AAPS Interlaboratory Study

Tier 1 Method: Size Exclusion Chromatography with UV Detection (and optional MALS)

Introduction

This protocol provides a brief procedure to measure size variants in mAb samples (unstressed control and stressed) using size-exclusion chromatography (SEC) with UV and an optional multi-angle light scattering (MALS) detection. The primary reported results are the percentages of the various size variants as calculated by UV detection (and mass of size variants, if MALS is used).

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

- HPLC system with UV detectors (RI and MALS detectors, optional), degasser, pump, injector, guard column, column, column oven, data acquisition and analysis software
- Mobile phase recipes (provided by sample providers)
- Protein samples and formulation buffers supplied by sample originators (see Table 1)
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
- Autosampler vials and caps and micro-inserts
- Pipette and pipette tips
- Bottle top filter (0.1 µm or 0.2 µm)
- Bovine serum albumin as suitability standard or for conditioning a new column, as required
- Molecular weight standards, if required

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 Stir stressed, 1 mg/mL Light stressed, 1 mg/mL
NISTmAb IgG1	12.5 mM L-histidine, 12.5 mMol L-histidine HCl, pH 6.0	Unstressed, 1 mg/mL Stir stressed, 1 mg/mL Light stressed, 1 mg/mL

Procedures

Instrument Preparation

1. Set up HPLC with appropriate column and guard column and ramp up to the desired flow rate slowly.
2. Input HPLC, detector, system suitability standards, and other information into the appropriate cells of the **SEC Data** reporting template.

Sample Preparation and SEC Separation

1. Thaw the samples from Shipping #1 and aliquot the Tier 1 vials (for the unstressed, stir, and light stressed mAb samples) as described in the instructions into smaller aliquots for the various methods and freeze until ready to use. Be sure to follow the procedure for vigorously mixing the sample prior to aliquoting.
2. For this analysis, thaw the aliquots designated for SEC/SEC-MALS measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes. It is recommended that these samples be analyzed within 2 hours of thawing.
3. Prior to analysis, mix each aliquot in the following manner: pipet with a 1000 uL tip near the bottom of the sample tube 10 times in different directions but not touching the bottom or creating bubbles. Remove sample from near the bottom of the vial, from about ¾ of the depth of the tube from the top of the liquid before dispensing the sample into another container containing the buffer.
4. Review **SEC Data** reporting Template for the requested information before beginning the measurements and input set up information.
5. Set up runs for each sample according to Table 2 (flow rate, injection volume, run duration, etc.), which have been provided by the protein suppliers. Perform the runs according to the instrument manufacturer instructions and instrument familiarity. Avoid starting/stopping

pump between injections or sequences by recirculating mobile phase at constant flow rates. Set sample rack/autosampler temperature to (4 to 10) ˚C.

6. Perform at least one blank (mobile phase or formulation) 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.
7. Depending on the column used, some samples for SEC injection may need to be concentrated if the starting concentration of 1 mg/mL is too low. If this is the case, Amicon Ultra 10K centrifugal filters or equivalent can be used for concentrating the sample. Refer to the manufacturer's instructions on the use of these filters. After spinning the sample, remeasure the concentration of the filtrate and inject (50-100) µg depending on the column size. You can use theoretical extinction coefficient of 1.42.
8. Perform 3 injections of each sample and corresponding buffer.
9. Process the data according to the analysis software manufacturer's instructions. Include the following peaks 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.
    - High Molecular Weight (dimer and higher)
    - Monomer
    - Low Molecular Weight
10. For each peak, determine the data listed below. Calculate the average and standard deviation among the three injections of each sample and input the result in the appropriate cells of the **SEC Data** reporting template.
    a. UV Peak Area
    b. Relative peak area (%) (peak area/total area) *100
    c. Optional: weight-average molar mass (M_w) by MALS
11. Standard and mobile phase injections may be repeated at the end of the sequence to assess system drift.

SEC Conditions

The SEC conditions for each molecule are summarized in the table below. This protocol assumes the conditions have been optimized to achieve good separation between monomer and aggregates and to reduce column interactions.

Table 2: SEC conditions previously used for each mAb (provided by the originator labs) from published papers. Your parameters might be a little different based on your column and instrument set-up.

mAb	Column	Mobile Phase	UV Detection wavelength (nm)	Column Temp. (°C)	Run Duration (min)	Flow Rate (mL/min)	Injection (µg)
NISTmAb(1)	UPLC Protein BEH 1.7 µm particle size, 200 Å pore size, 4.6 mm × 150 mm length	100 mM sodium phosphate + 250 mM NaCl, pH 6.8	280	25	10	0.3	60
Amgen IgG2(2)	HPLC, Tosoh Bioscience TSK-Gel G3000SWxl (7.8 x 300 mm, 5 µm particle size)	100 mM sodium phosphate + 250 mM NaCl, pH 6.8	280	25	35	0.5	100

(Optional) If using MALS detection in addition to UV detection, follow the steps below

Addition of multi-angle light scattering (MALS) and differential refractive index (RI) detection
MALS and RI detection may be added downstream of virtually any SEC chromatograph for quantifying absolute molar mass using light scattering.

1. After setting up the SEC-MALS system and equilibrating the column and detectors, perform at least one standard injection to validate the instruments. Determine normalization, alignment, and band broadening corrections according to manufacturer recommendation.
2. Introduce samples to perform SEC separation and collect MALS data using the appropriate software for the MALS detector. If available, RI may be used to quantify species.
3. Analyze data using instrument parameters established earlier. Define baselines and peaks to assess molar mass and quantify species (i.e. monomer and each oligomer population). Add measurement parameters and analyzed results to **SEC Data** reporting template.

Understanding Results
The concentration of eluting species can be determined using the UV detector (MALS detectors are optional). In the **SEC Data** reporting template, specify the detection method. You may use the software available with your instrument for calculating the areas of the various peaks (low molecular weight, monomer, high molecular weight species). Ensure the buffer chromatograms 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 of the raw data so they could be retrieved in the future, if needed.

For integration, the main peak corresponds to the monomeric peak of the untreated sample. High molecular weight (HMW) peaks include everything in the range between the excluded volume and the start of the main peak, and low molecular weight (LMW) peaks include all peaks up to excipient buffer peak.

Troubleshooting
The protocol assumes the optimal method for resolving monomers and oligomers is being used for each molecule. An overview of common issues is given in Table 3, with additional details given below.

Table 3: Troubleshooting overview

Problem	Probable Cause	Resolution
Baseline Noise and Drift	Dirty detector flow cell	Clean flow cell (try in-situ cleaning first).

	Dirty HPLC system	Clean HPLC system per manufacturer's recommendation.
	Refractive index detector needs purging	Purge RI detector for at least 1 hour before running experiments.
	Column shedding or sample adsorption on the column	Clean or replace column.
Excessive Peak Broadening	Aging or contaminated column	Clean or replace column.
	Mobile phase contamination or deterioration	Replace mobile phase, use a clean solvent reservoir and solvent filter, and clean HPLC and detectors if necessary.
	Slow leaks in the HPLC system or inside detectors	Locate leaks, clean spills, and replace tubing and fittings as necessary.
	Excessive tubing length or tubing I.D. too large	Reduce tubing length and use recommended tubing I.D.

References

1. Turner A, Yandrofski K, Telikepalli S, King J, Heckert A, Filliben J, et al. Development of orthogonal NISTmAb size heterogeneity control methods. Anal Bioanal Chem. 2018;410(8):2095-110. doi: 10.1007/s00216-017-0819-3.
2. Joubert MK, Luo Q, Nashed-Samuel Y, Wypych J, Narhi LO. Classification and characterization of therapeutic antibody aggregates. J Biol Chem. 2011;286(28):25118-33. doi: 10.1074/jbc.M110.160457.

Further Information

For any specific questions regarding this method, please contact Sophia Kenrick at skenrick@wyatt.com and Katharina Yandrofski at katharina.yandrofski@nist.gov. Please copy aapsinterlab@nist.gov on your emails.