

AAPS Interlaboratory Study

Tier 1 Method: Light Obscuration

Introduction

This protocol describes the method of using light obscuration 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 a designated set of diameters.

Important Notes: This protocol 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

- Light obscuration instrument appropriate for small-volume sampling (< 1 mL) of aggregated protein solutions. Diameter range of approximately 1 µm to 150 µm or 200 µm is preferred, if available.
- Protein aggregate samples and formulation buffers supplied by sample originators
 - a. Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
 - b. Stir-stressed samples from Shipping #2
- Commercial or in-house-prepared microsphere concentration standards
- Vacuum and desiccator for degassing samples
- 5 mL depyrogenated sterile glass vials, pipets/pipette tips for preparing samples
- Syringes, needles, and filters for formulation buffer (e.g. 0.2 µm cellulose acetate filter)
- Particle free water/de-ionized ultrafiltered 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.) Adjust software and hardware settings for the particle counting instrument as appropriate for protein aggregates.
- 2.) Verify the cleanliness of the instrument by running filtered water blanks. Particle concentrations for the blanks should be within limits established by past experience on the same instrument (e.g. < 10 particles/mL for $\geq 2 \mu\text{m}$ particles).
- 3.) Verify the size and count accuracy of the instrument by measuring commercial or in-house microsphere concentration standards. The measured concentrations should be within the limits for the standards and have size distributions consistent with experience on the instrument.
- 4.) Flush the microspheres from the instrument and re-verify the cleanliness of the instrument by measuring filtered water blanks between each sample measurement.
- 5.) Adjust software and hardware settings for the particle counting instrument as appropriate for the experiment. Input setup details into the **Light Obscuration Data** Excel file. Diameters for reporting particle concentrations: $\geq [2, 5, 10, 15, 20, 25, 50, 100] \mu\text{m}$. Set 4X injections, 0.5 mL per injection, discard first injection for quantification.
- 6.) Check filtered buffers provided to ensure they are clean (e.g. < 20 particles/mL for $\geq 2 \mu\text{m}$).

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 light obscuration 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 $\frac{3}{4}$ 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.

Clarification added Sept 20th regarding sample preparation for LO:

For samples in Shipment #2 only, perform one measurement with 4 sub-runs for the degassed set of samples and for the non-degassed set of samples. The instructions request participants to perform 2 replicate measurements under degassing and non-degassing conditions but the 5 mL sample would not be sufficient to do two measurements for both conditions.

For samples in Shipment #1, please follow original directions and do 2 replicates for both degassed and un-degassed condition. There is enough sample for both sets of measurements after 100 fold dilution of the stock material.

Sample Measurements

- 1.) Review the **Light Obscuration Data** reporting template for the requested information before beginning the measurements. Please follow sample order as shown in the template.
- 2.) Prior to each sample measurement, resuspend protein particles by gently tipping the vial to move the vapor bubble in the vial from one side to the other and back to the original side, and repeat 10 times. Do not entrain air bubbles in the sample.
- 3.) Promptly after resuspension, measure particle concentrations for each sample. Ensure the probe is submerged to about ½ fluid height in the vial. Flush and verify cleanliness by measuring filtered water between each sample.
- 4.) Discard the first run. Record averages and standard deviations of runs 2, 3, and 4 for each sample in the **Light Obscuration Data** Excel file.

Understanding Results

All results should be reported as the number concentration of particles per milliliter for particles greater than or equal to the specified diameters.

Data should be entered in the **Light Obscuration Data** reporting template. 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 the instrument software. If any corrections to these concentrations are performed (i.e. dilutions), report the magnitude and basis of the corrections.

Troubleshooting

As with other samples of aggregated proteins, the protein aggregates may settle, adhere to surfaces, or change over time. Indications of possible problems and recommended solutions include:

- 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.
- 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.
- 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. Please copy aapsinterlab@nist.gov on your email.