

# Jasco J-815 Spectropolarimeter: Getting Started Guide to Circular Dichroism

## Introduction

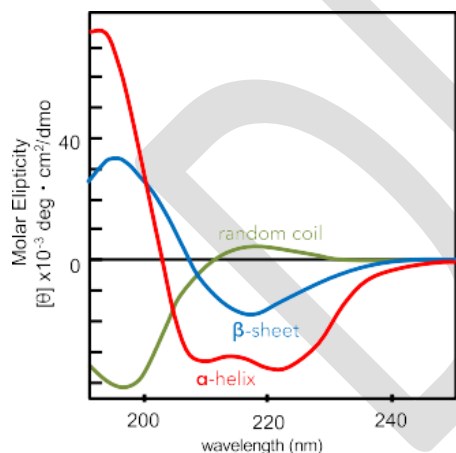
Circular Dichroism (CD) Spectroscopy is used to determine the optical isomerism and **secondary structure of molecules**. Circular dichroism (measured in molar ellipticity) is the difference in absorption of left-handed and right-handed circularly polarized light and can be observed in optically active molecules with chiral centers. Proteins have many chiral centers. CD spectra in the Far-UV region (185 - 250 nm) can be used to determine protein secondary structure. Characteristic peaks for Thermal stability ( $T_m$ ) can be measured by following changes in molar ellipticity with increasing temperature.

## Instrument Overview

The **J-815 CD Spectropolarimeter** from JASCO is equipped with a Peltier temperature controller and single cuvette holder and SpectraManager 2 software for data collection and analysis.

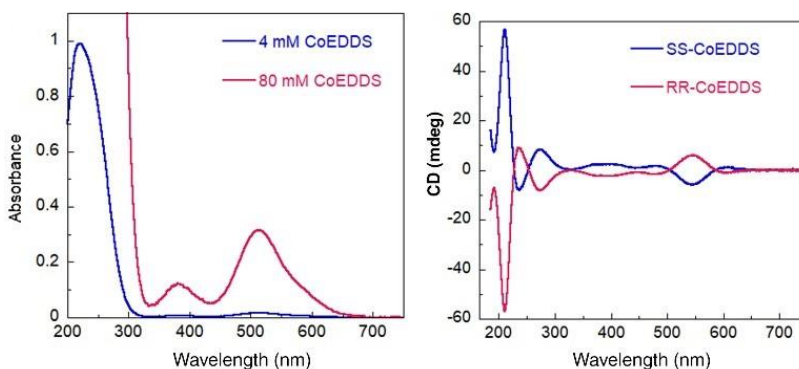
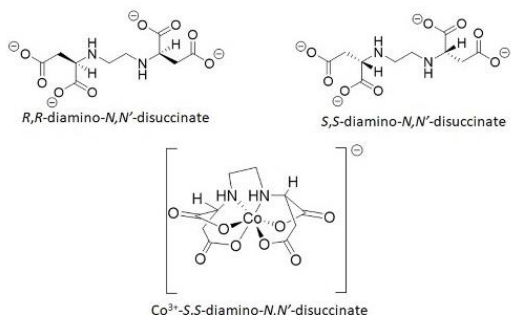
## Applications

- Variable wavelength (163 - 900 nm) and variable temperature (4 °C - 95 °C) measurements
- Characterization of protein secondary structure and folded state
  - Far-UV CD spectra (185 - 250 nm) for secondary structure
  - Near-UV spectra (> 250 nm) for tertiary structure fingerprint
- Detection of structural changes due to mutagenesis
- Protein unfolding
- Conformational stability in response to changes in:
  - Temperature ( $T_m$ )
  - Denaturant concentration
  - pH
  - Buffer composition



Poly-L-lysine in three conformations: alpha helix, b-sheet and random coil. Far-UV region of the CD spectra reveal peaks characteristic of these secondary structure elements, with helices having negative peaks at 222 nm and 208 nm and a positive peak around 190 nm,  $\beta$ -sheet proteins have a negative peak around 218 and a positive peak around 195 nm, and a random coil has a negative peak around 200 nm (as does poly-proline II helix).

Adapted from N. Greenfield, Biochemistry 1969.



## **Basic Operation**

### ***Experimental Design Tips***

- Most CD experiments are collected with two signals: CD and HT.
- A good CD signal will not dip below -50 mdeg.
  - CD signal is proportional to mass, so adjust concentration to increase or decrease signal.
- HT signal should be below 600 V or the CD signal will be too noisy to use.
  - HT voltage is proportional to absorbance.
  - Solvent and buffer absorbance or excess protein absorbance contribute to higher HT.
  - Adjust buffer composition or decrease protein concentration to reduce HT voltage.
- Absorbance can also be measured directly in a CD experiment.
  - To do so, add a 3<sup>rd</sup> channel to the general parameters and select absorbance as channel 3.
  - You MUST include a baseline correction when measuring absorbance.
    - Enable baseline subtraction correction in *Measure menu* → *Parameters*.

### ***Startup***

1. Turn on the nitrogen. **Never perform experiments without nitrogen gas.** Wait 2 minutes for N<sub>2</sub> purge.
  - Open the valve on the nitrogen tank (max 10 psi) so that the pressure gauge on the left side of the CD instrument reads ~ 15 units.
2. For variable temperature measurements, turn on the water bath (usually set at 20 C, but may be set to 25 C).
3. Turn on the Peltier temperature control unit.
4. Turn on the Jasco J-815 CD instrument. Wait 30 minutes for instrument to warm up.

### ***Load the Sample***

1. Fill cuvette with sample (~3/4 full), remove air bubbles
2. Place the cap (optional) on the cuvette and clean it with a Kim wipe or lens paper.
3. Open the hinged lid on the right side of the top of the CD.
4. Remove the rectangular metal cap on the Peltier cell holder.
  - a. Underneath this cap is a 1 cm rectangular cell holder.
  - b. When using a 1 mm path cuvette, place the black rectangular cuvette spacer in the cell holder. Make sure that the side with the hole is down when you place it in the holder, and that light can pass through it.
5. Place the cuvette in the cell holder.
6. Replace the metal cap and close the hinged lid.
7. You may want to leave this for a few minutes, then remove the cuvette and check for bubbles that have formed due to the temperature change. If any have formed tip the cuvette 45 degrees, and tap it carefully to dislodge the bubbles

### **Spectral Scan Data Collection**

1. Open the program ***Spectrum Manager 2*** on the desktop.
2. To measure the spectrum, double click *Spectra Measurement*.
3. Instrument will be initialized and self-tested.
  - a. Allow the N<sub>2</sub> purge step to complete.
  - b. Lamp will be automatically turned on.
  - c. Allow about 30 minutes for lamp to warm up.
4. Connect the **Peltier Cell Holder Accessory**
  - a. Control Menu → Select Accessory
  - b. Check box for Peltier Type FDCD Cell Holder
  - c. Click ok

5. Set temperature from settings in temperature control window or Control Menu → accessory PFD-425S.
6. **Set parameters:**
  - a. Go to *Measure menu* → *Parameters*
  - b. General standard settings
    - i) Photometric mode
      - (1) channels # 2
      - (2) channel 1 CD
      - (3) channel 2 HT (voltage)
    - ii) Sensitivity Standard
    - iii) D.I.T. (integration time) 1-2 seconds (longer times may mask finer features)
    - iv) Band Width 1 nm (try 2 nm if signal is low, especially for  $\beta$ )
    - v) Start max. 900 nm
    - vi) End min. 163 nm
    - vii) Data Pitch 0.1-0.5 nm
    - viii) Start mode Immediate
    - ix) Scanning Mode Continuous
    - x) Scanning Speed 50-100 nm/min
    - xi) Accumulation 3-5 (depending on signal-to-noise)
      - (1) Accumulations/Repeat Mode: On = average spectra; Off = save each spectrum separately.
  - c. Control
    - i) Correction None  
(baseline subtraction not recommended or necessary for standard CD measurements that do not record absorbance spectrum)
    - ii) Make sure checkbox to Open the Shutter is checked
  - d. Accessory
    - i) Control Temp 20 °C
    - ii) Control sensor holder
    - iii) Monitor sensor holder
    - iv) Start conditions start immediately or keep within  $\pm 0.3$  °C for 3-5s
  - e. Information add experimental details (optional)
  - f. Data
    - (1) Enable AutoSave (optional, but recommended).
    - (2) Chose AutoSave directory and select name format (e.g. date, number).
  - g. **Collect data:**
    - i) In the Spectra Measurement program, Go to *Measure* → *Sample* (or click the S button)
      - (1) Go to *Measure* → *Baseline* (or click the B button) before absorbance measurements
    - ii) The data will be graphed from right to left.
    - iii) The top graph is the CD data, the bottom is the HT (voltage).
      - (1) If the CD goes below -50 mdeg or the HT gets above 600 V before 200 nm, it may mean that the concentration of the sample is too high or there is an absorbing component in the buffer (such as chloride).
    - iv) Scans can be viewed in the Spectra Analysis module.

### **Spectral Scan Data Analysis**

1. Open the program *Spectrum Manager 2*.
2. To analyze data, double click *spectra analysis*.
3. Open buffer file: *File* → *open* → <your buffer blank>.

4. Open sample file (it will be active, if not, select sample trace to designate active spectrum).
5. Overlay data: Drag one spectrum onto another view to overlay spectra.
6. Subtract baseline.
  - a. Overlay sample and baseline spectra.
  - b. Go to *Processing* → *Subtraction*.
  - c. A subtracted file is created in memory.
  - d. Select the subtracted file and *Save As*.
7. Save data in Jasco format
8. Export data as .txt or .csv
9. For publication and for secondary structure estimation algorithms you'll need to perform a molar transformation of your data from raw mdeg ellipticity.

#### Common Data Transformations:

Delta Epsilon $\Delta\epsilon$ ( $M^{-1}\cdot cm^{-1}$ )	$\Delta\epsilon = \frac{\theta(mdeg) \cdot MRW}{10 \cdot C \left(\frac{mg}{mL}\right) \cdot l(cm) \cdot 3298}$
Molar ellipticity $[\theta]$ ( $deg \cdot cm^2 \cdot dmol^{-1}$ )	$[\theta] = \frac{\theta(mdeg)}{10 \cdot C \left(\frac{mol}{L}\right) \cdot l(cm)}$
Mean residue ellipticity $[\theta]_{mrv}$ ( $deg \cdot cm^2 \cdot dmol^{-1}$ )	$[\theta] = \frac{\theta(mdeg)}{10 \cdot C_{residue} \left(\frac{mol}{L}\right) \cdot l(cm)}$ <p>or</p> $[\theta]_{mrv} = \frac{MRW \cdot \theta(mdeg)}{10 \cdot c \left(\frac{g}{L}\right) \cdot l(cm)}$

- MRW = mean residue weight = molecular mass/N-1,  
 N = number of amino acids (~110 for most proteins)  
 $\theta$  = ellipticity (mdeg)  
 C = concentration (in units specified)  
*C<sub>residue</sub>* =  $n \cdot C_{polymer}$   
 l = pathlength (cm)

#### Shutdown

1. Remove cuvette from the CD instrument, replacing the lid and cap afterwards.
2. Wash shared cuvettes thoroughly. Return shared cuvettes to the drawer.
  - Run a 190-260 nm scan of water to verify that the cuvette is clean.
3. Close the SpectraManager software.
4. Turn off the CD instrument, Water Bath, and Temperature Control Unit, **but DO NOT turn off the gas.**
5. Let the gas run for **five minutes** to prevent ozone accumulation as the lamp cools, then shut off the N<sub>2</sub>.
6. Clean up in and around the instrument.

## **Sample Preparation**

### ***Assay Buffers***

Buffer selection is critical for accurate CD measurements. Solvent absorbance can severely interfere with the CD signal. Many commonly used buffers and additives absorb in the far UV region used for CD measurements.

- The ideal *CD experiment is performed in a buffer:*
  - in which your protein is well behaved and soluble
  - with no buffer absorbance through the range of the CD spectrum.
- **Always take a scan of your buffer blank to determine whether absorbance interferes with your region of analysis.**
  - Ensure that the buffer blank is well matched to the final dilution of your protein (even trace amounts of some solvents will interfere with the CD measurements).
- Phosphate buffers with little-to-no NaCl are recommended. 10 mM phosphate buffer is ideal.
- **Ideal aqueous solutions contain as little chloride as possible.**
  - NaCl and Tris buffers are not recommended but can be tolerated at low concentrations.
  - If salt is required,  $\text{SO}_4^{2-}$  or  $\text{F}^-$  are preferred counter ions, as they are transparent in the far UV. Potassium fluoride may be a good substitute for chloride-containing salts.
  - Chloride ions will result in a loss of signal below about  $200 \pm 5$  nm, which affects secondary structure estimation, but generally will not block detection of the alpha-helical peaks (208 nm and 222 nm) or the beta-sheet peak (218 nm).
- DTT,  $\beta$ -ME, or EDTA can be present at low concentrations (1 mM).
- Some detergents are fairly transparent in the far UV (e.g. Chaps and octylglucoside). Triton detergents should be avoided, as they can oxidize readily and form UV-absorbing materials.
- DMSO, formamides, and imidazole absorb strongly in the far UV region, and thus should be avoided in a CD experiment.

### ***Samples***

- An accurate protein concentration is required for all CD experiments.
  - Over-estimating or under-estimating the protein concentration interferes with signal
  - Algorithms that estimate secondary structure depend on correct protein concentrations
- The amount of sample required for a CD experiment depends on the size, cuvette pathlength and type of measurement being performed.
- **1 mm pathlength cuvettes hold 300  $\mu\text{l}$**  and a 1 cm cuvette holds 3 ml.
- Required protein concentration is inversely proportional to the cuvette pathlength, and thus a 1 mm path cuvette requires 10x the concentration of a 1 cm cuvette.
- **Recommended concentrations for Far-UV measurements of protein secondary structure are:**
  - **0.2 mg/mL in 1 mm path cuvette**
  - **0.02 mg/mL in 10 mm path cuvette**
  - $\text{Mol/L} = 115 / (\text{MW} * 7000) * 10 / \text{pathlength}(\text{mm})$ .
  - For alpha-helical proteins you may need  $\frac{1}{2}$  this concentration. For beta-sheet proteins, you may need double the concentration. If possible, make a concentrated stock solution of your protein (at least 2X) and dilute as needed.

- Protein aggregates can interfere with the CD signal.
  - Assess protein heterogeneity via light scattering.
  - Purify protein samples with soluble aggregates by size-exclusion chromatography.
- Samples can be recovered from the cell. This is not recommended for thermal melts, unless you know your thermal denaturation is reversible.

### **Thermal Stability Data Collection**

There are two methods for collecting thermal melt (denaturation) data. A **Variable Temperature Measurement** measures one or more wavelengths as a function of temperature. A **Temperature Interval Measurement** measures an entire spectral scan at each temperature. Experimental run time is typically hours for each of these, depending on the temperature interval, the incubation times at each temperature, and the number of wavelengths measurements (or the scan range).

### **Variable Temperature Measurement**

Perform the [Getting Started](#) protocol as outlined above for Spectra Measurement for setup and sample loading. Always perform a spectral scan before starting a variable temperature measurement.

#### **Variable Temperature Data Collection**

1. Open the program *Spectrum Manager 2* on the desktop.
2. Double click *Variable Temperature Measurement*.
3. Instrument will be initialized and self-tested.
  - a. Lamp will be automatically turned on.
  - b. Lamp will need approximately 30 min to stabilize.
4. **Set parameters:**
  - a. Go to *Measure menu* → *Parameters*
  - b. Temp

	Standard settings
i) Start temp	20 C
ii) Sampling C	1 C
iii) Target C	95 C
iv) Ramp Rate	1 C/min
v) Wait	6 - 60 sec (longer for slow unfolding kinetics)

    - (1) Multiple ramp stages can be combined if the approximate  $T_m$  is known.
 

e.g.	Stage 1: measure every 5 C from 20-40 C.
	Stage 2: measure every 1 C from 40-60 C.
	Stage 3: measure every 5 C from 60-95 C.
  - c. Start/End.
    - i) Start condition      Keep within  $\pm 0.2$  C for 3 sec.
    - ii) End condition      Return to start temp (or 20 C)
  - d. General.
    - i) Photometric mode.
 

(1) Channels #	2
(2) Channel 1	CD
(3) Channel 2	HT (voltage)
    - ii) Wavelengths      Wavelength of interest (222, 218, 208, etc)
    - iii) Sensitivity      Standard
    - iv) D.I.T.      2 sec
    - v) Bandwidth      1 nm
  - e. Control, Info and Data
    - i) As for *Spectra Measurement*

- ii) If you do NOT use AutoSave, you must remember to "Save All Data" when collecting more than one wavelength. "Save Data" will only save the active wavelength
- 5. Go to *Measure* → *Sample* (or click S button)
- 6. If you did not select AutoSave, then Save ALL Data (to save all wavelengths) or Save Data (to only save the active wavelength)
- 7. When the run is finished, shut down as described above in the "Shutting Down and Clean Up" protocol, as for *Spectra Measurement*.
  - a. NOTE: these temperature measurements can take a while depending on how the parameters are set.
  - b. NOTE: after thermal denaturation experiments, proteins are more likely to precipitate on the cuvette, and thus, wash the cuvette carefully.

### **Variable Temperature Data Analysis**

1. Open the program *Spectrum Manager 2*.
2. To analyze data, double click *spectra analysis*.
3. Open sample file: *File* → *open* → <your sample file>.
4. Truncate Data.
  - a. *Processing* → *Corrections* → *Truncate Data*.
  - b. Click apply for default parameters.
  - c. Default method: 3<sup>rd</sup> order spline.
  - d. Temperature range may be truncated, as needed.
5. Calculate the 1<sup>st</sup> Derivative.
  - a. *Processing* → *Operations* → *Derivatives*.
  - b. Click ok to apply default methods.
  - c. Defaults.
    - i) Algorithm: Savitzky-Golay.
    - ii) Order of derivatives: 1<sup>st</sup> order.
    - iii) Order of polynomial: 1<sup>st</sup> order.
    - iv) Data points: 7 (adjust to increase smoothing).
6. Find T<sub>m</sub> peak.
  - a. *Processing* → *Peak Processing* → *Peak Find*.
  - b. Select Parameters: Top.
  - c. Click apply.
  - d. The peak center is the melting temperature (T<sub>m</sub>).
7. Save and export data.
8. Perform **Shutdown** as for Spectra Measurement
  - Don't forget to clean thoroughly and run a scan of water to verify that the cuvette is clean.

## **Temperature Interval Measurement**

A *Temperature Interval Measurement* measures an entire spectral scan at each temperature. Therefore, the data takes longer to acquire than for a *Variable Temperature Measurement* due to the delay in collecting a complete scan (or multiple scans in accumulation mode). For this reason, a wider sampling interval (2-5 °C) is recommended.

Perform the [Getting Started](#) protocol as outlined above for Spectra Measurement for setup and sample loading. Always perform a spectral scan before starting a variable temperature measurement.

### ***Temperature Interval Data Collection***

1. Open the program *Spectrum Manager 2* on the desktop.
2. Double click *Temperature Interval Measurement*.
3. Instrument will be initialized and self-tested.
  - a. Lamp will be automatically turned on.
  - b. Lamp will need approximately 30 minutes to stabilize.
4. Set parameters:
  - a. Go to *Measure menu* → *Parameters*
  - b. Temp

	Standard settings
i) Start/End	20-95 C
ii) Data Pitch	(2-5 C)
iii) Temp. Gradient	1-2 C/min
iv) Delay time	6 sec
v) Start condition	Keep within $\pm 0.2$ C for 3 sec
vi) End condition	Return to start (or move to 20 C)
  - c. All Other tabs As for *Spectra Measurement*
5. Set wavelengths to monitor:
6. Measure tab → Monitor Parameters
  - a. Enter up to 4 wavelengths to monitor during data collection.
  - b. Check boxes next to wavelengths to be used.
7. Save and export data.
8. Perform [Shutdown](#) as for Spectra Measurement
  - Don't forget to clean thoroughly and run a scan of water to verify that the cuvette is clean.



## **CD Resources**

**DichroWeb** is a free online tool for determining protein secondary structures based on CD spectra. Academic users may apply for a username and password. Commercial use of DichroWeb is not permitted. DichroWeb provides five different calculation algorithms and a variety of reference datasets. To use DichroWeb, export data as a .txt file.

**BeStSel** (Beta Structure Selection) is a free online tool based on a novel method for the secondary structure determination and fold recognition from protein circular dichroism spectra. It is specifically designed to analyze beta-sheet rich proteins, but can be used for any soluble protein. To use BeStSel, export data as a .txt file.

**CDToolX** is a free, downloadable software program that enables processing, displaying, archiving, calibrating, comparisons, and analyses of circular dichroism (CD) and synchrotron radiation circular dichroism (SRCD) spectroscopic data. It is compatible with Windows 10 and can be used on Mac-based Windows emulators such as Microsoft Remote Desktop. To use CDToolX, export data from Jasco as a .txt file.

**ValiDichro** tests the quality of CD data based on based on common characteristics of CD protein spectra observed in the literature. Datasets are given a designation of a pass (P), a flag (F) for small deviations from the norm, or a fail (X) if the deviation is more severe.

The **PCDDDB** (Protein Circular Dichroism Data Bank) **YouTube channel** provides video tutorials on a variety of CD-related topics, including the successful measurement of a CD spectrum and analysis using DichroWeb and CDToolX.