AAPS Interlaboratory Study

**Tier 2 Method: Extrinsic Fluorescence Spectroscopy using ANS**

**Introduction**
This protocol briefly describes a procedure for monitoring changes in surface hydrophobicity (i.e. indicative of changes in tertiary structure) using extrinsic fluorescence spectroscopy. Fluorescent dyes such as 8-Anilino-1-naphthalene-sulfonic acid (ANS) bind with high affinity to hydrophobic surfaces and may be used to report the exposure of hydrophobic regions of proteins due to conformational changes and/or unfolding. The fluorescence peak positions and intensities will be monitored and compared to those of the unstressed control to compare the extent of surface hydrophobicity among samples.

**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**
- Spectrofluorimeter
- 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
- Pipets and pipette tips
- 1-cm path length quartz cuvettes
- 8-Anilino-1-naphthalene-sulfonic acid fluorescence dye (ANS)
- Dimethyl sulfoxide (DMSO) to make concentrated dye solution
- Particle free water/deionized ultra-filtered water
- PVDF syringe filters

**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.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Formulations</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amgen IgG2</td>
<td>10 mM sodium acetate, pH 5.0</td>
<td>Unstressed, 1 mg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stir stressed, 1 mg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light stressed, 1 mg/mL</td>
</tr>
<tr>
<td>NISTmAb IgG1</td>
<td>12.5 mM L-histidine, 12.5 mMol L-histidine HCl, pH 6.0</td>
<td>Unstressed, 1 mg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stir stressed, 1 mg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light stressed, 1 mg/mL</td>
</tr>
</tbody>
</table>

**Procedures**

**Instrument Setup and Sample Preparation**

1. Thaw the samples from Shipping #1 and aliquot the Tier 2 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 extrinsic measurements by bringing them to room temperature for no more than 15 minutes.

3. Turn on the instrument and allow it to warm up for 30 minutes or as suggested by the manufacturer.

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 µL 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. Under a laminar flow hood, dilute the protein samples to a final concentration of about 0.1 mg/mL in their appropriate buffers. Before adding ANS, verify that the protein concentration is around 0.1 mg/mL using UV spectroscopy measurement with light scattering correction. Refer to the UV Visible Spectroscopy protocol to measure the protein concentration.

6. Prepare a 0.1 M ANS stock solution in DMSO and add 50 µM of ANS solution to the 0.1 mg/mL protein solution. Incubate the solution in the dark for 5 minutes before measurements. Since a high concentrated ANS stock was prepared, only a very small volume of ANS needs to be used—this also won’t affect the concentration measurements made before addition of the ANS.

7. Prepare a blank containing the same amount of ANS and buffer but without the protein.

**Data Acquisition**
1. Review the **Extrinsic Fluorescence Data** reporting template for the requested information before beginning the measurements.
2. Use the experimental parameters in Table 2 to collect the data.

### Table 2: Extrinsic fluorescence parameters

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><strong>Excitation wavelength</strong></td>
<td>372 nm</td>
</tr>
<tr>
<td><strong>Emission wavelength</strong></td>
<td>400 nm to 600 nm</td>
</tr>
<tr>
<td><strong>Path length</strong></td>
<td>1 cm</td>
</tr>
<tr>
<td><strong>Integration time</strong></td>
<td>Determined by the individual lab to provide optimal S/N</td>
</tr>
<tr>
<td><strong>Step size</strong></td>
<td>1 nm</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>20 °C ± 2 °C</td>
</tr>
<tr>
<td><strong>Number of replicates</strong></td>
<td>1</td>
</tr>
</tbody>
</table>

3. Use automatic slit adjustments, if available on your instrument. Otherwise, you may need to adjust the excitation and emission slits on the instrument so the peak intensity of the samples is optimal (not too low that there is a lot of noise in the peaks and not too high that it saturates the detector).

4. All measurements should be done within 5 min of sample mixing. Based on your instrument set-up, you may not be able to add the dye to all of the samples at once. If you can only do one run at a time, you can get all of your cuvettes filled with the diluted protein, but wait to add the ANS. When you are ready to do the run, add the ANS to the cuvette, incubate for 5 minutes in the dark, and start the run.

### Data analysis & Data Reporting

1. Enter instrument parameters and the final analyzed data in the Extrinsic Fluorescence tab of the accompanying Excel template. If any corrections to the data are performed, report the magnitude and basis of the corrections.
2. Subtract the buffer blank (containing ANS) emission spectrum from each sample (protein + ANS) spectrum using automated data analysis provided by the manufacturer.
3. For each sample, record the buffer corrected emission intensity over the wavelength 400 nm to 600 nm (wavelength vs. fluorescence intensity). Compare both the fluorescence intensities and peak wavelengths of the samples relative to the unstressed control. This will give you information regarding the extent of apolar exposure of the samples relative
to the unstressed monomer (more/less exposed and red/blue-shift of the fluorescence signal).

**Understanding Results**

A high ANS fluorescence intensity indicates that the protein is structurally disrupted or aggregated. In addition to an increase in fluorescence intensity, interactions with hydrophobic environments are often accompanied by a blue shift (peak maximum moves towards smaller wavelength) in the fluorescence spectrum. Hydrophilic environments typically lead to decreases in fluorescence intensity and a red shift (peak shifts towards a larger wavelength) of the fluorescent peak.

**Troubleshooting**

1) Ensure there are no bubbles in the cuvette. This could distort the results.
2) Ensure the cuvette is placed in the proper orientation in the instrument, and same orientation for all samples.
3) If automatic slit adjustments are not available on your instrument, select proper slit widths so you do not obtain too much noise or saturate the signal.

**Further Information**

For any specific questions regarding this method, please contact aapsinterlab@nist.gov.