AAPS Interlaboratory Study

Tier 1 Method: FTIR

Introduction

This protocol briefly describes a method using Fourier-transform infrared spectroscopy (FTIR) to obtain information regarding the secondary structure of the protein in the unstressed and stressed monoclonal antibodies used in this study. This document describes sample handling, instrument set up, and data reporting of the results associated with this method. The primary reported results are the wavenumber versus absorbance for each sample. The measurements should be performed both in solution and on the particles present in the stressed 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 as it is not specific to the needs of each instrument/make/model. 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

- FTIR instrument and associated consumables for analysis of solution and analysis of particles in solution
- Protein samples and formulation buffers supplied by sample originators
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
- Pipette and pipette tips
- Liquid nitrogen (when LN_2 cooled MCT detector is used)
- Detergent or cleaning solutions, deionized ultra-filtered water, acetone or ethanol and vacuum or compressed N_2 for cleaning between runs
- PVDF syringe filters
- Pipets and pipette tips

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 1 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 FTIR measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes.

3.) After thawing, particle concentration may change with time, even if the sample is maintained at (2-8)˚C. This change may be mitigated by carefully planning the data acquisition to minimize the period of data acquisition, preferably to one day.

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.

Instrument Setup

1.) Follow routine set-up procedures provided by the equipment manufacturer. Use the most recent software version available for collecting and analyzing data. Ensure the sample chamber and interferometer is purged with nitrogen to remove water vapor and CO_2, as they can obscure important peaks in the spectrum.

2.) The samples provided will not need any further dilution for analysis. FTIR analysis should be performed in solution for the unstressed and stressed materials as well as on the particles (isolated from the stressed material) to elucidate the secondary structure conformation of protein in solution or within the particle of interest, respectively.
   a.) As these solutions have low protein concentration, the analyst can select appropriate FTIR procedures to collect the FTIR spectrum.

b.) For analysis of particles (proteinaceous or non-proteinaceous) in solution, a microscopic FTIR system in reflectance mode can be used to obtain spectra of isolated particles.

Solution Sample Measurement

1.) Review the **FTIR Data** template for the requested information before beginning the measurements and input the set-up information.
2.) All sample solutions including formulation buffer should be filtered using a 0.22 µm syringe filter to minimize the contribution from the particle to the measured spectrum.
3.) Since these solutions have low protein concentration, the analyst can select appropriate FTIR sampling method to collect the FTIR spectrum. For example, ATR cell can be loaded with excess volume of sample solution (30 to 50 µL) than the minimum required volume to minimize the impact of sample evaporation during measurement or in a transmission cell with a pathlength of ≤ 6 µm.
4.) Background measurements should be acquired before every sample measurement. Often instruments will allow you to specify a background measurement and will save the data accordingly. Subsequently, perform the sample measurement and collect the data using the parameters shown in Table 2. Some instruments will allow an automatic background subtraction and display the resulting sample absorbance spectrum.
5.) If the protein is sticky, you may need to use detergents and/or additional cleaning procedures to clean the sample chamber prior to loading the next buffer blank. When a series of runs are being performed, it is always good practice to inspect your buffer blank spectra and ensure that it looks "clean" (i.e. spectrum doesn't look like the protein spectrum of the previous protein sample). Only then load the corresponding protein sample.

Table 2. Recommended instrument parameters for FTIR in solution

Number of Sample Scans	256
Number of Background Scans	256
Wavenumbers (cm^{-1})	600 to 4000
Result Spectrum	Absorbance
Resolution (cm^{-1})	1
Temperature	20 ˚C
Replicates	1

Particle Sample Measurement using FTIR Microscopy in Reflectance Mode

1.) This analysis is appropriate for any particles (proteinaceous or non-proteinaceous) that are present in the samples and that can be collected by filtration. For example, particles

can be filtered in a gold coated polycarbonate track edge filter (0.8 or 3 µm pore size) by the application of vacuum.

2.) Follow the filtering procedure employed in your lab to isolate particles for analysis, and apply washing procedures without compromising the integrity of the particles.
3.) In addition to purging the detector, you must also purge the microscope with nitrogen. Set up the instrument as recommended by the manufacturer and check that the signal intensity is within the range prescribed by your manufacturer.
4.) Select reflectance mode when collecting data. On a particle free surface (identical to the one where the particle is located on), start the reference measurement by selecting the background option on your software. Once the background region spectrum is acquired, move to the sample and focus using the binocular. Select the particle of interest by moving the stage in the x and y-direction. Adjust the focus and image contrast appropriately. Start the sample collection. Take a picture by optical microscope and collect an FTIR spectrum for each particle you observe. Be sure to save the particle image and corresponding FTIR second derivative spectrum of that particle using easily identifiable names.
5.) Repeat for at least 10 particles, if available.

Understanding Results

Results from FTIR should inform us about the secondary structure of protein in solution or of the secondary structure of the protein and/or any non-protein component within the particles.

After correcting your raw spectra, load the files and perform automatic atmospheric compensation (to eliminate the H_2O and CO_2 bands) and baseline correction (automatic preferred). Use the Savitzky-Golay algorithm (built into the software) to calculate the second derivative spectra and perform smoothing (use 9 as the number of smoothing points). Perform normalization of the spectra.

Report the second derivative signal intensity over wavenumbers (from 1700 cm^{-1} to 1500 cm^{-1}) along with instrument parameters in the **FTIR Data** template or **FTIR Microscopy Data** template for data acquired in solution (unstressed samples) or for data acquired on proteinaceous particles (stressed samples), respectively. If the particles imaged and analyzed are non-proteinaceous, the analyst should identify the particles as such and choose the appropriate spectral range for reporting these types of particles. For data acquired on particles, paste images of those particles above the corresponding fully analyzed FTIR data on the **FTIR Data** template**.**

Further Information

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