# LIPID NANOPARTICLE SYNTHESIS PACK

ELVEFLOW
PLUG & PLAY MICROFLUIDICS

DOCUMENT REF: UGLNP-18102023

inside Therapeutics

# TABLE OF CONTENTS

## SYMBOLS USED IN THIS DOCUMENT

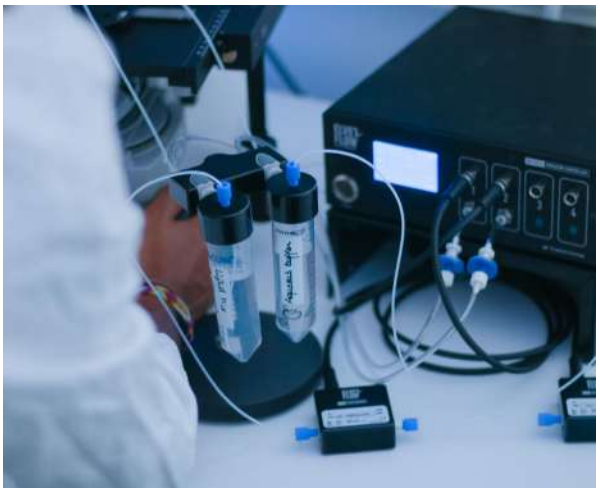
**IMPORTANT INFORMATION.** Disregarding this information could increase the risk of damage to the equipment, the risk of personal injuries, or degrade your user experience.

**HELPFUL INFORMATION.** This information facilitates the understanding of the system!

TIP **TIP** Use those tips to optimize your system use and performances

# INTRODUCTION

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



Simple and intuitive instructions are provided to quickly and smoothly make organic nanoparticles by nanoprecipitation with fine control over the synthesized nanoparticle physicochemical 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 size distribution (polydispersity index/PDI), using the numerous tips provided throughout the document. To finish, this guide provides 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, Lipid nanoparticles (LNP), PLGA nanoparticles, and solid lipid nanoparticles (SLNs),** see Figure 2. More generally, it can be used for the synthesis of most types of lipid-based and polymeric nanoparticles by a solvent-antisolvent nanoprecipitation. The main applicative example here is given for LNPs as they are now widely used in biomedical applications for the delivery of oligonucleotides and drugs. They serve as nanocarriers thanks to their excellent biocompatibility, high encapsulation efficiencies, and the possibility to optimize them for a variety of different targets. The principal fields of applications, among others, are 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.

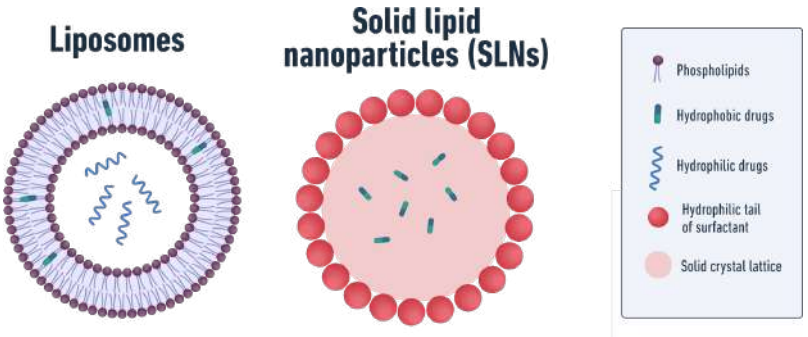


Figure 2: Illustration and composition of liposomes and solid lipid nanoparticles

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 pharmacokinetics. 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.

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 an LNP fabrication method.

| | Batch Methods | High Energy | Macrofluidics/ T-junctions | Microfluidics |
|---|---|---|---|---|
| |  |  |  |  |
| **Size control** | Poor | Poor | **Average** | **Excellent** |
| **Homogeneity** | Low (PDI ~0.5) | Low (multi steps) | **Average** | **Great (PDI <0.2)** |
| **Encapsulation efficiency** | Low | Poor | **Good** | **Excellent (>95%)** |
| **Repeatability** | Low | Low | Low | **Great** |
| **Achievable volumes** | µL/mL | µL/mL | **mL/L** | **µL/mL/L** |
| **Commercial available** | **Yes** | **Yes** | **Yes** | **Yes** |
| **Main characterstics** | **+ Affordable**<br>- Poor NP control | - Complex/Expensive/ Payload alteration | **+ Good scalability**<br>- Repeatability/ NP control | **+ Best NP control/ Scalability**<br>**- Solvent** |

***Table 1** : Nanoparticle synthesis methods summary*

## How does lipidic-based and polymeric nanoparticle generation work?

There are a few ways to generate lipid-based and polymeric nanoparticles in microfluidics, the most common one being solvent-antisolvent nanoprecipitation.

The fundamental principle of this method relies on the fast mixing of two phases (solvent and aqueous), leading to a drop in solubility of the lipids in the solvent, which start to agglomerate to form nanoparticles.

As one can observe, the mixing speed has a major influence on the synthesized nanoparticle size. The faster the mixing, the smaller the nanoparticle is. Also, the more efficient and homogeneous the mixing is, the better the control over the size and distribution.

Therefore, ensuring a uniform and constant mixing time throughout the process is crucial to optimize your final nanoparticle size control and homogeneity. More information can be found in our [organic nanoparticle formation review](#).

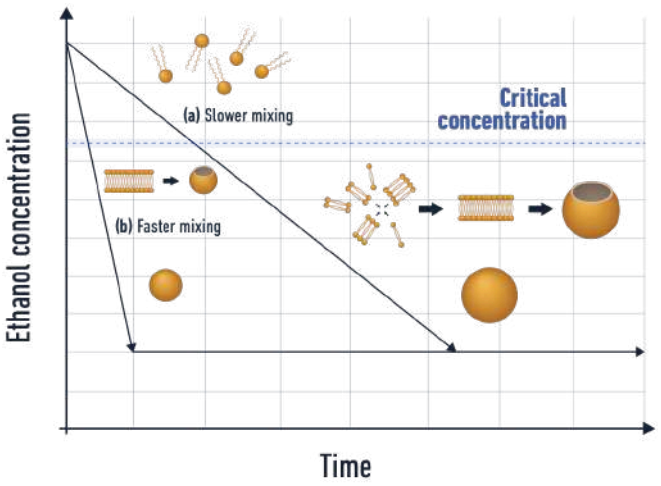
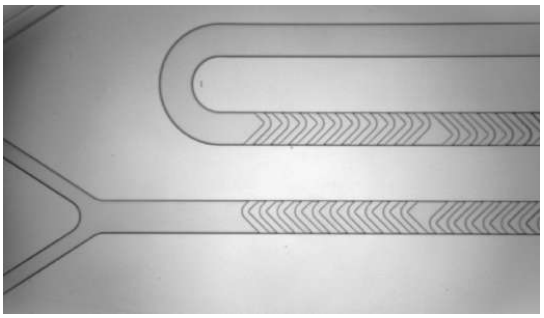



*Figure 3: Illustration of the mixing time influence over the lipid nanoparticle size*

In this context, microfluidics is the ideal solution thanks to its unique ability to maintain the fluid flow in laminar conditions, thus permitting excellent reproducibility of the mixing condition for optimal control of the final nanoparticle physicochemical parameters.

## Microfluidic mixing

Multiple strategies can mix fluids in microfluidics such as T-junction, baffle, recombination, and flow focusing (2D or 3D)... More information can be found on our website in our introduction to mixing for LNP synthesis



In this pack, a herringbone mixer has been chosen as this mixing method based on chaotic - though repeatable - flows offers the best performances in terms of mixing time control (thus achievable nanoparticle size), repeatability through the process (thus nanoparticle uniformity) while offering a very large accessible flow rate range (easy work with small and large volumes)

*Figure 4: Pictures of a herringbone mixer microfluidic chip*

The mixing principle of a herringbone mixer relies on the creation of micro-vortices in a ridged microchannel, where the 2 streams of reagents are injected. Those micro-vortices induce a "folding" of the 2 liquid phases on themselves, greatly increasing the exchange surface between the 2 phases.

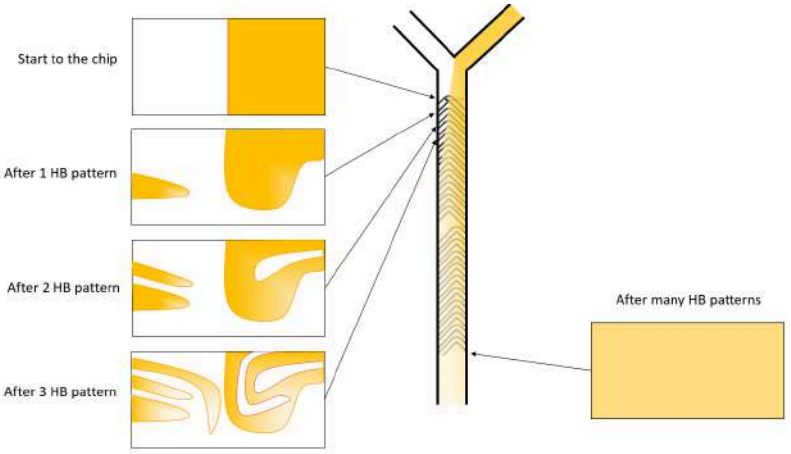



*Figure 5: Herringbone mixer mixing behavior*

## What are the most important parameters influencing the size of your nanoparticles?

In addition to 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), the physicochemical parameters of your nanoparticles are also dependent on the synthesis conditions (characteristics of the chips, flow rate conditions...)
We will now give an overview of these important parameters and how they influence LNP size.

**The flow rate ratio (FRR)** is an important parameter to play with when generating LNPs using a herringbone microfluidic mixer as it impacts both size and encapsulation efficiency.
FRR is defined as the ratio between the flow rate of the aqueous phase and the flow rate of the organic phase. For example, an 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. FRR impacts size at a low flow rate - especially before dialysis - while it has a lower to no impact at high flow rates. In addition to this, FRR has a major impact on encapsulation efficiency - EE% in a herringbone mixer (see characterization results)

**The total flow rate (TFR)** is the parameter that impacts the final NP size most. It is defined as the sum of the flow rates of the aqueous phase and the organic phase. For example, if you set the flow rate of the lipids to 100 µL/min and use an FRR of 5:1, the TFR in your system will be 600 µL/min. This parameter significantly impacts the herringbone chip as the liquids are mixed by chaotic mixing. This means that increasing the TFR will decrease the mixing time, thus decreasing the size of LNPs.

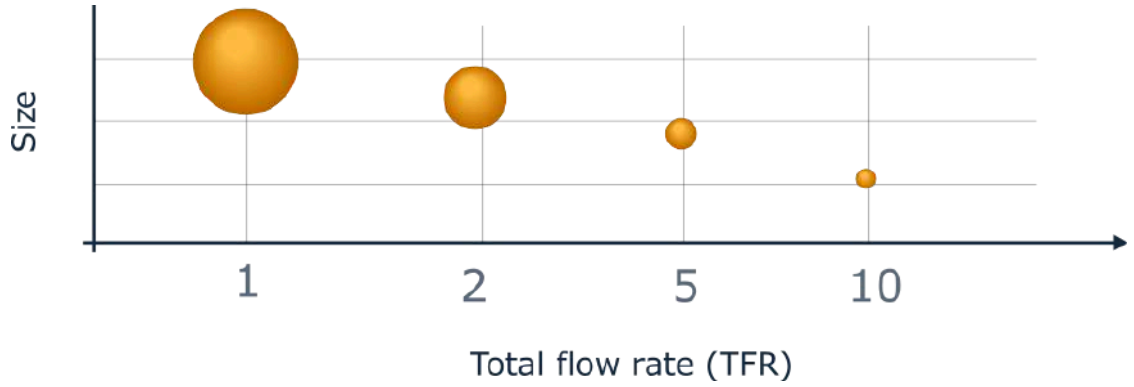



*Figure 6: Size variation vs TFR in a microfluidic herringbone mixer*

The **lipid formulation** and the **concentration of lipids** in the organic phase are crucial factors 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.

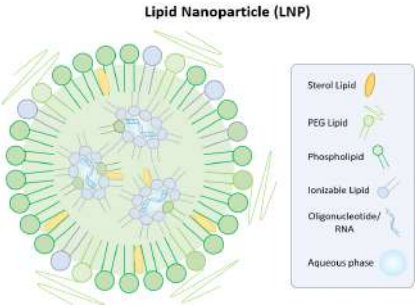



*Figure 7: Illustration and composition of Lipid nanoparticles (LNP)*

## 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, ionizable...) |
| **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 |

# Design and characteristics of the chips

Chips used are made with Zeonor, a thermoplastic polymer, which is a hydrophobic material helping prevent agglomeration. An important feature of this material is that it is compatible with polar solvents, such as acetone and ethanol. Chips also have excellent surface roughness to help minimize particle agglomeration.

## Herringbone chip

2 designs are available per chip. It is advised to use Design A for your synthesis.

| | | |
|---|---|---|
| Interface type | | Mini Luer |
| Lid thickness | | 188 µm |
| Material | | Zeonor |
| Design A | Channel depth | 200 µm |
| | Channel Width | 600 µm |
| Design B | Channel depth | 100 µm |
| | Channel Width | 400 µm |

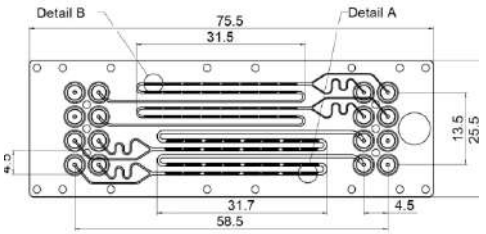



*Fig. 8: Layout of the Fluidic 1460 herringbone chip by microfluidic ChipChop*

The herringbone chip is also available in Polycarbonate (PC). PC is slightly less hydrophilic than Zeonor and can potentially reduce LNP adsorption on the walls - though no experimental results have shown it. However, PC chips tend to induce more cloggings.

Although these chips are said to be “single-use”, you can reuse them after carefully washing them.

# Fluidic setup

Two alternative options are available for the system offering different trade-offs in terms of performance, the flexibility of solvents used, and throughput. The below table summarized the difference between the 2 to help you choose the appropriate one depending on your requirements.

## Which setup is best for you?

| | Regular | Advanced |
|---|---|---|
| **Achievable size** | 50 - 400 nm | |
| **Homogeneity** | Very high (<0.2) | |
| **Encapsulation Efficiency** [a] | >90% | |
| **Repeatability** | Good | Excellent |
| **Flow rate (max TFR)** | 8 mL/min | 60 mL/min |
| **Solvent used** | Ethanol | Any solvent compatible with Zeonor |
| **Automation** | Yes | |
| **Compatible chip** | Herringbone (Any) | |
| **Internal volume** | ~100µL | ~500µL |
| **Dead volume** | ~20µL | ~20µL |
| **Achievable volumes** | 1 mL to L | 1 mL to L |

**Table 2:** Comparison table between regular and advanced pack

a Value provided for RNA encapsulation into LNP - can change with other compounds

The items included in the regular and advanced pack are identical except the flow sensor.
The regular pack can be later upgraded into the advanced one on request. Reach out to contacft@insidetx.com should you have any questions

# Regular setup

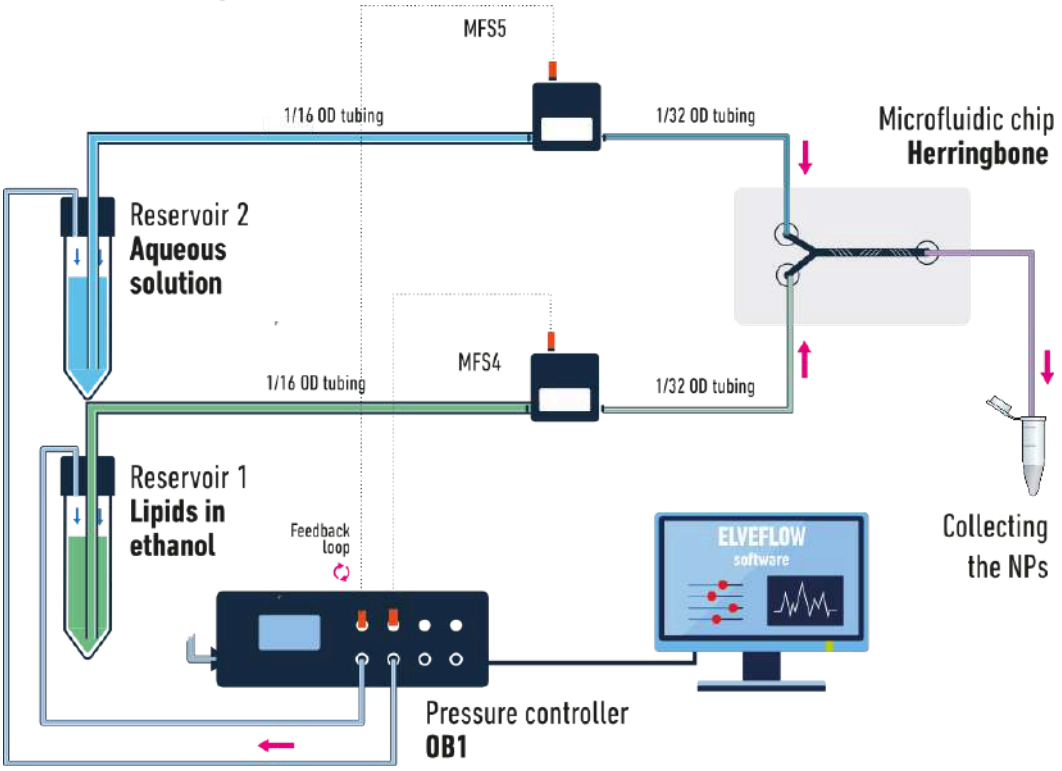



*Fig. 9: Sketch of the regular Lipid nanoparticle synthesis setup*

# Advanced setup

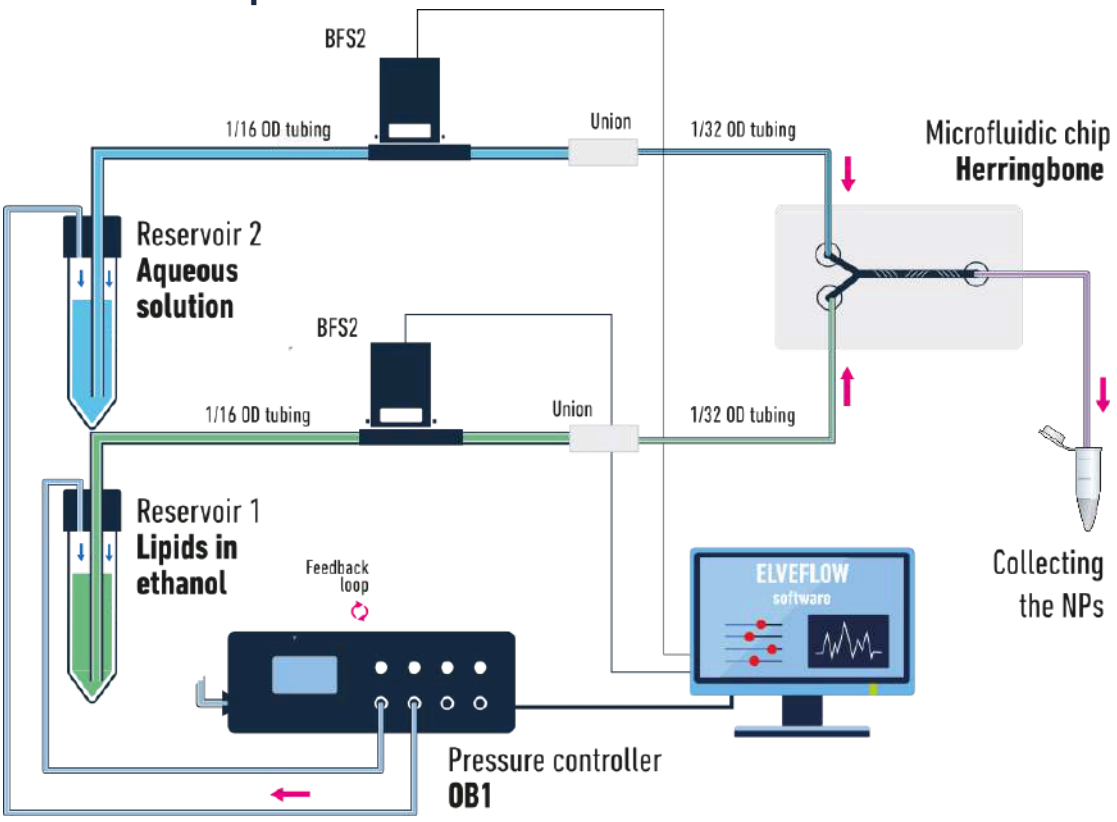



*Fig. 10: Sketch of the advanced Lipid nanoparticle synthesis setup*

# Characterization results

You are only a few steps away from making your own LNPs. In this section, we will guide you on how to set up your reagents, collect your LNPs after their fabrication and characterize your sample properly.

Typically, the process involves a first **mixing** step of the two phases, organic and aqueous, followed by an **equilibration time** after the collection of the LNPs and finally, a **buffer exchange** step. Each of these steps has an impact on the final LNPs size and characteristics and thus needs to be properly controlled. During the mixing step, you will be able to play with the flow parameters (TFR and FRR) and the reagent composition to adjust the size of the collected NPs. 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, and diluted, within 24 hours post-fabrication.

The buffer exchange, or solvent removal step can be done by various purification techniques: dialysis, rotary evaporator, ultra-filtration,... This generally leads to a decrease in nanoparticle size, such as described in the research article by Vargas et al.[1] Thus it is recommended to measure your LNP size prior to and after the purification step.

LNP's are typically purified by dialysis with 12-14k MWCO membranes and an aqueous buffer (PBS, Tris...).

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).

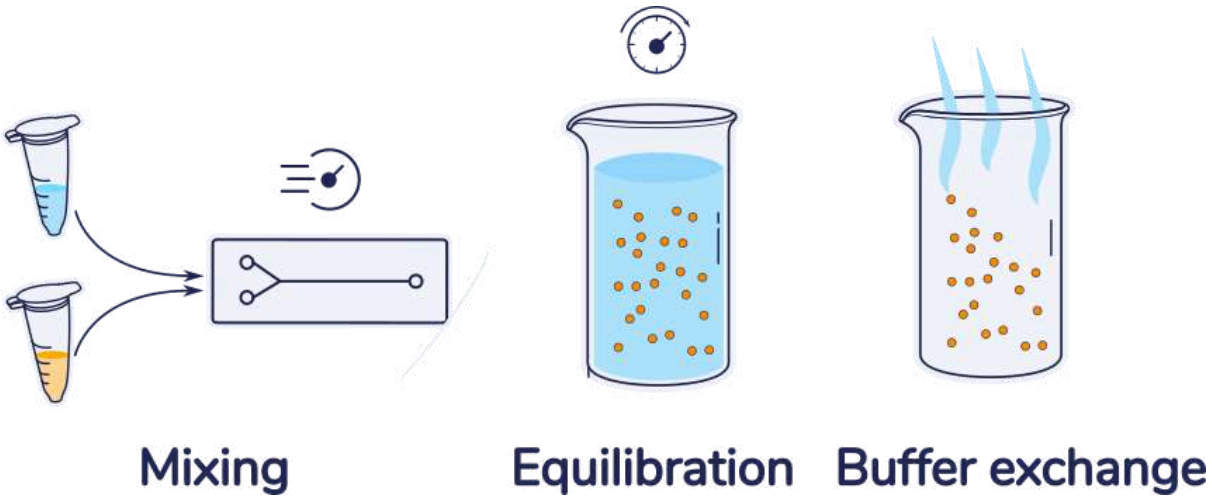



*Fig. 11: Illustration of the nanomedicine fabrication process*

[1] *Vargas, R. et al., (2023). Dialysis is a key factor modulating interactions between critical process parameters during the microfluidic preparation of lipid nanoparticles. Colloid and Interface Science Communications, 54, 100709. https://doi.org/10.1016/j.colcom.2023.100709*

# 1. Set up the reagents/Formulation used

**Aqueous phase:** Attach your reservoir (1,5, 15 or 50 ml) filled with your aqueous buffer / RNA to the pressurized reservoir cap and connect it to the supplied 1/16" OD tubing and fittings.

**Organic phase:** Attach your reservoir (1,5, 15 or 50 ml) filled with Ethanol (or other solventst) / Lipid formulation to the pressurized cap and connect it to the supplied 1/16" OD tubing and fittings.

Plug both reservoirs to the reservoir holder and to the corresponding OB1 pressure controller outlet following the system installation steps (see **System Installation** section)

This pack was tested with different lipid formulations for the fabrication of empty liposomes and lipid nanoparticles encapsulating siRNA. The table hereafter summarizes the recipes of the different lipid mix and their respective aqueous buffers. All lipids mix were dissolved in ethanol.

You may use a vortex mixer and a few minutes of sonication to ensure all lipids are well dissolved.

| Name | Lipid composition | Aqueous buffer |
|---|---|---|
| **RNA-LNPs** | DSPC : Chol : DOTAP : DSPE-mPEG200 at molar ratio 10 : 48.5 : 40 : 1.5 and 2.5 mM concentration | Citrate buffer 5 mM + RNA (N/P ratio of 3) |
| **Cationic liposomes (DDAB or DOTAP)** | DSPC : Chol : DOTAP or DDAB : DMG-PEG2000 at molar ratio 10 : 50 : 38 : 2 and 3 mM concentration | Citrate buffer 10 mM, pH 6.0 |
| **Neutral liposomes** | DOPC : Chol : DSPE-PEG2000 at molar ratio 94 : 5 : 1 and 3.84 mg/mL concentration | NaCl 0.9 mg/mL in water |
| **Lipoid liposomes** | Lipoid S75 at 40 mg/mL concentration | NaCl 0.9 mg/mL in water |

***Table 3**: Experimental material used for LNP pack tests*

# 2. Introduction to nanoparticle characterization

This section will describe the characterization methods used to measure the main LNPs **Critical Quality Attributes** (CQA). There are four main quantities that you need to measure to characterize your sample properly :

- Size of the LNPs (in nm)
- Polydispersity index or PDI (in a.u.)
- Zeta potential (in mV)
- Encapsulation efficiency (in %)

## 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 or a Vasco Kin from Cordouan Technologies. 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 determine 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.

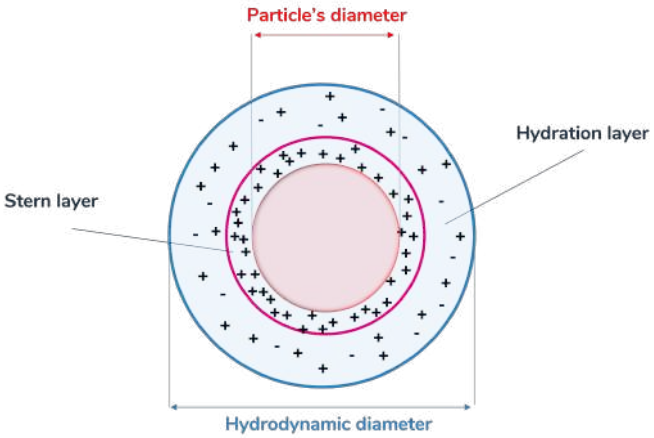



*Fig. 12: Hydrodynamic diameter of a nanoparticle*

Besides 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).

**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.

**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.

*Fig. 13: Sample preparation prior to DLS measurement*

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.

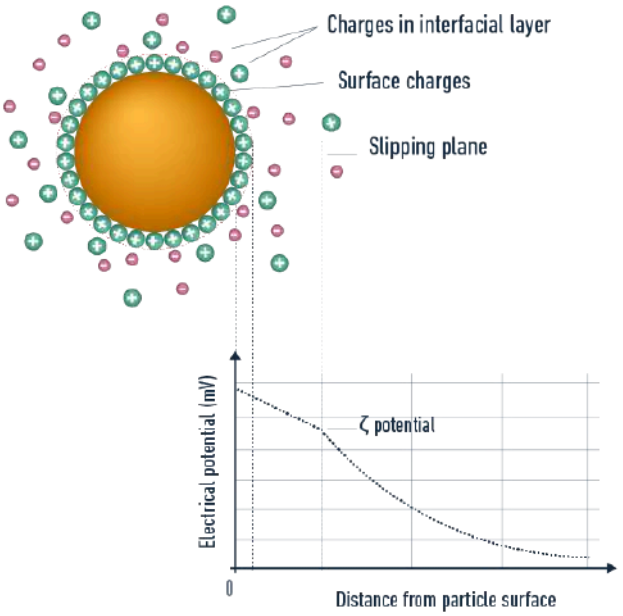



***Figure 14:*** *Zeta potential illustration*

## Drug encapsulation efficiency

This quantity refers to the percentage of the initial drug amount that has been encapsulated in the LNPs. It is typically measured after having isolated the LNPs from the surrounding medium (by ultrafiltration or other purification process), then analyzing the amount of drug that is left in this supernatant. You get the encapsulation efficiency by applying the following formula: $1 - (\frac{Unencapsulated\ drug}{Total\ drug})\ x\ 100$ .

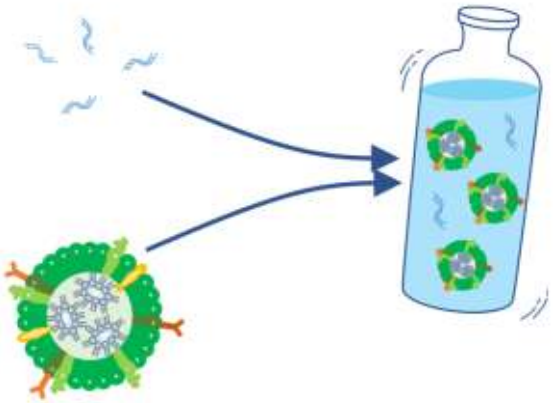


***Figure 15:*** *Encapsulation efficiency illustration*

[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.*

## 3. LNP Pack results

The following table shows the testing conditions and results obtained with this pack for RNA-LNPs. The samples were measured as collected, with an additional dilution step to be in the optimal concentration upon DLS measurement.

| | | TFR (µL/min) | FRR (µL/min) | Aqueous (µL/min) | Lipids (µL/min) | LNP size (nm) | PDI | EE% |
|---|---|---|---|---|---|---|---|---|
| Sample # | 1 | 1 | 3 | 750 | 250 | 143,2 | 0,11 | 93,4 |
| | 2 | 2 | 3 | 1500 | 500 | 121,7 | 0,12 | 94,3 |
| | 3 | 5 | 3 | 3750 | 1250 | 114,8 | 0,12 | 94,5 |
| | 4 | 1 | 5 | 833,33 | 166,67 | 140,5 | 0,07 | 94,6 |
| | 5 | 2 | 5 | 1666,67 | 33,33 | 125,2 | 0,11 | 94,7 |
| | 6 | 5 | 5 | 4166,67 | 833,33 | 95,9 | 0,09 | 95,5 |
| | 7 | 1 | 9 | 900 | 100 | 126,1 | 0,06 | 94,3 |
| | 8 | 2 | 9 | 1800 | 200 | 119,6 | 0,09 | 94,3 |
| | 9 | 5 | 9 | 4500 | 500 | 83,5 | 0,09 | 93,1 |

***Table 4:*** *SiRNA in LNP results table recap*

All zeta potential values were between 0 and + 1,5 mV. Although the ZP is typically taken as an indicator of colloidal stability, this information needs to be treated carefully. In this case, the negative charge on the citrate in the buffer is compensating for the positive charge of the cationic lipids, leaving the ZP to be almost neutral. This does not mean that the LNPs are unstable, as the PEGylated lipid added in the formulation acts as a stabilizing agent.

## TIP

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

## Effect of the flow parameters on Size and PDI

The following graph illustrates how the flow rate parameters influence the size of cationic liposomes. Using a herringbone chip, the TFR has a greater influence than the FRR. The FRR also changes the final concentration of liposomes.

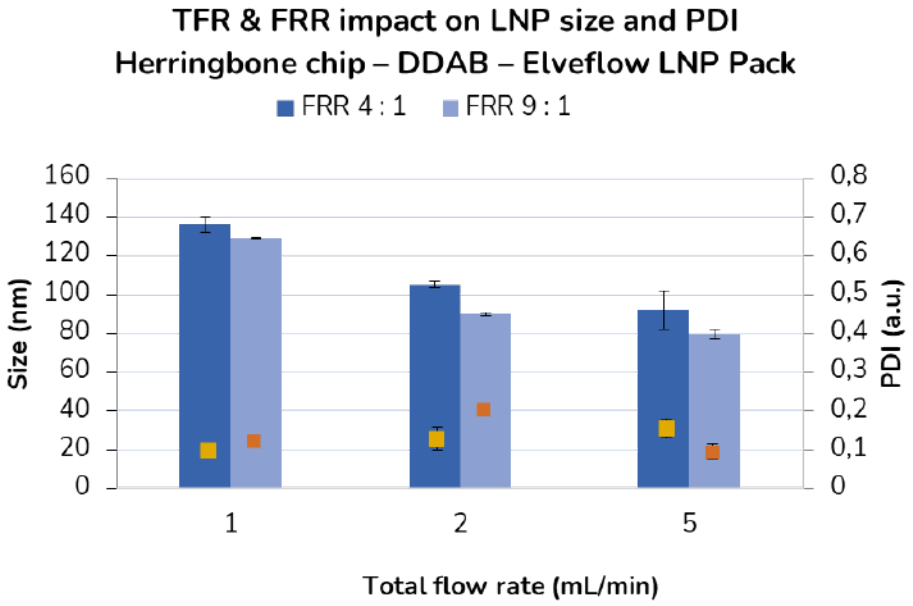



***Figure 16:*** *TFR & FRR influence on size and PDI*

## Effect of the composition and TFR on size and PDI

Here we compared three different formulations for the formation of liposomes: two based on cationic lipids and one without any charged lipids. We obtained significantly different sizes among all three formulations. This experiment clearly shows the importance of the formulation on the final size of the LNPs.

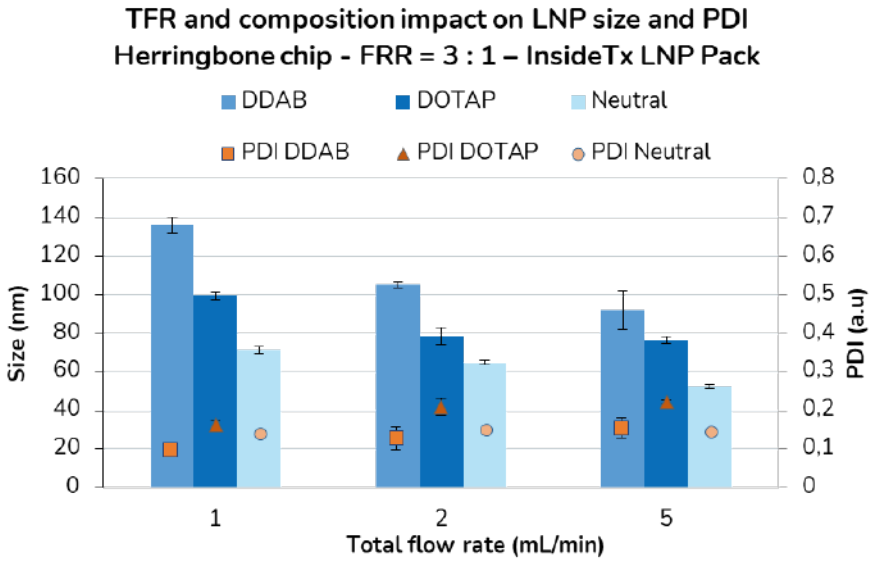



***Figure 17:*** *Composition impact on LNP size and PDI*

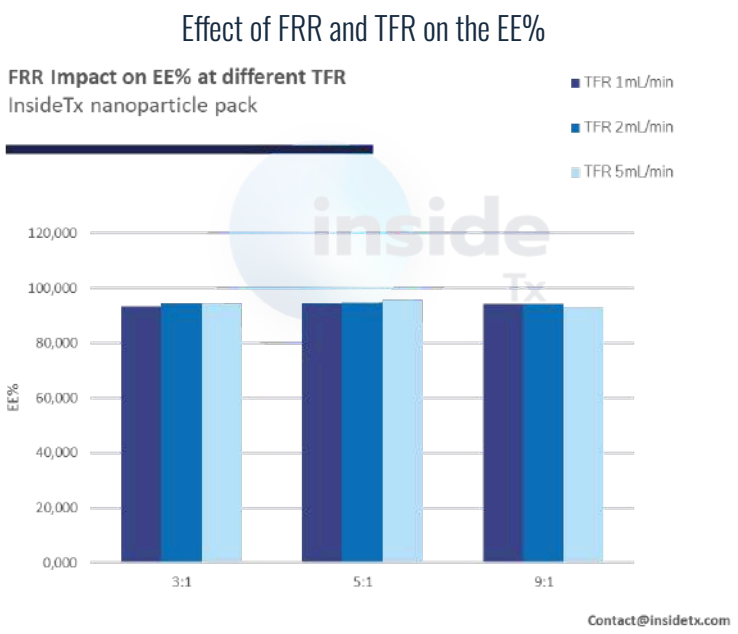



***Figure 18:*** *TFR and FRR influence on EE% (for SiRNA in LNP)*

## Effect of the buffer exchange step

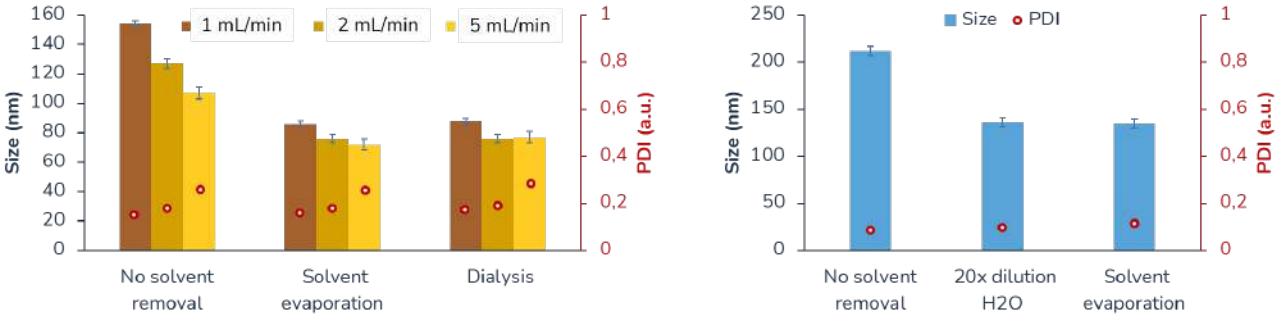



***Figure 18 a - b :*** *Buffer exchange effect on LNP parameters. Influence of different buffer exchange methods*

## Parameters influence recap

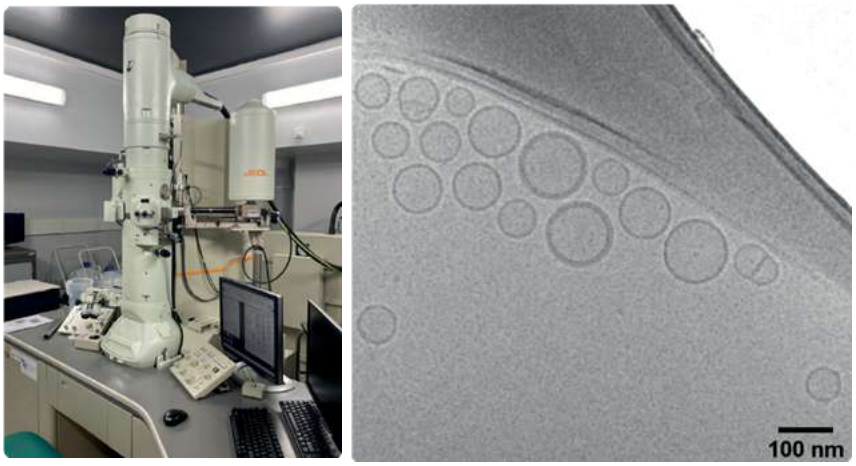
More generally, as introduced in the article of R. Vargas and all. " Dialysis is a key factor modulating interactions between critical process parameters during the microfluidic preparation of lipid nanoparticle " using a nearly identical system, the synthesis parameters influence can be summarized as follows

| Synthesis parameter | Nanoparticle characteristics | | | | | |
|---|---|---|---|---|---|---|
| | Average Size | | Zeta potential | | PDI | |
| | Before dialysis | After dialysis | Before dialysis | After dialysis | Before dialysis | After dialysis |
| TFR Increase | ↘↘ | ↘↘ | – | – | ↗↗ | ↗↗ |
| FRR Increase | ↘ | – | ↗↗ | ↘ | ↗ | ↗ |

***Table 5:*** *TFR, FRR and dialysis influence on Size, Potential and PDI in the LNP pack*

# 4. 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.




Briefly, fresh samples of cationic LNPs (DDAB) were fabricated using the setups described in this Pack. 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 19: Cryo-TEM setup (left) and example image (right) of the produced LNP using a Herringbone micromixer.*

Images were further analyzed using the ImageJ software. One example of the analysis is provided in Figure 22.

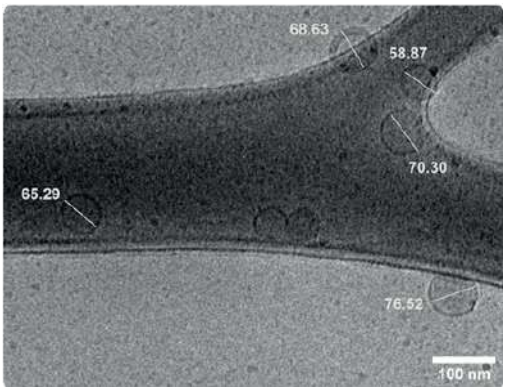
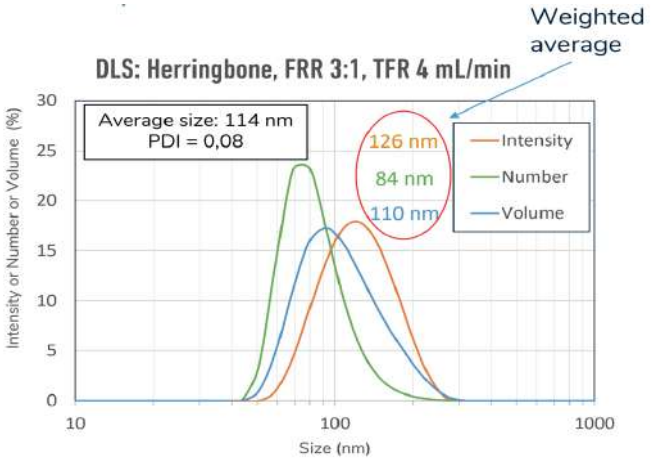



*Figure 20: Cryo-TEM image analysis example using ImageJ line selection tool (units in nm).*

# Results comparison and LNP size discussion

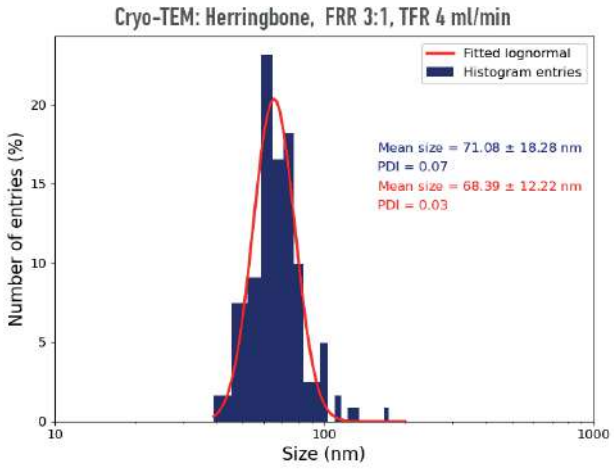
As mentioned previously, in addition to the intensity profile, the Zetasizer software also computes two other types of profiles, by volume and by number. Given that you introduce the correct material properties (absorbance and refractive index), these profiles can give you useful information on your 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 21: 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 22: 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 21 and 22). 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). 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.

# Contents of the Lipid nanoparticle pack

## 1 OB1 MK4 Pressure-driven flow controller

2 channels from 0 to 8 bar

The pressure controller is the centerpiece of the setup, enabling accurate control over the pressure difference across the microfluidic system and thus, fine control over the liquid flow in the microfluidic device.

## 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: **ΔP = RxQ**

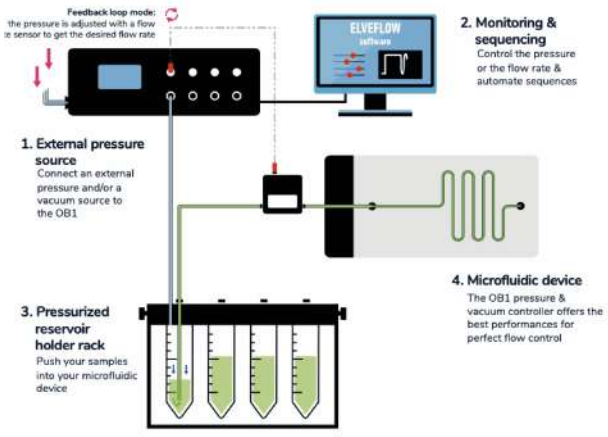
For instance, the resistance of a circular cross channel is

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

**R** depends on the fluidic system's 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 the same flow rate in a system with longer or thinner tubing or with a more viscous liquid.

$$R \propto \frac{\mu L}{r^4}$$

Viscosity, Length, radius




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 Flow rate sensors

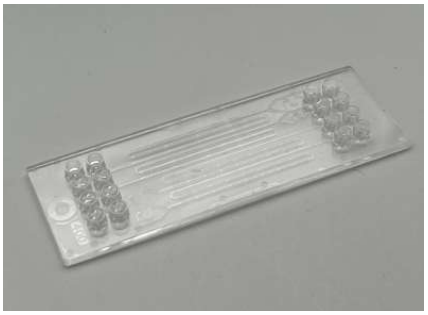
The flow sensors are used to measure the flow rate of the liquid going through the sensor. Combined with the pressure controller, it allows the user to monitor and precisely control the flow rate.
The 2 packs are offered with different flow sensors, to accommodate different requirements.

| Pack type | Solvent flow sensor | Aqueous flow sensor |
|---|---|---|
| **Regular pack**<br>- Up to 10 mL/min | **MFS4**<br>• 100 - 3500 µL/min with Ethanol calibration<br>• 5% accuracy<br>• Needs to be manually recalibrated for other solvents - performance not guaranteed | **MFS5**<br>• 200 - 5000 µL/min with Water calibration<br>• 5% accuracy<br>• Calibrated for water |
| **Advanced pack**<br>- Up to 60 mL/min | **BFS2 (or BFS3)**<br>• 0.2 - 33 mL/min (1 to 500mL/min)<br>• 0.2% accuracy<br>• Work with any solven: Calibration free | **BFS2 (or BFS3)**<br>• 0.2 - 33 mL/min (1 to 500mL/min)<br>• 0.2% accuracy<br>• Work with any solven: Calibration free |

# 2 Herringbone microfluidic chips

## Fluidic 1460, Microfluidic ChipShop

Made of Zeonor (COP), the micromixing chip is where aqueous buffer and lipids meet to make nanoparticles.



# 6 Reservoirs

## with pressurized reservoir caps

Reservoirs are used to hold your liquids and are closed with specialized black reservoir caps that contain ports to connect the tubing. The pressure imposed by the OB1 pressure-driven flow controller in the reservoir forces the liquid to leave the reservoir through the pressurized cap and flow through the microfluidic system. Pressurized caps are autoclavable and infinitely reusable.
6 reservoirs are provided in the pack:

- 2 in size S for Eppendorf tubes (1.5 mL)
- 2 in size M for 15 mL falcon reservoirs
- 2 in size L for 50 mL falcon reservoirs.

The S caps are compatible with any microcentrifuge tubes (1.5 and 2 mL) but the M and L caps are only compatible with Falcon™ and VWR™ brands.

# 1 Tubing 1/16" OD (30 m)

The liquid passes through the tubing to go from one component (such as reservoirs, MFS, chip...) to another. Its internal diameter is 800 µm (1/32")
The 1/16" OD tubing is used to connect the reservoir to the flow sensor.
Fittings used to connect it are ¼-28 to 1/16 OD

## 1 Tubing 1/32" OD (20 m)

The liquid passes through the tubing to go from one component (such as reservoirs, MFS, chip...) to another. Its internal diameter is 300 µm.
The 1/32" OD tubing is used to connect the flow sensor to the microfluidic chip.
Fittings used to connect it are mini luer to 1/32 and mini luer to ¼-28 adapters

## Pneumatic tubing

To connect the OB1 outputs to the pressurized reservoirs.

The fittings used to connect it to reservoirs are: 3/32 barb to ¼-28

## 1 Microfluidic connection kit

These pieces are required to connect the different components with the tubing.

From left to right: Set of fittings used to connect the OB1 to a pressure supply (pressure source connexion kit), 3/32 barb to ¼-28 (reservoir air connectors), ¼-28 to 1/16 OD tubing and its ferrule (connectors for 1/16" OD fluidic tubing), ¼-28 to mini luer and mini luer to 1/32 tubing connector (connectors for 1/32" OD fluidic tubings and chip)

# 1 Air filter

This air filter is placed between the pressure generator (compressor for instance) and the OB1 inlet. It keeps the OB1 clean from dust and humidity.

# 1 Tubing cutter

This cutter is useful to properly cut the tubing (perpendicular and clean section),which helps to prevent leaks in your setup.

## TIP

Tubing cutters are great for 1/16 OD tubings but can be difficult to use with 1/32" tubings. For those, you can also use a razor blade or sharpened pair of scissors.

# ESI software

The OB1 and the flow sensors are controlled by the software. Its interface allows you to easily select the pressure or the flow rate you want to set in the system. The ESI software also contains a useful tool to automate your droplet generation: the ESI sequence scheduler. It allows you to define a set of actions and to connect them in sequence.


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. Here, a pressure supply of 8.5 to 10 bar is suggested. Refer to the OB1 user guide for more information. Feel free to contact us at contact@insidetx.com to help you pick the optimal pressure source for your system, or to provide the pressurized air source if needed.

# System installation

Let's start installing the system together!
The full assembled system should look like the one depicted in the picture here.
The following steps are required for the system installation:

1. Software installation
2. OB1 installation
3. Reservoir assembly
4. MFS flow sensor installation (4bis for BFS)
5. Complete system installation

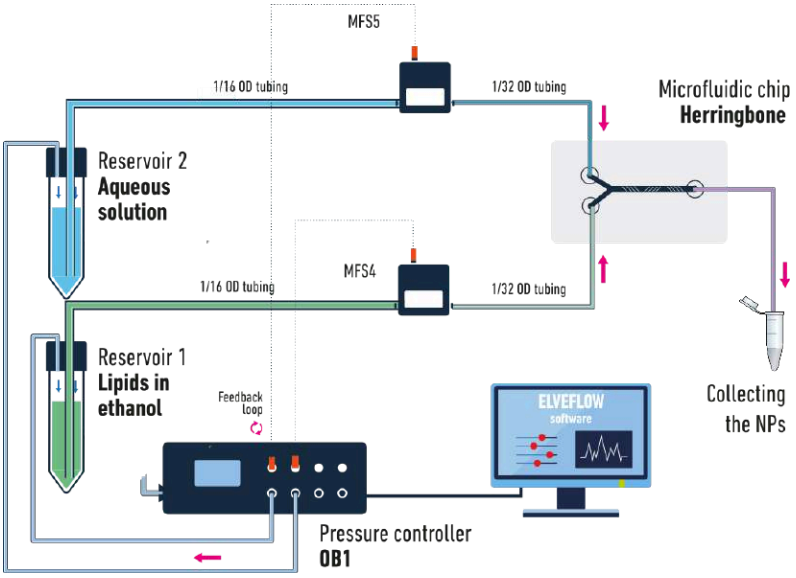



*Figure 23: Lipid nanoparticle Pack regular version*

## 1. Software installation

The **minimum computer requirements** to install the ESI software are the following :

- Processor: 3.0 GHz Pentium 4
- RAM: 1 GB
- USB 2.0
- 1 GB of free hard disk space
- OS Compatibility : Windows 10, Windows 8

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


*You can refer to this video.*

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

They can be downloaded from the support section of Elveflow's website

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

**REFER TO ANOTHER USER GUIDE**
ESI software user guide | Installation

## 2. OB1 installation

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 into the power source.

*Refer to **Appendix 1** to see how to connect all the components together.*

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 MK4, 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.


*You can refer to this video.*

**REFER TO ANOTHER USER GUIDE**
Refer to the OB1 user guide for detailed instructions | **Instrument connection**

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

## 3. Reservoir assembly

Now that the OB1 is duly installed, it is time to connect the reservoirs.
Pick the reservoir size you decide to use (1.5, 15, or 50 mL) and follow the following steps:

Start by connecting some 4mm tubing to the outlet of the OB1 (the filter is not necessarily required for the first installation)

Connect the other end of the tubing to the reservoir by attaching it to a 3/32 barb to ¼-28 connector, and screw it to the chosen reservoir cap as depicted bellow.



Once the reservoir is connected to the OB1, install the ¼-28 to 1/16” OD tubing fitting (blue) to the 1/16” OD tubing. Once done, connect it to the reservoir. Make sure the tubing is going all the way to the bottom of the reservoir.


## TIP

**To scale up your production, larger bottles (up to 5L) can also be used with this system using the optional GL45 caps. Note that those reservoirs should be used with pressure-resistant bottles up to 10 bar minimum.**

# 4. MFS installation (Regular pack version)

*Add the MFS to the system and calibrate it. For the advanced version, please check 3. bis below*

## A/ MFS Software connexion:

First, physically connect the two flow sensors to the OB1. Use the provided electrical connector with an orange end to connect to hook it up to the front panel of the OB1

Add the flow sensors (MFS) to your ESI software by

- **Pressing add sensor \ select flow sensor \ digital**
- Giving it a name
- Selecting to which device and channel the sensor is connected and pressing OK to save the changes.

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

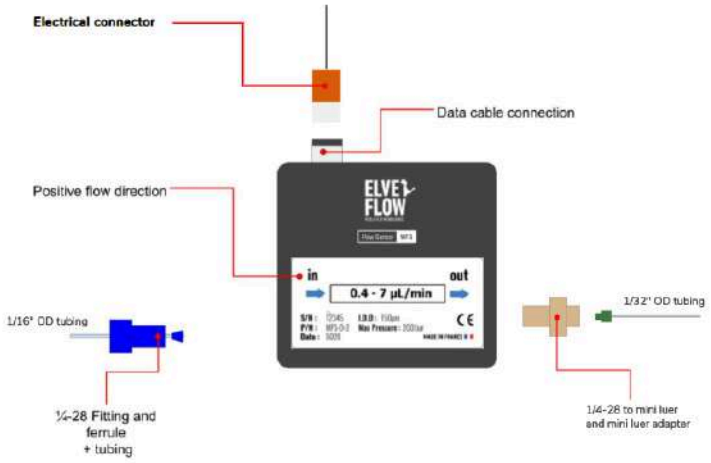



*Figure 24: MFS and connectors hardware connection*

**Any questions?**

**REFER TO THE MFS USER GUIDE**
**Electronic connection**

**REFER TO THE ESI 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.

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


**Lipids** | MFS-4

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


**Water** | MFS-5

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

## Why is it necessary to calibrate the MFS?

The flow sensors measure the flow rates of a liquid by locally warming it and by measuring the temperature differences in different locations of the sensor capillary. The relation between temperature measurement and the flow rate highly depends on the physical properties of the liquid passing through the MFS. That is why calibration is required. Two calibrations are implemented directly in the MFS: Water and Isopropyl. The Water calibration is suited for all aqueous solutions. The calibration Isopropyl is appropriate for all the carbon chains (fluorinated oil), with an additional linear adjustment.

For the aqueous phase flow sensor, simply add the Flow Sensor and choose the “H2OWater” Calibration without changing the Scale factor and offset (Figure 25, A-C).

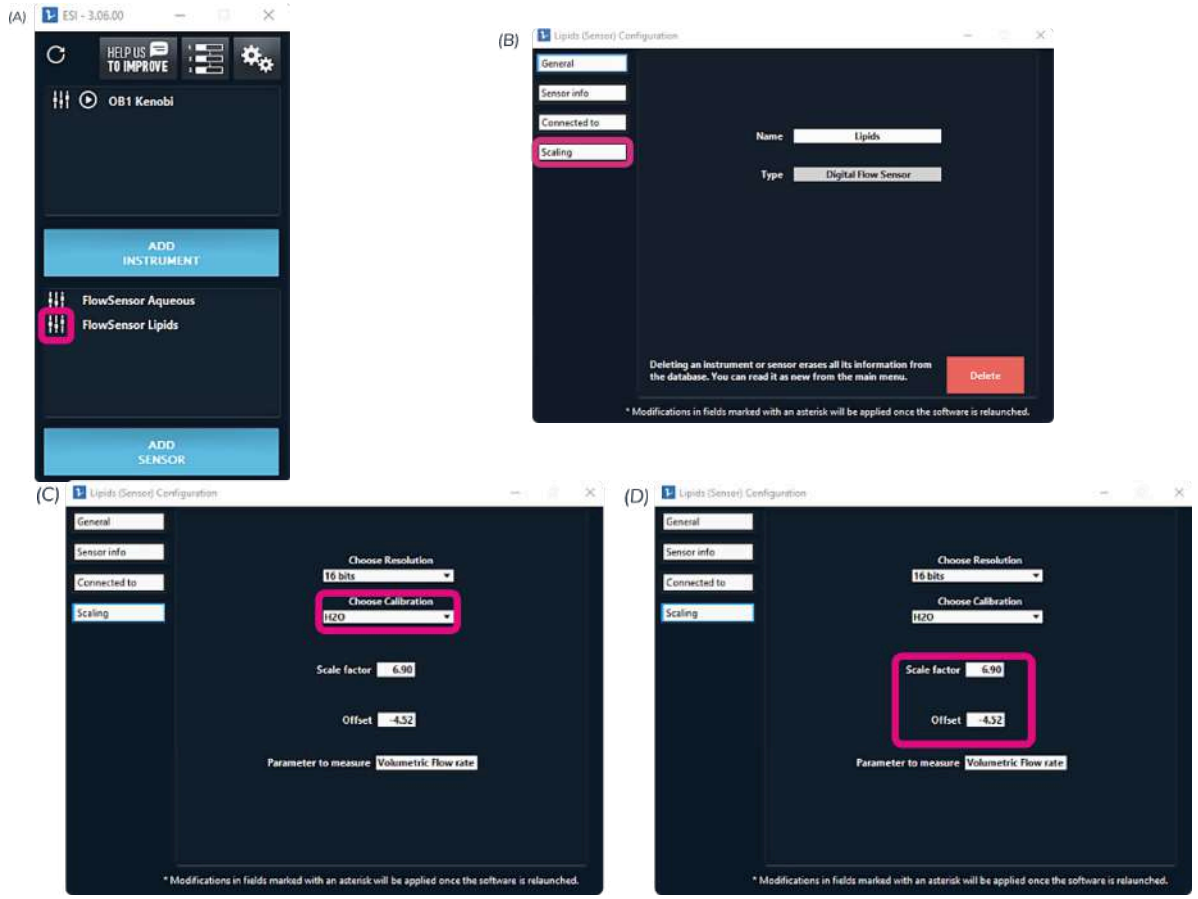



*Fig.25 : How to add an MFS Flow sensor and set the appropriate calibration in the ESI. (A) Add a Flow Sensor, (B) Access the calibration parameters, (C) Choose the calibration type, (D) Modify the scale factor and offset.*

For the lipid phase sensor, a recalibration is required. The following steps illustrate how to change the calibration of the flow sensors for lipids or water (Figure 25, A-D).

To calibrate the lipid flow sensor for ethanol, we advise you to use the following values of offset and scale factor:

| | MFS4 |
|---|---|
| SCALE FACTOR | 4.05 |
| OFFSET | -80.09 |

## TIP

**For other solvents, please proceed to the calibration following process available here: *https://support.elveflow.com/support/solutions/articles/4800116307***
**Please bear in mind that performances are likely to be degraded for other solvents and flow rate range is liquid dependent.**

### B/ MFS Fluidic connexion

The MFS sensor uses different connectors for its inlet and outlet as different tubing size are used: 1/16 OD for the inlet (coming from the reservoir) and 1/32" OD for the outlet (going to the chip).

The 1/16"OD tubing should be connected using the provided ¼-28 to 1/16 OD blue fittings and corresponding ferrule.

Instead, the 1/32" OD tubing should be connected using a ¼-28 to mini luer adapter (Grey) and a mini luer to 1/32 OD tubing adapter (green)

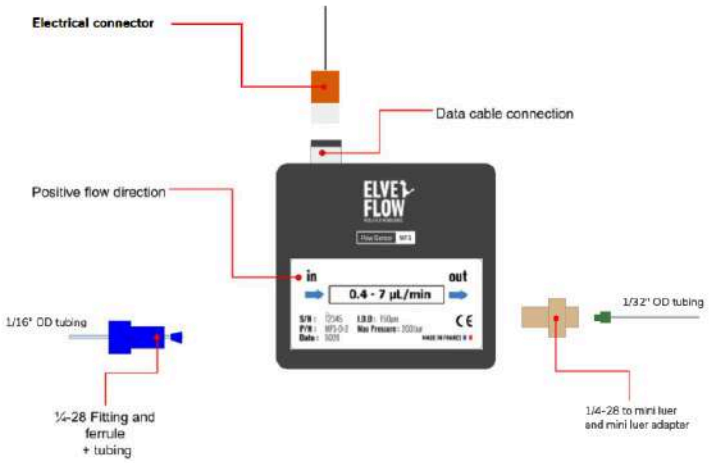



*Figure 24: MFS and connectors hardware connexion*

## TIP

When connecting the 1.4-28 to 1/16 OD tubing, make sure that the tubing end is going all the way through the ferrule, but does not extend out by more than 2-3 mm

# 4bis. BFS installation (Advanced pack version)

## A/ BFS Software connexion:

Start by connecting the BFS sensor to the computer using the provided RS-232 cable, and RS232 to USB converter.
Please also connect it to the power supply using the plug-in power supply.

Once the BFS flow sensor is physically connected to the computer, first please make sure that the drivers located in the folder are well installed
C:\Program Files (x86)\Elvesys\driver (look for driver_MUX_distAndBFS.exe)

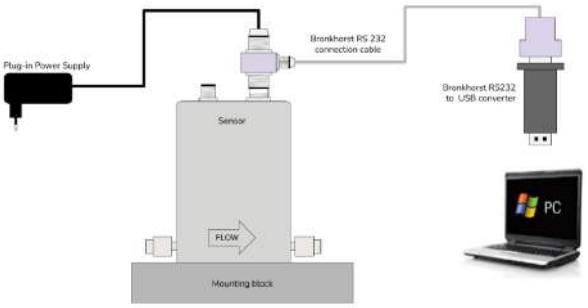
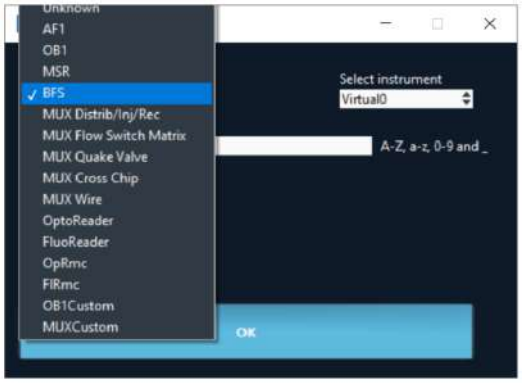



*Figure 26: BFS software connexion*

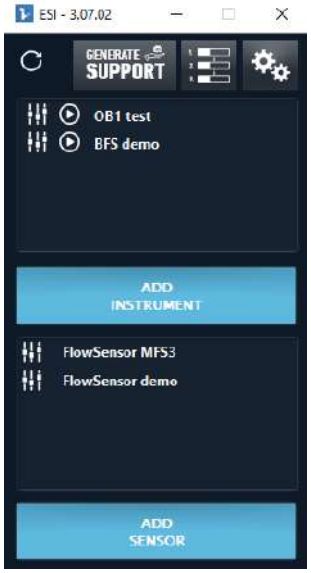



Following this, open the ESI software and add the BFS sensors by:

- **Pressing add instrument \ select BFS (figure 27 A)**
- Giving it a name

**Your flow sensor should now be both on the list of recognized instruments and sensors.**

As the BFS sensor is calibration-free, no further calibration is required.




Once the BFS sensor is added as an instrument, it should appear both in the instrument section and the sensor section (figure 27. B)

You should now connect the BFS sensor to the OB1. To do so:

- Click on the BFS sensor in the instrument section
- Select connected to (figure 27. C)
- Select in the left window to which channel do you want to connect the BFS
- Click on the right arrow to add the BFS to the correct displayed location (Figure 27.D)

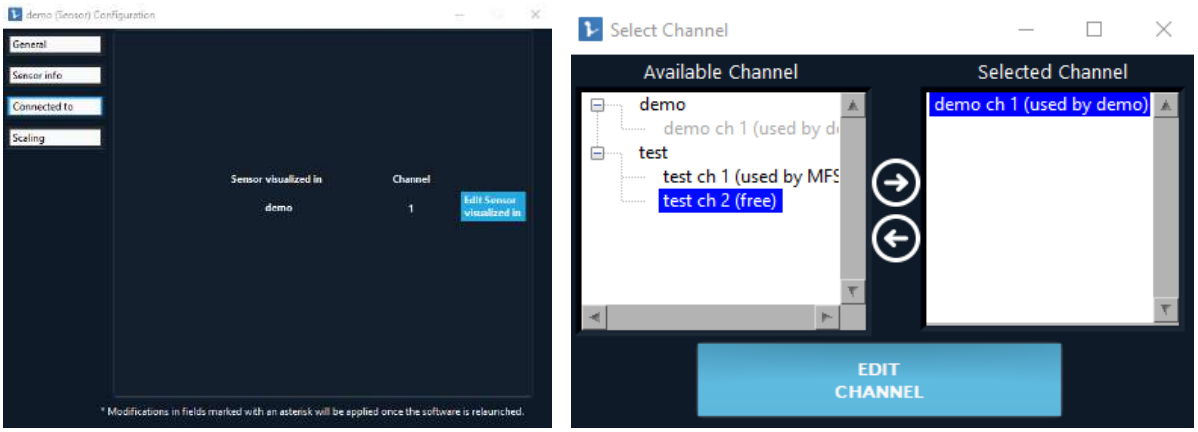



*Fig.27 : How to add and name a BFS Flow sensor (A) Open the sensor parameters (B) Select "Connected to" (C) and Select the display windows (D)*

## B/ BFS Fluidic connexion

The BFS sensor uses the same connectors for its inlet and outlet:1/16 OD tubing.
Then the outlet 1/16 OD tubing should be connected to a 1/32 OD tubing using a union.

To start with, connect the BFS to the 1/16" OD tubing using the premounted metallic nut.
Make sure you will replace the metallic ferrule with a red plastic one to ensure proper connexion
Do the same on both sides.

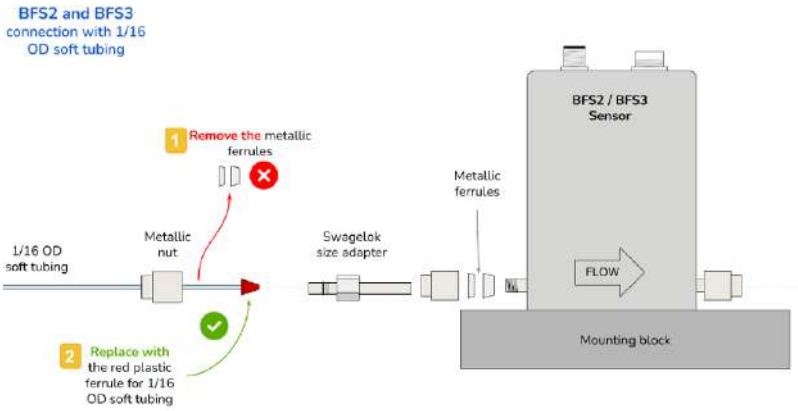



*Figure 28: BFS fluidic inlet connexion (to 1/16" OD tubing)*

# TIP

**Use an 11 mm wrench to screw/unscrew the metallic nut holding the swagelok size adapter. When screwing them, do not hesitate to screw them very tight to ensure no leakage can happen and the swagelok size adapter is immobile. The metallic nut between the 1/16 OD tubing and the swagelok size adapter needs to be tightened by hand: do not overtighten to avoid squeezing the soft tubing.**

Once the BFS sensor is connected to the 1/16 tubing on both sides, connect the outlet to the 1/32"OD tubing.
To do so, connect the 1/16" OD tubing to a union using a ¼-28 to 1/16 OD fitting (blue fitting).
Then connect the 1/32" tubing to the union using a ¼-28 to mini luer adapter (Black) and a mini luer to 1/32 OD tubing adapter (green)

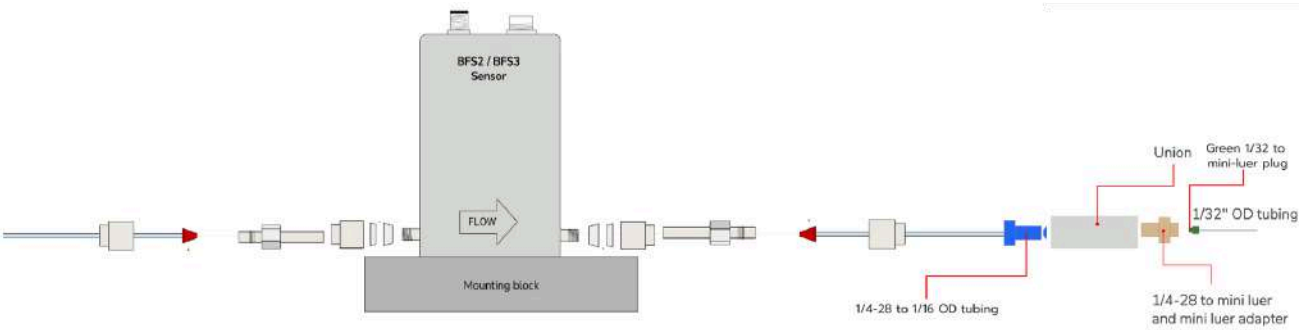


*Figure 29: BFS fluidic outlet connexion (to 1/32" OD tubing)*

## 5. Chip connexion

The chip connection should be done to the 1/32 OD tubings.
Use the green Mini-luer to 1/32" OD tubing connectors to connect them to it.


*Figure 30: Chip connection to 1/32 tubing*

## 6. Setup assembly

Once the OB1 is installed, the reservoir is assembled and 2 flow sensors are connected to both the computer & the tubing, set up the following system for the regular pack (replace the MFS with the BFS sensor in the advanced pack) and ensure that the chip is correctly connected

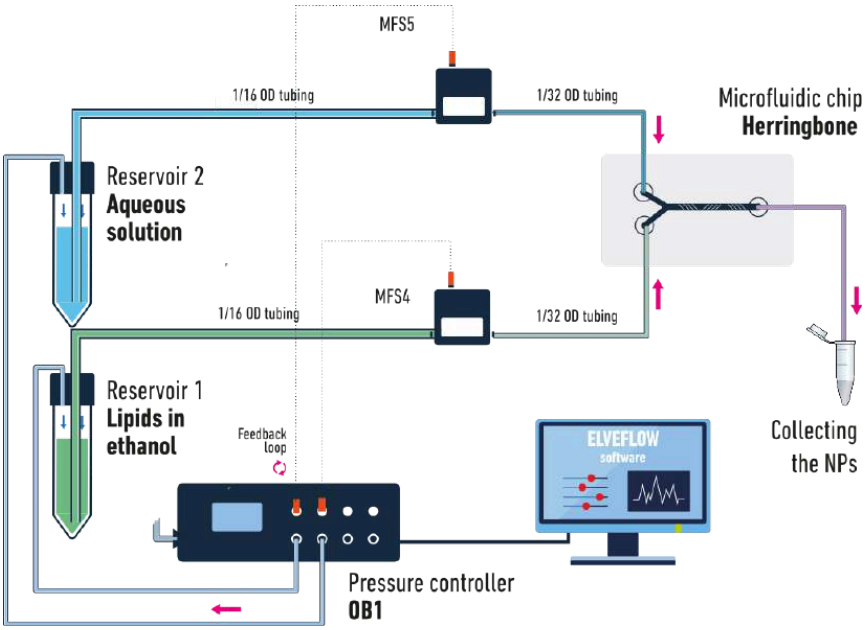



*Figure 31: LNP pack regular setup*

Bear in mind to use 1/16" OD tubing prior to the chip, and 1/32" OD tubing after it! Use **30 cm** of 1/32" tubing **for the aqueous phase**, and **45 cm for the lipid phase** to ensure proper **fluidic resistance**.

## 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 changes in 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.

## TIP

The connectors used in the system are:

- Air tube coming from the OB1 to the reservoir: 3/32 barb to ¼-28 connector
- Flow sensor: See fluidic connexion in 3 or 3 bis
- 1/32" OD tubing to the chip: Use a ¼-28 to mini luer adapter (Black) and a mini luer to 1/32 OD tubing adapter (green)

More information on connexion is provided in appendix

# Now let's start making nanoparticles

## 7. Fill the microfluidic chip

Once the system is fully assembled, it should be filled a first time to ensure all the elements are correctly put together and no leakage happens.

When doing this for the first time, use water and pure ethanol as your aqueous and organic phases, respectively, this will help minimize sample loss while you get your first in-hand experience with the system. Once your setup is complete and workflow mastered, change your tubes 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 positioned very close to the bottom of the reservoirs. Turn on the OB1 and launch the ESI software. Open the OB1 in the ESI:

Set the pressure of both channels to 500 mbar until the chip is filled (it should take less than a minute). 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).

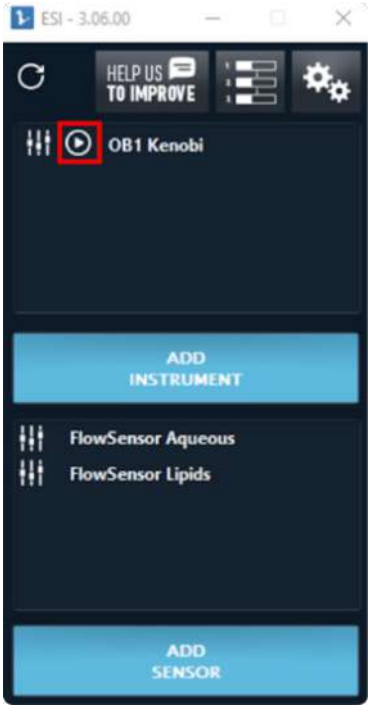



*Figure 32: OB1 launch*

### 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.

### 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. To prevent leakage around the mini luer connectors, wrap a piece of Teflon seal thread tape around the base (as shown in the troubleshooting section)

# 8. Improving TFR & FRR control by switching to flow control mode

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!

However, 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. The following 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:

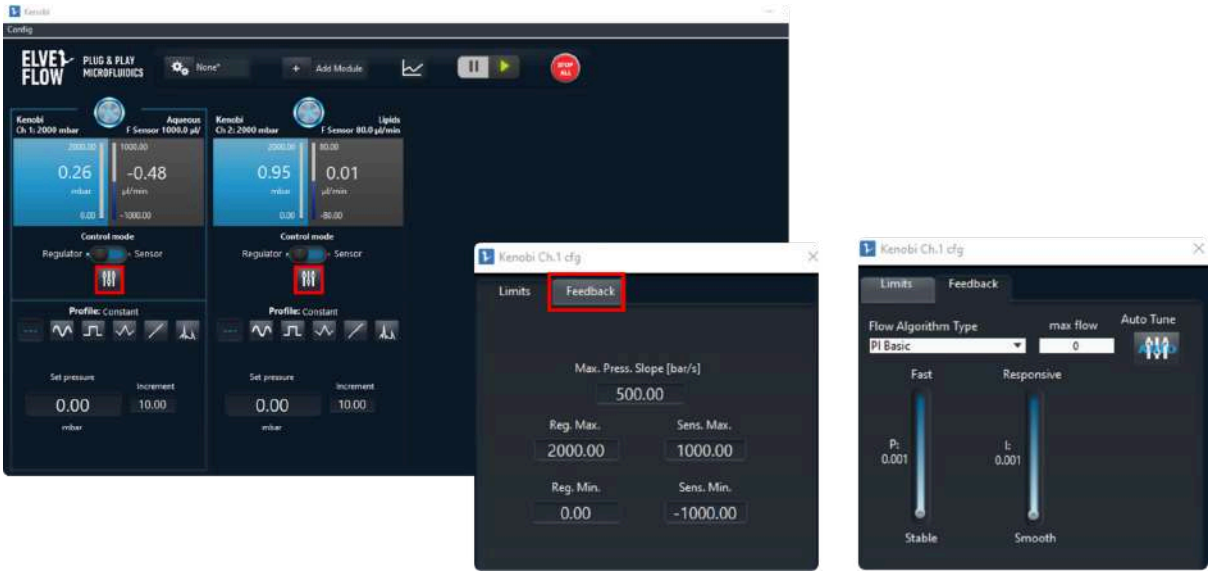



*Figure 33: How to set the P & I feedback loop parameters in the ESI (A) Feedback loop parameters access from the main window, (B) Parameters window*

For the **regular version of the pack**, we advise you to begin with the following values for the feedback parameters:

| Regular Pack | MFS4<br>*Solvent* | MFS5<br>*Aqueous* |
|---|---|---|
| P | 0.09 | 0.01 |
| I | 0.06 | 0.065 |

While we suggest using the following values for the advanced pack version

| Advanced Pack | BFS2<br>*Solvent* | | BFS2<br>*Aqueous* | |
|---|---|---|---|---|
| | High flow rates (> 1 mL/min) | Low flow rates (< 1mL/min) | High flow rates (> 4 mL/min) | Low flow rates (<4mL/min) |
| P | 0.5 | 0.3 | 0.2 | 0.05 |
| I | 0.35 | 0.25 | 0.2 | 0.15 |

These values are appropriate **for all couples of aqueous buffer and lipid flow rates.** They give a good compromise between stable flow rates and high responsiveness of the system. We recommend you start by using these values when getting familiar with the setup and 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 low responsiveness and it might take several minutes for the flow rates to equilibrate at the desired values.

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

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):

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

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


*Figure 34: Switch from pressure to flow control*

For example, you can set the aqueous buffer flow rate to 1000 µL/min and the lipids flow rate to 300 µL/min to produce your first nanoparticles.

You can refer to the next section for the 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.**

## 9. Between samples : Washing and Loading

Once you have successfully collected your LNP sample, you have two options before starting your next run:

1. Start with your next sample/flow rate conditions and collect the next sample after discarding the remaining of the previous sample to the waste. This option is the simplest but does not prevent any risk of cross-contamination between samples.
2. Perform a washing step before collecting your next sample. This ensures minimal cross-contamination but requires some additional steps, which will be detailed below.

To perform an efficient washing step, we recommend switching your inlet reservoirs with one containing cleaning solutions (e.g. ethanol and water) and start flushing your system at relatively high pressure (e.g. 2000 mbar on both channels). We recommend washing your system with a volume equivalent to 10 times the internal volume (tubing + chip) so around a couple of mL. Once this step is done, exchange the reservoirs for empty ones and apply the same pressure until the full system is dry.

Before starting your next run - if you intend to work with flow rate control - you will need to load your reagents up to the chip. To do this simply and manually : remove the tubings from the chip inlets and apply a relatively low pressure (~100-200 mbar) until you see the first drop coming out of the tubing; stop the pressure and plug the tubings back into the chip : your system is now ready for its next run!

## 10. Creating a sequence for specific volume collection

Once you are familiar with how the system works, you can start improving your workflow with these helpful tips and tricks. The first one is to use the **Sequencer** to program a semi-automatic run. The sequencer is like a simplified programming module that allows you to automate some repetitive actions.

In this example, you want to produce 1,3 mL of LNPs at a TFR of 1,3 mL/min and FRR of 4.3, then you want to turn off the pressure and record the graph of the experiment (flow rates and pressure)..

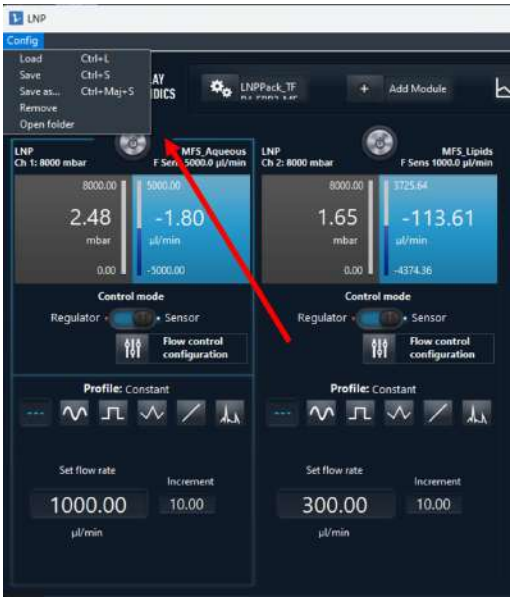



*Figure 35: Save a configuration*

To do this, you will first need to save the *configurations* corresponding to the flow rate conditions of your run. Here is an example of how to save a certain configuration.

By clicking on the **config** button you can "**save**" or "**save as...**" your flow rate conditions (you can leave the channels turned off when you do this). This will save all parameters associated with this page : the control mode, the pressure or flow rate set for each channel, the P and I values, etc.

At this point, you can create two distinct configurations : one with your target flow rates + one with the pressure off - which will correspond to the end of your run.

Once this first step is done, you can open the sequencer and start building your sequence. Figure 36 shows you the steps to (1) open the sequencer, (2) add a command about the OB1, (3) select the OB1 in the list of instruments and (4) load the configuration you want to start with (the one you saved previously).

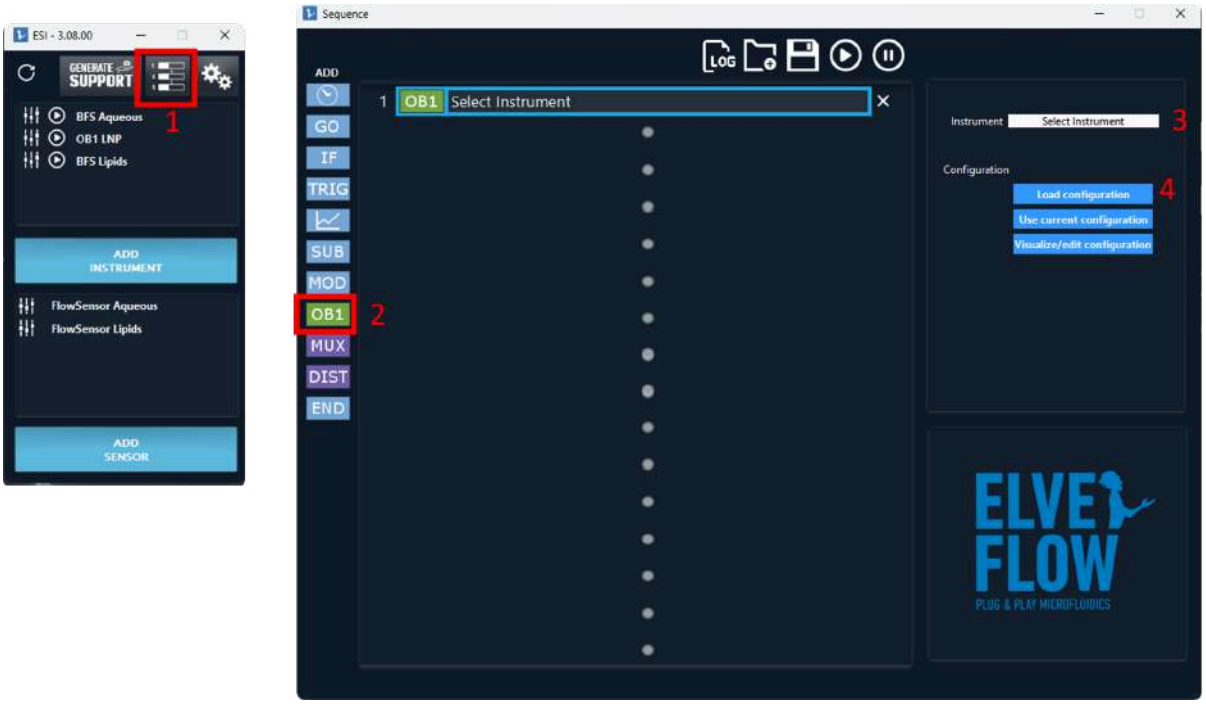



*Figure 36: Open the sequencer and load a configuration*

## TIP

**You can modify a configuration - for example to turn on the channels - in the sequencer. Simply make sure to save your changes by clicking on "config" then "save". Keep in mind that all changes will overwrite the existing config file, it will not create a new configuration.**

Figure 37 illustrates how the full sequence looks like. In this example, the command are as follows :

1. **Start graph** (optional) : if you want to record the data of the whole run in a .txt file, this command indicates when to start the recording.
2. **Run :** the configuration corresponding to the LNP formulation flow rates starts now.
3. **Wait** (optional) : this will allow you to wait for the flow rate to stabilize before collecting your sample
4. **Collect** : this command will hold the configurations set at step 2 for a dedicated time. Here, at a TFR of 1,3 mL/min, in 1 min we will collect 1,3 mL of sample.
5. **Stop** : a new configuration is loaded, the pressure is turned off.
6. **Stop graph** (optional) : if you decided to record the data, this command stops the recording.

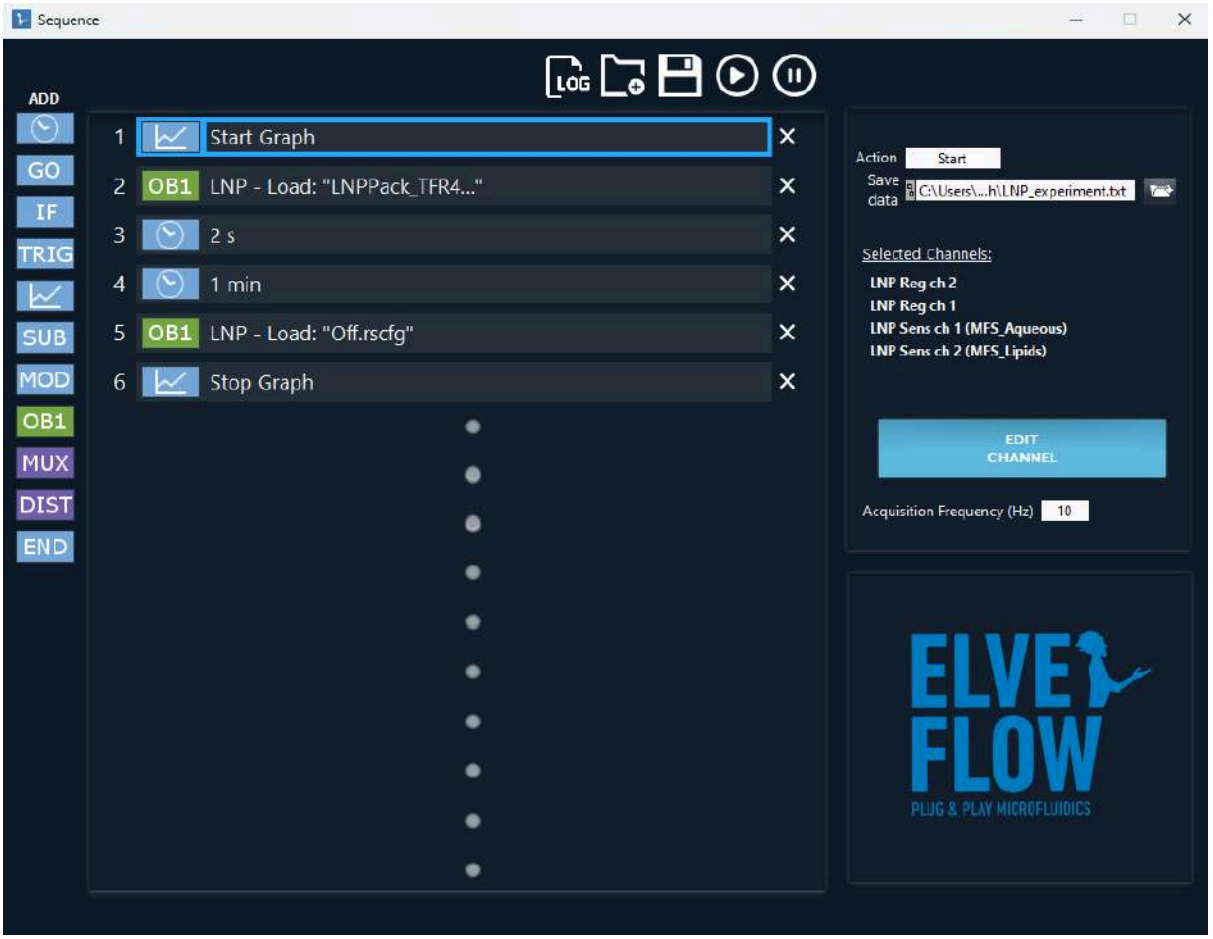



*Figure 37: Create the full sequence*

The figure below describes how to select the data you wish to record (which channels, which sensors...). This step is mandatory if you want to include a graph recording step.

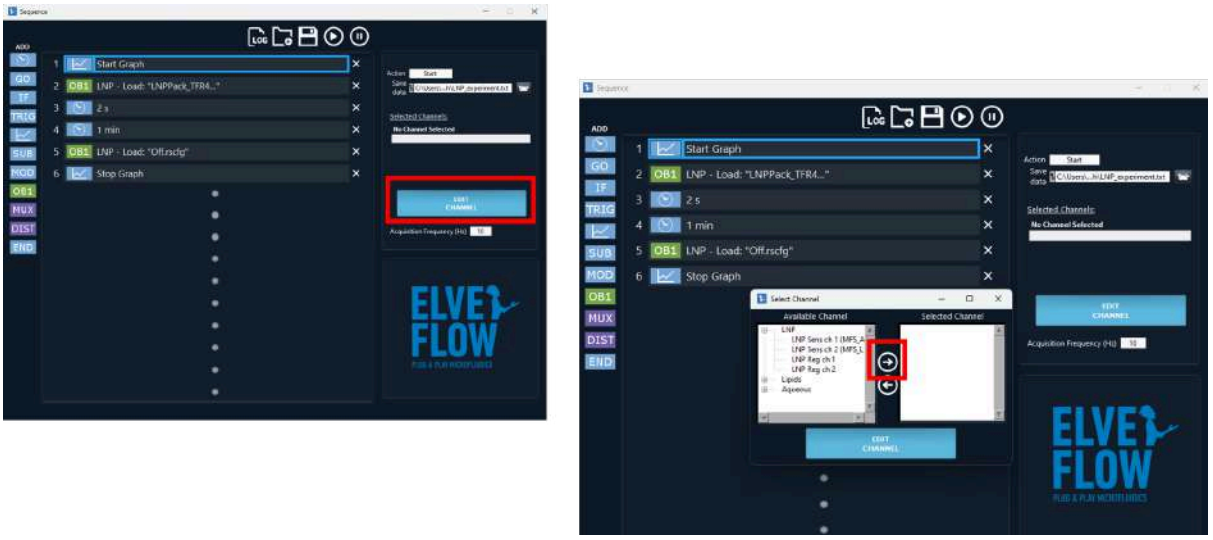



*Figure 38: Edit the graph settings*

Once you are satisfied with your sequence, you can save it and load it during your next use of the system !

Make sure that your system - especially the flow sensors - is filled with liquid before you start your run with the sequencer. If you are experienced with the system, you can add an automated loading step prior to your run - e.g. loading at low pressure or low flow rate with low PDI values.

# 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 or BFS. The MFS should be connected in the right direction (indication of inlet/outlet are depicted on top of the sensor). If both BFS 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 BFS 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 to the Control of the flow rate section)

## 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

If your OB1 starts making loud noises, 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 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 200 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 user to user.
The provided diagrams are indicative and only valid for the specific lipid formulation and concentration used in this user guide.

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 flow sensors can't precisely measure. It is possible to reach these low flow rates thanks to the control of the pressure with the OB1. Still, 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. An easy way to increase the flow rate control in your setup is to higher the resistivity in your whole system by adding longer tubings to it. You can change the microfluidic tubing for longer 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 up to at least 6 mL/min with 2 bar pressure channels - higher flow rate values can be achieved using 8 bar pressure channels (up to 15 mL/min).

## TIP

Changing the TFR in the herringbone chip will tend to reduce the size of your LNP. This is true up to a certain TFR threshold that needs to be assessed experimentally. Bear this in mind when changing flow rates

Please feel free to reach out to our technical sales team **contact@insidetx.com** for more information about it.

# 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 tailor 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

### Connect the reservoir to the OB1



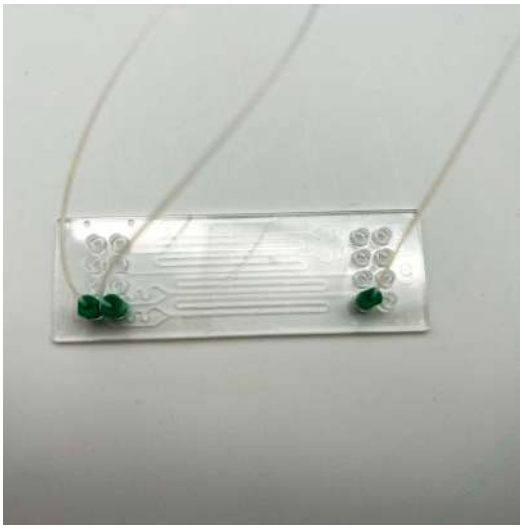




## Connect the reservoirs

## Connect the MFS


## Connect the tubing to the chip



# APPENDIX 2 Optimize your PID by changing 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 InsideTx 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 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.

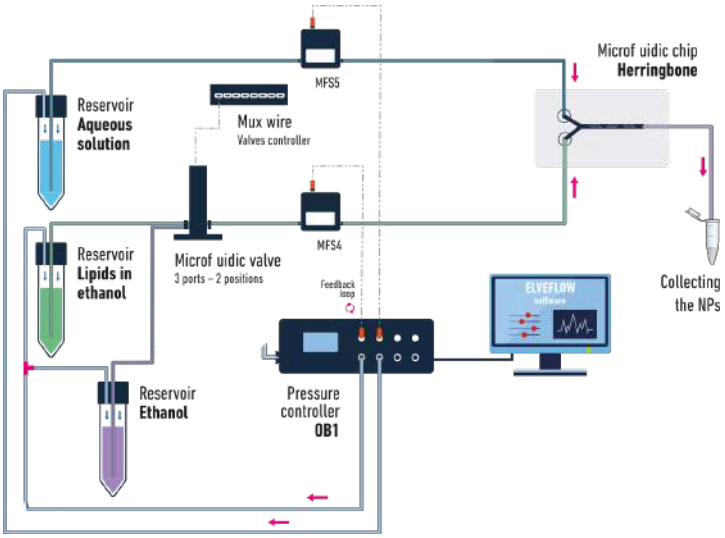
If when requesting a low flow rate (for example, 2 µl/min for the aqueous buffer) the system is really too slow to stabilize, carefully increase the value of **I**.

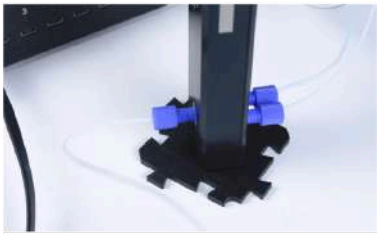
Increasing the value of **P** could increase the responsiveness of the system, and sometimes reduce the oscillation of the flow rate, but if it is too high, the flow rate will not stabilize.

# 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:

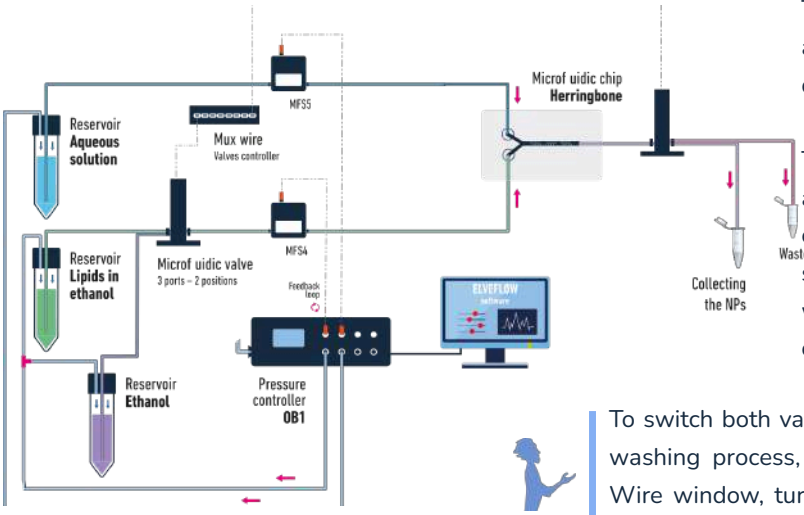
1. Install your setup as described in this user guide and start producing your first LNPs
2. Switch the valve to perform a washing step. Optional: you can increase the flow rate of the ethanol for a faster and more efficient washing.
3. Once you are satisfied with the washing step, change your flow rates 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

The side of the valve with only one port is always 'open', while the other side has two ports labeled 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.

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.

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.

## APPENDIX 4 Automated sample collection with an additional 3/2 valve

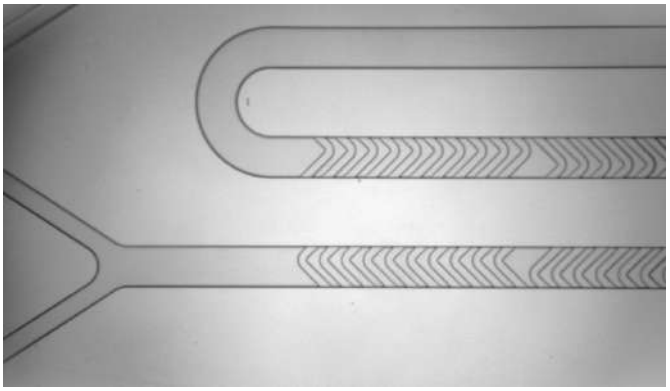


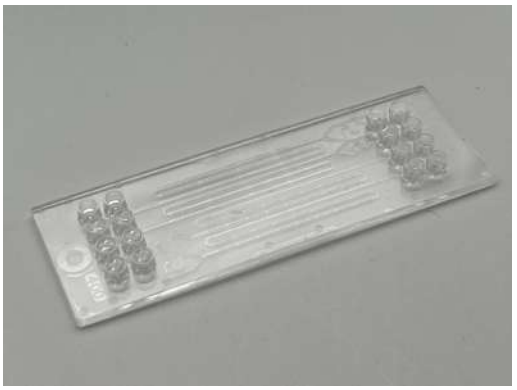

This setup goes a step further and adds a second 3/2 valve to the previously described microfluidic setup.

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.

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 microfluidics chips





*Herringbone micromixer 1460 from microfluidic chipshop*

# SUPPLEMENTARY INFORMATION

## 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

InsideTx 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. The 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 a safety or emergency stop device or in any other application where a 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 datasheet and user manual. Failure to comply with these instructions could result in death or serious injury. If the buyer purchases or uses Elveflow® and InsideTx products for any unintended or unauthorized application, the buyer shall defend, indemnify and hold harmless Elveflow®, InsideTx 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® and InsideTx 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® and InsideTx pressure pump due to the use of pressurized equipment. This is particularly important when hazardous liquids are used.

### Electricity Advice

Use Elveflow® and InsideTx instruments with the provided power unit only. Maintenance should only be attempted by qualified Elveflow® or InsideTx 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, and 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® and InsideTx 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.

ELVE FLOW
PLUG & PLAY MICROFLUIDICS


**Technical support**
customer@elveflow.com

**General information**
contact@elveflow.com
+33(0).184.163.807

**ELVESYS – Microfluidics innovation center**
172 Rue de Charonne,
75011 Paris, FRANCE


www.elveflow.com

Product:
Elveflow.com/microfluidic-products/microfluidics-application-packs/easy-droplet-generation/

MAY THE FLOW BE WITH YOU