Soup-up AAV & LNP sample prep with Big Tuna

Introduction

Optimizing the production of delivery vehicles is a critical area of gene therapy development. Compared to monoclonal antibodies and other biologics, the characterization and formulation of delivery vectors like lipid nanoparticles (LNPs) and adeno-associated viruses (AAVs) are still new areas of research, and the production of these molecules is challenging with many opportunities for optimization and scale up.

Desalting or buffer exchange for nucleic acid payloads is a fundamental sample preparation step. LNPs can be synthesized by solvent injection into the aqueous drug-containing solution. After LNPs are formed, solvents such as ethanol should be quickly removed to maintain drug encapsulation and LNP integrity. Dialysis is commonly used but it is time consuming and requires a secondary concentration step to reduce the sample volume. AAVs are often cleaned and concentrated using ultrafiltration devices, but it can be labor intensive and difficult to manage at large sample numbers.

All gene therapy related molecules require rapid buffer exchange and preparation methods that do not compromise the integrity of the sample and have high recovery rates. Current exchange methods are slow and require a great degree of manual time and effort and are inadequate for scale up and efficient analytical characterization. Big Tuna has optimized processes tailored to each sample type, which automates the process to increase throughput and maintaining sample integrity.

Figure 1: Big Tuna automates buffer exchange for up to 96 unique samples with Unfilter 96 or up to 24 unique samples with Unfilter 24.

Big Tuna was developed to address gaps in low-volume, high-throughput buffer exchange and sample cleanup (Figure 1). Big Tuna uses a pressure-based ultra-filtration/diafiltration (UF/DF) method to remove and replace buffer. During the 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 Big Tuna is highly customizable and adaptable, allowing for buffer exchange of up to 96 or 24 unique samples and formulations in a single experiment. Unchained Labs developed two filter plate formats for this process (Figure 2). Unfilter 96 can process up to 96 samples ranging from 100-450 µL; Unfilter 24 can process 24 samples

Figure 2: Big Tuna can accommodate both Unfilter 96 and Unfilter 24. **A:** Unfilter 96 allows for up to 96 samples to be buffer exchanged simultaneously at volumes of 100-450 µL per well. **B:** Unfilter 24 allows for up to 24 samples to be buffer exchanged simultaneously at volumes of 0.45-8 mL per well.

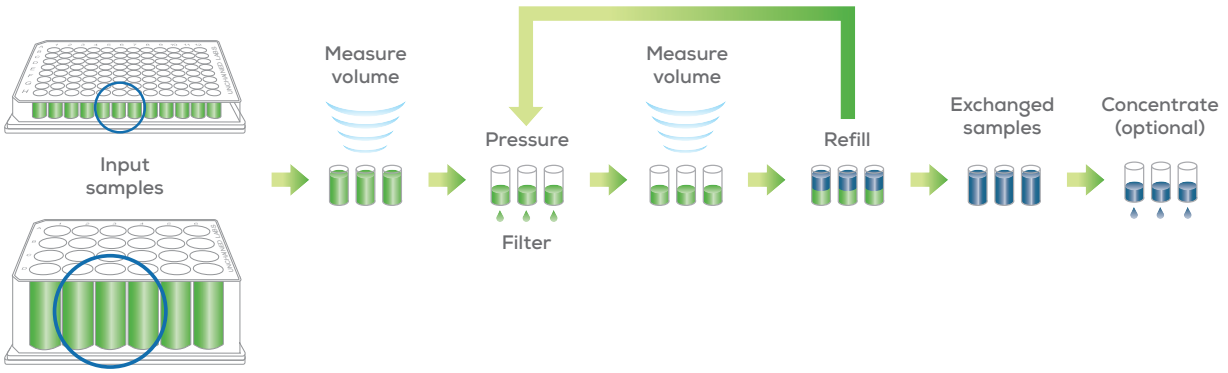

Figure 3: Big Tuna uses a pressure-based UF/DF method with gentle orbital mixing to buffer exchange proteins with the Unfilter 96 or Unfilter 24.

ranging from 0.45-8 mL in a single run. Before the run, the Unfilter 96 or Unfilter 24 is filled with the sample to be exchanged and placed in the exchange chamber. The new buffer is placed on the deck. During the run, Big Tuna alternates between filtration, volume measurement, and new buffer addition to buffer exchange samples (Figure 3).

Big Tuna has three 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. Finally, the Reduce Sample Volume application reduces up to 24 dilute samples in parallel from ≤48 mL to 8 mL. Each method can be run independently or consecutively to achieve the desired results.

In this application note, we will demonstrate how Big Tuna can be used to perform key applications in gene therapy and vaccine sample prep and purification using specific presets that have optimized parameters developed for each molecule type. For LNPs, we will remove ethanol as part of the exchange process. 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 downstream applications.

Methods

LNP buffer exchange and concentration

Firefly Luciferase mRNA encapsulated LNPs (Fluc mRNA-LNPs) were provided by Precision NanoSystems Inc. (Vancouver, BC). 78.4 µg/mL Fluc mRNA-LNP stock in PBS was diluted 3-fold into 10% EtOH in PBS. 1345 µL of the diluted LNP was transferred into 2 wells of a 100 kDa Unfilter 24.

Key buffer exchange parameters are outlined in Table 1. The experiment used the LNP preset exchange parameters. The buffer exchange protocol was set to 99% total exchange per well with a target volume removal per cycle of 50% at 60 psi. To reach a final concentration of 78.4 µg/mL, final well volume was targeted at 450 µL.

Parameter	LNP settings
Target exchange %	99%
Target volume removed per cycle	50%
Initial LNP concentration	26.1 µg/mL
Initial well volume	1345 µL
Target final concentration	78.4 µg/mL
Target final well volume	450 µL

Table 1: Key buffer exchange parameters used for LNP buffer exchange and concentration performed in a 100 kDa Unfilter 24. Wells were run in duplicate.

Big Tuna Client was used for experimental design and execution. Total processing time, initial and final well volumes, and final percent exchange were reported by the software.

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

9.4E13 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 a 30 kDa Unfilter 96 or Unfilter 24. AAVs were exchanged into PBS, pH 7.0 with 0.001% Pluronic F-68 and then concentrated 3-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 well with a target removal of 33% per well at 15 psi.

Parameter	AAV settings	
	Unfilter 96	Unfilter 24
Target exchange %	96%	96%
Target volume removed per cycle	33%	33%
Initial AAV conc. (cp/mL)	7.89E12	8.17E12
Initial well volume	450 µL	1.5 mL
Target final conc. (cp/mL)	2.38E13	2.45E13
Target final well volume (µL)	150	500

Table 2: Key buffer exchange parameters used for AAV buffer exchange and concentration in 30 kDa Unfilter 96 and Unfilter 24. Wells were run in triplicate.

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

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. 8 mL of the diluted AAV9 were manually pipetted into 3 wells of a 30 kDa Unfilter 24. 10 mL of the AAV9 stock were manually pipetted into 3 wells of a 4x6 reservoir. Diluted AAV was reduced from 18 mL to a final volume of 500 µL in a two-step process. First, 18 mL of dilute AAV9 was reduced to 8 mL with the Reduce Sample Volume application on Big Tuna Client. A Concentrate Only experiment using the same Unfilter 24 plate concentrated each well to 500 µL, for a total 36-fold concentration from the 18 mL stock.

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

Parameter	AAV settings
Initial AAV concentration	5E11 cp/mL
Initial volume	18 mL
Target final concentration	1.8E13 cp/mL
Target final well volume	500 µL

Table 3: Targeted initial and final concentration and volume used to concentrate dilute AAV up to 36-fold in a 30 kDa Unfilter 24. Wells were run in triplicate.

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

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 Unfilter 96 or Unfilter 24. 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 well with a target removal of 50% per well at 60 psi.

Parameter	DNA settings	
	Unfilter 96	Unfilter 24
Target exchange %	96%	96%
Target volume removed per cycle	50%	50%
Initial conc. (mg/mL)	2	2
Initial well volume	450 µL	3 mL
Target final conc. (mg/mL)	6	6
Target final well volume (µL)	150	1000

Table 4: Key buffer exchange parameters used for DNA concentration in 30 kDa Unfilter 96 and Unfilter 24. Wells were run in six replicates.

The UV/Vis application on Lunatic was used to analyze concentration before and after exchange and concentration.

Results

LNP buffer exchange and concentration

A target percent exchange of 99% was set per well, and 99.7 ± 0.0% exchange was achieved (Table 5). The final volume after the concentration step was ultrasonically measured as 436.5 ± 12.0 µL, at the target of 450 µL per well. The final LNP concentration was calculated based on the final volume measured from Big Tuna at 83.5 ± 2.3 µg/mL with the target concentration expected at 78.4 µg/mL. A RiboGreen® assay was used to determine % encapsulation of LNP before and after the buffer exchange and concentration process. After exchange, each well was measured in duplicate and average % encapsulation was reported as 96.5 ± 0.1% compared to 98% before buffer exchange. 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	Target final	Actual final
Conc. (µg/mL)	26.1	78.4	83.5 ± 2.3
% encapsulation	98.0	98.0	96.5 ± 0.1
Volume (µL)	1345	450	436.5 ± 12.0
% exchanged	-	99	99.7 ± 0.0

Table 5: LNP solution concentration and % encapsulation before and after buffer exchange and concentration in a 100 kDa Unfilter 24.

AAV buffer exchange: Unfilter 96

A target percent exchange of 96% was set per well, and 97.0 ± 0.2% exchange was achieved based on the diavolume added to each well (Table 6). The final volume per well was ultrasonically measured as 149 ± 1 µL, at the target of 150 µL per well. The final AAV concentration was determined by Stunner at 2.37E13 ± 1.10E12 cp/mL with the target concentration expected at 2.38E13 cp/mL. The AAV empty/full ratio was determined by Stunner. The AAV before buffer exchange had an empty/full capsid ratio of 23/77. After buffer exchange the empty/full ratio was measured at 28/72. 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	Target final	Actual final
Conc. (cp/mL)	7.89E12	2.38E13	2.37E13 ± 1.10E12
Well volume (µL)	450	150	149 ± 1
% exchanged	–	96	97.0 ± 0.2
Capsid empty/full ratio	23/77	23/77	28/72

Table 6: AAV concentration and empty/full ratio before and after buffer exchange and concentration in a 30 kDa Unfilter 96.

AAV buffer exchange: Unfilter 24

A target percent exchange of 96% was set per well, and 96.2 ± 0.2% exchange was achieved based on the diavolume added to each well (Table 7). The final volume per well was ultrasonically measured as 524 ± 23 µL, at the target of 500 µL per well. The final AAV concentration was determined by Stunner at 3.13E13 ± 9.54E11 cp/mL with the target concentration expected at 2.45E13 cp/mL. The AAV empty/full ratio was determined by Stunner. The AAV before buffer exchange had an empty/full capsid ratio of 29/71. After buffer exchange the empty/full ratio was measured at 36/64. The sample was slightly more concentrated than the targeted value. 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	Target final	Actual final
Conc. (cp/mL)	8.17E12	2.45E13	3.13E13 ± 9.54E11
Well volume	1.5 mL	500 µL	524 ± 23 µL
% exchanged	–	96	96.2 ± 0.2
Capsid empty/full ratio	29/71	29/71	36/64

Table 7: AAV concentration and empty/full ratio before and after buffer exchange and concentration in a 30 kDa Unfilter 24.

Dilute AAV: Reduce sample volume

In this experiment, we first concentrated 18 mL of AAV9 to 8 mL using the Reduce Sample Volume application. We then concentrated the reduced volume sample in the same Unfilter 24 well plate with the Concentrate Only application down to a 500 µL final volume for a total 36-fold concentration.

The 18 mL was reduced to 7.913 ± 0.022 mL with the Reduce Sample Volume application. The same wells were further concentrated with the Concentrate Only application and reached a volume of 523.7 ± 4.2 µL, compared to the target of 500 µL/well (Table 8). The final AAV concentration was determined by Stunner at 1.88E13 cp/mL with a target concentration expected at 1.74E13 cp/mL. The AAV before concentrating had an empty/full capsid ratio at 33.3/66.7. After concentrating the empty/full ratio was measured at 24.5/75.5. No significant differences in capsid empty/full ratio were observed.

AAV parameter	Initial	Target final	Actual final
Conc. (cp/mL)	4.82E11	1.74E13	1.88E13
Well volume	18 mL	500 µL	523.7 ± 4.2 µL
Capsid empty/full ratio	33.3/66.7	33.3/66.7	24.5/75.5

Table 8: AAV sample volume reduced 36-fold in a 30 kDa Unfilter 24.

Desalting DNA: Unfilter 96

A target percent exchange of 96% was set per well, and 97.8 ± 1.4% exchange was achieved (Table 9). The final volume per well was ultrasonically measured as 137 ± 9 µL at the target of 150 µL per well. The final DNA concentration was determined by Lunatic at 7.163 ± 0.352 mg/mL with target concentration expected at 6.243 mg/mL.

Unfilter 96	Initial	Target final	Actual final
DNA conc. (mg/mL)	2.081	6.243	7.163 ± 0.352
Well volume (µL)	450	150	137 ± 9
% exchanged	–	96	97.8 ± 1.4

Table 9: Desalting DNA into nuclease-free water in a 30 kDa Unfilter 96.

Desalting DNA with Unfilter 24

A target percent exchange of 96% was set per well, and 98.5% ± 0.2 exchange was achieved (Table 10). The final volume per well was ultrasonically measured as 1.022 ± 0.027 mL, at the target of 1 mL per well. The final DNA concentration was determined by Lunatic at 6.707 ± 0.204 mg/mL with target concentration expected at 6.243 mg/mL, slightly more concentrated than the target.

Unfilter 24	Initial	Target final	Actual final
DNA conc. (mg/mL)	2.081	6.243	6.707 ± 0.204
Well volume (mL)	3	1	1.022 ± 0.027
% exchanged	–	96	98.5 ± 0.2

Table 10: Desalting DNA into nuclease-free water in a 30 kDa Unfilter 24.

Conclusion

AAVs, LNPs, nucleic acids, and proteins are highly varied in size and conformation. 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 preset processing parameters on Big Tuna alter the UF/DF conditions, namely pressure and sample removal rates based on the specific molecule type and its concentration. This ensures that all sample types can be optimally exchanged and concentrated while retaining sample integrity.

For assembled molecules like AAVs and LNPs, exchange needs to be fast and must retain the 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 Big Tuna to exchange these larger assemblies as well as smaller proteins and nucleic acids makes it an ideal fit for gene therapy sample preparation laboratories.

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