AAPS Interlaboratory Study

Tier 1 Method: Near-UV CD Spectroscopy

Introduction

This protocol describes the method of using near-UV circular dichroism (CD) spectroscopy to analyze the protein conformation of mAb samples. The CD spectrum in the near UV region, between 250 nm to 350 nm, reflects the environment around the aromatic amino acid side chains and disulfide bonds and typically reflects the tertiary and/or quaternary structure of the proteins. For each mAb sample, the reported results should be the wavelength (250-350 nm) and corresponding mean residue ellipticity.

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

- CD spectrometer
- Measurement cell (UV-transparent)-cuvette 0.5 to 1.0 cm pathlength
- Protein samples and formulation buffers supplied by sample originators
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
- Detergents or cleaning solutions for cleaning cuvettes between runs

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 selection

Select your preferred CD spectrometer that allows the measurement of undiluted mAb samples according to the settings specified in the table below.

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 sample prior to aliquoting.
2. For this analysis, thaw the aliquots designated for near-UV CD measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes.
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 standard PP-tubes for CD analysis.
4. If the sample is not transparent/clear visually, use adequate centrifugation to remove any suspended particles. Because the near-UV CD signal of most proteins is much weaker than far-UV CD, typically a 10-fold higher concentration is needed for the near-UV CD range to collect good quality CD spectra. Use the UV Visible Spectroscopy protocol to determine the exact concentration of the diluted solution at 280 nm using the designated extinction coefficient.

Sample measurement

1. Review the **Near UV CD Data** template for the requested information before beginning the measurements and input the set-up information.
2. Set up the instrument according to Table 2 below. It is important to obtain an accurate protein concentration prior to starting a CD experiment since an incorrect concentration may result in poor CD values due to signal masking or low signal. Additionally, an accurate concentration is needed to calculate normalized CD values. Refer to the UV absorbance protocol.
3. For each mAb sample, collect spectra for the formulation buffer and mAb sample with identical acquisition parameters, sample volumes, and measurement cell orientation. If necessary, clean and dry the measurement cell between each sample and check the formulation buffer spectra for contamination from previous mAb samples. Take care to avoid the formation of air bubbles and prevent evaporation of the sample (e.g., use lids,

covers, seals, and measure promptly). Measure the spectra of the samples as specified in the Table 2 below.

Table 2. Recommended instrument parameters.

Wavelength of interest	250-350 nm
Cell pathlength	0.5 cm to 1 cm
Number of spectra acquisitions per sample	10
Number of replicates	1
Measurement temperature	20 °C
Sample volume	1-2 mL
Sample concentration	1 mg/mL
Sample required	1 mL
Mode	Continuous

Data reporting

1. Record the instrument details and measurement parameters in the **Near-UV CD Data** Excel file.
2. Use the instrument's software or manually average the 10 individual spectra for each sample. Subtract the buffer spectra from the corresponding sample spectra. Using the known protein molecular weight and the number of amino acids residues, calculate the mean residue ellipticity (MRE). See the equation below to calculate the MRE in deg.cm^2.dmol^{-1}

$$MRE = \frac{(MRW * \Theta_{obs})}{10 * d * c} \text{ deg . cm}^2\text{. dmol}^{-1}$$

θ_{obs} = observed ellipticity in degrees

MRW (mean residue weight) = molecular weight in g/mol / (N - 1); N = number of amino acid residues.

d = pathlength in cm

c = concentration in g/mL

3. Report average near-UV CD spectra (in mean residue ellipticity, MRE) for each mAb sample in the **Near-UV CD Data** Excel file.

Understanding Results

- The buffer subtracted near-UV CD spectra of proteins (250-350 nm) combine contributions from aromatic amino acid side chains which are sensitive to their local environments. Therefore, near-UV CD spectra provide ensemble reports on the folded structure of the proteins being interrogated.
- Strong signals generally reflect well-defined structures, whereas flatter signals typically indicate unfolded, misfolded, and "molten-globule" protein structures.
- Roughly attributable signals: phenylalanine ~250-270 nm; tyrosine ~ 270-290 nm; tryptophan ~ 280-300 nm; disulfide bonds ~broad weak signals throughout spectrum.

Troubleshooting

- Insufficient signal or signal-to-noise ratio measure undiluted samples and/or increase cell pathlength, adjust acquisition time, increase nitrogen pressure as appropriate for instrument
- Samples were not homogenized re-homogenize samples immediately before measuring
- Absorbance/OD above linear range of instrument be sure to stay within the linear range of Beer-Lambert's law during measurements by selecting the right instrument and settings
- Readouts not corrected for the pathlength report pathlength-corrected values (per cm)
- Evaporation of samples during data collection prevent evaporation by sealing cuvette
- Artifacts from air bubbles prevent air bubbles by careful pipetting, gentle degas if needed
- Artifacts from dirty cells clean cuvette and/or use disposable cuvettes

Further Information

For any specific questions regarding this method, please contact Tim Menzen at tim.menzen@coriolis-pharma.com. Please copy aapsinterlab@nist.gov on your emails.