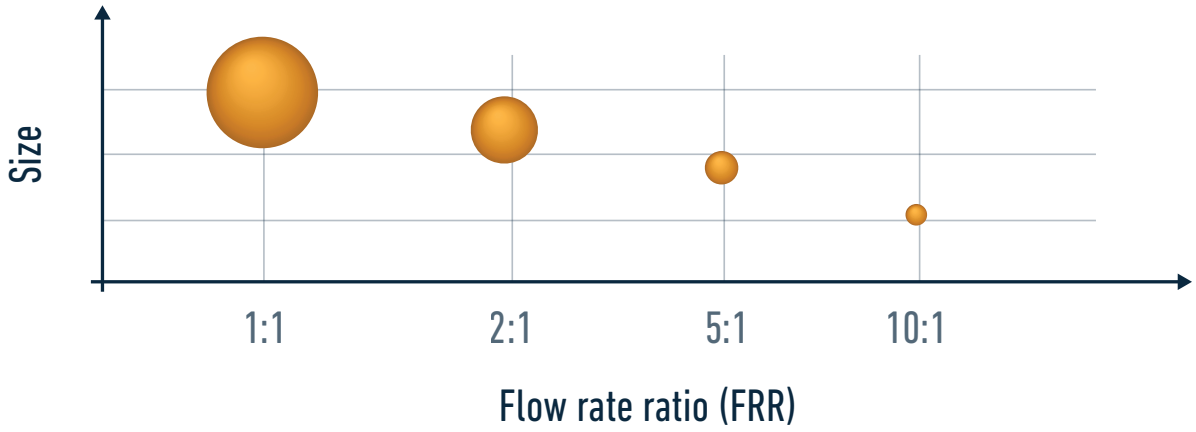


Figure 6: Impact of aqueous:organic phase flow rate ratio on LNP size

The total flow rate (TFR) is defined as the sum of the flow rates of the aqueous phase and of the organic phase. For example, if you set the flow rate of the lipids to 100 µL/min and use a FRR of 5:1, the TFR in your system will be 600 µL/min. This parameter has very little impact when using the flow focusing method, thus increasing the TFR (and the production rate at the same time) will not affect LNP size because FRR is the most important parameter in this case. On the other hand, as the liquids in the herringbone chip are mixed by chaotic mixing, increasing the TFR will decrease the mixing time, thus decreasing the size of LNPs.

The **lipid formulation** and the **concentration of lipids** in the organic phase is a crucial factor determining the final size of the LNPs. In general, a higher lipid concentration leads to smaller LNPs, especially when working at high aqueous:organic FRR. The lipid formulation used in this Pack is composed of 4 different lipids that are similar to those used in vaccine and drug delivery development (Figure 7).

This Pack provides the exact recipe to replicate this formulation and the range of sizes we obtained.

Keep in mind that working with different lipids, molar ratios and concentrations **will change the size of the obtained LNPs.**

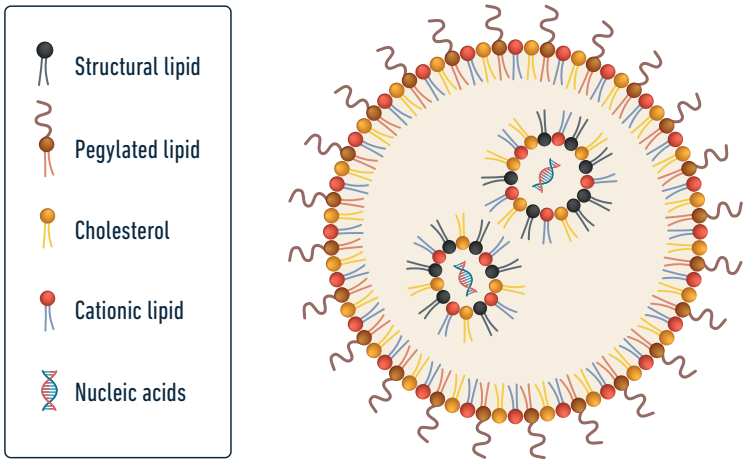


Figure 7: Illustration example of a LNP formulation

A few other parameters are summarized in the following table:

pH	The influence of this factor will depend on the type of lipids used in your formulation (neutral, cationic, ionisable...)
Temperature	Higher temperature during mixing generally leads to smaller LNPs
Buffer composition	Certain buffers can cause your LNPs to agglomerate (e.g. PBS will increase aggregation of cationic LNPs)
Geometry of the microchannel	A faster mixing after the contact point of the two streams leads to smaller LNPs

Examples of how these parameters influence LNP size using a typical lipid formulation are available in **5** Characterization section.

Which microchip is best for you?

Answer the following questions and check which microfluidic chip is best for your needs. This helps and guides you to choose the appropriate micromixer.

You can choose between one or the other, or even ask for both systems in the same Pack. Nevertheless, details matter and our expert team is here to advise.

☐ I want to produce LNPs with an average hydrodynamic diameter between 110 and 200 nm	☐ I want to produce LNPs over a broad average hydrodynamic diameter range (e.g. from 80 to 300 nm)
☐ I am working with low volumes of samples (< 1 mL) and/or very expensive lipids/drugs	☐ I know which formulation to use and I want to collect larger sample volumes to perform characterization studies
☐ I want to try many different formulations of lipids	☐ I want to scale up my LNP production for clinical trials
☐ I am working with highly concentrated solutions and only need to collect a few µL of LNPs	☐ I am working with dilute solutions and/or I am not limited in the volume of buffer containing hydrophilic drugs (> 1 mL)
The flow focusing chip is for you!	**The Herringbone chip is for you!**

For a given system (chip, lipid formulation and buffer), **the characteristics of the NPs** will depend on the flow rates of the two liquid phases.

The setup and protocol will allow you to finely tune the flow rate of the two liquids in order to obtain the desired NPs.

The material provided with this Pack will work with other chips, solvents or volumes and the protocol detailed in this document can be easily adapted for that purpose.

please refer to the going further section

Characteristics of the microfluidic chips

These chips are made with Zeonor, a thermoplastic polymer, which is a hydrophobic material. An important feature of this material is that it is compatible with polar solvents, such as acetone and ethanol.

Flow focusing chip - low sample volumes (< 1 mL) and low flow rates (< 500 µL/min), indicative LNP size from 110 to 200 nm

Interface type	Mini Luer
Nozzle sizes	10 - 20 - 30 - 50 µm
Lid thickness	188 µm
Material	Zeonor

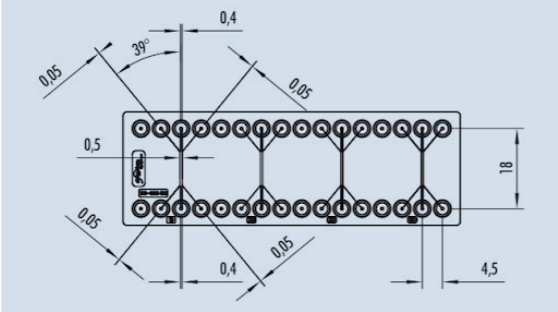


Figure 8: Drawing, dimensions and specifications of the flow focusing microchip

Herringbone chip - high sample volume (> 1 mL) and higher flow rates, indicative LNP size from 80 to 300 nm

Interface type	Luer
Channel depth	200 µm
Lid thickness	188 µm
Material	Zeonor

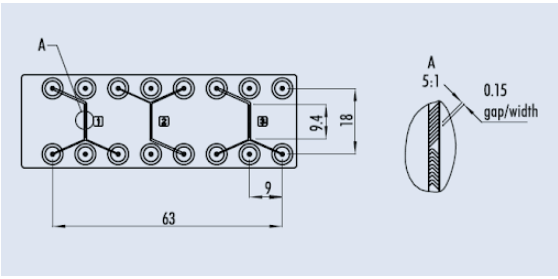


Figure 9: Drawing, dimensions and specifications of the herringbone microchip

TIP

Although these chips are said to be “single use” chips, you can reuse them after careful washing steps. We recommend washing them with an organic solvent (e.g. ethanol, isopropanol, acetone) followed by a wash with clean DI water and drying with air.

TIP

The herringbone microfluidic chip is also available in polycarbonate (PC). PC is slightly less hydrophobic than Zeonor and can potentially reduce LNP adsorption on the microchannel walls, although no significant difference has been found in our experiments.

Figure 10: Picture of the Herringbone chip

Contents of the lipid nanoparticle pack

1 OB1 MK3+ Pressure-driven flow controller

2 channels 0-2000 mbar (one for each phase to be mixed)

The pressure controller is the centerpiece of the setup. It controls the pressure difference across the fluidic system and thus, controls the liquid flow.

PRINCIPLE OF PRESSURE-DRIVEN FLOW RATE CONTROL

Elveflow instruments use pressure to drive liquid flows in fluidic systems

The elementary principle is that a pressure difference across a fluidic line (between the outlet and the inlet of the fluidic system: ΔP= Pout-Pin) generates a motion of the liquid in the system characterized by its volumetric flow rate. The flow rate Q is proportional to the pressure difference, and the proportionality coefficient R is called the fluidic resistance:

$$\Delta P = R \times Q$$

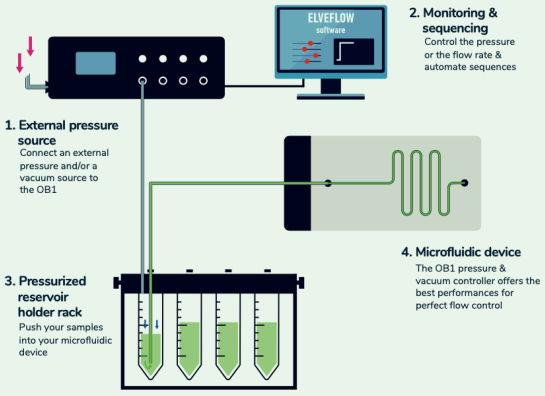
R depends on the fluidic system geometrical characteristics and on the properties of the liquid.

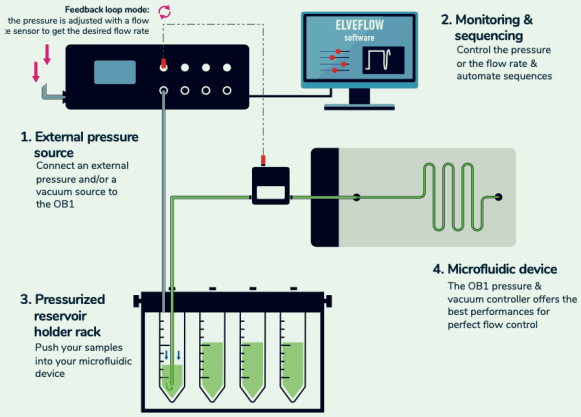
For instance: the longer or the thinner the tubing is or the more viscous the liquid is, the greater is the resistance. It means that a higher pressure difference is required to obtain a same flow rate in a system with longer or thinner tubing or with a more viscous liquid.

For instance, the resistance of a circular cross channel is $R \propto \frac{\mu L}{r^4}$ (Viscosity, Length, radius)

Each element of the circuit (chip, tubing, sensors, ...) contributes to the whole resistance R of the system.

The **OB1 pressure controller** creates and controls the pressure difference by pressurizing the air in a sealed reservoir that contains the liquid at the inlet of the system. The outlet pressure Pout most often equals the atmospheric pressure, so the OB1 controls **ΔP**.





A **flow rate sensor** can be added in line to measure and monitor the flow rate Q.

IN PRESSURE CONTROL: the user controls the pressure difference (the command is a pressure) and it generates a flow rate.

IN FLOW RATE CONTROL: the user sets the flow rate (the command is a flow rate) and the system continuously adapts the applied pressure difference so that the Q meets its targets.

You can refer to this video to learn more about pressure-driven flow control

https://youtu.be/niWfINDUub4

2 or 3 Flow rate sensors

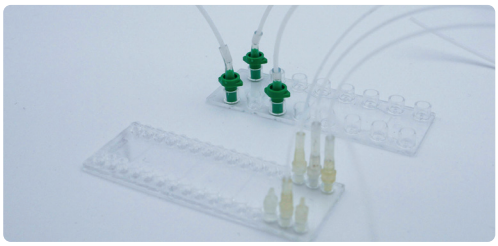
The MFS measures the flow rate of the liquid going through the sensor. Used with the pressure controller, it allows control of the flow rate. 3 options are available:

Chip	MFS3	MFS4	MFS5
Flow focusing	• ~25 - 500 µL/min with Ethanol calibration	• 30 - 1000 µL/min with Water calibration	
Herringbone		• ~100 - 3500 µL/min with Ethanol calibration	• 200 - 5000 µL/min with Water calibration
Both systems	•	•	•

1 or 2 commercial micromixing chips

Fluidic 187, Microfluidic ChipShop and/or Fluidic 386, Microfluidic ChipShop

The micromixing chip is the place where aqueous buffer and lipids meet to make nanoparticles.



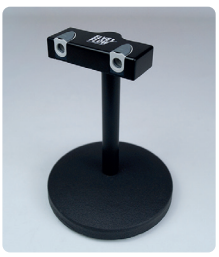
1 pack of Mini Luer connectors & plugs and/or 1 pack of Luer connectors + silicone tubing

The male Luer fluid connectors are designed to connect the tubing to the ChipShop Luer compatible microfluidic chips. The male Mini Luer plugs are designed to block the unused ports of the ChipShop microchips.

6 reservoirs and pressurized reservoir caps

The pressure imposed by the OB1 in the reservoir forces the liquid to leave the reservoir through the pressurized cap and flow through the microfluidic system. Pressurized caps are autoclavable, infinitely reusable and are available for eppendorf tubes (1.5 mL) and falcon reservoirs (15 mL and 50 mL). Two of each type are included inside this pack.

Option: Other reservoirs are available upon request (bottles with GL45 caps).



1 reservoir holder with 2 push-in connectors

A specific reservoir holder has been designed to securely hold your reservoirs and solutions.

It can hold up to two reservoirs with an easy-to-plug push-in connector ensuring a secure connection of your tubing to the reservoir in order to avoid any spill or leak from your system.

2 flow resistor systems KFR-23/24 series

Flow resistors are PEEK capillaries with small internal diameters. These resistors are used to increase the resistivity of the microfluidic system to improve the stability and control of the flow rate in the system.

This Nanoparticle Pack comes with 2 ready-to-use Flow Resistors, both allowing for operation over the full range of the pressure controller (0 to 2000 mbar).

Flow focusing	Lipid phase	MFS3	KFR-23-A
	Aqueous phase	MFS4	KFR-24-B
Herringbone	Lipid phase	MFS4	KFR-24-A
	Aqueous phase	MFS5	KFR-25-B

Starter Pack Kit

The following items are included in our Starter Pack. They will enable you to easily set up your system and connect all components together:

Tubing 1/16" OD X 1/32" ID (50 m) and/or 1 tubing 1/32" OD x 300 µm ID (30 m)

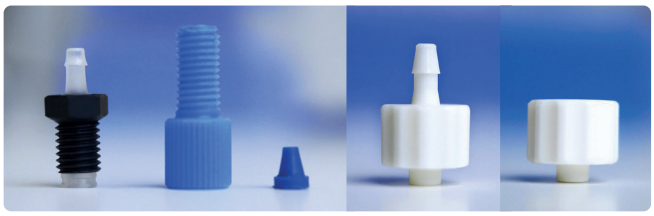
Tubing is used to connect all components of the flow path (e.g. reservoirs, MFS, microfluidic chip). The internal diameter (ID) of the thicker tubing is 1/32" or 794 µm. The outer diameter is 1/16" or 1590 µm. If using the flow focusing chip, we recommend using the thinner 1/64'' ID (400 µm) PTFE tubing to reduce the dead volume in the tubing. This smaller tubing is in principle also compatible with the herringbone chip but it will limit the range of flow rates that can be used as it significantly increases the resistance.

1 Microfluidic connection kit

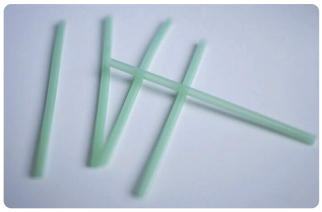
These pieces are mandatory to connect the whole system.

Refer to **appendix 1** for a visual guide to connections

Pressure connection kit



Fittings

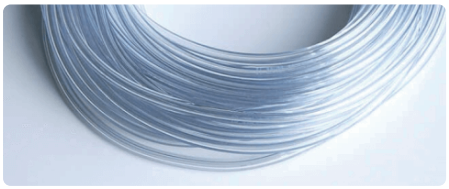


Sleeves

Backflow blocker

Pneumatic tubing

This tubing is used to connect the OB1 outputs to the reservoirs.



1 air filter

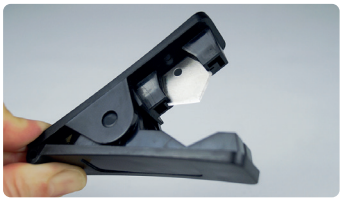
The air filter is placed between the pressure generator (e.g. compressor) and the OB1 inlet.

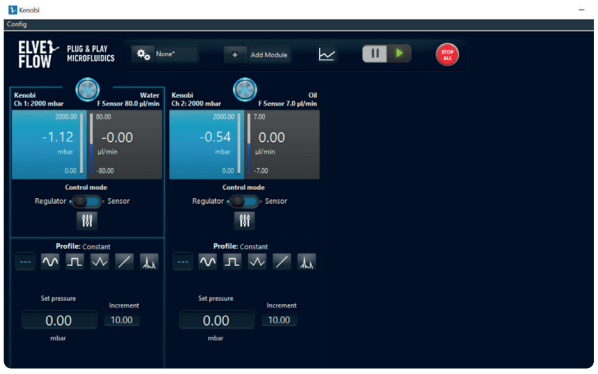
It keeps the OB1 clean from dust and humidity.

1 Tubing cutter

The cutter is useful to properly cut the tubing (perpendicular and clean cut).

This helps to prevent leaks in your setup.





ESI software

All the elements of the system can be controlled by the ESI software, including the OB1 parameters, the switching of a valve position, and many more. The ESI interface allows you to easily set the pressure or flow rate you wish to apply to the system, as well as select a rotary valve port to inject a selected liquid. It also includes a built-in sequencer for easy system automation. The provided set of SDKs in C, LabVIEW, Matlab and Python can be used for further automation and integration into larger systems.

DOWNLOAD THE LATEST VERSION OF ESI SOFTWARE FOR FREE

This pack does not contain a pressure source.
The OB1 should be connected to a pressure supply. The optimal pressure source has to deliver a pressure higher than the maximum pressure of your OB1 channel (or at least 1.5 bar), and a maximum of 10 bar. For example, if you are using a 0-2 bar pressure channel, working with a pressurized source delivering 2,5 bar is required to cover the whole pressure range.
Refer to the OB1 user guide for more information. Feel free to contact us at contact@elveflow.com to help you pick the optimal pressure source for your system, or to provide the pressurized air source if needed.

Here are two Pressure Sources available from Elveflow's catalogue:

Bench-top Pressure Source

Noiseless & portable.

Specifically designed to fit OB1 single or two 0/2000 mbar channels.

Air compressor

A perfect pressure source to use with any positive pressure OB1 configuration to get the best out of your pressure controller

Before starting the experiment

1

To begin with, make sure you have installed the software. Launch it.

You can refer to this video

https://www.elveflow.com/microfluidic-applications/setup-microfluidic-flow-control/ob1-pressure-controller-install-box/#esisoftwareinstall

REFER TO ANOTHER USER GUIDE

ESI software user guide | **Installation**

User safety

Before manipulating any instrument, **please read its specific user guide.**

They can be downloaded from the support section on the our website.

Each user guide describes the good practices to handle Elveflow's equipment.

2 Add the OB1 to the software and calibrate it

Connect your OB1 pressure controller to an external pressure source using pneumatic tubing, to a computer using a USB cable and plug it to the power source.

Once the OB1 is correctly connected, switch it on and close all the channels using the plug fittings.

Add the OB1 to your ESI software by pressing *Add instrument \ choose OB1 \ set as MK3+*, set pressure channels if needed, give a name to the instrument and press OK to save changes. Your OB1 should now be on the list of recognized devices.

REFER TO ANOTHER USER GUIDE

Refer to the OB1 user guide for detailed instructions | **Instrument connection**

You can refer to this video

https://www.elveflow.com/microfluidic-applications/setup-microfluidic-flow-control/ob1-pressure-controller-install-box/#firstcalib

An OB1 calibration is required for the first use. Once calibrated, it is not necessary to do so before each experiment, unless it drifts slightly or if you change the pressure source. Once it is calibrated, you can connect it to any other component of the system to perform experiments.

Don't forget to **set up the provided filter** between **the pressure source** and the **OB1**.

Refer to **appendix 1** to see how to connect all the components together

3 Add the MFS on the software and calibrate it

Connect the **two flow sensors** to the **OB1** using the appropriate cable. You can then add them on the software:

- Add the flow sensors (MFS) to your ESI software by pressing Add sensor \ select flow sensor \ analog or digital (choose the working range of flow rate for the sensor if you have an analog one).
- Give a name to the sensor
- Select to which device and channel the sensor is connected and press OK to save the changes.

Your flow sensor should be on the list of recognized devices.

REFER TO ANOTHER USER GUIDE

MFS user guide | **Electronic connection**

ESI software user guide | **Add a new sensor**

In this user guide, the MFS measuring the lipid solution flow rate will be called **Lipids**, and the MFS measuring the aqueous buffer flow rate will be called **Water.**

The digital MFS has **two available calibrations:** Water and Isopropyl alcohol.

Since the lipid is dissolved in ethanol and since no pre-existing calibration exists for that particular solvent, we recommend calibrating both flow sensors with the calibration Water and manually adjusting the scale factor and offset values for the ethanol/lipid phase flow sensor. This will ensure an accurate value of the measured flow rate.

Lipids | MFS

measure the lipid solution flow rate | calibration **Water**

Water | MFS

measure the aqueous buffer flow rate | calibration **Water**

WHY IS IT NECESSARY TO CALIBRATE THE MFS ?

The flow sensor measures the flow rate of a liquid by locally warming it and measuring the temperature differences between different locations of the sensor capillary.

The relationship between temperature measurement and the flow rate highly depends on the physical properties of the liquid passing through the MFS. That is why a calibration is required. Two MFS calibrations can be implemented directly using the ESI: Water and Isopropyl.

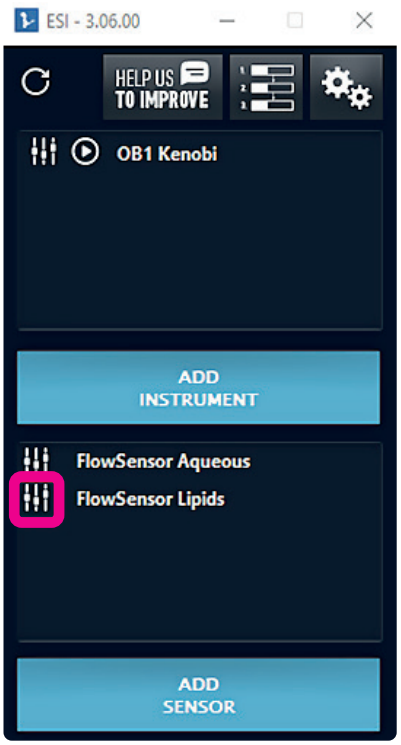
The Water calibration is suited for all aqueous solutions.

The following steps illustrate how to calibrate the flow sensors for lipid or water (Figure 11, A-D).

For the aqueous phase flow sensor, simply add the Flow Sensor and choose the "Water" Calibration without changing the Scale factor and offset (Figure 11, A-C).

Values of Scale Factor and Offset calibration corrections for the Lipid flow sensor are provided below.

(A)



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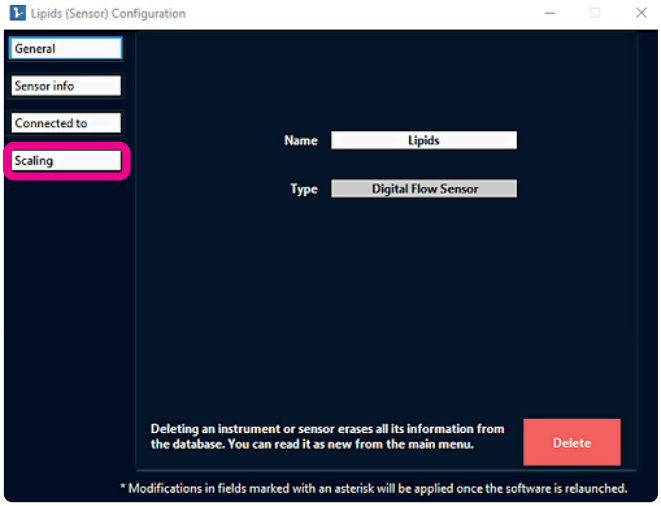


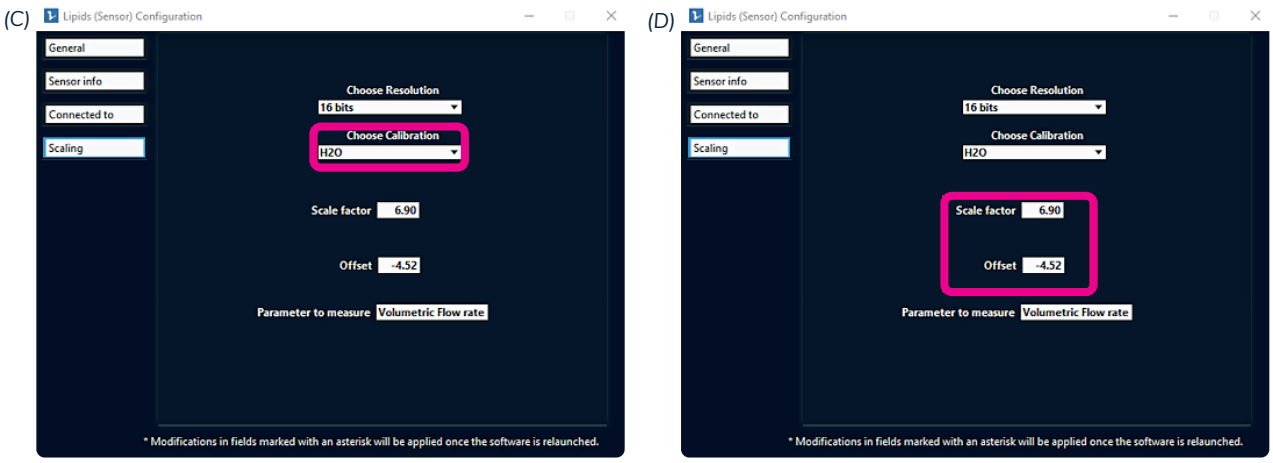


Figure 11: How to add a Flow sensor and set the appropriate calibration in the ESI.
(A) Add a Flow Sensor, (B) Access the calibration parameters, (C) Choose the calibration type, (D) Modify the scale factor and offset.

Elvesys advises using the following values of offset and scale factor **to calibrate the Lipid flow sensor** (MFS3 or MFS4, depending on your pack):

	MFS3	MFS4
SCALE FACTOR	6.9	4.05
OFFSET	-4.52	-80.09

I NEED TO RECALIBRATE MY MICROFLUIDIC FLOW SENSOR
https://support.elveflow.com/support/solutions/articles/48001163077

↓ Let's start making nanoparticles!

Let's start making nanoparticles!

1 Set up the reagents

This pack was fully tested using the following citrate buffer and lipid formulation.

For your first experiments, we advise you to start with this specific formulation to get used to the system before moving forward to work with your own recipe.

Lipid mixture

- 1,2-distearoyl-sn-glycero-3-phosphocholine (**DSPC**) as the structural lipid
- Cholesterol
- dimethyldioctadecylammonium (**DDAB**) as the cationic lipid
- 1,2-dimyristoyl- rac-glycero-3-methoxypolyethylene glycol-2000 (**DMG-PEG2000**) as the pegylated lipid.

DSPC:Cholesterol:DDAB:DMG-PEG200 at a molar ratio of 5.6:62.7:29.4:2.09, working concentration of 3.54 mM.

First, prepare 10 mL of stock solution at 17.7 mM.

In a clean beaker, add:

- 7.9 mg of DSPC
- 43.1 mg of Cholesterol
- 32.8 mg of DDAB
- 9.3 mg of DMG-PEG2000

Add 10 mL of pure ethanol and use a vortex mixer and sonication until all the lipids are dissolved.

Then, dilute an aliquot to 3.54 mM: Mix 2 mL of the stock solution and 8 mL of absolute ethanol in a 15 mL reservoir.

TIP

Use a 0.22 µm syringe filter to filter and clean your ethanol prior to use.

Citrate buffer, 100 mM, pH 6

To prepare 50 mL of solution, add 1.213 g of Sodium citrate dihydrate and 168 mg of Citric acid to 40 mL of ultrapure deionized water. Mix well and adjust pH to 6 by slowly adding 0.1 N NaOH or HCl. When you reach the desired pH, complete the volume to 50 mL with DI water.

TIP

Use a pH meter or pH strips to measure the pH of your solution and filter the buffer with a 0.22 µm membrane prior to use.

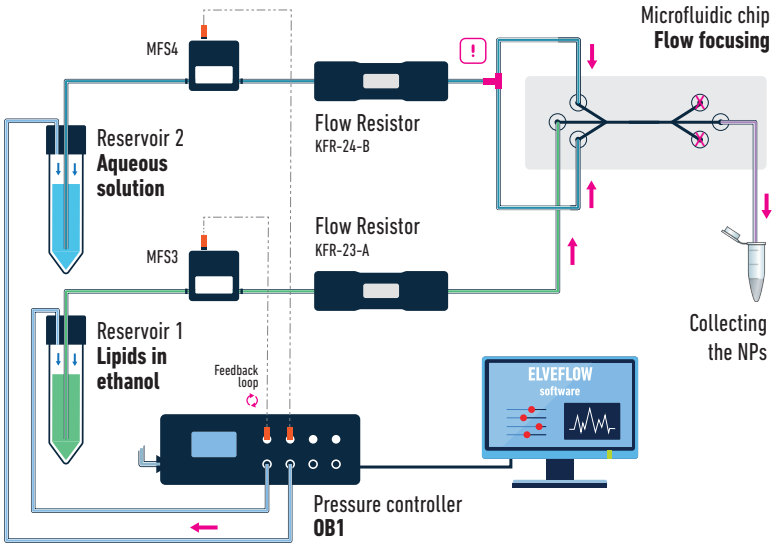
Figure 12: Elveflow's reservoir holder

Plug both reservoirs to the reservoir holder and to the corresponding OB1 pressure controller outlet.

Refer to **appendix 1** to see how to connect all the components together

2 Set up installation

Set up the following system if using a **flow focusing chip:**



For this setup, use **PTFE 1/32" OD tubing**

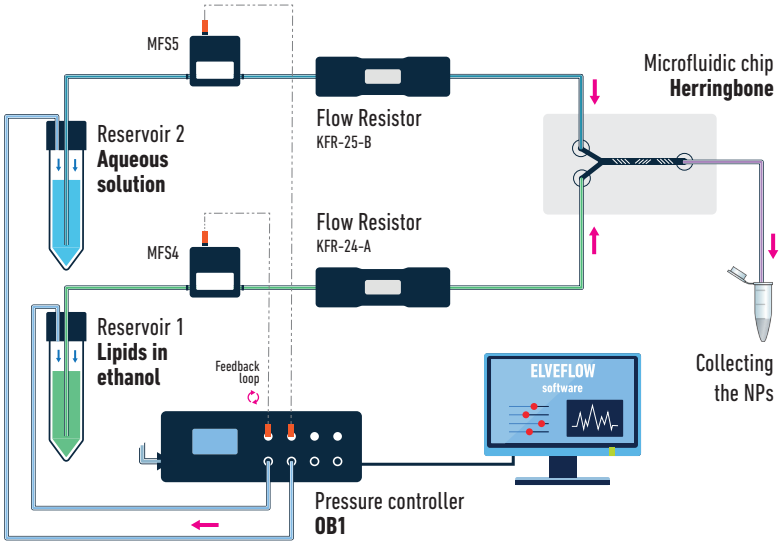
Refer to **appendix 1** to see how to connect all the components together

TIP

If you want to increase the total flow rate in your setup you can replace the flow resistor in the aqueous line with an equally long piece of 1/16" ID tubing.

Important: the two pieces of tubing after the T-junction on the aqueous phase line must be of equal length, otherwise the flow might not be divided equally in both side inlets of the microfluidic chip.

Set up the following system if using a **Herringbone chip:**



For this setup, use **PTFE 1/16" OD tubing**

Refer to **appendix 1** to see how to connect all the components together

TIP

The flow resistor should always be placed downstream of the MFS (between the MFS and the chip) to get more stable measurements.

TIP

If you want to collect a relatively low volume of samples (<2 mL), prepare and label a set of 1.5 - 2 mL eppendorf tubes and place them in a rack close to the outlet tubing for easy collection. To avoid sudden change of flow rates, you should minimize the outlet tubing displacement from your waste reservoir to your collection reservoirs.

TIP

For a more automated sample collection, you can add a microfluidic valve with 3 ports and 2 positions between the outlet of your chip and your waste / sample reservoirs. An example of how to add valves into your system is described in the Going Further section.

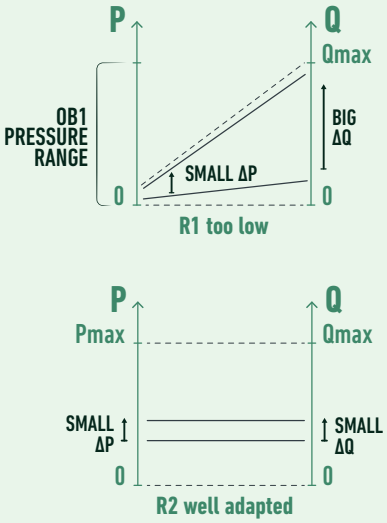
WHY IS IT IMPORTANT TO WORK WITH RESISTANCES?

Adding the right amount of fluidic resistance in the fluidic path is important as it is the way to finetune the system's whole resistance R to obtain the best performance in terms of flow rate control. Ilt allows an adjustment of R so that the range of accessible ΔP (given by the range of the OB1 channel output) matches the range of the flow rate sensor used. Doing so, the system uses the whole dynamic range of both the sensor and the regulator to control the flow rate.

For instance in a system (with a 2 bar OB1 channel) where R=R1 is too low, few mbar of ΔP generate a high flow rate that can saturate the flow rate sensor.

In other terms, if a small part of the accessible ΔP matches the full range of the sensor, it can lead to unstable flow control: a small ΔP variation triggers a huge flow rate variation. The solution is to add the right amount of fluidic resistance to increase R=R2 so that most of the range of the pressure regulator is used: a higher pressure is required to saturate the sensor and consequently a small ΔP variation triggers a small flow rate variation, allowing a stable system.

TO LEARN MORE
https://www.elveflow.com/microfluidic-applications/setup-microfluidic-flow-control/microfluidic-flow-restrictors/



3 Fill the microfluidic chip

When doing this for the first time, use water and pure ethanol as your aqueous and organic phase, respectively. This will ensure that you don't waste your sample in case you need to troubleshoot. Once your setup is complete, change your reservoirs for those containing your lipids and reagents to encapsulate.

First, make sure your reservoirs are filled with their respective liquids and that the tube endings are are positioned very close to the bottom of the reservoirs. Turn on the OB1 and launch the ESI software. Open the OB1 in the ESI:

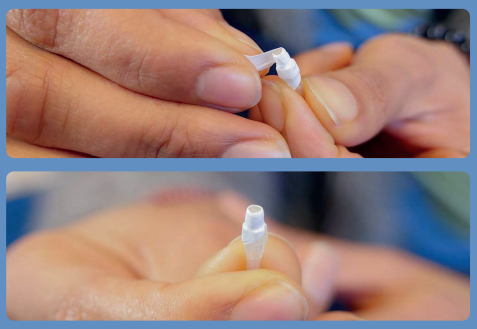
Figure 13: How to set the pressure in the ESI software

Set the pressure of both channels to 500 mbar until the chip is filled (it should take less than a couple of minutes). You can see that the chip is filled when the liquid is passing through the outlet after having passed through the chip. At this stage, you can check your setup for leaks (pressure or fluid).

Refer to **Troubleshooting** if this does not work

TIP

Fluid leaks are usually located at the connection between tubing and other components of your setup. They can be discrete and you might only notice them after a few minutes. Pay attention to leaks around the connectors to your microfluidic chip, especially for the flow focusing chip. To prevent leaks around the mini luer connectors, wrap a piece of Teflon seal thread tape around the base (as shown in the troubleshooting section)



TIP

To reduce the formation of bubbles and shorten the initial filling time, it can be useful to fill the tubing from the reservoir to the chip with liquid before connecting the chip (set low mbar command on the OB1 and watch the liquid interface going through the tubing, set the pressure to zero when the interface reaches the tubing extremity then connect the extremity to the chip. If you have a low volume of reagents, use pure solvent to perform the filling.

You are now **controlling the pressure.** Once the filling is done, you may already replace the reservoirs with the ones containing your reagents.

This is enough to make nanoparticles!

But if you want to control the flow rate and thus have more precise control over the size of the nanoparticles, there are few last things to set up.

WHY CONTROL THE FLOW RATE RATHER THAN THE PRESSURE?

The characteristics of your nanoparticles are determined by the flow rates of both dispersed and continuous phases. Requesting a flow rate instead of imposing a pressure has mainly two advantages:

- The flow rate stays constant throughout time, even though the experimental conditions slightly change (if a bubble or clogging occurs, the system tries to compensate and to maintain a constant flow rate.)
- It compensates for the hydrostatic pressure changes. The inlet pressure of the fluidic system is equal to the atmospheric pressure, plus the pressure imposed by the OB1 on the air above the liquid in the sealed reservoir, plus the pressure exerted by the water column (height of liquid between the water/air interface in the reservoir and the tube inlet at the bottom of the reservoir) around 1 mbar per cm of water. So when the liquid level decreases, the ΔP decreases over time and so will Q, when using pressure control. In Flow rate control the ΔP is adjusted to maintain a constant flow rate.

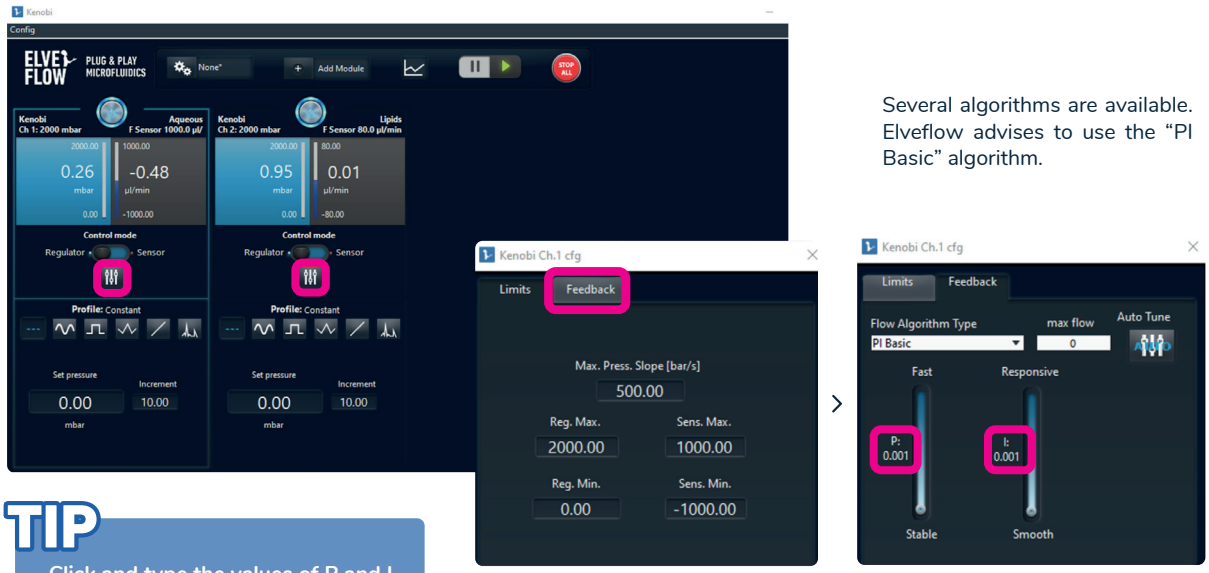
Even if your setup changes from one experiment to another, using the same flow rates will lead to the same nanoparticles (setup changes will change the fluidic resistance and using constant pressure will lead to a different flow rate).

4 Ensure a proper control of the flow rate for accurate TFR and FRR monitoring

This section explains how to directly control the system in flow rate, through a feedback loop between the pressure controller and the flow sensor.

In **feedback control mode** ("Sensor" mode), you can set a target flow rate, and the inlet pressure will automatically adapt to reach it. The feedback loop relies on a PID algorithm. The D parameter is preset but the gain parameter (P) and the integration parameter (I) can be tuned. We advise you to use the "PI Basic" algorithm that will work for most applications. The user can use the autotune module to automatically find P and I parameters, or set these parameters manually to better match the requirements in terms of responsiveness and stability.

To set the **feedback parameters**, follow these illustrations:



Several algorithms are available. Elveflow advises to use the "PI Basic" algorithm.

TIP

Click and type the values of P and I, it will be easier

Figure 15: How to set the P & I feedback loop parameters in the ESI (A) Feedback loop parameters access from the main window, (B) Parameters window

For the **flow focusing system**, we advise you to begin with the following values for the feedback parameters:

SENSOR TYPE	P	I
MFS3 measuring lipids flow rates	0,02	0,01
MFS4 measuring buffer flow rates	0,05	0,035

These values are appropriate for the **flow focusing setup and for all couples of aqueous buffer and lipid flow rates.** They give a good compromise between stable flow rates and a high responsiveness of the system. We recommend you to start by using these values when getting familiar with the setup and the nanoparticle generation.

Lower values may give **more stable flow rates**, thus in principle increasing the monodispersity of your collected lipid nanoparticles, but your system will have a low responsiveness and it might take several minutes for the flow rates to equilibrate at the desired values

For the **herringbone mixer setup**, we recommend starting with this set of values :

SENSOR TYPE	P	I
MFS4 measuring lipids flow rates	0,1	0,08
MFS5 measuring buffer flow rates	0,03	0,015

To learn more about the tuning of the **P and I parameters**, please refer to the **going further section**.

If you have another setup (especially, if you have different resistances) and/or depending on your needs (if you want to increase the responsiveness for instance), you can finetune the values of the feedback parameters.

Now, you can start the feedback loop of flow rate control by **choosing the sensor control mode** (flow rate control instead of pressure control), and setting the flow rate you want to have (you are now controlling the flow rate instead of the pressure):

Don't forget to make sure that the whole fluidic system is filled with liquid before switching to flow rate control mode.

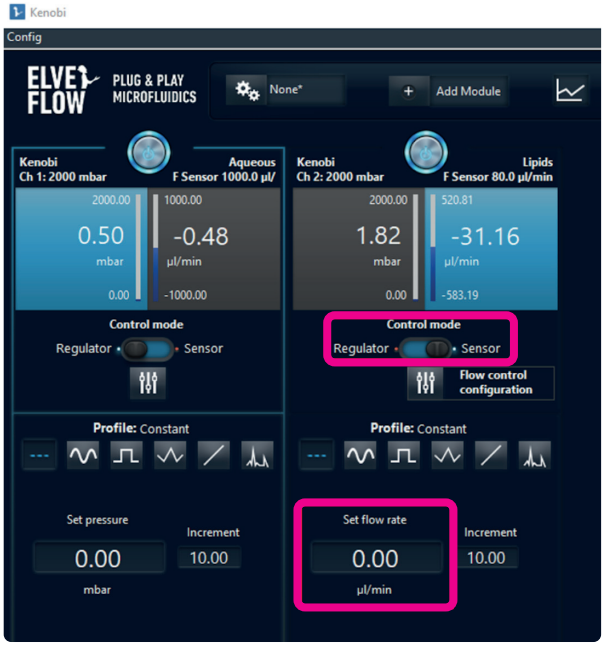


Figure 16: How to switch from pressure control to flow rate control in the ESI software

For example, you can set the aqueous buffer flow rate to 200 µL/min and the lipids flow rate to 100 µL/min to produce your first nanoparticles.

You can refer to the next section for given sets of flow rates that have been tested.

In general, the flow rate of the aqueous phase should be equal to or higher than the flow rate of the phase containing the lipids.

← Switch the control mode to sensor

Soon after the chip is filled with your reagents and your nanoparticles start nucleating, you might see them adsorb onto the walls of the microfluidic chip where the interface of mixing is.

In a short time period this usually doesn't affect the performance of the chip but in some cases it is necessary to get rid of these adsorbed particles.

TIP

While the tubing is filled with air, you should not control the flow rate but the pressure. Indeed, the MFS filled with air will not measure any flow rate, and the OB1 will keep increasing the pressure until either the fluid finally reaches the MFS or the pressure reaches its maximum, which should be avoided.

Once the fluid has reached the MFS, it is safe to control the flow rate.

How do I know I have adsorbed/agglomerated LNPs?

Agglomeration or adsorption should be clearly visible under a microscope, with a magnification of at least 5x. Here is an example photo of the flow focusing microfluidic chip with a lot of adsorbed LNPs versus the same chip after a washing step:

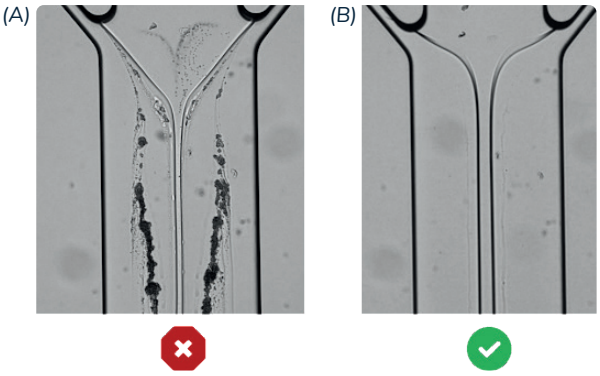


Figure 17: Picture of absorbed LNPs in a chip (A) versus the same chip after a washing step (B)

TIP

As this phenomenon is invisible to the naked eye, we recommend monitoring the fabrication of your nanoparticles under a brightfield microscope with low numerical aperture.

TIP

One efficient way to clean the microchannel of these agglomerates is to flush the entire width of the channel with only the organic phase (ethanol) followed by water or the aqueous phase. You have a few options: you can do this with the OB1 by applying a higher pressure on the Lipids channel (here, channel 2) until it prevents the aqueous solution from entering the central channel or you can disconnect the inlet tubings and inject pure solvent with a syringe. A quick and easy way that has been proven even more effective is to very briefly switch off the pressure in the Aqueous channel and turn it on again, repeat it a couple of times until the channel appears clear of these agglomerates.

TIP

Lipid nanoparticles usually interact more strongly with hydrophobic surfaces. Using a more hydrophilic material for the microfluidic chip or applying a coating in the microchannels might reduce the adsorption phenomena.

The complete characterization of the sizes and polydispersity index of the lipid nanoparticles depending on the FRR and TFR used can be found in the following section.

After each use we recommend cleaning your setup (tubing and flow sensors included) with 1) pure ethanol (instead of lipids) and filtered water (instead of the aqueous buffer) and 2) air, following this procedure:

"ETHANOL/LIPID" CHANNEL	"AQUEOUS" CHANNEL
Plug a reservoir filled with pure ethanol	Plug a reservoir filled with filtered water
Use the "Regulator" mode of the OB1	Use the "Regulator" mode of the OB1
Set Pressure at 2000 mbar and run for 2 minutes	Set Pressure at 2000 mbar and run for 2 minutes
Unplug, empty and plug the reservoir again	Unplug, empty and plug the reservoir again
Set Pressure at 2000 mbar and flush liquid for 2 minutes	Set Pressure at 2000 mbar and flush liquid for 2 minutes

if you are using an additional channel, follow the same steps using the appropriate washing solution.

For this, change the reservoirs and use the 'Regulator' mode to start flushing the appropriate cleaning solutions (ethanol or water) at the highest pressure (2000 mbar) for a couple of minutes until the channels look clean. Then, stop the pressure and retract the tubing plunging in the liquid so that it hangs in the air, while still being locked in the reservoirs (see picture X). Pressurize with 2000 mbar once again until all tubings are empty.

TIP

Make sure that the outlet reservoir is not totally empty, and that the outlet tubing plunges into the solution, otherwise there will be dripping, which will harm the stability of the flow rate.

5 Results & characterization of nanoparticles

Introduction to LNP characterization

Once you have collected your LNPs, it is advised to purify them to extract the solvent and/or filter them to ensure that they are sterile as well as remove any aggregates. These steps have not been performed in this user guide and the LNPs were all characterized, as collected, within 24 hours post-fabrication. This section will describe the characterization methods used and the results obtained for a given set of flow rates.

TIP

LNPs are typically purified by dialysis with 12-14k MWCO membranes and an aqueous buffer (PBS, Tris...). This step may impact the final characteristics of your LNPs, hence it is recommended to measure your LNPs size prior and after the purification step.

TIP

You can add an additional step of filtration with a 0.22 µm syringe filter in PVDF to eliminate undesired biological microorganisms. Keep in mind that this step will also remove any particles bigger than 220 nm (e.g. aggregates).

Dynamic Light Scattering as a commonly used technique

Dynamic light scattering (DLS) is the most commonly used technique for qualitative and quantitative analysis of organic or inorganic spherical nanoparticles in suspension. It is usually done with an instrument from Malvern panalytical called a Zetasizer. This method consists in sending a laser on a suspension of particles and recording the intensity of light that is scattered by the sample. DLS measurements will give you the average hydrodynamic diameter of the particles in your samples as well as an indicator of the monodispersity of your solution : the polydispersity index (PDI). Typically, a sample with a PDI below 0.2 is considered monodispersed. DLS is a very convenient method to characterize the PDI of your sample as well as determining its reproducibility. However, as this is a dynamic measurement (i.e. correlating the motion of particles in the solvent with their size), the size dispersion can be high (10-20 nm) and it is recommended to use another method to precisely characterize the size of your LNPs. Typically, microscopy techniques such as transmission electron microscopy are best to characterize the true size of your LNPs. We will now describe a few elements that need to be considered when using DLS for the first time.

Beside the average hydrodynamic diameter and PDI of your sample that are directly computed by the software on the Zetasizer, other values are available and shouldn't be neglected when analyzing the results. Some advice is given by the software after each measurement to assess the quality of the readout (e.g. "Good quality", "Multiple scattering", "Multiple populations", etc.) and three types of size plots can be displayed : **intensity, number and volume.** The intensity curve is a representation of the scattering intensity fraction relative to the size of the particles. As for number and volume curves, they are computed based on the intensity curve. More precisely, these two other curves are called "number weighted" and "volume weighted" and take into account the physical characteristics of the NPs material (refractive index and absorption coefficient).

Figure 18: Illustrations of the DLS instrument used to characterize the LNPs produced using this Pack

To measure nanoparticle sizes, it is recommended to use a backscattering measure (angle of the detector ~173°) as larger particles will mainly diffuse/scatter light in the forward direction.

To have more information on your sample, especially if multiple populations are present, **multi-angle DLS** measurement is recommended.

Other important factors to think about are:

Solvent: it should be ultrapure water or at least another pure solvent of known composition, viscosity and refractive index. Be careful to input the correct solvent parameters in the software.

TIP

To improve the monodispersity of your LNPs, one possibility is to add extra steps of size separation after the fabrication.

Sample concentration: in most cases, you need to dilute your sample prior to DLS measurement, the color must be clear (not turbid). Some useful indicators to know if your concentration is adequate:

- Attenuator: if this parameter is set up automatically, it needs to be between 7-8 to have a reliable and precise measurement
- Mean count rate: this value is usually displayed at the end of a measurement and should be between 200-500 kcps

HELPFUL INFORMATION ON AGGLOMERATION

The quantity measured by DLS is the scattering intensity. As the scattering intensity is proportional to the power of 6 of the size, this means that even a low amount of larger agglomerates can overshadow the most occuring size of nanoparticles in your sample. In other words, if you have 1 million LNPs of 10 nm, the intensity of the scattered light will be equivalent to one LNP of 100 nm. If both are present in the same sample, you will get two distinct peaks on your intensity profile. However, if you have different size populations in your sample, scattering intensity measurements will give you wide peaks and an average size closer to the larger sized particles. It is recommended to use microscopy techniques such as TEM to get the real particle size.

Zeta potential as a quality and stability LNPs marker

Zeta potential (ZP) is a measure of the charge in the slipping plane around particles. Knowing the zeta potential of your LNPs can give you very useful information and is part of the standard control parameters. This quantity is measured with most Zetasizers and is a good indicator of the stability of your sample. Stable LNPs usually have a ZP of ± 30 mV, depending if they are composed of positively or negatively charged lipids.[2] However, the presence of surfactants in the formulation will reduce the charge without necessarily affecting the stability. Therefore, the condition for stability and preventing agglomeration is to have LNPs with enough repulsive interactions among one another, whether it's steric or electrostatic repulsions.

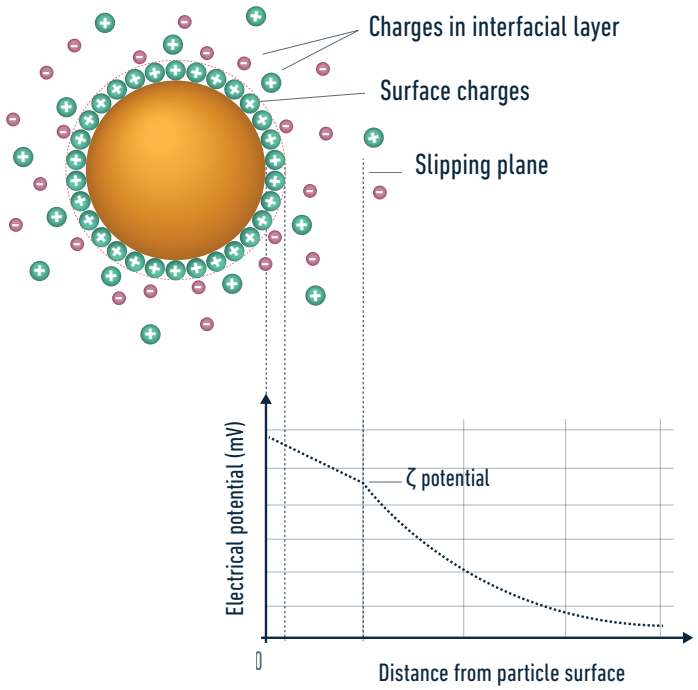


Figure 19: Illustration of the different layers of ions surrounding a positively charged nanoparticle.

Elveflow LNPs synthesis pack characterization results

DLS size and PDI measurements

The following tables and figures represent the different conditions of flow rates (FRR and TFR) used for the fabrication of LNPs and the resulting sizes and polydispersity index given by the zetasizer software. All samples were diluted with ultra-pure water to adjust the concentration and reduce the fraction of ethanol down to less than 1%.

[2] *S. Scioli Montoto, G. Muraca, and M. E. Ruiz, "Solid Lipid Nanoparticles for Drug Delivery: Pharmacological and Biopharmaceutical Aspects," Front. Mol. Biosci., vol. 7, no. October, pp. 1–24, 2020, doi: 10.3389/fmolb.2020.587997.*

Flow focusing	FRR	TFR (µL/min)	Aqueous (µL/min)	Lipids (µL/min)	LNP size (nm)	PDI
#1	20:1	210	200	10	131,7 ± 1,9	0,09 ± 0,01
#2	10:1	220	200	20	156,6 ± 9,5	0,19 ± 0,05
#3	7:1	228,6	200	28,6	168,4 ± 0,6	0,06 ± 0,01
#4	5:1	240	200	40	174,1 ± 0,7	0,08 ± 0,04
#5	2:1	300	200	100	191,4 ± 2,7	0,05 ± 0,03

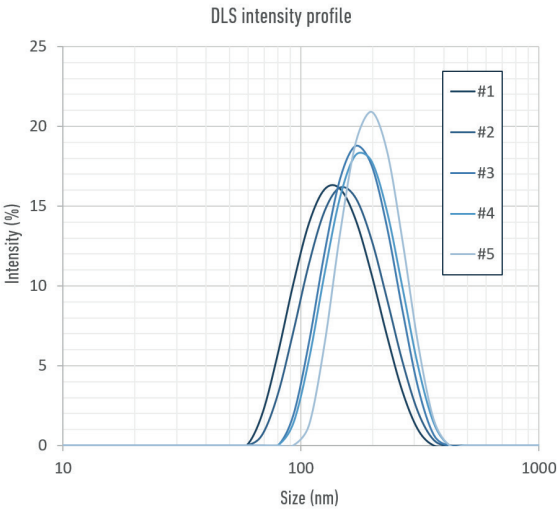


Figure 20: Nanoparticle generation parameters using a Flow focusing micromixer and size characterisation using DLS. (A) Table of the five conditions used to generate LNPs and corresponding size and PDI. (B) DLS intensity profiles obtained for each of the 5 conditions.

Flow focusing	FRR	TFR (mL/min)	Aqueous (µL/min)	Lipids (µL/min)	LNP size (nm)	PDI
#1	10:1	4,4	4000	400	112 ± 1,9	0,25 ± 0,03
#2	5:1	4,8	4000	800	94,4 ± 0,9	0,19 ± 0,02
#3	5:1	2,4	2000	400	150,4 ± 1,2	0,11 ± 0,02
#4	5:1	1,2	1000	200	183,9 ± 2,5	0,14 ± 0,01
#5	2:1	6	4000	2000	119,8 ± 1,6	0,07 ± 0,02
#6	2:1	3	2000	1000	142,6 ± 2,2	0,08 ± 0,01

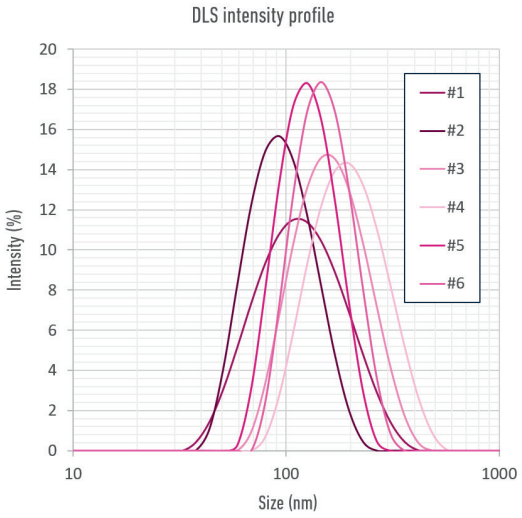


Figure 21: Nanoparticle generation parameters using an Herringbone micromixer and size characterisation using DLS. (A) Table of the six conditions used to generate LNPs and corresponding size and PDI. (B) DLS intensity profiles obtained for each of the 6 conditions.

Impact of the buffer pH and TFR on LNPs size

The following figure shows the impact of the buffer pH and the TFR on the LNPs size. It can be observed that the TFR has a lot more impact in the herringbone system than the flow focusing one.

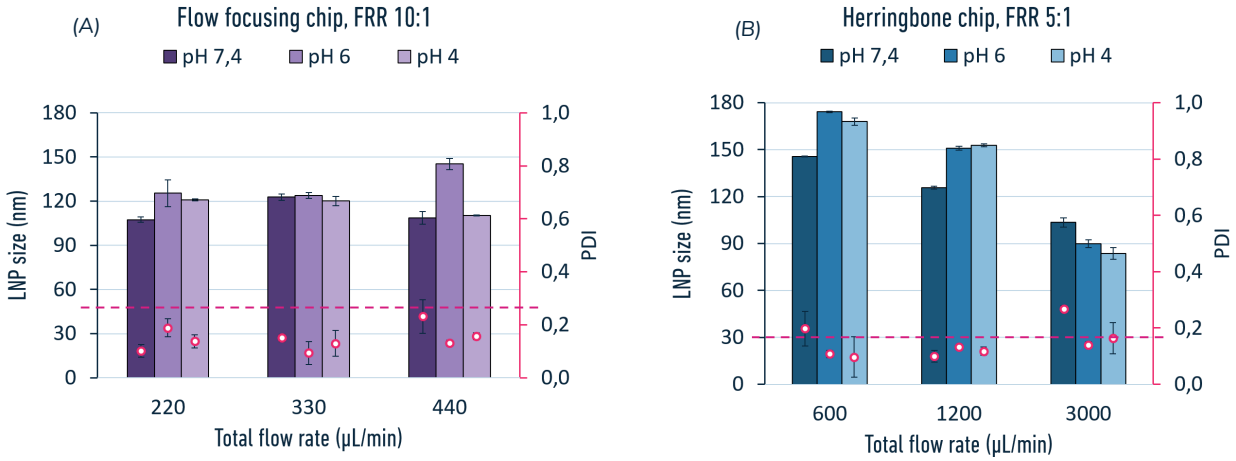


Figure 22: pH buffer impact on LNPs size and PDI under various flow rate and buffer pH conditions (A) Flow focusing micromixer. (B) Herringbone micromixer.

The LNP produced with buffer citrate at pH 6 were found to be very stable even a week after their production (when stored at 4°C). Further tests of stability are ongoing for longer storage duration.

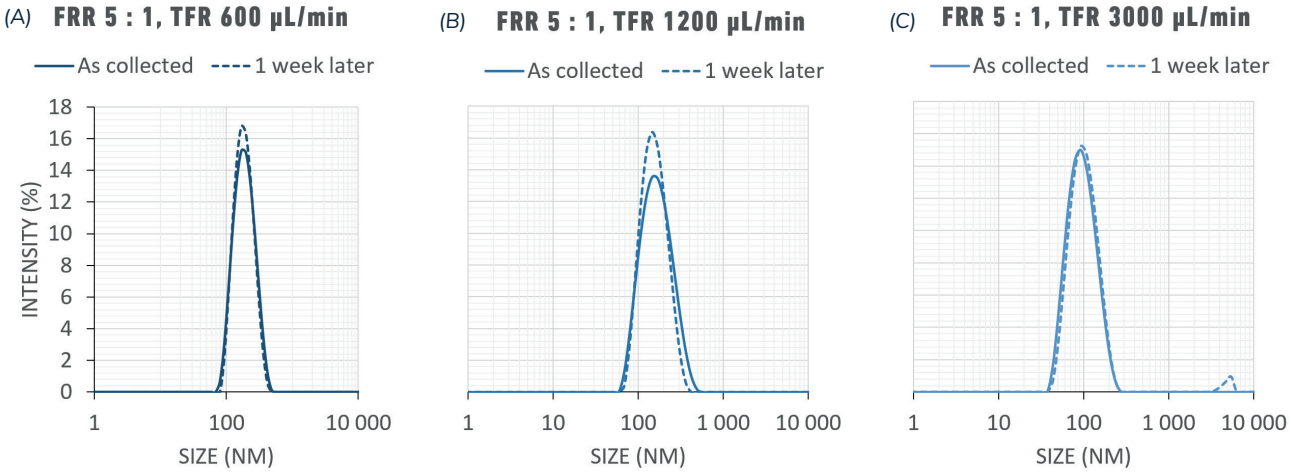
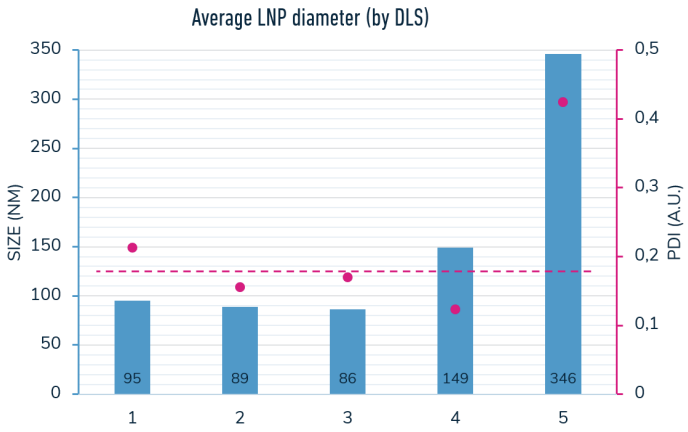


Figure 23: Long storage stability tests of LNPs generated at pH buffer 6 and various flow rate conditions (A) FRR 5:1, TFR 600 µl/min. (B) FRR 5:1, TFR 1200 µl/min. (C) FRR 5:1, TFR 3000 µl/min

Impact of an alternative lipid composition on LNP generation

The following figure shows the results obtained with the herringbone system after changing the type of lipid entirely. Here, a phospholipid called 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine or POPC was dissolved in ethanol at a concentration of 10 mg/mL and phosphate buffer saline (PBS) at pH 7,4 was used as the aqueous solution. Changing the type of lipid has a drastic effect on the size of LNP obtained.

(A)



(B)

	FRR	TFR	Aqueous	Lipids
1	20:1	1,05	1000	50
2	10:1	1,1	1000	100
3	5:1	1,2	1000	200
4	2:1	1,5	1000	500
5	1:1	2	1000	1000

Figure 24: Nanoparticle generation using the herringbone micromixer and a different type of lipid, POPC. (A) Sizes and PDI measured by DLS and (B) flow rates conditions used to produce the NPs.

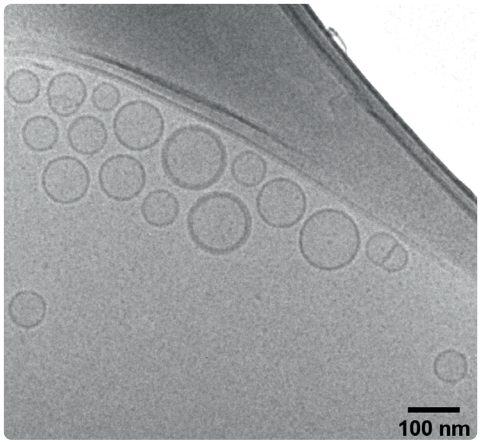
In order to further investigate the LNP size, we also carried out Cryo-TEM measurements on some samples which are shown and discussed in the next section.

Cryo-TEM observation

As discussed above, because DLS is not a precise quantitative measurement method, another round of characterization has been carried out using a Cryo-TEM microscope for a precise measurement of the LNP size.

(A)

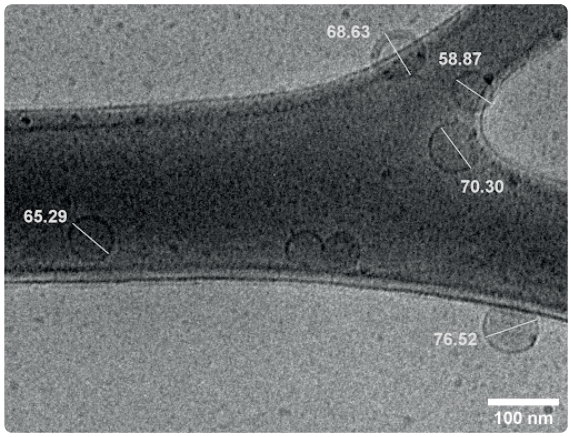
(B)



Briefly, fresh samples of LNPs were fabricated using the setups described in this Pack and a different lipid and aqueous buffer formulation. We used DSPC:Chol:DDAB:DMG-PEG2000 at 10:50:37:3 molar ratio and 5 mM concentration for the lipids and 10 mM citrate buffer at pH 4 for the aqueous solution. The same samples were analyzed with DLS and Cryo-TEM within 72h post-fabrication.

Cryo-TEM has the advantage of imaging the LNPs in solution, thus avoiding integrity loss that comes with drying the sample.

Figure 25: Cryo-TEM setup (A) and example image (B) of the produced LNP using a Herringbone micromixer.



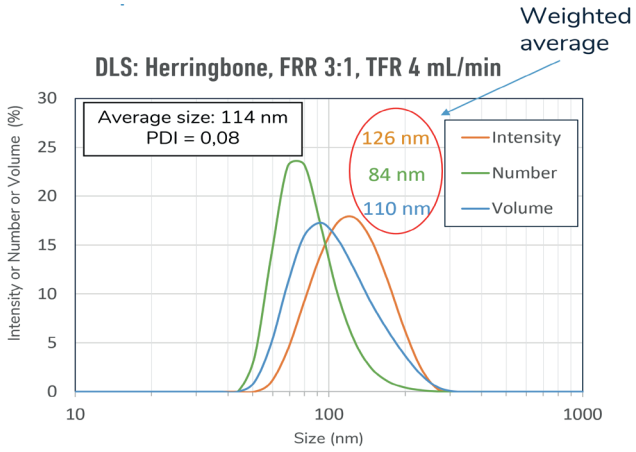
Images were further analysed using the ImageJ software. One example of the analysis is provided in the figure 26.

Figure 26: Cryo-TEM image analysis example using ImageJ line selection tool (units in nm).

Results comparison and LNP size discussion

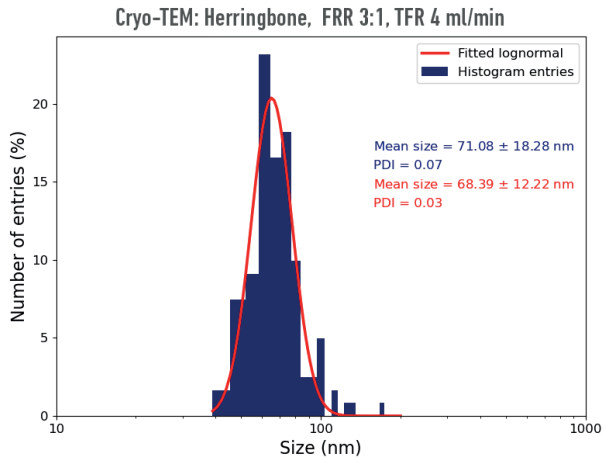
As mentioned previously, in addition to the intensity profile, the Zetasizer software also computes two other types of profiles, by volume and by number. Given that you introduce the correct material properties (absorbance and refractive index), these profiles can give you useful information on your sample. For monodisperse samples with no agglomeration, the number, volume and intensity profiles should be almost identical. However, if you have agglomeration in your sample, the intensity profile is less reliable and the number weighted profile is more representative of the real size distribution of the LNPs (which will then appear smaller than the size given by the intensity profile).

In order to compare both techniques (DLS and cryo-TEM), we analyzed the cryo-TEM images using ImageJ and the size distribution was plotted for comparison with the DLS size distribution.



The average size given by the DLS software is 114 nm. However a large discrepancy can be observed between the weighted average intensity (126 nm), volume (110 nm) and number (84 nm).

Figure 27: Intensity (yellow), number (green) and volume (blue) profiles of LNPs generated using the Herringbone micromixer with a 3:1 FRR and a 4 ml/min TFR.



The weighted average from the Cryo-TEM images is 71 nm +/- 18 nm, showing that the majority of LNPs generated using this Pack range from 55 to 90 nm.

Figure 28: Plot of ImageJ analysis of LNPs size generated using the Herringbone micromixer with a 3:1 FRR and a 4 ml/min TFR. Herringbone micromixer with a 3:1 FRR and a 4ml/min TFR.

We therefore observed up to approximately a 40 nm difference between the average size given by the DLS software and the weighted average from the Cryo-TEM images (Figure 35 and 36)). However, the difference was much smaller (up to 15 nm) with the average size (71 nm) computed from the number weighted profile of DLS (84 nm). From this, we have recalculated the sizes the LNPs produced in this user guide at different flow rates based on their number weighted curves. The results are displayed in the following figure and should, based on our findings, represent more closely the real size of the LNPs produced. These results mean that a lot of precaution needs to be taken when characterizing LNPs size and that different methods should be used to have a full comprehension of the samples (size, monodispersity, stability, etc). It is also important to keep in mind that quality and homogeneity of the samples can be further improved with the addition of purification and filtration steps prior to the characterization.

(A)

Flow focusing	FRR	TFR (µL/min)	Aqueous (µL/min)	Lipids (µL/min)	LNP size (nm)	PDI
#1	20:1	210	200	10	94,77	0,09 ± 0,01
#2	10:1	220	200	20	103,97	0,19 ± 0,05
#3	7:1	228,6	200	28,6	136,51	0,06 ± 0,01
#4	5:1	240	200	40	141,09	0,08 ± 0,04
#5	2:1	300	200	100	166,62	0,05 ± 0,03

(B)

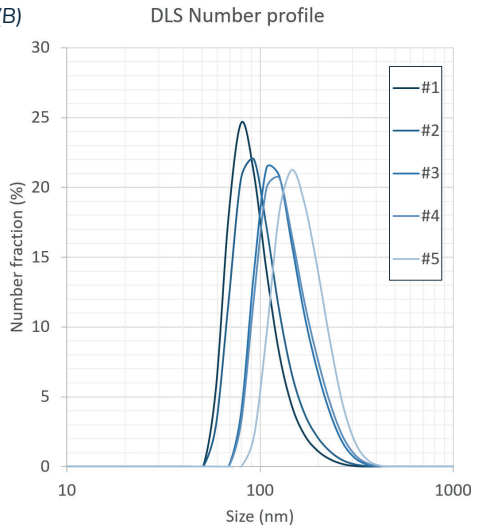


Figure 29: Nanoparticle size characterization based on the DLS number wieghted values for different generation parameters using a Flow focusing micromixer. (A) Table of the five conditions used to generate LNPs and corresponding size and PDI. (B) DLS number profiles obtained for each of the 5 conditions.

(A)

Flow focusing	FRR	TFR (mL/min)	Aqueous (µL/min)	Lipids (µL/min)	LNP size (nm)	PDI
#1	10:1	4,4	4000	400	54,45	0,25 ± 0,03
#2	5:1	4,8	4000	800	60,12	0,19 ± 0,02
#3	5:1	2,4	2000	400	102,31	0,11 ± 0,02
#4	5 :1	1,2	1000	200	128,9	0,14 ± 0,01
#5	2:1	6	4000	2000	89,22	0,07 ± 0,02
#6	2:1	3	2000	1000	110,80	0,08 ± 0,01

(B)

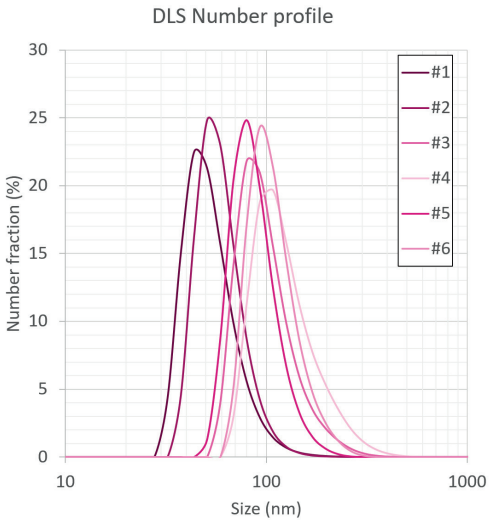


Figure 30: Nanoparticle size characterization based on the DLS number wieghted values for different generation parameters using an Herringbone mixer. (A) Table of the six conditions used to generate LNPs and corresponding size and PDI. (B) DLS number profiles obtained for each of the 6 conditions.

Troubleshooting

The flow rate measured by my MFS suddenly drops down for a short duration

The reason is probably that you have air bubbles in your system. When an air bubble passes through the MFS, the sensor measures a zero flow rate while the bubble hasn't left. To get rid of air bubbles, just wait for it to get out by maintaining a constant pressure or flow rate in both channels. The initial filling of the system is critical to avoid bubbles.

The flow rate doesn't stabilize

If the flow rate can't stabilize itself on the fixed value, first check that you haven't forgotten to put the flow resistor in the fluidic system.
If it's not the cause of the problem, you should consider changing the values of P and I.
The lower the values of P, the more the flow rate will be stable. The **"going further"** section details this solution.

Another way of solving this problem is to increase the microfluidic resistance. The **"going further"** section details this solution.

I see only one phase (aqueous buffer or ethanol) in my chip

Check both your MFS. The MFS should be connected in the right direction (indication of inlet/outlet are depicted on top of the sensor). If both MFS are connected in the right direction and are measuring a positive flow rate, and if the situation is lasting too long, check all your connections, there must be a leak.

If one of the values measured by the MFS is negative:

- if you are controlling the pressure, increase the pressure of the channel flowing in the wrong direction
- if you are controlling the flow rate, the flow rate should gradually increase to the fixed value. If it takes too much time, you can open the flow control configuration of the channel flowing in the wrong way, and gradually increase the value of I in the PI Basic algorithm (refer 4 Control of the flow rate p.18).

I have dust in my chip/my chip is clogged

In this case, there are two possible scenarios:

- In case your microfluidic chip starts to clog with solid material (dust, bigger particles, etc.), try to increase the pressure (or flow rate) of both phases to send the dust away. Alternatively, you can exchange the outlet and inlet connectors to reverse the flow in your chip. If it doesn't work, you should change the chip you're working with. The presence of dust in the chip should not be ignored. The monodispersity of the nanoparticles can't be ensured in this case, and their size could significantly change from the expectations of the user. Working with "clean" solutions and reservoirs is essential to prevent chip clogging.

- In case your microfluidic chip is clogged with an agglomerate of lipid particles, flush the channels with pure ethanol first, then water, at high pressure or with a syringe. You might have to wait a few seconds before the clog starts to dissolve.

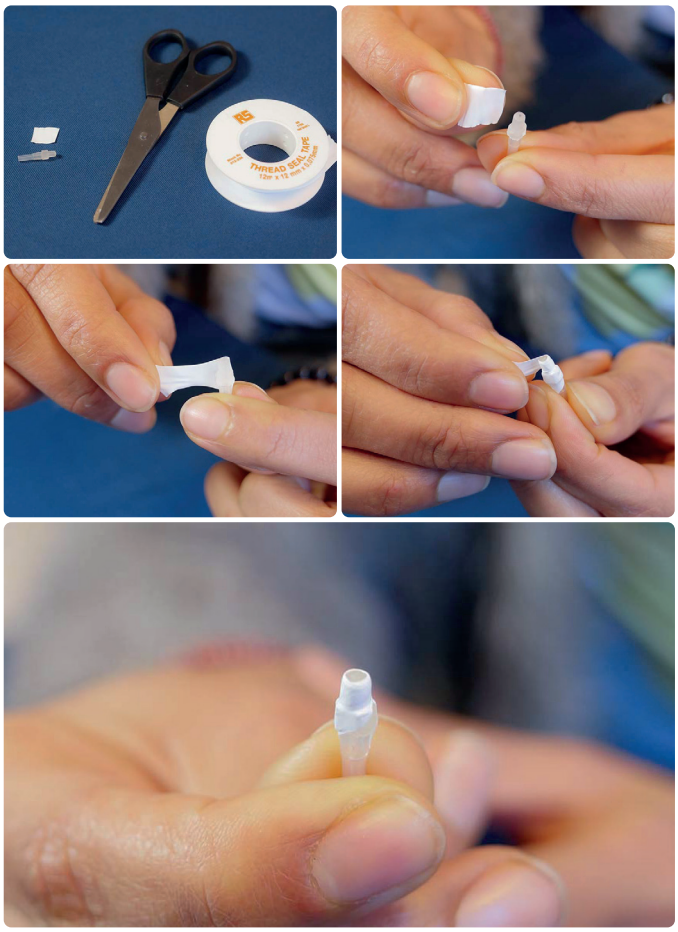
My OB1 is very noisy, what is the problem?

If your OB1 starts making loud noise, it's probably because of a leak in your system: the pressure source tries to permanently compensate for the lack of pressure due to the leak.
Check all the connections of the system (using teflon tape is often useful to avoid leaks on fluidic and pneumatic connections).

This PDF is dedicated to the problem
https://support.elveflow.com/support/solutions/articles/48001143505-fix-ob1-continuous-noise-or-a-leak

I have leaks from the mini Luer connectors to the flow focusing chip

This case is very common and a simple solution exists for you. Cut a piece of PTFE seal thread tape and wrap it carefully around the base of the connector. Be careful not to block the opening in the middle. Then insert the connector back into the female mini luer port and try again to flow liquid into the chip. This step might need to be repeated, perhaps with another connector, but once the leak is blocked, it is highly stable in time and will hold during the full duration of your experiment.



I don't have the same results as those provided in the diagram

If your nanoparticles don't have the size you expected, the reason could be a light systematic error of the MFS or the chip dimensions. You should consider calibrating the MFS once again.

This PDF is dedicated to the problem
https://support.elveflow.com/support/solutions/articles/48001163077-calibrate-mfs-flow-sensor-with-different-liquids

Be careful when controlling very low lipids flow rates (lower than 100 µl/min) with the MFS4, even though the measurements of the MFS are highly repeatable, they could lack accuracy in this range of flow rates.
The difference could also come from the purification or dilution step as this involves manual manipulations (pipetting) ; it may slightly vary from one user to another.
The provided diagram is indicative.

Refer to the MFS user guide for more details

I would like to produce nanoparticles with lower or higher flow rates, is it possible with these chips?

It is possible to reach lower flow rates, but you will have to reach very low aqueous buffer and lipid flow rates that the MFS can't precisely measure. It is possible to reach these low flow rates thanks to the control of the pressure with the OB1, but the stability of the flow rates (and thus the monodispersity of the nanoparticles) could not be as good as the stability with the control of the flow rates.

The maximum TFR you can expect from the flow focusing chip is around 500 µL/min with 2 bar pressure channels and 1/32" ID tubing. An easy way to increase the flow rates in your setup is to lower the resistivity in your whole system. You can change the microfluidic tubing and the resistance tubing for higher internal diameter ones and adapt the P and I values.

To learn more about the importance of resistances
https://www.elveflow.com/microfluidic-applications/setup-microfluidic-flow-control/microfluidic-flow-restrictors/

The maximum TFR in the herringbone chip can be much higher, up to at least 6 mL/min with 2 bar pressure channels. You can upgrade your OB1 pressure controller to 8 bar if higher flow rates are needed.
To reach higher flow rates, other sensor types might be suitable for you, such as ou BFS (below). Please contact us to discuss this case.

Changing the TFR in the flow focusing chip will have little effect on the LNP size for a given FRR, thus this chip is convenient when you wish to scale up your process and keep the LNP size constant. However, this is not the case with the herringbone chip as higher TFR will tend to reduce the size of your LNP. This is true up to a certain TFR threshold that needs to be assessed experimentally.

BFS: principle, advantages and specifications

The Bronkhorst Flow Sensor (BFS) is based on the Coriolis technology and presents unique benefits for your lipid synthesis setup.

- **Versatility**: it works with any liquid and doesn't require any calibration to ensure its compatibility;
- **High accuracy**: up to 0,2% of the flow rate;
- **Broad flow rate range**: a single sensor measures from µL/min to mL/min (several ranges available from 1.6 µL/min to 500 mL/min) to easily scale up your system.

Be aware that this flow sensor is added to the software **as an instrument** and not as a sensor. To learn more, refer to the dedicated webinar page and the Bronkhorst website.

Going further

The lipid nanoparticle generation Pack is very flexible. Depending on your needs, you could find it beneficial to change the setup: for example, working with another chip, with other resistances, or even other liquids.

You must be aware that, even though it is not hard to adapt your setup to new experimental conditions, there always are a few elements to adjust.

This section is here to introduce further upgrades and improvements to the system to perfectly taylor it to your specific experiment!

Appendix 1 Microfluidic connector guide

Connect the OB1 to the pressure source

Connect your OB1 pressure controller to external pressure supply using pneumatic tubing

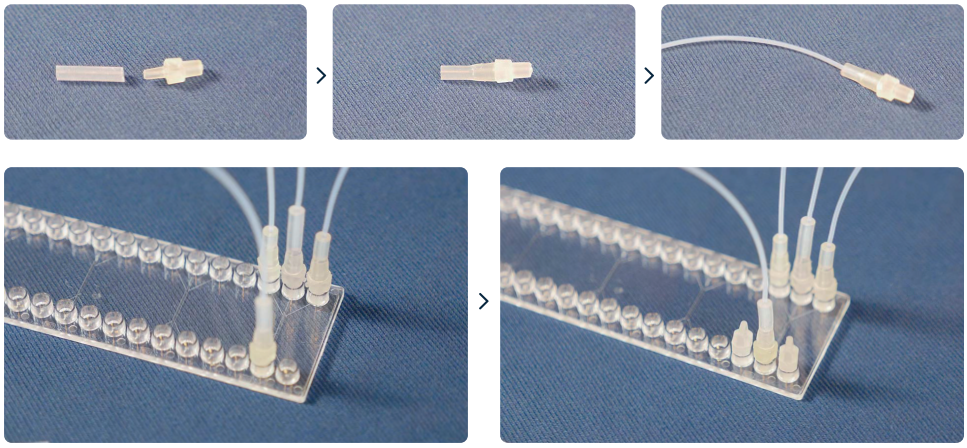
REFER TO AN OTHER USER GUIDE

Refer to the OB1 user guide for detailed instructions

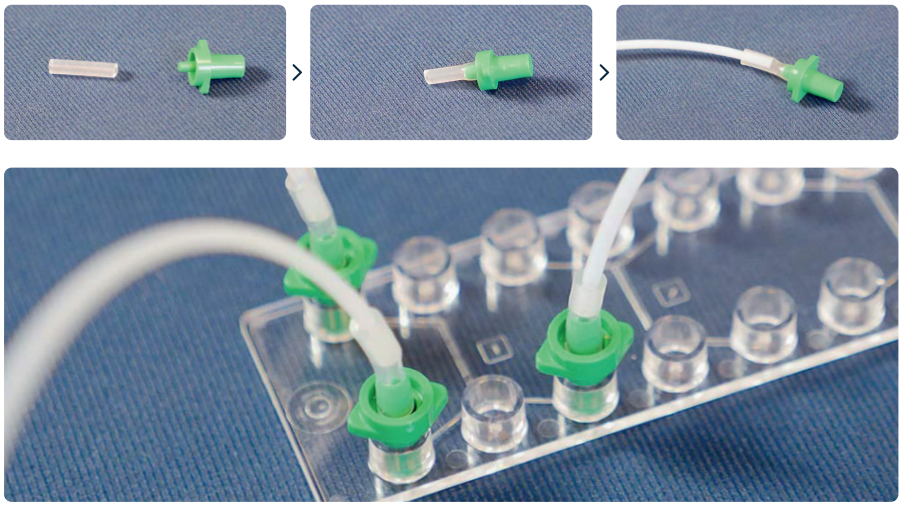
Prepare the MFS

Connect the reservoir to the OB1

Connection Tubing - Flow focusing chip

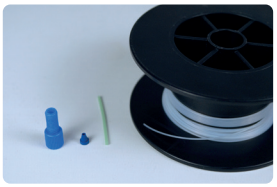


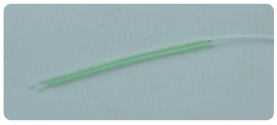
Connection Tubing - Herringbone chip



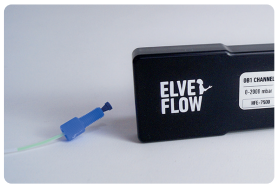
Connection Tubing - MFS

Add a sleeve on the tubing





Prepare the resistances



Appendix 2 Change the values of P and I in the PI Basic algorithm

Whenever your setup changes (new liquids, new chip...), you will have to check that your feedback parameters **P** and **I** are still appropriate.
Even if you keep the same setup, you could find benefit in changing the values of **P** and **I**.
The values of **P** and **I** Elveflow advises to set for the PI Basic algorithm are adapted for most combinations of aqueous buffer and lipids flow rates for this particular chip. Nevertheless, they can't be optimized for all combinations. If you want to make nanoparticles of a specific size for which you know the generation flow rates, you can gradually change the values of **P** and **I** until you find more optimized ones for for these flow rates (more responsiveness or more stability).

To learn where and **how to change and choose** the values of P and I in the PI algorithm, refer to 4 Control of the flow rate **p.18**

To learn more about the tuning of the feedback parameters
https://www.elveflow.com/ /support/solutions/articles/48001142611-set-a-flow-control-feedback-loop-resistance-and- -tuning

Be careful when changing the values of the parameter **I**. Suddenly increasing the value of the parameter **I** of the PI Basic algorithm will induce an overflow. You can avoid this overflow by changing the value of **I** while controlling the pressure instead of the flow rate.

Generally speaking, the higher the value of **I**, the more responsive the system will be, but the the less stable it will be once it has reached the requested value of the flow rate. If the value of **I** is too high, the flow rate will not stabilize.

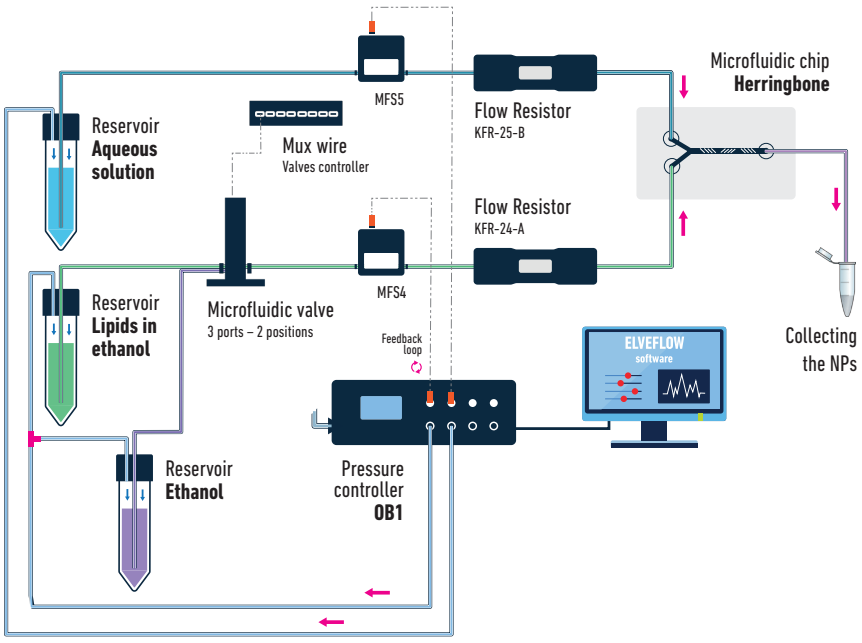
If when requesting a low flow rate (for example, 2 µl/min for the aqueous buffer) the system is really too slow to stabilize, carefully increase the value of **I**.

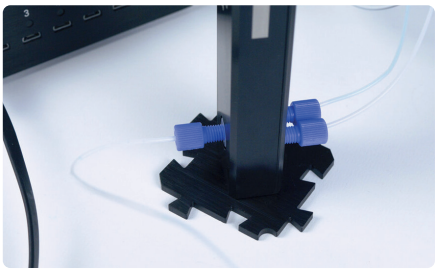
Increasing the value of **P** could increase the responsiveness of the system, and sometimes reduce the oscillation of the flow rate, but if it is too high, the flow rate will not stabilize.

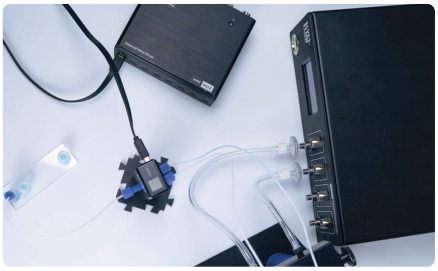
Appendix 3 Automated cleaning steps with a 3/2 valve and a mux wire

The following setup (based on the herringbone setup) is an example of how to integrate cleaning steps with pure ethanol in a more automated way. This feature is especially useful when you want to produce LNPs at different FRR or TFR and avoid cross contamination between your conditions. It will also help in the removal of adsorbed LNPs on the microchannel walls. To make this setup you will need the addition of two components:

- 1 microfluidic 3/2 valve : the 3/2 valves is a 3 ports valve with a shared inlet or outlet and enables the direction of fluid to or from different microfluidic lines.
- 1 MUX Wire valve controller : this instrument from the Elveflow product line enables you to control the valve, i.e. switch its position from port A to port B.







When controlling your setup with flow rates, you do not need to switch off the pressure on the first channel of the OB1 before switching the valve. In a typical LNPs fabrication experiment, we recommend the following steps:

1. Install your setup as described in this user guide and start producing your first LNPs
2. Switch the valve to perform a washing step. Optional: you can increase the flow rate of the ethanol for a faster and more efficient washing.
3. Once you are satisfied with the washing step, change your flow rates conditions corresponding to your next generation of LNPs
4. Switch the valve again and wait until lipids have reached the chip
5. Collect your LNPs
6. Repeat

TIP

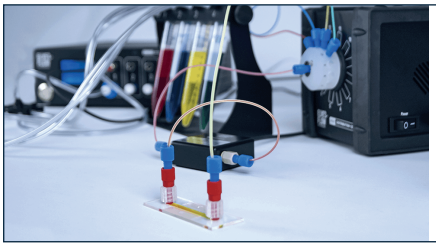
The side of the valve with only one port is always 'open', while the other side has two ports labelled N.C., for Normally Closed, and N.O., for Normally Open. To avoid confusion, decide on a port as your ethanol inlet and the other for your lipids and always keep the same configuration. When the valve on the software is open (green tick), the liquid is passing through the N.C. port. When the valve on the software is closed (red cross), the liquid is passing through the N.O. port.

TIP

The same principle applies if you would like to add a second valve at the outlet of the setup to automatically send your fluid either to the waste reservoir or to the collection reservoir. This is illustrated in the next section. The MUX Wire can control up to 8 valves.

TIP

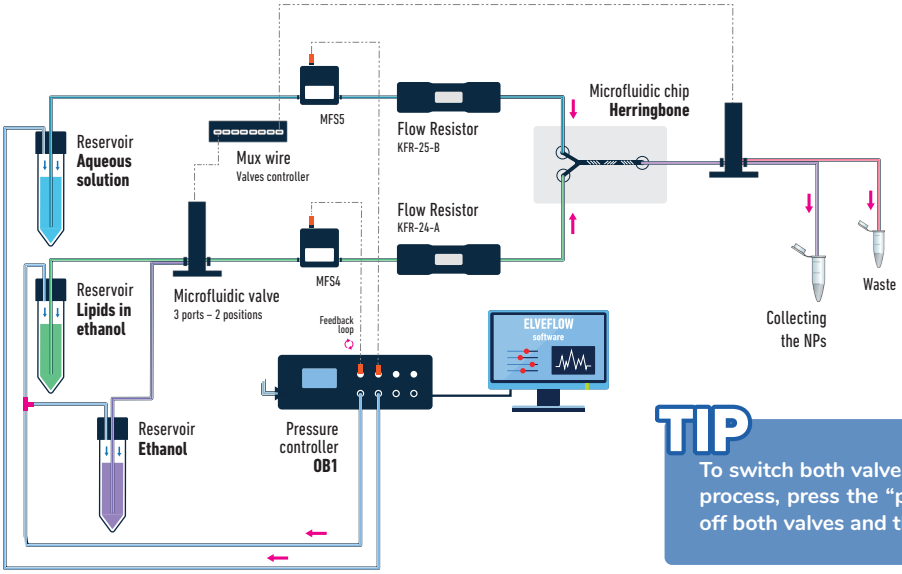
Adding valves will add a bit of resistance to your system, if your flow rate control becomes too slow, you might need to adjust the PI values in the software.



If you want to inject different solutions sequentially in your system, check out our sequential injection pack!

www.elveflow.com/microfluidics-application-packs/microfluidic-packs/sequential-fluid-injection-pack/

Appendix 4 Automated sample collection with an additional 3/2 valve



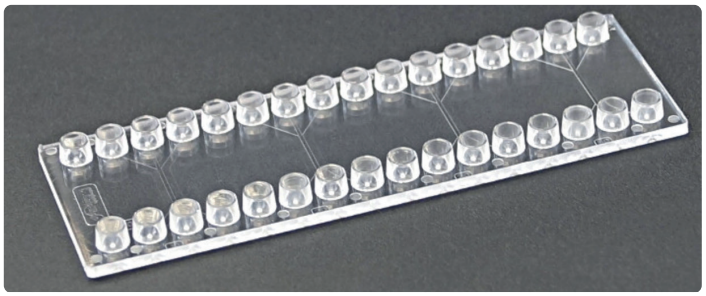
This setup goes a step further and adds a second 3/2 valve to the previously described microfluidic setup.

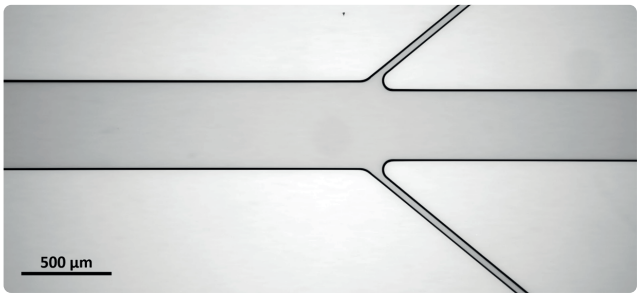
This additional valve allows you to automatically discard any unwanted output fluid (contaminated, flow stabilization time, washing steps, ...) without having to manually move the outlet tubing.

TIP

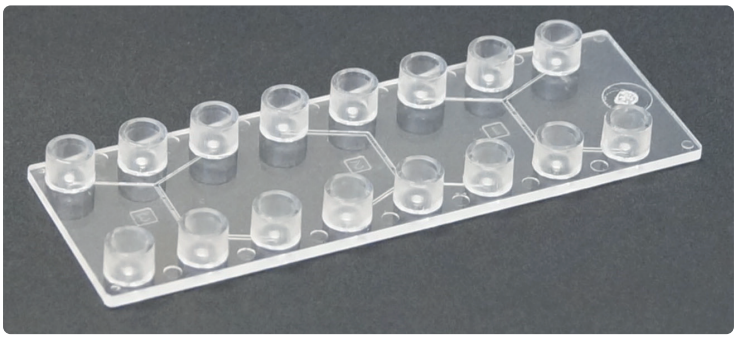
To switch both valves at the same time when starting the washing process, press the "pause" button on the MUX Wire window, turn off both valves and then press "play" again.

Appendix 5 Detail of microfluidic chips





Flow focusing chip



Herringbone chip

Supplementary information

Conditions of use

Terms and conditions of use

We strongly believe in the intrinsic quality of our microfluidic instruments line and we hope that you will be pleased with your purchase. However, in the unlikely event that you should receive damaged or incorrect goods in your delivery, please notify us within 7 days.

You will be offered the option of a refund or an exchange (provided the goods are in stock).

You may be asked to return goods for inspection. In this case we will refund the shipping fees.

Should the damaged or incorrect item be no longer available, you will be given the option of a refund. Please note that goods that become damaged or broken after 7 days of receipt cannot be returned.

Unwanted items

If for any reason you do not wish to keep your purchase and would like a store credit, then please notify us within 7 days.

We cannot accept unwanted returns that have been opened, used or damaged by the customer.

For unwanted goods, we allow up to 14 days for the return of goods. We will only issue a credit upon receipt of all returned goods.

Please note that we are unable to refund your costs in returning unwanted goods or the delivery costs of sending the goods to you in the first place.

Cancellations

If you wish to cancel your order please email us immediately: contact@elveflow.com.

Privacy Policy

Customer details remain private and confidential and will not be released to a third party unless required to do so by law.

We use the information we collect about you to process orders, to provide a more personalized shopping experience and, if you request it, to notify you about new products, special offers or other information that may be of interest to you. We do not sell or pass on any personal information to any other Companies or Organizations.

Payments & Procedures

Payment from private customers must be paid upfront. Trade orders from registered companies or organizations can be invoiced. Payment is due strictly within 30 days of the invoice date.

Products & Prices

Please note that some goods may vary in style, color or detail from the image shown. We reserve the right to change prices at any time.

Transport and storage

Be careful not to harm or shake Elveflow® products while moving. Elveflow® products must not be transported when plugged in. Store products in standard conditions in an adapted box (typically the one used to send you the product).

Humidity and temperature must not exceed those of the specifications.

Exclusive remedies

The remedies provided herein are the customer's sole and exclusive remedies. Elveflow® shall not be liable for any direct, indirect, special, incidental, or consequential damages, whether based on contract, tort, or any other legal theory.

Safety Information

THE FOLLOWING GENERAL SAFETY PRECAUTIONS MUST BE FOLLOWED DURING ALL PHASES OF OPERATION, SERVICE, AND REPAIR OF THIS INSTRUMENT. FAILURE TO COMPLY WITH THESE PRECAUTIONS OR WITH SPECIFIC WARNINGS ELSEWHERE IN THIS MANUAL VIOLATES SAFETY STANDARDS OF DESIGN, MANUFACTURE, AND INTENDED USE OF THE INSTRUMENT. ELVESYS ASSUMES NO LIABILITY FOR THE CUSTOMER'S FAILURE TO COMPLY WITH THESE REQUIREMENTS.

Important advice

Elveflow® products are for research use only.

No liquid should get into the OB1, otherwise this would void the warranty.

The pressure source connected to the OB1 must be dry, dust and oil free, and of a maximum of 10 bar. Please take the required action to ensure that these conditions are met and maintained.

Conditions of use

This instrument is intended for indoor use. It is designed to operate at a maximum relative humidity of 60% and at altitudes of up to 2000 meters. Operating temperature range is +5 °C to 50 °C.

Do not operate in wet/damp conditions: to avoid electric shock, do not operate this product in wet or damp conditions.

Do not operate in an explosive environment. Do not operate the equipment in the presence of explosive or flammable gases or fumes.

Warning: Do not use this product as safety or emergency stop devices or in any other application where failure of the product could result in personal injury. The protective features of this product may be impaired if it is used in a manner not specified in the operating instructions. Before installing, handling, using or servicing this product, please consult the data sheet and user manual.

Failure to comply with these instructions could result in death or serious injury. If the buyer purchases or uses Elveflow® products for any unintended or unauthorized application, the buyer shall defend, indemnify and hold harmless Elveflow® and its officers, employees, subsidiaries, affiliates and distributors against all claims, costs, damages and expenses, and reasonable attorney fees arising out of, directly or indirectly, any claim of personal injury or death associated with such unintended or unauthorized use, even if Elveflow® is allegedly negligent with respect to the design or the manufacture of the product.

Pressurized Equipment

Care must be taken when the Elveflow® pump is pressurized to ensure that the instrument is not damaged in any way.

Protection

Safety glasses and lab coats should be worn at all times when using an Elveflow® pressure pump due to the use of pressurized equipment. This is particularly important when hazardous liquids are used.

Electrictity Advice

Use Elveflow® instruments with the provided power unit only. Maintenance should only be attempted by qualified Elveflow® personnel. Removal of the back panel may invalidate any warranty.

Before applying power: verify that the line voltage matches the product's input voltage requirements and that the correct fuse is installed. Use only the specified line cord for this product and make sure the line cord is certified for the country of use.

Fuses: only fuses with the required rated current, voltage, and specified type (normal blow, time delay, etc.) should be used. Do not use repaired fuses or short-circuited fuse holders. To do so could cause a shock or fire hazard.

Keep away from live circuits: operating personnel must not remove instrument covers. Component replacement and internal adjustments must be made by qualified service personnel. Do not replace components with a power cable connected. Under certain conditions, dangerous voltages may exist even with the power cable removed.

To avoid injuries, always disconnect power, discharge circuits and remove external voltage sources before touching components.

ESD precautions: the inherent design of this component causes it to be sensitive to electrostatic discharge (ESD). To prevent ESD-induced damage and/or degradation, take customary and statutory ESD precautions when handling this product.

Maintenance advice

Maintenance should only be attempted by qualified Elveflow® personnel. Removal of the back panel will invalidate any warranty.

Do not service or adjust alone: do not attempt internal service or adjustment unless another person, capable of rendering first aid and resuscitation, is present.

Do not substitute parts or modify the instrument: because of the danger of introducing additional hazards, do not install substitute parts or perform any unauthorized modification to the instrument.

Return the instrument to an Elveflow® Technologies Sales and Service Office for service and repair to ensure that safety features are maintained.

Instruments which appear damaged or defective should be made inoperative and secured against unintended operation until they can be repaired by qualified Elveflow® personnel.

CE compliance

This product meets the essential requirements of applicable European Directives, as amended for CE marking, as follows:

Electromagnetic Compatibility

COUNCIL DIRECTIVE 89/336/EEC of 3 May 1989
This directive has been amended by the following Council Directives:
1. 92/59/eec of 29 June 1992 (General Product Safety)
2. 93/68/eec of 22 July 1993 (CE Marking directive)
3. 99/5/ec: Directive of Radio Equipment & Telecommunications Terminal Equipment (R&TTE).

ELVE FLOW

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Product:

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