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 CMI has a J-815 CD Spectropolarimeter from JASCO, with a Peltier temperature controller and single cuvette holder and SpectraManager 2 software for data collection and analysis.

Applications
- 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

Key Features
- CD scans are easy, rapid, and non-destructive (~10 min/sample)
- Label-free
- Variable wavelength and variable temperature measurements
- Wavelength Range: 163 – 900 nm
- Peltier Temperature Control: 4 C – 95 C

Required Supplies
- CD cuvette such as:
  - 1 mm pathlength (Hellma 110-1-40, style 110-QS, can purchase from Sigma-Aldrich)
  - 1 cm pathlength (Hellma 100-10-40, style 100-QS, can purchase from Sigma-Aldrich)
- (Recommended) Pipettes and tips (gel-loading tips can aid in sample recovery from 1 mm cuvette)
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, SO_{4}^2\text{−} or 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±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, β-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 μL 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
  - Mol/L=115/(MW*7000)*10/pathlength(mm).
  - For alpha-helical proteins you may need ½ 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.
  o Assess protein heterogeneity via light scattering.
  o 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.

Getting Started

Resources
Additional resources are available at the instrument, including original instrument manuals (paper only).

DichroWeb (academic-use only), for on-line analysis of protein CD spectra:
http://dichroweb.cryst.bbk.ac.uk/html/home.shtml
• You will need to go through an application process to get an account, which can take several days.

Experimental Design Tips
• Most CD experiments are collected with two signals: CD and HT.
• A good CD signal will not dip below -50 mdeg.
  o 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.
  o HT voltage is proportional to absorbance.
  o Solvent absorbance or excess protein absorbance contribute to HT.
  o Adjust buffer composition or decrease protein concentration to reduce HT voltage.
• Absorbance can also be measured directly in a CD experiment.
  o To do so, add a 3rd channel to the general parameters and select absorbance as channel 3.
  o You MUST include a baseline correction when measuring absorbance.
    ▪ In Control Panel, enable baseline subtraction correction.

General Care and Maintenance
• Never perform experiments without nitrogen gas.
• Clean cuvette thoroughly after use.
• Return shared cuvette to the drawer under the instrument computer.

Startup
1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (eCommons ID and password).
3. Turn on the nitrogen:
   • 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.
   • Please inform CMI staff if the attached nitrogen tank is less than ½ full.
   • If the tank is empty, then DO NOT PROCEED. Nitrogen gas is essential.
4. Turn on the water bath (usually set at 20 C, but may be set to 25 C for variable temperature measurements).
5. Turn on the Peltier temperature control unit.
6. Turn on the Jasco J-815 CD instrument.

**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 black circular 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 black 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 N2 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 β)
      v) Start  260nm
vi) End 190 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 5  

(1) Accumulations/Repeat Mode: Toggled = accumulation, measures 5 times and averages, Untoggled = repeat mode, saves each cycle separately.

c. Control  
i) Correction None  
   (baseline subtraction not recommended or necessary for standard 2 channel CD measurements that do not measure absorbance)  

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 ±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)  

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:**

<table>
<thead>
<tr>
<th>Transformation</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta Epsilon</td>
<td>( \Delta \varepsilon = \frac{\theta(\text{mdeg}) \cdot \text{MWR}}{C \left( \frac{\text{mg}}{\text{ml}} \right) \cdot l(\text{cm}) \cdot 3298} )</td>
</tr>
<tr>
<td>Molar ellipticity</td>
<td>([\theta] = \frac{\theta(\text{mdeg})}{10 \cdot C \left( \frac{\text{mol}}{\text{L}} \right) \cdot l(\text{cm})})</td>
</tr>
<tr>
<td>Mean residue ellipticity</td>
<td>([\theta]<em>{\text{mwr}} = \frac{\theta(\text{mdeg})}{10 \cdot C</em>{\text{residue}} \left( \frac{\text{mol}}{\text{L}} \right) \cdot l(\text{cm})}) or ([\theta]_{\text{mwr}} = \frac{\text{MWR} \cdot \theta(\text{mdeg})}{10 \cdot c \left( \frac{\text{g}}{\text{L}} \right) \cdot l(\text{cm})})</td>
</tr>
</tbody>
</table>

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

**Shutdown**
1. Remove cuvette from the CD instrument, replacing the lid and cap afterwards.
2. Wash the cuvette thoroughly. **Return shared cuvettes to the drawer.**
3. Close the SpectraManager software.
4. Turn off the CD instrument, Water Bath, Temperature Control Unit, and computer/monitor, but DO NOT turn off the gas.
5. Let the gas run for five minutes to prevent ozone accumulation as the lamp cools, and then shut off the gas.
6. Clean up in and around the instrument.
7. **Logoff from PPMS!**

Book time and Report Problems through the PPMS system: [https://ppms.us/hms-cmi](https://ppms.us/hms-cmi)
- rates are based on **booked and real-time usage.**

Contact [cmi@hms.harvard.edu](mailto:cmi@hms.harvard.edu) with questions.
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  
      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 Tm 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 ± 0.2 C for 3 sec.
      ii) End condition  Return to start temp (or 20 C)
   d. General  
      i) Photometric mode.  2
         (1) Channels #
         (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.
4. Truncate Data.
   a. Processing → Corrections → Truncate Data.
   b. Click apply for default parameters.
   c. Default method: 3rd order spline.
   d. Temperature range may be truncated, as needed.
5. Calculate the 1st Derivative.
   b. Click ok to apply default methods.
   c. Defaults.
      i) Algorithm: Savitzky-Golay.
      ii) Order of derivatives: 1st order.
      iii) Order of polynomial: 1st order.
      iv) Data points: 7 (adjust to increase smoothing).
6. Find Tm peak.
   a. Processing→ Peak Processing→ Peak Find.
   b. Select Parameters: Top.
   c. Click apply.
   d. The peak center is the melting temperature (Tm).
7. Save and export data.
8. Perform Shutdown as for Spectra Measurement
**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 ±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

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