AAPS Interlaboratory Study

Tier 2 Method: Intrinsic Fluorescence Spectroscopy

Introduction

This protocol describes the procedure for monitoring protein tertiary structural changes using intrinsic fluorescence. Protein fluorescence spectroscopy is dependent on the presence of aromatic amino acids like phenylalanine, tyrosine, and tryptophan. Tryptophan fluorescence (with excitation wavelengths between 280 and 300 nm) is used to monitor structural changes in the protein as its emission maximum (ca. 300 – 350 nm) is highly dependent on the polarity of the environment. The intensity of the emission and the wavelength of maximum intensity for tryptophan fluorescence are indicators of the average residue microenvironment and will be monitored to provide information on protein tertiary structure.

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 Materials List

- Fluorescence Spectrophotometer with a temperature control unit
- UV spectrophotometer
- Quartz cuvette (path length depends on system being utilized)
- Pipets and pipette tips as recommended by the manufacturer.
- Cuvette cell washer
- High purity ethanol
- Milli-Q grade water or equivalent
- 5% Contrad 70 (or equivalent detergent)
- Protein samples and formulation buffers supplied by sample originators (see Table 1)
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
- Appropriate dilution buffer for the mAbs

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

Sample preparation

1. Thaw the samples from Shipping #1 and aliquot the Tier 2 vials (for the unstressed, stir, and light stressed 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 intrinsic fluorescence measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes.
3. 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.
4. Under laminar flow hoods, dilute all samples to 0.5 mg/mL with 0.22 µm filtered designated buffer. Use the UV Visible Spectroscopy protocol to determine the exact concentration of the diluted solution at 280 nm using the designated extinction coefficient.
5. If the stressed sample is highly turbid, the sample may need to be briefly centrifuged. The "supernatant" of the centrifuged sample should be carefully separated from the vial and should be used for analysis. Here, we define the "supernatant" as 70% of the filling volume.

Instrument Setup

1. Turn on the instrument and temperature controller. Set temperature to 20°C +/- 2°C. Warm the lamp for at least 30 min.
2. Measure fluorescence of the background sample (buffer) prior to fluorescence of samples, using the parameters listed below in Table 2.
3. After collecting background, collect spectra of the sample.
4. Between each sample, wash the cuvette thoroughly with water and dry with ethanol rinse, followed by vacuum drying.

5. Turn off system in accordance with instrument manufacturer's instructions.

Table 2: Intrinsic fluorescence parameters

Excitation Wavelength (nm)	295
Emission (nm)	300 to 550
Em/Ex Slit width (nm)	Automatic, if possible
Scan speed (nm/min)	100
Step size (nm)	1
Accumulations	3
Temperature	20°C
# of Replicates	1

Sample Measurement

1. Review the **Intrinsic Fluorescence Data** reporting template for the requested information before beginning the measurements.
2. Subtract the buffer spectrum from the sample spectrum.
3. Buffer subtracted (corrected) value is then concentration-normalized by dividing the corrected fluorescence intensity value divided by the protein concentration determined by UV-Vis absorbance (A280).
4. Wavelengths of fluorescence peak maxima (λmax) are determined manually in Excel spreadsheet and confirmed visually.

Understanding Results

Enter instrument information and final data into the provided **Intrinsic Fluorescence Data** Excel sheet. Instrument biases can depend strongly on the instrument configuration. Document instrument type, model, and configuration and settings. Export the data as recommended by the instrument manufacturer.

Normalized fluorescence spectrum: Results are plotted as Normalized Fluorescence Arbitrary Units (A.U.) vs.Wavelength (nm).

Emission Maxima reporting: Round reported wavelength values (λmax) to the nearest whole number increment.

Troubleshooting

As with other samples of aggregated proteins, the proteins may adhere to the surface of the cell. Visually ensure no residual protein or residue streaks/smears remain on the cuvette. If the cuvette's background scans appear to contain unusually high absorbance signal, soak the cuvette with 5% Contrad 70 (or similar detergent) for 30 minutes. Rinse with Milli-Q water to remove all detergent and wash cell thoroughly.

Further Information

For any specific questions regarding this method, please contact Sarita Mittal at sarita.mittal@merck.com. Please copy aapsinterlab@nist.gov on your email.