## AAPS Interlaboratory Study

## Tier 2 Method: Asymmetric-Flow Field-Flow Fractionation

### Introduction

Asymmetric flow field-flow fractionation (AF4) is a technique that allows for separation and quantification of monomers, dimers, trimers, and higher order aggregates.

This protocol briefly describes a method to employ asymmetric flow field flow fractionation (AF4) to characterize submicrometer particles. It can also be used to separate monomer, dimer, and soluble aggregates in protein solutions as an orthogonal technique to SEC, with the key difference being the reversed elution order of species compared to SEC. For this study, the primary method describes submicrometer characterization and the reported results should be geometric radius (hydrodynamic radius if in-line DLS is installed); this is required data from the participants. Participants may also report quantities such as quantities (%) of various species (fragment, monomer, dimer, and aggregated species), and weight average molecular mass but this is optional.

Important Notes: This protocol is not a Standard Operating Protocol and assumes the required instrument is in good working order and the analysis is performed by experienced user(s). Required calibration or check standards should be run according to the manufacturer instructions. This protocol does not contain all the details of the analysis. The analyst should rely on their best judgment, routine practices, and knowledge of the technique to conduct the study; this protocol should be used as a guideline for the analysis. Samples should be analyzed immediately after thawing and preparation.

### Equipment and Material List

- AF4 system (including separation channel, membranes, etc.) including necessary fluidic system that may include pump(s) to run AF4, degasser, sample injection/autosampler, UV detector, MALS detector, DLS in-line (if equipped)
- Mobile phase (identical to the SEC mobile phase)
- Standards as recommended by the manufacturer
- Protein samples and formulation buffers supplied by sample originators (see Table 1)
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
- Pipets and pipette tips as recommended by the manufacturer.
- Particle free water/deionized ultra-filtered water
- Autosampler vials and caps

## Reagents and Solutions

**Table 1:** The following samples and their buffers will be provided by sample originators. Buffers, aliquots of unstressed, stir stressed and light stressed from Shipping #1 should be used for this analysis. All stressed material were generated at nominally 1 mg/mL.

| Proteins | Formulations | Samples |
|---|---|---|
| Amgen IgG2 | 10 mM sodium acetate, pH 5.0 | Unstressed, 1 mg/mL<br>Stir stressed, 1 mg/mL<br>Light stressed, 1 mg/mL |
| NISTmAb IgG1 | 12.5 mM L-histidine, 12.5 mM L-histidine HCl, pH 6.0 | Unstressed, 1 mg/mL<br>Stir stressed, 1 mg/mL<br>Light stressed, 1 mg/mL |

## Procedures

The AF4 conditions to be used for each molecule are summarized in Table 2 below. The primary method describes the set-up to obtain information regarding the submicrometer content in each sample. The secondary, or optional, method describes the set-up to obtain information regarding the soluble aggregate content in each sample. Depending on the channel design, membrane, spacer, and flow conditions, it is possible to achieve suitable separation under a variety of conditions. The subsequent instructions assume the conditions have been optimized by the sample providers to achieve good separation between the species within the samples.

**Table 2:** Recommended AF4 conditions for each mAb(1)

| | **Primary Method (Submicrometer Particles)** | | **Secondary Method (Soluble Aggregates)-Optional** | |
|---|---|---|---|---|
| | NISTmAb | Amgen mAb | NISTmAb | Amgen mAb |
| **Mobile Phase** | 25 mM His/L-His HCl, pH 6.0 | 100 mM sodium phosphate + 250 mM NaCl, pH 6.8 | 25 mM His/L-His HCl, pH 6.0 | 100 mM sodium phosphate + 250 mM NaCl, pH 6.8 |
| **Channel Type** | Short | Short | Short | Short |
| **Spacer Thickness** | 250 or 190 µm Thick Mylar | 250 or 190 µm Thick Mylar | 350 µm Thick Mylar | 350 µm Thick Mylar |
| **Membrane Type/MWCO** | 10 kDa Regenerated Cellulose | 10 kDa Regenerated Cellulose | 10 kDa Regenerated Cellulose | 10 kDa Regenerated Cellulose |
| **Detector Flow (mL/min)** | 1.0 | 1.0 | 1.0 | 1.0 |
| **Focus-Inject** | | | | |
| *Position* | 12% channel length | 12% channel length | 12% channel length | 12% channel length |
| *Flow-Rate (mL/min)* | 0.5 | 0.5 | 1.5 | 1.5 |
| *Duration (min)* | 3.0 | 3.0 | 5.0 | 5.0 |
| **Separation** | | | | |
| *Crossflow Initial (mL/min)* | 0.5 | 0.5 | 3 | 3 |
| *Crossflow Final (mL/min)* | 0 | 0 | 0 | 0 |
| *Ramp Type and Time (min)* | Linear, 45 min | Linear, 45 min | Linear, 45 min | Linear, 45 min |

*Prepare the AF4 system for experiments*

This protocol assumes the HPLC/fluidics system driving the FFF separator is in good working order and equilibrated with the desired mobile phase. Ensure all inline filters, pump frits, and fluid lines are clean and free of microbial growth. Ensure that the channel membrane has been conditioned and the AF4 system pressures are normal as specified by the manufacturer.

1. Prepare at least 2 L mobile phase. Filter the mobile phase to 0.1 µm into a clean bottle using a bottle top filter (0.1 – 0.2 µm) that has been pre-flushed to a waste bottle with ~100 mL ultrapure water or mobile phase to remove particles. Maintain sterility but change mobile phase every few days and replacing the bottle instead of topping off with mobile phase.
    a. Mobile phase for NISTmAb: 25 mM Histidine buffer, pH 6
    b. Mobile phase for Amgen mAb: 100 mM sodium phosphate + 250 mM NaCl, pH 6.8
2. Install a new membrane in the channel, follow the manufacturer guidelines for disconnecting the channel from the system, disassembling the channel, installing new membranes or other consumables, reassembling the channel, and subsequently purging, conditioning, or otherwise preparing the channel for experiments.
3. Membranes should be passivated by optimally running a large quantity of BSA in PBS buffer (Recommended: 7.5 mg BSA at 10 mg/mL) at a low detector flow rate (Recommended: 0.25 mL/min) with a high constant cross flow (Recommended: 3.0 mL/min on 350 µm WS; 2.0 mL/min on 250 µm WS) for 20-30 min to passivate the membrane. If an alternative membrane passivation procedure is implemented, please include details in your report.
4. The crossflow should be terminated and the detector flow should be reduced to 1.0 mL/min (or whatever the run conditions will be) and the detectors allowed to equilibrate under the rinse.
5. Continue flowing to waste until detector signals are equilibrated, and air bubbles and other contaminants have been flushed out. The detector signals can be monitored to assess the current state of equilibration.
6. (Optional) dRI detection may be added downstream for quantifying absolute molar mass using light scattering. Purge or flush all optional detectors according to manufacturer specifications.
7. After setting up the system, conditioning the channel, and equilibrating the channel and detectors, perform at least one standard injection to validate the instruments. Determine normalization, alignment, and band broadening corrections per the manufacturer's recommendation. Save these parameters to the method to be used for data analysis. Recommended quality control is BSA in PBS mobile phase at 10 mg/mL with a total injection mass of at least 50 µg to not more than 200 µg to assess peak shape, quantitative recovery, and normalization constant accuracy. Quantitative recovery of the injected BSA standard should be > 80 % of injected mass. Following this, if all parameters look okay then the system can be purged and flushed with the corresponding buffer listed above for each mAb molecule.

*Perform separations for analysis*

1. Use the method created or the system parameters from "*Prepare the AF4 system for experiments*" for data collection. Follow the manufacturer recommendations for method creation, including appropriate flow equilibration steps, duration for focus or injection steps, and other method requirements for executing the AF4 method. The total experiment time should be long enough to ensure that all sample signals elute and may include additional flushing steps without cross flow to reduce carryover peaks into the next injection as per manufacturer recommendations.

2. Set up runs for each sample according to Table 2 for appropriate flow rates for focus or injection steps. Refer to your manufacturer's instructions for setting up injections and sequences.

3. Perform at least one blank (mobile phase) injection and at least one standard injection (e.g., BSA) at the beginning of the sequence to assess system suitability and identify possible system peaks. Ensure that the results obtained from these runs are consistent with expectations before proceeding to inject the mAb samples. Mobile phase or standard injections may be repeated at the end of the sequence to assess system drift.

4. Perform 3 injections of each experimental sample. Blank injections can also be acquired for baseline correction of the RI signal, if used, to improve the concentration determination.

5. Process the data according to the analysis software manufacturer's instructions. Include the following information listed below in the analysis. Ensure the boundaries of each peak extend from baseline-to-baseline or from inflection point to inflection point if baseline-resolution is not achieved.

   Primary method (required), see 1st tab of **AF4 Data Reporting Template**:

   - geometric radius (nm)

   Secondary method (optional), see the 2nd tab of the **AF4 Data Reporting Template**:

   - High Molecular Weight-HMW (dimer and higher)
   - Monomer
   - Low Molecular Weight (LMW)

6. Apply baseline subtractions, despiking, and alignment to your sample runs using the blank runs with an identical method to improve the RI detector signal, using your manufacturer's instructions. This can improve the accuracy of the molar mass calculations or concentration from RI detectors.

7. For each peak, determine the data listed below. Calculate the average and standard deviation (% CV) among the three injections of each sample and input the results in the appropriate cells included in the **AF4 Data Template**.

   Primary method (required):

   - Geometric radius (nm) at elution time (at peak max)
   - UV Peak Area

- UV % Peak Area (=Peak Area/Total Area)

Secondary method (optional):

- UV Peak Area
- UV % Peak Area (=Peak Area/Total Area)
- Weight-average molar mass (Mw) by MALS
- Optional: RI Peak Area and RI % Peak

## Understanding Results

The concentration of eluting species can be determined using the UV and MALS detector. In the **AF4 Data Reporting Template**, specify the detection method. You may use the software available with your instrument for calculating the areas of the various peaks (hydrodynamic radius, fragment, monomer, and high molecular weight species, etc.). Ensure the buffer fractograms are subtracted from the sample (if they contribute to the signal) prior to calculating the peak areas. While you do not have to report mobile phase runs or standard BSA runs, please ensure that you perform them and that the results are consistent with expectations before running the protein samples. Be sure to save all raw data so they could be retrieved in the future, if needed.

## Further Information

For any specific questions regarding this method, please contact Sophia Kenrick at skenrick@wyatt.com and Sean Lehman at sean.lehman@nist.gov.

## Appendix

**Table 3:** General recommendations for good AF4-MALS data quality

| Settings | Recommendations |
|---|---|
| General | a) Change the HPLC pump frit according to the manufacturer's specification.<br>b) Fully flush the sample loop, needle seat, and injection needle.<br>c) Make sure your sample is fully in solution. Sample filtration may be necessary. |
| Mobile Phase | a) Use a clean solvent reservoir.<br>b) Filter mobile phase, particularly when containing salt. |

| | |
|---|---|
| | c) Suppress bacterial growth (antibacterial agent or frequent mobile phase change).<br>d) Clean contaminated HPLC system according to the manufacturer's recommendation.<br>e) Use an inline filter between pump and autosampler/injector. |
| **AF4 Separator & Channel Considerations** | a) Follow manufacturer recommendations for replacing frits, filters, and other consumable components.<br>b) Observe system and channel pressures to assess the performance per manufacturer guidelines.<br>c) Replace the membrane at least once a month but often more frequently depending on fouling or performance with standard.<br>d) Condition new membranes with a suitable standard.<br>e) Ensure adequate flushing, conditioning, and purging of the system for optimal signal baselines. |

## Reference(s)

1. Lehman SE, Karageorgos I, Filteau JR, Vreeland WN. Effect of Azide Preservative on Thermomechanical Aggregation of Purified Reference Protein Materials. J Pharm Sci. 2021;110(5):1948-57. doi: 10.1016/j.xphs.2021.01.013.