Micro Scale Separation: Optimizing the Bio-Rad NGC Chromatography System for Low Volume Size Exclusion Chromatography

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Introduction:

Structural biology is the three-dimensional study of complex biological molecules, like proteins, to determine their intricate structures at atomic or near-atomic resolution. The structural characterization of complex proteins gives researchers a glimpse into their in vivo mechanistic functions and complex chemistries. In the case of drug development, structural information of proteins involved in disease pathways is crucial for downstream rational design of novel therapeutics. The field of structural biology is currently undergoing a renaissance due to significant advances in cryogenic electron microscopy (cryo-EM) following the emergence of direct electron detectors and advanced computational platforms. Protein structures can now be determined to the near-atomic level using this technique and many researchers are looking to add cryo-EM into their research repertoire.

Recent advances in cryo-EM now enable near-atomic resolution of protein structures without many of the pain points experienced in traditional crystallography. First, cryo-EM experiments require significantly less protein sample as compared to crystallography. Cryo-EM uses cryogenic techniques to immobilize the protein sample in a vitreous aqueous environment without the need for crystallization, using only a few microliters of monodisperse protein for each cryo-EM experiment. Another enticing reason researchers are leaning toward cryo-EM is the significant time-savings. Protein samples can be cryogen-ically fixed and imaged with the electron microscope all in one day, allowing for much faster time-to-results and higher sample throughput. Cryo-EM also allows the user to fix each sample in a biologically relevant buffer composition, enabling structural determination of some of the most elusive proteins and protein complexes which have been incapable of analysis through standard crystallographic techniques.

Laboratory-scale protein purification systems are primarily utilized for batch-production of recombinant proteins that are then employed in downstream in vitro applications. The Bio-Rad NGC Chromatography System is ideal for purifying recombinant proteins at laboratory scale; however, the same machine can easily be transformed to accomplish high-resolution and low-volume size exclusion chromatography (SEC) separations to generate monodisperse protein samples for downstream cryo-analysis. Here we present how simple tubing adaptations on the Bio-Rad NGC Chromatography System enables the use of high-resolution size exclusion columns for Cryo-EM applications. These adaptations allow for the purification of low volume samples, at high resolution, with 8 µl fraction drops and a total delay volume of less than 45 µl.

Adapting a NGC Chromatography system for low volume applications

To prepare a NGC Chromatography System for low volume, high-resolution, SEC based separations all PEEK tubing after the injection valve to the drop-head will need to be plumbed with a narrower internal diameter tubing of choice. All tubing before the injection valve does not need to be altered. Standard NGC systems come configured with green or orange PEEK, but there are several viable choices to decrease system volume, shown in **Table 1**.
We recommend the use of a low-volume Hamilton syringe with a blunt-tip 22 guage needle to inject sample into a 25 µl static loop. From the injection valve to the top of the column, attach the high-resolution SEC column with the shortest length of tubing possible without any kinking. Connect the bottom of the column directly to the detector module equipped with a 10 mm flow cell. To reduce swept volume further, systems that have a separate conductivity monitor on their detector can be completely bypassed, with the ports on the conductivity module plugged if not in use. Attach PEEK tubing from the outlet of the flow cell of the detector module to the BioFrac diverter valve. If you would like to decrease the delay volume further, the diverter valve of the

PEEK Type	ID (in)	ID (mm)	µl/cm
Green	0.030	0.76	4.56
Orange	0.020	0.51	2.03
Blue	0.010	0.25	0.51
Yellow	0.007	0.18	0.25
Purple	0.006	0.15	0.18
Red	0.005	0.13	0.13

Table 1. PEEK tubing compatible with the NGC Chromatography system. Green and orange are standard PEEK tubing provided with the NGC. To reduce the overall swept volume, consider using blue, yellow, purple, or red.

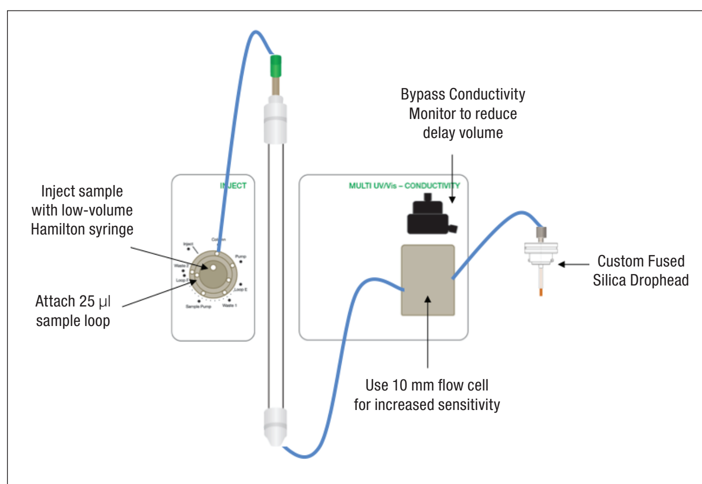

Figure 1. Example of plumbing changes to minimize overall swept volume on the NGC Chromatography system. Narrower ID PEEK tubing,such as blue or smaller, should be used from the injection valve through to the the fused silica drop head.

BioFrac can be bypassed as well, saving 12 µL in delay volume, as depicted in Figure 1. Remove the standard drop head from the BioFrac as well as the tubing between the drop head and diverter valve. To make the custom fused silica drop head, strip the outer tubing from BioFrac Microplate Drop Head Kit (7410088), remove the Delrin nut, and cut a 3 cm length of the internal 1/16" tubing

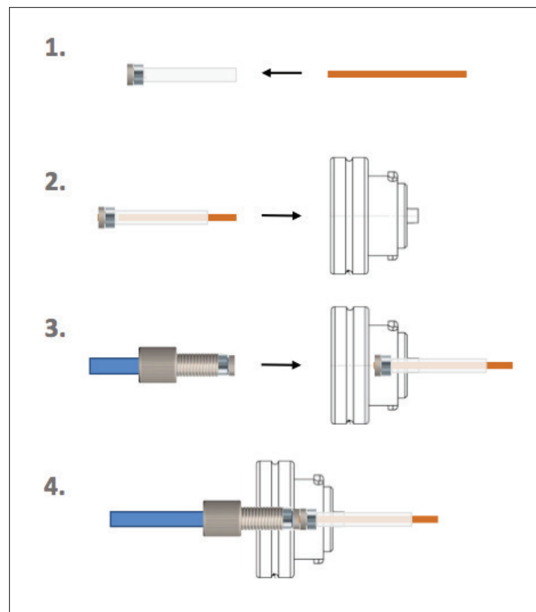

Figure 2. Stepwise instructions for custom fused silica drop head for BioFrac collector.

starting from the ferrule and lock ring end, **Figure 2** details this procedure. **(1)** Insert a 4 cm length of fused silica at non ferrule end until it is flush with the end of the ferrule, **(2)** then insert carefully into the top of the drop head holder, **(3)** and then connect the PEEK tubing ontop to create a tight union. The internal assembly of the custom fused silica union is depicted in **(4)**.

Manually calculate the delay volume based on the tubing ID chosen and enter it in the system settings. **Table 1** can be used as a reference. Typical delay volumes with minimized pathlength can be 25 to 68 µl. The software does not have the ability to convert the length of narrower than standard ID tubing to a volume, so it needs to be entered as additional volume. 10 cm of purple tubing = 1.78 µl. So in the above example, 18 µl for the flow cell, plus 28 cm of tubing between the detector and the diverter valve is 5.0 µl, plus 12 µl (the BioFrac diverter valve volume) plus 8.0 µl (diverter valve to drop head tubing volume) for a total of 43 µl, as represented in **Figure 3**.

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Location	Tubing ID (mm)	Tubing length (cm)	Vol (µl)
Purple PEEK			
MWD to diverter	0.15	28	5.0
Diverter to Drophead	0.15	45	8.0
Diverter Valve			12.0
10 mm flow cell			18.0
Total delay volume			43 µl
Blue PEEK			
MWD to diverter	0.25	28	13.7
Diverter to Drophead	0.25	45	22.1
Diverter Valve			12.0
10 mm flow cell			18.0
Total delay volume			65.8 µl

Figure 3: Delay volume as a function of tubing internal diameter for the microfluidic adaptation of BioFrac attached to NGC.

Drop size is determined by the outer diameter at the exit of the tubing and fused silica is commonly used to generate drop sizes of less than 10 µl. NGC Micro allows users to simply and quickly attach a standard ¼-28 connection to the fused silica drop head (**figure 2**). At both 63 microlitres/min & 125 microlitres/min 100 µl fractions were counted to be composed of 13 drops which equates to 7.7 microlitres per drop. The software default minimum fraction size is 30 µl, but lower fraction sizes can be collected via consulation with a Bio-Rad NGC specialist. To collect into 96 well plates on the BioFrac, we recommend the Ice Bath/Microplate Rack (7410017).

SEC sample resolution increases as tubing ID decreases

Using the NGC Quest 100 Chromatography System to conduct SEC separation of gel filtration standards, we directly observe the influence of PEEK tubing ID on the overall resolution of protein separation of 25 µl of gel filtration standard applied to the Superdex 3.2/300 GL column in PBS buffer. Three different PEEK tubing IDs were evaluated: 0.03” (0.75 mm) ID (green), 0.02” ID (0. 5 mm orange), and 0.01” ID (0.25 mm blue). Green and orange PEEK are the standard tubing that are provided with the NGC 100 and NGC 10 systems, respectively.

The first difference observed in chromatograms that are overlayed is a shift of peak retention toward the left as the ID decreases. This shift results from a decrease in overall swept volume between the injection valve and the column when using a comparatively narrower ID tube. It is also observed that the peak height increases as the tubing ID decreases and we attribute this to the minimized swept volume between the injection valve and the column. The smaller the swept volume between the injection valve and the top of the column, the less sample diffusion will occur in the mobile phase prior to reaching the column. These chromatograms were integrated using ChomLab 6.0 Software, as seen in **Figure 4**. The integrated areas of each respective peak are comparable among all three runs, independent of tubing used. For example, the relative area for peak three for ovalbumin displayed 12.9% for 0.03” ID, 12.2% for 0.02” ID, and 12.4% for 0.01” ID. These data indicate that the same amount of sample was in-fact introduced to the SEC column in each independent run, and changes in peak height, half-width, and retention are solely due to changes in tubing ID.

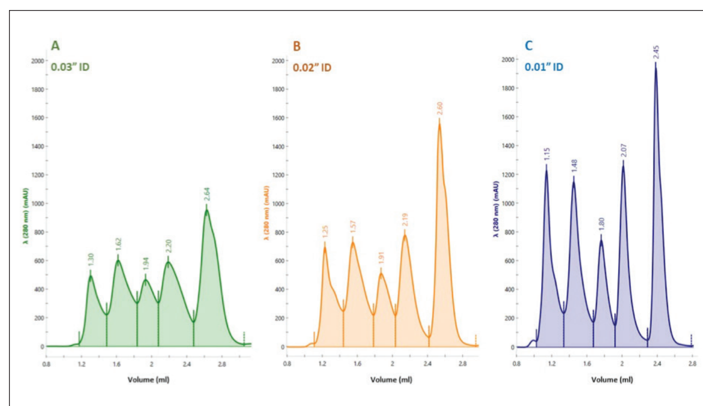

Figure 4: Peak integration of absorbance at 280 nm was performed using ChromLab 6.0 Software. The PEEK tubing used on the NGC System had different IDs, while the same 25 µl of sample was run as described in the method section. The PEEK tubing on the NGC Chromatography System was altered

from: A, 0.03” ID (green 0.75 mm), B, 0.02” ID (orange 0.5 mm), C, 0.01” ID (blue 0.25 mm), while all tubing lengths remained identical. Peaks from highest to lowest molecular weight (left to right): thyroglobulin (670 kD); bovine γ-globulin (158 kD); ovalbumin (44 kD); myoglobin (17 kD); and vitamin B12 (1.35 kD).

Pressure considerations of low ID tubing:

As liquid flows through tubing of different internal diameter, but at the same speed, the pressure increases within the tubing as the diameter decreases. With respect to a chromatography system, a decrease in the tubing ID will increase the pressure seen by the column. The Bio-Rad micro NGC Chromatography System adaptation allows users to run the Superdex 200 increase column at the maximum recommended flow rate of 150 µl/min, whilst generating pressure at less than half of the recommended pre-column pressure of the S200 increase column (**see Figure 5**).

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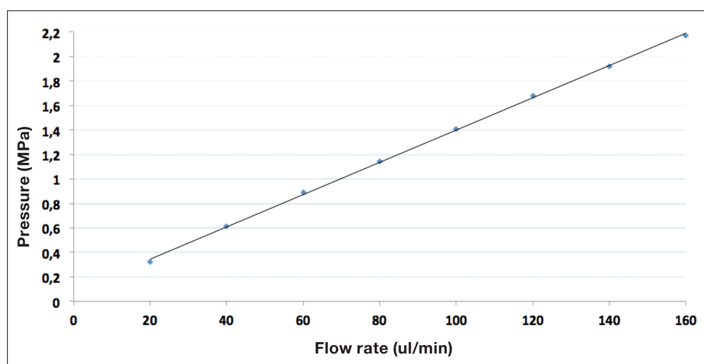

Figure 5. Pressure vs flow rate for the purple micro fluidics set up, pressure readings taken with the full fluidic path (including drop head) in line. For the Superdex 200 increase 3.2/300, the recommended flow rate is 75 microlitres/min and a max flow rate is 150 microlitres/min, with a maximum delta P limit of 4.5 MPa.

Use of Superdex 200 increase 3.2/300 column

Use of low volume tubing produces characteristically sharp peaks due to the lack of sample dilution as the purified materials passes through the tubing. **Figure 6** shows the application of 5 microlitre (5 ug) sample of the Bio-Rad gel filtration standards to the S200 Increase 3.2/300 GL column. The Bio-Rad standards are a mix of thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12, and the 18 mg total mass was reconstituted so the sample was 1 mg/ml. Following establishing a stable baseline prior to sample application, **Figure 6** shows limits of detection of less than 1 microgram of protein can be achieved. This data was produced with a 5 mm flow cell, with the use of a 10 mm flow cell expected to generate a limit of detection well below 1 µg for a protein sample, seen in **Figure 6**.

Application of sample volumes less than 25 microlitres require users follow the following sequence. **(1)** Using a 20 microlitre syringe draw 10 microlitre of load buffer into syringe. **(2)** Draw 10 microlitre of sample into syringe. **(3)** Load the 10 microlitre & 10 microlitre sample onto 25 µl loop. The 10 µl of buffer ensures the sample is transferred along the internal connection between injection port & loop.

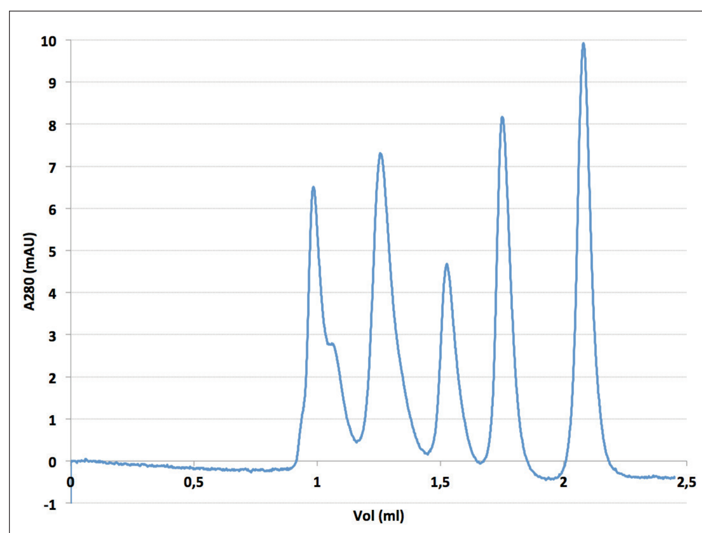

Figure 6. Showing the injection of a 5 microlitre sample (1ug/µl) of BR gel filtration standards, with 0.22 micron filtered PBS as the running buffer at 40 µl/min.

Use of very high resolution size exclusion columns

Some applications require the use of very high resolution size exclusion columns with bead sizes less than 5 microns and **Figure 7** shows the same Bio-Rad gel filtration standards, this time with a total mass of 270 micrograms applied to an eight ml SEC column with 3.5 micron beads for increased resolution. More peaks are generated compared to Figure 6 for the same mix of gel filtration standards as the lower particle size of the column resolves thyroglobulin into its different oligomeric states.

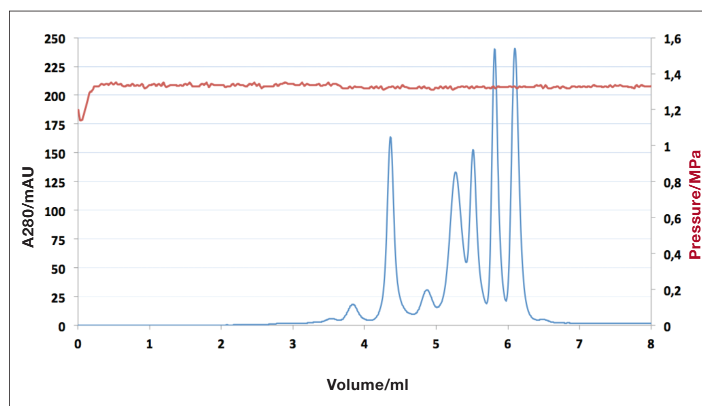

Figure 7. Showing the sharp peaks for the application of a 30 microlitre sample of the Bio-Rad gel filtration standards to a Waters X Bridge protein BEH, SEC 450A, at a flow rate of 200 µl/minute, using 0.22 micron filtered PBS as the buffer.

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Conclusion:

The NGC Chromatography System is a versatile platform that grows with your ever-changing research needs. We show here that one system typically used for preparative protein purification can also be used for low-volume high-resolution SEC purifications by simple tubing modification to satisfy the multiple chromatographic needs of those in the field of structural biology with one chromatography platform.

For more information about the NGC Chromatography system, please visit: **www.bio-rad.com/NGC**

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Bio-Rad Materials

Catalog	Description
7885023	NGC UV Flow Cell 10 mm (Compatible with the NGC Single-Wavelength and Multi-Wavelength detector modules)
12012531	NGC UV and Conductivity Flow Cell, 10 mm (compatible with the NGC Multi-Wavelength II Detector Module)
7885025	PEEK Nut 1/16”
7500556	Ferrule and Lock ring, for 1/16” OD (1.6 mm) tubing
7410017	BioFrac Ice Bath/Microplate Rack
7410088	BioFrac Microplate Drop Head Kit
7500563	Tefzel Plug

Other Materials

Other Materials	Source	Catalog #
PEEK Tubing:		
Purple – 1/16" OD x 0.15 mm ID x 1.5 m	Cytiva	18-1156-59
Blue – 1/16" OD x .010" ID x 5 ft	IDEX	1531B
Red – 1/16" OD x 0.005” ID x 5 ft	IDEX	1535
Yellow –1/16" OD x 0.007” ID x 5 ft	IDEX	1536
Fused Silica Tubing:		
0.39mm OD x 0.15 mm ID X 2 m	Thermo	00106-10498
360µm OD x 150 µm ID x 2 m	IDEX	FS-115
Columns:		
Superdex Increase 200 3.2/300 SEC	Cytiva	28990946
Waters X Bridge protein BEH SEC	Waters	176003598

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