AAPS Interlaboratory Study

Tier 1 Method: Flow Imaging

Introduction

This protocol describes how flow imaging can be used to obtain the size distribution of particles in dilute protein solutions. The primary reported results are the particle concentrations (number of particles/mL) for particles with sizes above designated diameters.

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.

These samples are time sensitive with regards to particles but depending on the time requirement, analyst, and instrument availability, it might be possible to do some measurements simultaneously (i.e. run the samples prepared at the same time on light obscuration and MFI/ FlowCam simultaneously). Please coordinate if it is a general practice to do these measurements concurrently.

Equipment and Materials List

- Flow imaging particle counting instrument appropriate for small-volume sampling of aggregated protein solutions. Flow Imaging instruments should use magnifications in the range (4× to 10×) and cell depths of not more than 100 µm.
- Protein samples and formulation buffers supplied by sample originators
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
    - Stir-stressed samples from Shipping #2
- Commercially available size and count particle standards-at least 1 of the following: 2 µm, 5 µm, or 10 µm
- Cleaning solutions or detergents to run between samples, as recommended by the sample originators
- Vacuum and desiccator for degassing samples
- Pipette and filtered pipette tips
- 15 mL sterile falcon tubes or 5 mL sterile glass vials for preparing samples
- Syringes, needles, and filters for filtering buffer (e.g., 0.22 µm cellulose acetate filter)
- Particle free water/deionized ultra-filtered water

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 and stir-stressed samples from Shipping #2 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 Setup

1.) Autofocus the flow cell. If automatic focusing is not available, perform manual focusing using beads.

2.) Verify the cleanliness of the instrument by running ultra-filtered water blanks. The particle concentration of the blanks should be within limits established by prior blank runs on the same instrument.

3.) Adjust software settings of the instrument as appropriate for microsphere standards. Verify the size and count accuracy of the counting instrument by measuring commercial or in-house microsphere concentration standard. The particle size distribution (PSD) of the microsphere standard should have a single peak with a width consistent with prior experience on the instrument. For Flow Imaging instruments with adjustable thresholds, set thresholds to values appropriate for microsphere beads. The reported size for adjustable threshold Flow Imaging instruments may depend on the thresholds chosen; the observed size should be consistent with prior experience.

4.) Flush the microspheres from the instrument, and re-verify cleanliness of the instrument by measuring filtered-water blanks.

5.) Adjust software settings of the particle counting instrument as appropriate for protein aggregates. Input setup details into the **Flow Imaging Data Reporting Template** Excel file. Note the diameters for reporting particle concentrations: ≥ [1, 2, 5, 10, 15, 20, 25, 50] for the recommended diameter values for reported particle concentrations. For Flow

Imaging instruments with adjustable thresholds, set these thresholds to values appropriate for protein aggregates (for FlowCam, use Dark = 10, Light = 10, nearest neighbor = 10 µm).

6.) Check filtered formulation buffers to ensure they are clean.

Sample Preparation

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 contents of the vial prior to aliquoting.

2) For this analysis, thaw the aliquots designated for the flow imaging measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes. Thaw 1 vial each of the Amgen mAb and NISTmAb pre-diluted stir stressed samples from Shipping #2 by bringing them to room temperature for 30 minutes.

3) **These samples are time sensitive with respect to particle concentration.** After thawing, particle concentrations may change with time, even if the sample is maintained at (2-6)°C. This change may be mitigated by analyzing the samples immediately (within 2 hours) of thawing. It is recommended to perform all control measurements before the protein samples are fully thawed.

4) A proper mixing of the samples prior to analyzing is important to eliminate particle clumping. Prior to dilution of each sample, mix each aliquot (mentioned in #2) by pipetting the sample with a 200 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.

5) In a laminar flow hood, prepare a total of 6 mL of each sample (unstressed, light stressed, and stir-stressed) in sterile 15 mL conical tubes by diluting each aliquot 100-fold. For diluent, use a filtered formulation buffer that is provided by the originators for each of the mAbs. The pre-diluted stir stressed samples do not need to be diluted.

6) Split the prepared samples into two sets with each set consisting of 3 mL of the unstressed, stir stressed, and light stressed and 2.5 mL of pre-diluted stir stressed samples. Degas one set of the samples (e.g. 1 h, 75 Torr at room temperature). Run the other set immediately without degassing.

Sample Measurements

1.) Review the **Flow Imaging Data** reporting template for the requested information before beginning the measurements. Please follow sample order as shown in the template.

2.) Set up the instrument for sample measurements. Prime the instrument as recommended by the manufacturer.

3.) Ensure the sample is homogeneous by resuspending protein solutions by using a "mixing" option, if available on your instrument. If that is not available, you may gently swirl the vial in circular motion 10 times to distribute protein particles immediately before measuring the sample. Do not create air bubbles during resuspension.

4.) Initiate a measurement promptly after resuspension. A method can be created that has the following parameters: load volume of 0.7 mL; purge volume of 0.2 mL.

5.) After each run, flush the system with water and or filtered buffer.

6.) Obtain 3 replicates for each sample. Between each sample, verify the cleanliness of the instrument by running ultra-filtered water blanks.

7.) Record averages and standard deviations for the runs in the **Flow Imaging Data** Excel file.

Understanding Results

Data and selected instrument parameters used should be entered in the **Flow Imaging Data** reporting template. All results should be reported as the number concentration of particles per milliliter greater than or equal to the indicated equivalent diameter. Results from a FlowCam instrument should be reported as ECD or ESD. If data was not obtained for any diameters listed on the templates, enter "NA" in the cells for that diameter.

Most users will report particle concentrations as generated by instrument software. If any corrections to these concentrations are performed, report the magnitude and basis of the corrections. If the samples were diluted, please adjust the particle concentrations for the dilution factor.

Troubleshooting

As with other samples of aggregated proteins, the proteins may adhere to flow-cell walls or clog apertures. Indications of possible problems and recommended solutions are:

1. Clogging or partial clogging of flow cells. A partial or full clog will appear in the data as a reduction in counts, especially for the larger observed particle sizes. If such a decrease is observed, backflush the cell with detergent solution, then forward flush thoroughly with filtered water. Confirm flow-cell cleanliness with blank runs, and then recommence sample measurements.
2. Particles adhering to the walls of a flow-imaging flow cell can cause large increases in apparent counts. Follow manufacturer's recommendations for removal of stuck particles in final reported values.

3. Change in particle concentration. After thawing, the particle concentration will change with time, even if the sample is maintained at (2 to 6) °C. This change can be mitigated by carefully planning the data acquisition to minimize the period of data acquisition, preferably to within a couple of hours of thawing.

Further Information

For any specific questions regarding this method, please contact Dean Ripple at dean.ripple@nist.gov.