Crank up LNP & AAV sample prep with Unagi

Introduction

Buffer exchange is a critical step to developing optimal vectors and payloads. Compared to biologics, development of delivery vectors like lipid nanoparticles (LNPs) and adeno-associated viruses (AAVs) is a newer research area with different challenges and opportunities to address.

Desalting nucleic acid payloads through buffer exchange is a fundamental sample preparation step. After formulation, LNPs need to be buffer exchanged to remove organic solvents as a step to maintain LNP integrity. Although dialysis is commonly used to buffer exchange LNPs, it is slow and requires an additional step to concentrate diluted samples. Other common manual exchange methods require a large amount of hands-on time and can also deliver sub-optimal sample recovery.

AAVs, LNPs and related gene therapy vectors require rapid buffer exchange and preparation methods that do not compromise the integrity of the sample and have high recovery rates. Because of the complexity of AAV, sample clean-up and concentration can also be time intensive, and the problem only compounds when multiple samples are involved.

To address these challenges, Unagi was developed to automate buffer exchange and sample cleanup (Figure 1) with optimized processes tailored to multiple sample types to save user time and maintain sample integrity.

Unagi uses pressure-based ultra-filtration/diafiltration (UF/DF) technology to remove and replace buffer. During pressure-based filtration the plate is gently mixed, ensuring that samples cannot accumulate at the membrane surface while keeping flow more uniform and faster than dead-end filtration methods.

Buffer exchange with Unagi is highly customizable and adaptable, allowing for buffer exchange of up to 8 unique samples and formulations in a single experiment. Unchained Labs developed a single sample consumable, Una, for this process (Figure 2A). A single Una has a working volume range between 0.5 and 8 mL, with the option of 10, 30, or 100 kDa molecular weight cutoffs in the regenerated cellulose membrane. Before the run, the Una is filled with the sample to be exchanged and placed in the sample rack (Figure 2B) and then placed in the exchange chamber. The new buffer is placed on the deck in 50 mL conical tubes. During the run, Unagi alternates between filtration, volume measurement, and new buffer addition to buffer exchange samples (Figure 3).

Figure 1: Unagi is your automated benchtop buffer exchange and concentration solution for up to 8 unique samples between 0.5 – 48 mL.

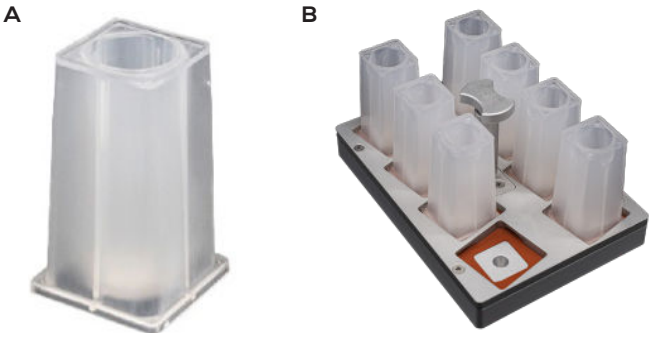

Figure 2: (**A**) Unagi processes samples in up to eight Una consumables. Each Una has a working volume range of 0.5 – 8 mL, with capacity for an additional 40 mL per sample on deck. (**B**) The sample rack holds 1 – 8 Unas and can be used to transport samples to and from Unagi. The sample rack locks into place in the buffer exchange chamber on Unagi.

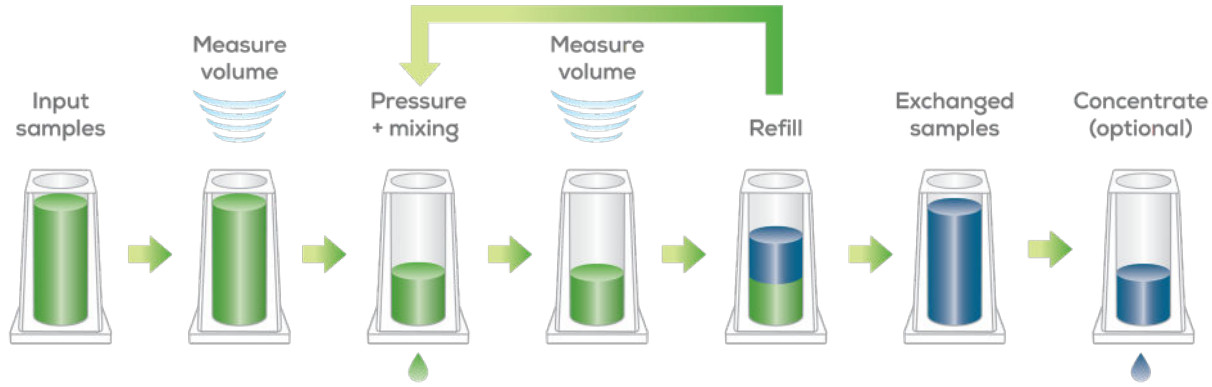

Figure 3: Unagi's automated pressure-based UF/DF technology uses ultrasonic volume measurement of each sample to monitor flow rate and adapt the pressure cycle time accordingly. The sample volume measurement enables precise target exchange percentages and concentration values, since the amount of retentate is known at every step.

Unagi has four user-selectable applications designed for different processing needs. The Buffer Exchange application automates the buffer exchange process with the option to further concentrate the sample after the exchange is complete. The Concentrate Only application concentrates samples to a new target volume without going through buffer exchange. Buffer Exchange + Concentrate will first exchange and then concentrate samples. Finally, the Reduce Sample Volume application reduces up to 8 dilute samples in parallel from ≤48 mL to 8 mL (Figure 4). Each method can be run independently or consecutively to achieve the desired results.

In this application note, we will demonstrate how Unagi can be used to perform key applications in viral vector and LNP sample prep and purification using specific presets that have optimized parameters developed for each molecule type and concentration range. For LNPs, we will remove ethanol as part of the exchange and concentration process. LNPs used in this study were manufactured on Nunchuck (Figure 5A), which is the first and only platform that combines two

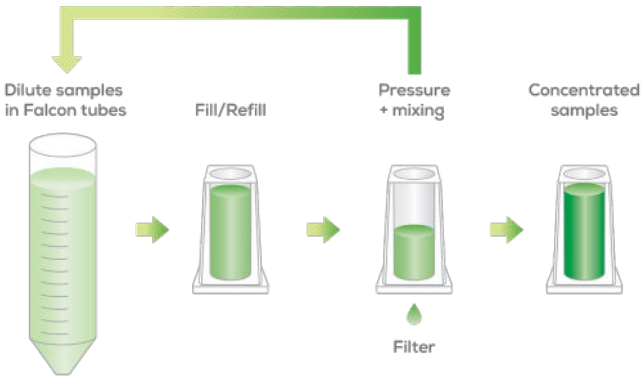

Figure 4: The Reduce Sample Volume application on Unagi utilizes the on-deck Falcon tubes to concentrate larger volumes of samples, up to 48 mL, down to 8 mL in a single run. Subsequent concentration experiments will bring the final volume down to as low as 0.5 mL, for a 96x final sample concentration factor.

A

B

Figure 5: (**A**) Nunchuck combines total flow rate screening and lipid nanoparticle formulation. Screen 8 total flow rates (TFRs) and make between 0.5 - 200 mLs of LNPs. (**B**) Stunner combines UV/Vis quantification, Dynamic Light Scattering (DLS) and Static Light Scattering (SLS) data from the same 2 µL sample.

key steps needed to make perfectly-sized LNPs – total flow rate screening and lipid nanoparticle formulation. Diluted and large volume AAVs will be concentrated into a manageable volume. Finally, we will clean up DNA to remove any salt and EDTA in preparation for storage or down-stream applications.

Methods

LNP buffer exchange and concentration

PolyA-LNPs were formulated on Nunchuck using Polyadenylic acid (polyA, Sigma Aldrich, GE27-4110-01) and the SM-102-containing Lipid Nanoparticle (LNP-102) Exploration Kit (Cayman Chemical, Item No. 35425) with a N:P ratio of 8, FRR of 3:1, and a TFR of 15mL/min. PolyA-LNP stock in 12.5% EtOH was diluted 4-fold into 6.25% EtOH in 1x PBS to an initial concentration of 30.99 µg/mL. 4 mL of the diluted LNP was transferred into a 100 kDa Una.

Key buffer exchange parameters are outlined in Table 1. The buffer exchange protocol was set to 98% total exchange per sample with a target volume removal per cycle of 25% at 30 psi. To reach a final target concentration of 120 µg/mL, final sample volume was targeted at 1 mL.

Parameter	LNP settings
Target exchange	98%
Target removal per cycle	25%
Initial concentration (µg/mL)	30.99 ± 0.02
Initial well volume (mL)	4
Target final concentration (µg/mL)	120
Target final well volume (mL)	1

Table 1: Key buffer exchange parameters used for LNP buffer exchange and concentration performed in a 100 kDa Una.

Unagi software was used for experimental design and execution. Total processing time, initial and final sample volumes, and final percent exchange were reported by the software.

Stunner (Figure 5B) was used to measure the concentration, size and polydispersity of polyA-LNPs. RiboGreen® (ThermoFisher) was used to determine the encapsulation efficiency before and after exchange with a SpectraMax i3 plate reader (Molecular Devices).

AAV buffer exchange and concentration

2.86E13 cp/mL AAV9-CMV-GFP (Virovek) were nominally diluted to 5E12 cp/mL in PBS, pH 7.0 with 0.001% Pluronic F-68. Diluted AAVs were manually pipetted into 30 kDa Unas. AAVs were exchanged into PBS, pH 7.0 with 0.001% Pluronic F-68 and then concentrated 2-fold.

Key experiment setup and parameters are outlined in Table 2. The experiment used the AAV preset exchange parameters. The buffer exchange protocol was set to 96% total exchange per sample with a target removal of 33% per sample at 15 psi.

The Stunner AAV Quant application was used to analyze concentration and capsid empty/full ratio before and after the exchange and concentration process.

Parameter	AAV settings
Target exchange	96%
Target removal per cycle	33%
Initial concentration (cp/mL)	5.00E12
Initial volume (mL)	2
Target final concentration (cp/mL)	1.00E13
Target final volume (mL)	1

Table 2: Key buffer exchange parameters used for AAV buffer exchange and concentration in a 30 kDa Una.

Dilute AAV: Reduce sample volume

AAV9-CMV-GFP (Virovek) was nominally diluted to 5E11 cp/mL in PBS, pH 7.0 with 0.001% Pluronic F-68. 12 mL of the diluted AAV9 were manually pipetted into 30 kDa Unas. Diluted AAV was reduced from 12 mL to a final volume of 1 mL in a two-step process. First, 12 mL of dilute AAV9 was reduced to 8 mL with the Reduce Sample Volume application on Unagi. A Concentrate Only experiment using the same Una concentrated each sample to 1 mL, for a total 12-fold concentration from the 12 mL stock.

Key experiment setup and parameters are outlined in Table 3. Both steps used the AAV preset exchange parameters.

The Stunner AAV Quant application was used to analyze concentration and capsid empty/full ratio before and after the concentration steps.

Parameter	AAV settings
Initial concentration (cp/mL)	5.00E11
Initial volume (mL)	12
Target final concentration (cp/mL)	6.0E12
Target final volume (mL)	1

Table 3: Targeted initial and final concentration and volume used to concentrate dilute AAV up to 12-fold in a 30 kDa Una.

Desalting DNA

Invitrogen UltraPure Salmon sperm DNA solution (ThermoFisher) was diluted to 2 mg/mL in Tris-EDTA, pH 8.0. Diluted DNA was manually pipetted into a 30 kDa Unas. Samples were exchanged into nuclease-free water and then concentrated 3-fold.

Key experiment setup and parameters are outlined in Table 4. The experiment used the Nucleic Acid preset exchange parameters. The buffer exchange protocol was set to 96% total exchange per sample with a target removal of 50% per sample at 60 psi.

The UV/Vis application on Stunner (Unchained Labs) was used to quantify DNA concentrations before and after exchange and concentration.

Parameter	DNA settings
Target exchange	96%
Target removal per cycle	50%
Initial concentration (mg/mL)	2
Initial volume (mL)	3
Target final concentration (mg/mL)	6
Target final volume (mL)	1

Table 4: Key buffer exchange parameters used for DNA buffer exchange and concentration in 30 kDa Unas.

Results

LNP buffer exchange and concentration

A target percent exchange of 98% was set per sample, and with the pre-dilution the EtOH concentration was reduced to 0.1%. The final volume after the concentration step was gravimetrically measured as 0.88 ± 0.03 mL. The final LNP concentration was measured on Stunner at 137.68 ± 9.73 µg/mL with the target concentration expected at 120 µg/mL (Table 5). Stunner measured an initial size of 76.76 ± 0.49 nm (PDI 0.09 ± 0.02) and a post experiment size of 80.78 ± 1.62 nm (PDI 0.06 ± 0.01). A RiboGreen® assay was used to determine % encapsulation of LNP before and after the buffer exchange and concentration process. After exchange, each sample was measured in duplicate and average % encapsulation was reported as 99.21 ± 0.37%. A 98.41 ± 3.01% sample recovery was determined by a combination of gravimetric and Stunner measurements. All runs took approximately 2 hours to complete on Unagi. Ethanol was removed from the LNP solution, concentration targets were hit, and % encapsulation was consistent before and after buffer exchange and concentration.

LNP parameter	Initial	Final
Conc. (µg/mL)	30.99 ± 0.02	137.68 ± 9.73
Size (nm)	76.76 ± 0.49	80.78 ± 1.62
PDI	0.09 ± 0.02	0.06 ± 0.01
Encapsulation efficiency (%)	-	99.21 ± 0.37
Recovery (%)	-	98.41 ± 3.01

Table 5: LNP solution concentration, size, PDI, % encapsulation, and recovery in a 100 kDa Una.

AAV buffer exchange

A target percent exchange of 96% was set per sample, and 96.5 ± 0.1% exchange was achieved based on the diavolume added to each Una (Table 6). The final volume per sample was ultrasonically measured as 0.98 ± 0.03 mL, at the target of 1 mL per Una. The final AAV concentration was determined by Stunner at 1.2E13 ± 3.6E11 cp/mL with the target concentration expected at 9.85E12 cp/mL. The AAV empty/full ratio was determined by Stunner. The AAV before buffer exchange had an empty/full capsid ratio of 21/79. After buffer exchange the empty/full ratio was measured at 18/82. A slight decrease in the capsid empty/full ratio was observed, potentially due to the removal of free DNA during the exchange process.

AAV parameter	Initial	Final
Conc. (cp/mL)	4.93E12 ± 2.9E11	1.2E13 ± 3.6E11
Volume (mL)	2.2 ± 0.03	0.98 ± 0.03
% exchanged	–	96.5 ± 0.1
Capsid empty/full ratio	21/79	18/82

Table 6: AAV concentration and empty/full ratio before and after buffer exchange and concentration in a 30 kDa Una.

Dilute AAV: Reduce sample volume

In this experiment, we first concentrated 12 mL of AAV9 to 8 mL using the Reduce Sample Volume application. We then concentrated the reduced volume sample in the same Una with the Concentrate Only application down to 1 mL final volume for a total 12-fold concentration.

The 12 mL was reduced to 8.1 ± 0.02 mL with the Reduce Sample Volume application. The same samples were further concentrated with the Concentrate Only application and reached a volume of 1.02 ± 0.02 mL, compared to the target of 1 mL (Table 7). The final AAV concentration was determined by Stunner at 5.9E12 cp/mL with a target concentration expected at 6.0E12 cp/mL. The AAV before concentrating had an empty/full capsid ratio at 10/90. After concentrating the empty/full ratio was measured at 15/85. No significant differences in capsid empty/full ratio were observed.

AAV parameter	Initial	Final
Conc. (cp/mL)	5.00E11	5.9E12 ± 1.6E11
Volume (mL)	8.1 ± 0.03	1.02 ± 0.02
Capsid empty/full ratio	10/90	15/85

Table 7: AAV sample volume reduced 12-fold in a 30 kDa Una.

Desalting DNA

A target percent exchange of 96% was set per sample, and 96.7 ± 0.2% exchange was achieved (Table 8). The final volume per sample was ultrasonically measured as 1 ± 0.02 mL at the target of 1 mL. The final DNA concentration was determined by Stunner at 6.7 ± 0.2 mg/mL with target concentration expected at 6.3 mg/mL.

DNA Parameter	Initial	Final
Conc. (mg/mL)	2.1 ± 0.006	6.7 ± 0.2
Volume (mL)	3.3 ± 0.03	1 ± 0.02
% exchanged	–	96.7 ± 0.2

Table 8: Desalting and concentrating DNA into nuclease-free water in a 30 kDa Una.

Conclusion

AAVs, LNPs, nucleic acids, and proteins vary in size and complexity. In addition, working concentrations and buffer conditions also vary between sample types, and these differences drastically impact the filtration rate and efficiency of buffer exchange and concentration methods. The processing parameters on Unagi alter the UF/DF conditions, namely pressure and sample removal rates based on the specific molecule type and its concentration and can be modified by the user to achieve optimal results, without compromising sample integrity.

For molecules like AAVs and LNPs, exchange needs to be fast and must retain payload integrity. AAVs are exchanged at a lower pressure because they flow faster, and the lower pressure ensures they can concentrate to the right level consistently. Larger molecules like LNPs, and higher concentrations of nucleic acids are exchanged at a higher pressure and exchange rate to increase processing speed. The flexibility of Unagi to exchange larger biomolecules as well as smaller proteins and nucleic acids makes it an ideal fit for gene therapy sample preparation workflows.

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