



Standard Operating Procedure

Title: Measurement of Circular Dichroism (CD) Spectrum of Proteins and Peptides on Jasco J-720 Spectropolarimeter and Estimation of Protein Secondary Structure Using *CDPro* Software

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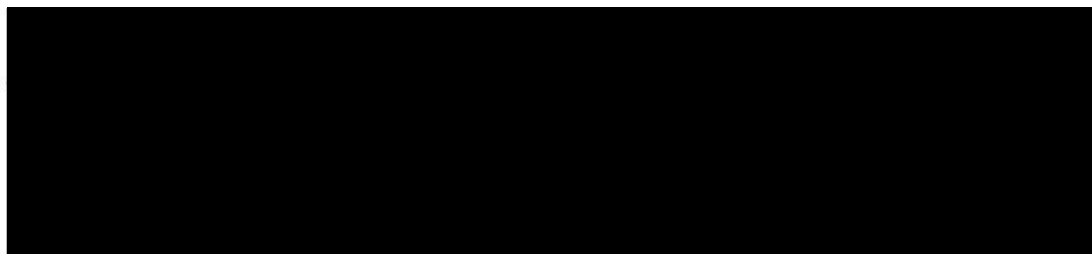


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1.0 Purpose

Circular dichroism (CD) spectroscopy is the difference in the absorption of left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL) and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups) and can be measured using the Spectropolarimeter. This Standard Operating Procedure (SOP) describes the basic operation of the J-720 Spectropolarimeter, from Jasco, data acquisition, and analysis using the Spectral Manager program. A brief description of the estimation of the secondary structure using *CDPro* software is also included.

2.0 Scope

This procedure applies to Process Analytics/Quality Control staff that uses the Jasco J-720 to characterize the conformation of protein/peptide or to study the conformational stability under different stress conditions.

3.0 Authority and Responsibility

- 3.1 The Director, Technical Operations, Process Analytics/Quality Control (PA/QC), and the Biopharmaceutical Development Program (BDP) have the authority to define this procedure.
- 3.2 PA/QC personnel are responsible for the implementation of this procedure.
- 3.3 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

4.0 Equipment and Accessories

- 4.1 Jasco J-720 Spectropolarimeter, MEF 74970.
- 4.2 Computer with Windows 7 or 8.1 (professional version) operating system.
- 4.3 Spectra Manager I (for spectrum measurement and analysis).
- 4.4 Spectra Manager II (for *CDPro* analysis).
- 4.5 Power supply unit.
- 4.6 Type 1 Ultra-Pure Sartorius Water System, MEF 3001-LWPS-015B or BDP-approved equivalent.
- 4.7 Nitrogen inlet source with pressure gauge.
- 4.8 Calibrated Neslab RTE7 Waterbath, MEF 82820 or BDP-approved equivalent.
- 4.9 Calibrated Mettler-Toledo balance, MEF 80190 or BDP-approved equivalent.
- 4.10 Vakuwash Spectrophotometer Cell Washer, Fisher Scientific PN 14-385-937 or BDP-approved equivalent.

- 4.11 10 mm path-length Rectangular Quartz Cell, Jasco PN 0553.
- 4.12 0.1 mm path-length Quartz Rectangular Mountable Cell, Jasco PN 0559.
- 4.13 Cell Holder, Jasco PN CH-2049.
- 4.14 Camphorsulfonic Acid NH₄ Standard, Jasco, BDP PN 31063.
- 4.15 Isopropyl Alcohol, BDP PN 30882 or BDP-approved equivalent.
- 4.16 Methanol, BDP PN 30883 or BDP-approved equivalent.
- 4.17 Water for injection, BDP PN 30295 or BDP-approved equivalent.
- 4.18 Eppendorf tubes, 1.5 ml, BDP PN 20394 or BDP-approved equivalents.
- 4.19 Calibrated Rainin pipettes 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl or BDP-approved equivalents.
- 4.20 Pipette tips, Rainin, 20 µl (BDP PN 21472), 200 µl (BDP PN 21470), 1000 µl (BDP PN 21471) or BDP-approved equivalents.

5.0 Procedure

5.1 Startup.

5.1.1 Purging with N₂ gas.

Open the valve of N₂ source and adjust the pressure gauge so that the top of the float reads 20 SCFH (standard cubic foot/hour) and purge the instrument for 10 minutes prior to start.

NOTE: Operating the instrument without N₂ gas can result in ozone build up on the optical mirrors and ruin the optics.

5.1.2 Set the water-bath temperature to 4°C and turn the pump 'ON' so that the water reaches the set temperature and the flow of water through the tubing starts cooling the Xenon lamp when operating.

NOTE: Coolant water must be in circulation prior to operating the Xenon lamp. Otherwise, overheating during the operation can damage the lamp.

5.1.3 Turn 'ON' the Power 1 switch on the power supply. This turns 'ON' the Xenon lamp. The Lamp Current indicator moves to 25 amps on the dial.

5.1.4 Turn 'ON' the Power 2 switch. Push the 'Start' button. The Line Voltage indicator moves to 100 V on the dial. Allow about 20 minutes for the lamp to warm-up.

5.1.5 Turn the computer 'ON'. Enter the User ID and password and open the 'Spectral Manager I' by double clicking on the icon. Double click on the 'Spectrum Measurement', on the right side (Figure 1). The instrument is now connected and will undergo its initialization process by checking the internal diagnostics.

NOTE: If any of the internal diagnostics fail, it is advised to contact Jasco to discuss the symptoms with a technician. It should not be assumed that the instrument can be continually run without further maintenance.

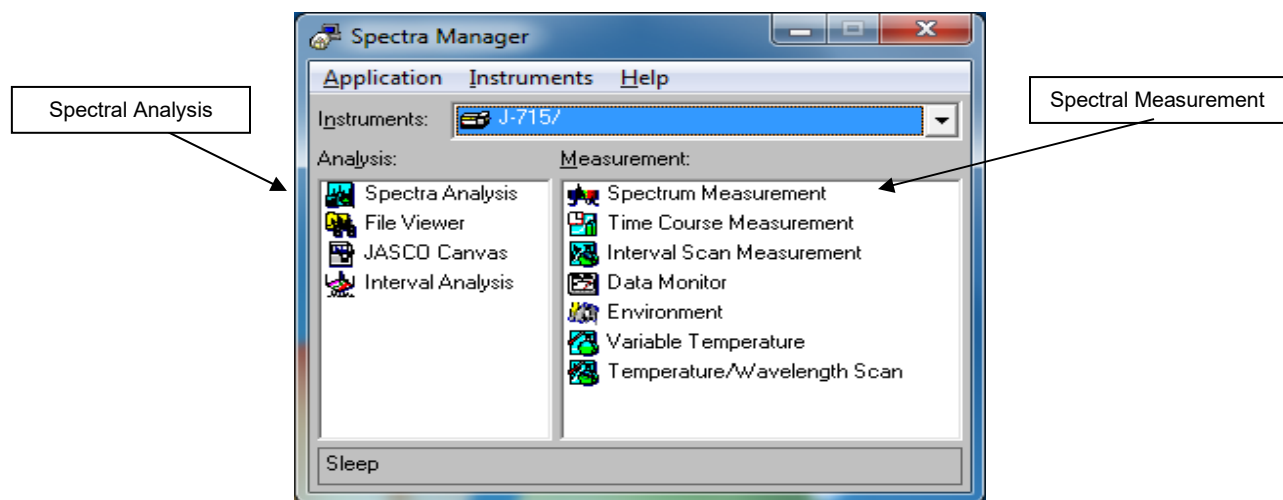


Figure 1. Spectra Manager I View.

5.1.6 The window in Figure 2 opens if it passes the internal diagnostics test.

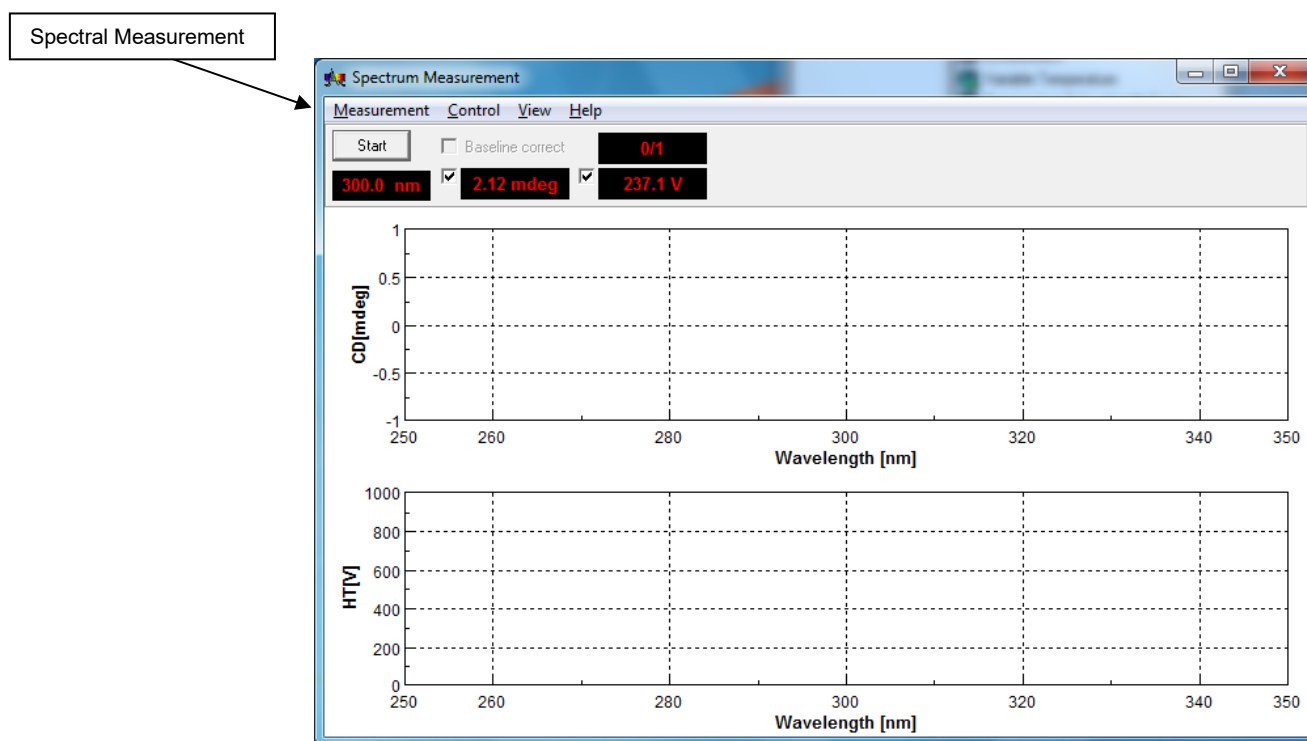


Figure 2. Spectrum Measurement Window.

5.2 Spectrum measurement.

5.2.1 To collect CD spectra, click on 'Measurement' on the menu, select 'Parameter' (Figure 3), and set required parameters for data acquisition.

5.2.2 Method parameters.

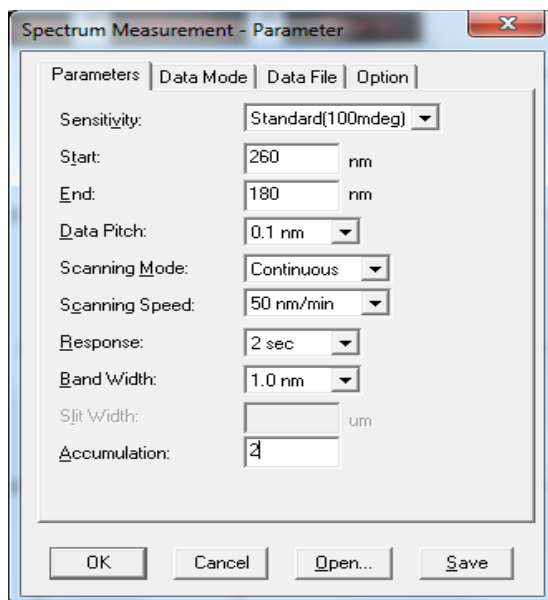


Figure 3. Setting Acquisition Parameters.

The basic parameters generally used are as follows:

1. Sensitivity: Standard (100 mdeg).
2. Start: 350 nm.
3. End: 190 nm.
4. Data Pitch (the interval that data points are collected at): 0.1 nm.
5. Scanning Mode: Continuous.
6. Scanning Speed: 50 nm/min.
7. Response: 2 sec.
8. Bandwidth: 1 nm.
9. Accumulation: 4.

NOTE:

- **Scan speed** is how quickly the data is acquired at e.g. 100 nm/min or 50 nm/min.
- **Response time** (DIT-Data Integration Time) is the time over which the data is integrated.

- Increasing the scan speed increases the number of points integrated. However, increasing the scan speed, depending on the DIT, may not necessarily integrate more data. A good rule of thumb when setting these parameters is to keep $DIT \times \text{Scan Speed} = < 200$.
- Accumulation** is how many scans per sample are acquired. If there is a rather noisy sample spectrum, taking multiple scans will help eliminate any noise (by averaging the background signal) that does not contribute to the actual CD signal of the sample.
- Data pitch** is the interval at which the data is acquired. The smaller the data pitch, the more points collected and the more noise. However, a larger data pitch could lead to critical data points (CD signal) being left out.

- 5.2.3 Under the 'Data Mode' menu 'Channels 1 and 2' are selected for monitoring the CD signal and the HT (voltage), respectively. It is important to monitor the HT signal as this gives a direct measure of the amount of light produced and passing through the sample. If the voltage exceeds 700, the signal will become noisy and the CD data will be unreliable. HT results greater than 800 V are invalid.

- NOTE:**
- A buffer blank must be run to check the absorbance over the intended range of wavelength, prior to sample preparation, to determine the extent the buffer contributes to the CD spectrum. The path-length of the cuvette used is also a contributing factor.
 - To estimate the secondary structure using *CDPro*, acquire the CD spectra within one or more of the following spectral ranges: 190 to 240 nm, 185 to 240 nm, 178 to 240 nm.

- 5.2.4 Select 'Data File' from the 'Parameter' menu and give the experiment folder a suitable name. Save the experiment folder in the local (C:) drive or the network. The result files will be saved in this folder with a *.jws extension. The 'Option' menu allows the user to record important sample information.
- 5.2.5 Once all this is completed, clicking 'OK' displays a message indicating that the parameters are being transferred to the main unit and opens the window in Figure 4. Click 'OK' to initiate data acquisition.

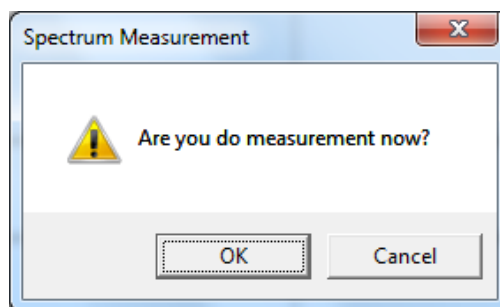


Figure 4. Initiating Data Acquisition.

- 5.3 System suitability assessment with d-10-ammonium camphorsulfonic acid (NH₄CSA).
 - 5.3.1 System suitability is determined with a 0.06% (w/v) solution of d-10-camphorsulfonic acid ammonium salt in WFI.
 - 5.3.2 Accurately weigh 60 mg of d-10-camphorsulfonic acid ammonium salt and dissolve it in 100 mL of WFI. Ensure that the contents are completely dissolved before use. The prepared solution is stable for at least six months, if stored at 2° to 8°C.
 - 5.3.3 The CD spectrum of the blank (in this case WFI) is measured for subsequent blank subtraction.
 - 5.3.4 About 1 mL of this solution is pipetted into a 10 mm path-length cuvette (Section 4.11) and placed in the sample compartment of the CD instrument. Close the compartment door after all the steps under Sections 5.1 through 5.2.4 have been executed before clicking 'OK' to initiate data acquisition.
- 5.4 Sample preparation and measurement.
 - 5.4.1 Buffer blank (formulation buffer) measurement.
 - 5.4.1.1 Prior to sample analysis, it is always recommended that the CD spectrum of the formulation buffer over the desired range of wavelength be measured because in some instances, depending on the contents in the buffer blank, there can be significant noise or CD signal.
 - 5.4.1.2 About 50 µl of buffer is pipetted on to the 0.1 mm path-length rectangular cell (Section 4.12) and gently covered with the cover cell ensuring that the liquid forms a thin film sandwiched between the two parts of the cell, when the window is in place, and that no air bubble is trapped in between. This is then gently slid into a cell holder (Section 4.13) to keep the cells together while standing inside the instrument cell holder within the sample compartment. The 'Start' button on the software is clicked to initiate measurement, see Section 5.2.5.
 - 5.4.2 Sample preparation.
 - 5.4.2.1 The protein concentration has to be adjusted so that the HT signal remains under ~ 700 V beyond which signal can get noisy and CD output can become unreliable/invalid. Typically, a protein concentration of 0.5 to 1 mg/ml can be used with a 0.1 mm path-length cell. Higher concentration samples need to be diluted appropriately in the diluent or formulation buffer, to get the best results.
 - 5.4.2.2 The diluted sample is, then, mixed well, by pipetting up and down a few times, before sample is loaded into the cell and the rest of Section 5.4.1.2 is followed.

5.4.3 Cleaning of cells.

- 5.4.3.1 At the end of each run, the cuvette/cells are removed, emptied, and washed. First, with Laboratory Type I (ultra-pure) water from a squirt bottle and, second, with methanol/isopropyl alcohol in a Vakuwash cell washer. Dry the cell before loading the next sample.

6.0 Data Analysis

- 6.1 The CD data acquired with Spectra Manager I is saved in the specific folder, created earlier. Each file is given a unique 'Name ID' and saved in the folder with a *.jws extension, e.g., ACS Std.jws for the system suitability standard.

6.1.1 System suitability assessment (d-10-camphorsulfonic acid NH₄ salt).

- 6.1.1.1 Open the ACS Std.jws results file for the system suitability standard, from Spectra Analysis > File. From the menu bar select 'View' > 'Select Graph' and uncheck 'Graph 2' so that only the CD (mdeg) graph is displayed in the window.

- 6.1.1.2 Blank subtraction for 'Standard' is carried out by subtracting the background spectrum (Section 5.3.3) from the sample.

- 6.1.1.2.1 With the ACS Std.jws file open, go to 'Processing' under menu and select 'Subtraction' which takes you to the 'Open New File' window. Open the blank run you wish to subtract. When the 'Subtraction' menu pops up (Figure 5), the scale on the x axis can be set to 250 to 320 nm and the y axis can be set to 0 to 200 nm range before clicking 'OK'.

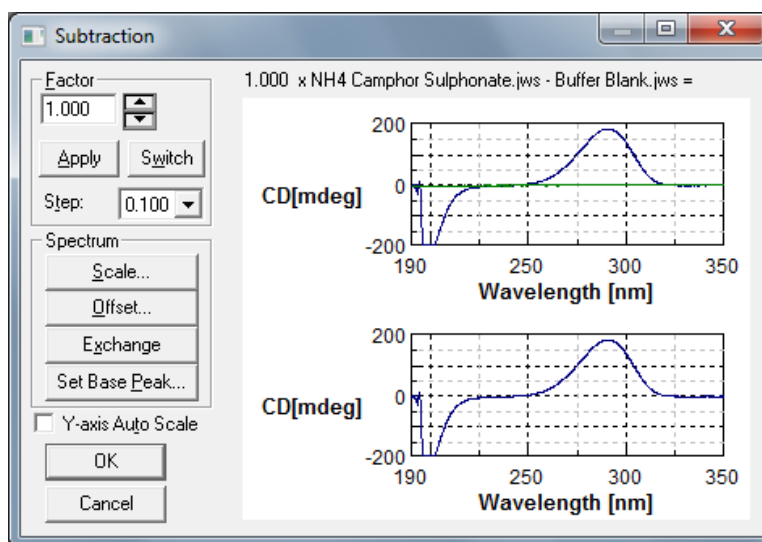


Figure 5. Blank Subtraction for ACS Std.jws.

- 6.1.1.3 Then go to 'Processing' in the menu and select 'Peak Process>Peak Find'. From that window, select 'Top' in the drop down menu for 'Peak', set the 'Noise' level to 0.5, and select 'Execute' (Figure 6). Save the file with appropriate ID.

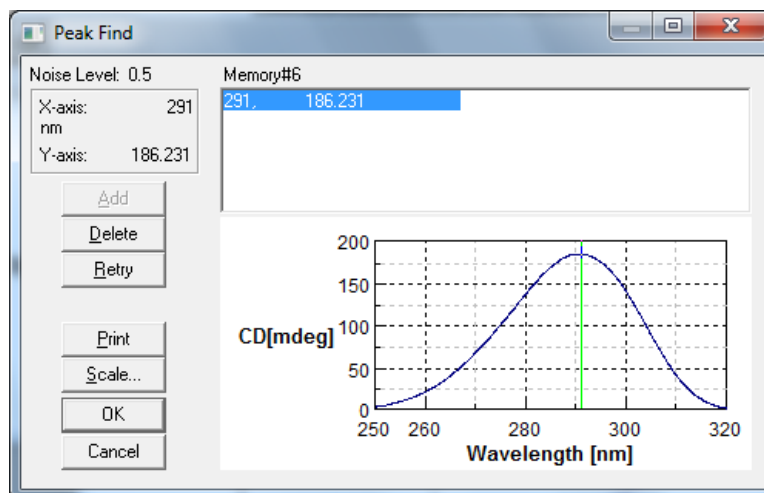


Figure 6. Processed d-10-Camphorsulfonic Acid NH₄ Salt.

- 6.1.1.4 When the CD spectrum of a standard solution is measured in the wavelength region of 350 to 220 nm in a 1 cm cell with a CD full scale of 200 mdeg, the CD peak value (290 nm) is + 190 mdeg \pm 1%.
- 6.1.2 CDPPro analysis for secondary structure estimation of samples.
- 6.1.3 Open Spectra Manager II, on the desktop, and double click on 'Spectral Analysis'. This opens a new window. Go under 'File' and select 'Import'.
- 6.1.4 Select the folder in which the data is located and double click the file you wish to analyze. This opens the file in a new window.
- 6.1.5 Go to the menu bar and select 'Processing', navigate to 'CD Options', and click on 'CDPro Analysis'.
- 6.1.6 Enter the cell path-length (cm) and concentration (mean residue concentration in moles/liter calculated from the molecular weight and amino acid residues in the sample molecule) in the appropriate slot (Figure 7) and click 'OK'.

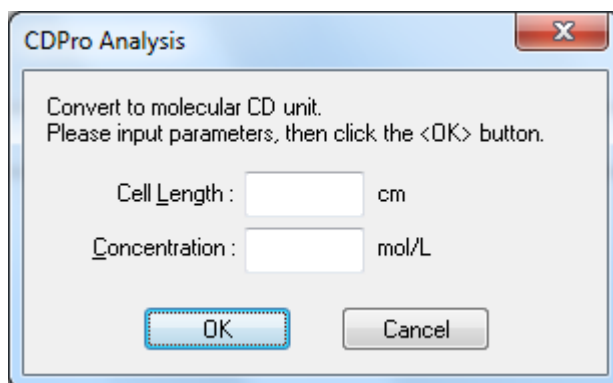


Figure 7. Conversion to CD Units.

6.1.7 A new window opens displaying the CD Spectrum in Molar Ellipticity units (Figure 8).

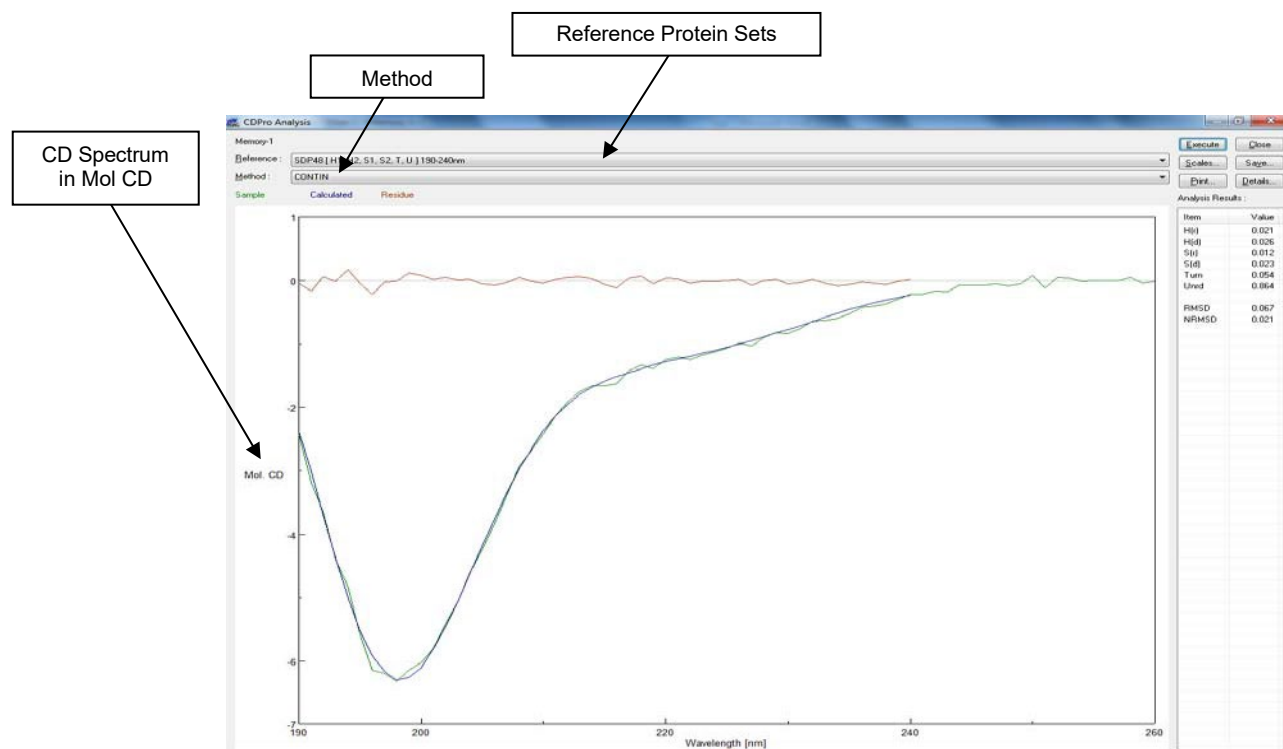


Figure 8. CD. Spectrum in Mol CD.

6.1.7.1 Select the 'Method' (CONTIN-LL, SELCON 3, CDSSTR) and 'Reference Proteins' to be used and click 'Execute', on the right hand corner. Try the fit for all the reference proteins in the method. Examine the Calculated and Sample fit and the NRMSD (normalized root mean square deviation) values, to compare the fit between different data. Check the Calculated and Sample fit with the other methods and their reference proteins.

NOTE: Where necessary, it is appropriate to average the different data base values for the different fractions [α_R (alpha-helix, regular), α_D (alpha-helix, distorted), β_R (beta-strand, regular), β_D (beta-strand, distorted), T (turns) and U (unordered)], although some CD databases do include soluble, insoluble, membrane and denatured proteins in their reference sets.

6.1.7.2 The results give the secondary structure composition in decimal fractions.

6.1.7.3 Click 'Scale' to scale the CD spectrum or 'Print' for printing results. The results can be saved as an *.xps extension file, in the appropriate folder, and printed later (Figure 9).

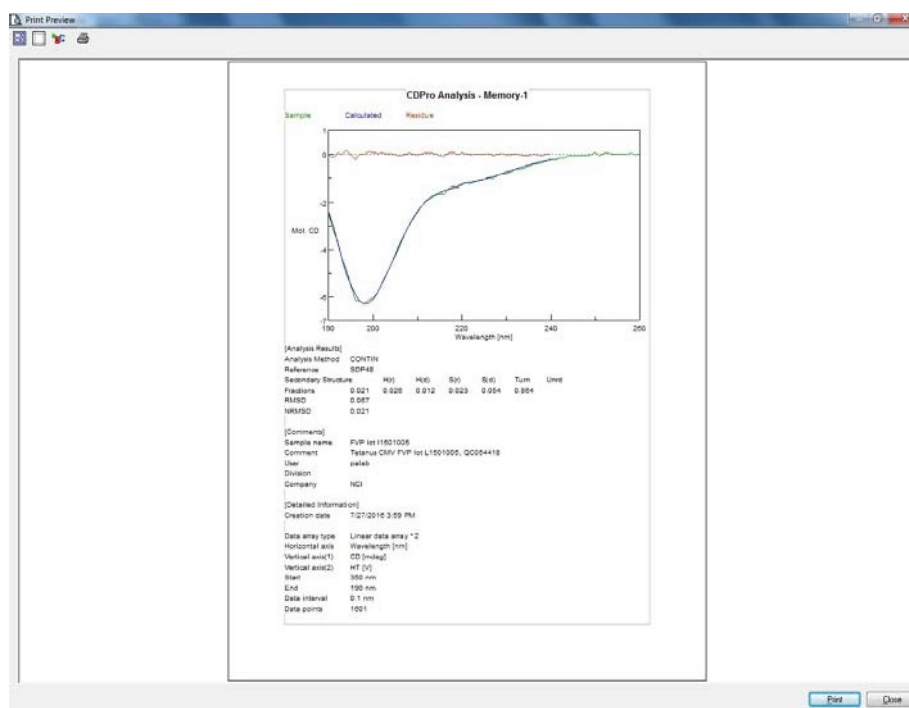


Figure 9. CDPro Report Printout.

7.0 Shutdown of System

7.1 At the end of the instrument run, turn 'OFF' the Power 2 switch followed by the Power 1 switch (Xenon Lamp). Let the water and N_2 run for about 10 more minutes before shutting them 'OFF'.

NOTE: The Xenon lamp coolant water must continue to circulate after shut-down to prevent damage to the lamp, from overheating.

8.0 Documentation

8.1 All use of instrument, calibration, maintenance, service or repair must be documented in an issued equipment logbook as per **SOP 21531 - Equipment Logs**.

8.2 Buffers and solutions prepared for this procedure must be documented as per ***SOP 22702 - Solutions Used in Process Analytics.***

8.3 All experimental and sample information are documented in the laboratory notebook as per ***SOP 21408 - Laboratory Notebooks Control and Use*** and ***SOP 21409 - Good Documentation Practices.***

9.0 References and Related Documents

9.1 CD-ORD Measurement Manual J-710/720.

9.2 Model J-710/720 Spectropolarimeter Instruction Manual.

9.3 Sreerama, Narasimha and Robert W. Woody, 2000. "Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Comparison of CONTIN, SELCON and CDSSTR Methods with an Expanded Reference Set." *Analytical Biochemistry* 287: 141–260.

9.4 **SOP 21408** *Laboratory Notebooks Control and Use.*

9.5 **SOP 21409** *Good Documentation Practices.*

9.6 **SOP 21531** *Equipment Logs.*

9.7 **SOP 22702** *Solutions Used in Process Analytics.*

10.0 Attachments

10.1 **Attachment 1** Form No. 16149-01, Assay Template.

10.2 **Attachment 2** Form No. 16149-02, Equipment/Materials/Reagent Information.

Attachment 1

CD Assay Template

FNLCR, BDP
Form No. 16149-01
SOP No.: 16149
Revision 00: SEP 30 2016

CD Assay Template

QCTR # _____

Date _____

Experiment # _____

Operator _____

1. Method parameters (*SOP 16149*, Section 5.2.2).

Spectrum Measurement Parameters Used	
Sensitivity Standard (100 mdeg)	
Wavelength Start (350 nm)	
Wavelength End (190)	
Data Pitch (0.1 nm)	
Scanning Mode (Continuous)	
Scanning Speed (50 nm/min)	
Response (4 sec)	
Bandwidth (1 nm)	
Accumulation 2	

2. Water-bath temperature (Section 5.1.2): _____ °C (4°C).

3. Ammonium camphor sulfonate calibration standard (Section 4.14).

Item	BDP P/N	BDP Lot Number	Manufacturer Lot Number	Expiration Date
Camphorsulfonic Acid NH ₄ Standard				

4. Preparation of 0.06% ammonium camphor sulfonate (NH₄CSA) in WFI (Section 5.3).

Amount of NH ₄ CSA (mg)	
Volume of WFI (ml)	
Date Prepared	
Solution #	
Storage	
Expiration	

Attachment 1 (Continued)

FNLCR, BDP
Form No. 16149-01
SOP No.: 16149
Revision 00: SEP 30 2016

CD Assay Template (continued)System Suitability Test NH₄CSA:

CD Peak value is: _____ (+190.4 ± 1mdeg @ 290.5nm)

Passed ☐ Failed ☐

If failed, reason: _____

Performed by: _____ Date: _____

5. Sample information.

Project Number:

Product Name:

Reference Standard/Lot #/Concentration (mg/mL):

Buffer Blank:

Sample ID/Name	Lot Number	Concentration (mg/mL)

6. Sample preparation.

Sample ID	Initial Concentration (mg/mL)	Final Concentration (mg/mL)	Diluent Used

Attachment 1 (Continued)

FNLCR, BDP
Form No. 16149-01
SOP No.: 16149
Revision 00: SEP 30 2016

CD Assay Template (continued)

7. Directory/File saved in:

Computer: *NCI #S075673*

Subdirectory C:\CD Data_____.

Sample ID	CD Data File Name

Performed by: _____

Date: _____

SOP Number: 16149

Revision Number: 00

Effective Date: SEP 30 2016

Title: Measurement of Circular Dichroism Spectrum of Proteins and Peptides on Jasco J-720 Spectropolarimeter and Estimation of Protein Secondary Structure Using *CDPro* Software

Attachment 2

Equipment/Materials/Reagent Information

FNLCR, BDP
Form No. 16149-02
SOP No.: 16149
Revision 00: SEP 30 2016

Equipment/Materials/Reagent Information

Items	MEF/BDP Number		Calibration Due Date
Jasco J-720			
Neslab RTE7 Waterbath			
Mettler-Toledo Balance			
Type 1 Ultra-Pure Sartorius Water System			
	BDP P/N	BDP Lot Number	Expiration Date
NH ₄ CSA			
WFI			
Eppendorf Tubes (1.5 ml)			
Isopropyl Alcohol			
Methanol			
Rainin 20 µl tips			
Rainin 200 µl tips			
Rainin 1000 µl tips			
	S/N	Calibration Due Date	
Rainin 10 µl			
Rainin 20 µl			
Rainin 100 µl			
Rainin 200 µl			
Rainin 1000 µl			

Recorded by: _____

Date: _____