1 INTRODUCTION

This Chapter contains a brief introduction to the Chirascan or Chirascan V100 spectrometer, its hardware, software and operation. It also describes the basics of making a good Circular Dichroism (CD) measurement.

1.1 *The Chirascan or Chirascan V100 spectrometer*

1.1.1 The Spectrometer Hardware

Figure 1.1: The Chirascan V100 spectrometer

The Chirascan or Chirascan V100 spectrometer (Figure 1.1) is designed for performance and versatility, allowing it to make high quality CD or other measurements with a broad range of accessories.

The spectrometer consists of the lamp housing on the left, the monochromator with accompanying optics and electronics in the center and a Sample Chamber and a light sensitive detector to the right. The spectrometer usually uses a 150-watt air-cooled xenon arc lamp as the light source and a vacuum Photomultiplier Tube (PMT) detector (Chirascan) or a solid state Large Area Avalanche Photodiode (LAAPD) detector (Chirascan V100), which is both more sensitive and has a broader wavelength operating range than a PMT.

In its standard configuration, the spectrometer uses a Single Cell Peltier Holder for sample positioning and temperature control, and that configuration is shown in this User Manual.

More detailed information on the spectrometer hardware is given in Chapter 3. A list of the accessories available for use with the Chirascan or Chirascan V100 is give in full User Manual.

1.1.2 The Spectrometer software

The Chirascan or Chirscan V100 software comprises two main programs, **Pro-Data Chirascan** and **Pro-Data Viewer**.
Acquisition is controlled with **Pro-Data Chirascan**, where measurement settings can be adjusted, saved and reopened. Experimental data is saved to a Datastore, and is viewed, analyzed, exported and imported in **Pro-Data Viewer**.

The general operation of **Pro-Data Chirascan** and **Pro-Data Viewer** in full User Manual in more detail.

1.1.3 The Datastore file structure

Measurement data are written to a results file called a Datastore, which is a proprietary format designed for handling multi-dimensional data. Its content depends on the type of measurement that is carried out. A

knowledge of the structure of Datastores is helpful for understanding some of the functionality of Pro-Data Viewer.

A Datastore contains all the data from a measurement, as well as measurement settings such as wavelength range, bandwidth, sampling times, etc. It can also include remarks and other information input by the user for reference.

The measurement data contained in a Datastore consists of *dimensions* and *properties*. The dimensions are the controlled (independent) experimental variables, such as wavelength or time; the properties are the measured (dependent) variables such as CD or absorbance; temperature may be either a dimension or a property or both, depending on the type of experiment. When data are plotted using Pro-Data Viewer, dimensions are always plotted on the x-axis, and the properties on the y-axis.

A Datastore can therefore have one or more dimensions, depending on the number of controlled variables. For example, a simple experiment in which CD is measured as a function of wavelength would generate a one-dimensional Datastore containing wavelength as the dimension, and CD, detector HV (Chirascan) or detector emulated HV (Chirascan V100), absorbance (if measured), transmission, temperature, the detector signal voltage (DC component), and the number of samples taken at each step (count), as properties.

If a temperature ramp is performed in which a series of spectra are measured while the temperature is changing, a two-dimensional Datastore would result, with wavelength and temperature as dimensions, and containing CD, HV etc. as properties. A titration experiment, in which a spectrum is measured at each of a series of concentrations, would also generate a two-dimensional Datastore, in this case containing properties as functions of wavelength and titrant concentration. A dimension is also added if measurements with repeat scans are performed.

Higher dimension Datastores can also be generated. If a stepped temperature ramp is carried out using the 6-cell Peltier Holder, then cell position becomes a third dimension and the Datastore contains properties as functions of temperature, wavelength and cell position. Substituting time for temperature would result in a Datastore containing properties as functions of wavelength, time and cell position.

All data from a particular measurement are written to a single Datastore. Subsequently, the Datastore can be reduced to subsets of the data.

Note that the Datastore file format is designed for Pro-Data multi-dimensional data acquisition and is not suitable for direct access outside the Pro-Data environment. A range of file export options is provided within Pro-Data, generating formats that are suitable for third-party applications.

1.2 *Operating principles*

This Section gives a short introduction to CD spectroscopy and describes the operating principles of the spectrometer.

1.2.1 Circular dichroism

Circular dichroism (CD) is the difference in absorbance between left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL) and occurs when a molecule contains one or more chromophores (light-absorbing groups) in chiral environments, i.e.:

$$CD = \Delta A(\lambda) = A(\lambda)_{L\text{-}CPL} - A(\lambda)_{R\text{-}CPL} \qquad 1.1$$

where λ is the wavelength and A is the absorbance.

Circular Dichroism spectroscopy is a technique where the CD of a sample is measured over a range of wavelengths. It is used extensively to study chiral molecules of all types and sizes, but it is in the study of large biological molecules where it finds its most important applications. A primary use is the analysis of the secondary structure and tertiary structure of macromolecules, particularly proteins. As protein conformation is sensitive to environment, e.g. temperature, pH, or the presence of ligands, CD can be used to observe how secondary or tertiary structure changes with environmental conditions or on interaction with other molecules. Structural, kinetic and thermodynamic information about macromolecules can be derived from CD spectroscopy.

Measurements carried out in the UV, visible and near infrared regions of the electromagnetic spectrum monitor electronic transitions, and, if the molecule under study contains a chromophore in a chiral environment, then one CPL state will be absorbed to a greater extent than the other, and the CD signal over the corresponding wavelengths will be non-zero.

The spectrometer measures alternately the absorbance of L- and R-CPL, at a frequency of 50 kHz, and then calculates the CD signal. A CD signal can be positive or negative, depending on whether L-CPL is absorbed to a greater extent than R-CPL (CD signal positive) or to a lesser extent (CD signal negative).

Simultaneously with the CD measurement, the spectrometer can measure the sample absorbance, but note that, unlike on conventional CD spectrometers, the absorbance measurement is *exact* when performed on the Chirascan V100, as the LAAPD detector gain is known precisely. The Chirascan and Chirascan V100 can both be used in direct absorbance mode as single-beam absorbance spectrometers.

1.2.2 How the spectrometer works

The spectrometer scans across a range of wavelengths, from high to low. The wavelength does not change continuously but is selected step-wise by the monochromator, with a measurement being taken after each step.

At any step, light with a certain spread in wavelength is allowed to pass through the sample rather than light of a single wavelength. This spread is the spectral bandwidth. For example, with the monochromator set to 260 nm and a bandwidth set to 1 nm, the light arriving at the sample will be between 259.5 and 260.5 nm (approximately – the situation is slightly more complicated than that).

At each step, photons are collected by the detector for a given amount of time, the time-per-point. After the scan across the specified wavelength range is completed, the scan may be repeated multiple times with the same settings.

In summary, the settings that need to be defined for spectral acquisition are the wavelength range, step size, time-per-point, bandwidth, number of repeats and, usually, temperature.

For example, with the wavelength range set to 260 to 180 nm, the step size to 1 nm, and the time-per-point to 1 s, the monochromator remains stationary for 1 s for a reading at 260 nm, then it moves to 259 nm for another reading for 1 s, then moves to 258 nm, and so on.

This will give a total of 81 data points, so the total measurement time will be 81 seconds. The monochromator will spend a little time moving between wavelengths (about 25% of total measurement time), so the total scan time will be about 100 seconds. An approximate scan time is shown on the spectrometer control panel when the settings are defined. If more than one repeat is used, the acquisition time increases in proportion.

1.3 *The basics of making a good CD measurement*

This Section describes how to design a CD measurement and optimize the experimental conditions to obtain high quality data. The actual operation of the spectrometer is described in later Sections. Though this Section focuses on proteins, the approach to making a good CD measurement is the same for other samples, although the settings may be different.

1.3.1 The importance of the absorbance

The simple rule of making an efficient CD measurement is not to allow the absorbance to be too high. As CD spectroscopy is an absorbance technique, the CD signal is linear with sample concentration only if total sample absorbance obeys the Beer-Lambert law. If the total absorbance, including that of the buffer, is much above about 2 AU, then the light level is too low and reliable CD measurements cannot be made.

1.3.2 The signal-to-noise ratio

The most important factor when designing a CD experiment is the signal-to-noise ratio, S/N. For any measurement made using electronic equipment, there are always two parts to the response. One part is the signal, which is the "true" part of the response, due to the sample. The other is the superimposed random part, the "noise", due to the irregular emission of photons from a light source and the irregular movement of electrons in electrical circuitry. The noise can never be eliminated, although it can be reduced by good instrument and experimental design. The important consideration is not the actual noise level, but the ratio of the signal to the noise. For CD measurements, this is given by [W.C Johnson in *Circular Dichroism and the Conformational Analysis of Biomolecules*, ed. G.D. Fasman, Plenum, 1996, pp 635 to 652]:

$$S/N \propto (I_0 \cdot Q \cdot t \cdot 10^{-A})^{0.5} \Delta A \qquad 1.2$$

where I_0 is the intensity of the incident light, Q is the quantum efficiency of the detector (i.e. the fraction of incoming photons that produce electrons), t is the sampling time, A is the absorbance and ΔA is the difference in absorbance between L- and R-CPL as given in Equation 1.1.

1.3.3 Optimal absorbance

Equation 1.2 maximizes when $A = \log_{10} \exp(2) \approx 0.87$, which is therefore the optimal absorbance for making CD measurements. Figure 1.2 shows the S/N as a function of wavelength, normalized to a maximum of 1. The S/N is greater than 0.8 over the range between about 0.4 to 1.6, and the absorbance should be within this range for as much of the spectrum as possible. There is insufficient light to make CD measurements when the absorbance is much above about 2 AU.

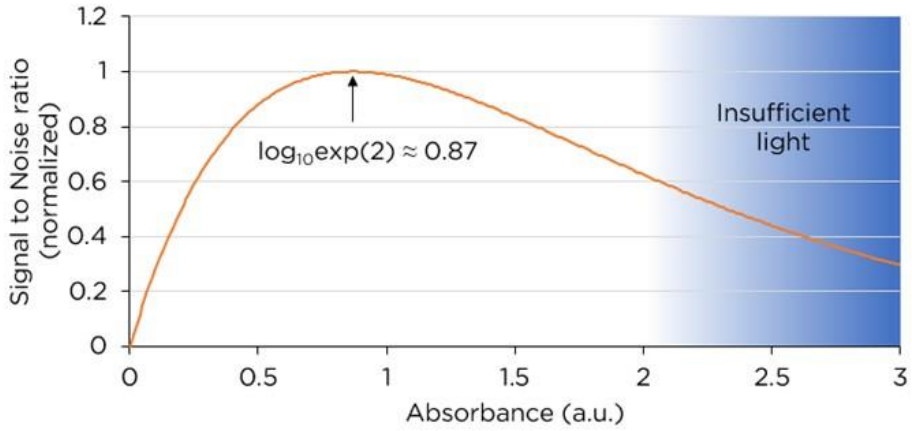

Figure 1.2: Signal-to-noise ratio as a function of absorbance

1.3.4 Measuring the absorbance

To obtain the total absorbance of a buffer or sample, a reference measurement in air is required beforehand. To this end, a background is recorded with the cuvette holder (and adaptor for short pathlength cuvettes or demountable cells, if necessary) in place, but with no cuvette. The background measurement must be performed over the same or a broader wavelength range as that used for the buffer or sample.

1.3.5 Adjusting the absorbance

The Beer-Lambert law (Equation 1.3) is used to adjust the sample concentration, the buffer type, and the cuvette pathlength to give an absorbance that is appropriate for a given sample.

$$\Sigma A = \Sigma(\varepsilon \cdot c) \cdot l \qquad 1.3$$

where ε is the extinction coefficient of each species, c is the concentration of that species, l is the pathlength, and the summation sign Σ indicates that the contributions of all the species present are added up.

This means that the total absorbance can be adjusted in three ways:

- by changing the pathlength (i.e., using a different cuvette)
- by changing the buffer and hence ε for the buffer (e.g., by dialysis).
- by changing the concentration of the buffer or the sample (i.e., by concentrating or dilution)

The extinction coefficient of the sample cannot be changed, as that would mean changing the sample. To obtain a high CD signal, the sample must be as concentrated as possible, but to keep the total absorbance low, the buffer absorbance must be as low as possible. To meet both these criteria, a short pathlength cuvette is used, preferably with a low absorbance buffer, and the sample concentration is adjusted to give the optimum absorbance.

1.3.6 Cuvette pathlength

Applied Photophysics recommends using CD or CD/fluorescence cuvettes from Hellma GmbH, Müllheim, Germany, or Starna Group, Hainault, United Kingdom.

Standard cuvettes are available in pathlengths from 0.5 mm to 10 mm. For CD measurements in the far-UV region (< 260 nm), 0.5 or 1 mm pathlength cuvettes are recommended, as water and aqueous buffers absorb strongly in this region, particularly at low wavelengths, and short pathlength cuvettes are used to keep the buffer absorbance low.

In the near-UV region (> 260 nm) the pathlength is usually less important because water and most buffers do not absorb strongly in this region, so a 10 mm pathlength cuvette can be used. Specially designed cuvettes must be used if fluorescence measurements are intended; these normally have pathlengths of at least 2 mm.

For more information about available cuvettes, please consult the Cuvettes and Holders Selection Guide available in the Related Products section on www.photophysics.com.

1.3.7 Buffer absorbance

Table 1.1 shows the absorbance cut-off for some common buffers in a 0.5 mm pathlength cuvette. At wavelengths lower than the cut-off, the absorbance of the buffer is greater than 1 AU.

Table 1.1: Absorbance cut-off of some common buffers in a 0.5 mm pathlength cuvette

Buffer	Cut-off (A > 1), nm
D_2O (0.1 mm demountable cell)	171
Water (0.1 mm demountable cell)	175
D_2O	174
Water	178
20 mM pH 7 phosphate	181
10 mM NaF	180
10 mM NaCl	185
1% DMSO	220
100 mM Tris	190
100 mM MES	201
100 mM HEPES	209
100 mM PIPES	210

1.3.8 Sample concentration

In the far-UV region, proteins have an extinction coefficient of about 7000 M^{-1} cm^{-1}, where M refers to the concentration of peptide bonds. For a 0.5 mm pathlength cuvette, this means that the protein concentration should be about 0.2 to 0.4 mg/ml. In the near-UV region, the absorbance depends mainly on the concentrations of the aromatic amino acids, particularly tryptophan. This results in an optimal concentration ranging from about 0.45 mg/ml for proteins with a high tryptophan content, such as lysozyme which has 6 tryptophan residues and a molecular weight of 14 kD, to more than 2.5 mg/ml for molecules with a much lower tryptophan content.

1.3.9 Summary

1. If possible, use a buffer that does not absorb strongly.
2. If necessary, use a short pathlength cuvette.
3. Adjust the sample concentration to keep the total absorbance below 2 AU and centered on about 0.9 AU.

2 QUICK START

This Chapter describes the basic operation of the spectrometer, covering the startup of hardware and software, and use of the spectrometer instrument control and data acquisition software to collect and manipulate CD spectra, and to export data to formats compatible with third-party programs. More detailed information on these operations is given in full User Manual.

2.1 *Hardware startup*

1. Put the black rocker switch for the **System** on the front panel (Figure 2.1) to the power on (I) position (the rocker switch for the **Lamp** should be left on permanently when using the ANMS, Section 3.3).

The instrument will automatically check its zero positions (you may hear the monochromator motors moving). Ensure that the computer and its monitor are powered on. The light-emitting diodes (LEDs) on the system panel (Figure 2.1) are labelled **Status**, **Tx** and **Rx**: Tx and Rx will illuminate when commands are being transmitted or data is being received, respectively. The status LED is illuminated when the instrument is powered on.

Figure 2.1: The front panel, including lamp panel (left) and system panel (right)

⚠CAUTION The interaction between UV light and oxygen leads to the formation of ozone, a very reactive gas that is damaging to health and may cause deterioration of the optical components of the instrument. If an ozone producing lamp is used, it is essential that the spectrometer is thoroughly purged with nitrogen before the lamp is ignited.

The spectrometer requires purging with high purity nitrogen gas before and during use, to eliminate oxygen from the light path .

2. Make sure that your nitrogen purge gas supply is stocked sufficiently to provide enough nitrogen for the planned measurements. Then turn on the nitrogen supply.

Purging is controlled by the Chirascan Active Nitrogen Management System (ANMS).

3. Briefly, start the ANMS software and click the **Connect** button on its main screen. If required, schedule purging on the **Schedule** tab. If the spectrometer has not been used for an extended period, purge overnight.
4. Click **Start Lamp Ignite Sequence** in the **Monitor** tab; the lamp will be ignited automatically once the instrument has been purged sufficiently.
5. After ignition, leave the lamp to stabilize for at least 20 min before acquiring data.
6. Turn on the water circulator.

7. Turn on the Peltier controller.

NOTICE Always make sure that the water circulator is switched on to remove heat from the Peltier device. Otherwise, the temperature of its heat exchanger rises until a certain cut-off value is exceeded and temperature control is automatically shut down to prevent damage to the unit.

2.2 *Software startup*

Before starting any software, make sure that the spectrometer including its peripheral hardware is turned on.

The programs need to be opened in the following sequence for correct communications to be established:

1. Open Pro-Data Chirascan (Chapter 4) by double-clicking the  desktop icon.

2. Open Pro-Data Viewer (Chapter 5). Pro-Data Viewer is launched on-line from the Pro-Data Chirascan Spectrometer Control Panel (SCP) by clicking on the Pro-Data Viewer launch icon  in the SCP toolbar.

Note that if Pro-Data Viewer has been launched by clicking on its desktop icon, then Pro-Data Chirascan will not know of its existence, and Pro-Data Viewer will not respond to the start of an experiment in the normal way. To make the connection between the programs, go to **Preferences > Go online…** in Pro-Data Viewer and select **Local** in the **Connect…** dialog.

In general, when working at the instrument, it is recommended that Pro-Data Viewer is launched from Pro-Data Chirascan using the toolbar icon as this will ensure that local communication is established, that Pro-Data Viewer will work correctly as a real-time display, and that the data will be saved in the current working directory.

3. Choose your current working directory.

All data acquired will be saved in the current working directory. To change the current working directory, first navigate in the Pro-Data Viewer file browser and open, or create, a folder for data collection. Then select the folder of your choice and click on the **Set Working Directory** toolbar icon. Alternatively, right-click on the folder of choice and choose **Set Working Directory Here** or use the **Set Working Directory** option on the **Directory** menu .

To set file names for background, buffer and sample spectra, click on the **File Names** icon  in the toolbar of the Pro-Data SCP or select **Configuration > Preferences > File Names**. This opens the **File Names** dialog where seed names and running numbers (four digits with leading zeroes) can be specified (Figure 2.2). The file name for the background is set in the **Background** input field, the file names for buffer or sample spectra are set in the **Spectrum** input field. Note that entering special characters is not allowed.

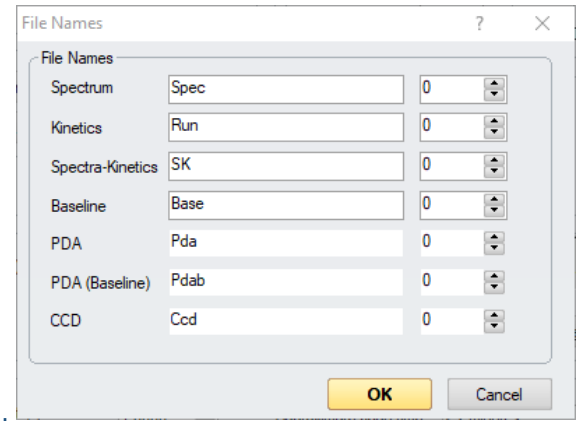

Figure 2.2: The File Names dialog

2.3 *Spectrum acquisition*

2.3.1 General sequence

To make a good CD measurement for a sample, follow the general sequence, which is explained in more detail in the following subsections.

1. **Adjust the measurement settings** (Section 2.3.2) as required – some preliminary work may be required to establish the optimal conditions.
2. **Acquire a background** (Section 2.3.3). The background enables you to measure the absorbance spectrum of your buffer.
    a. Mount the cuvette holder in the Single Cell Peltier Holder, but do not insert a cuvette.
    b. Set the file name for the background.
    c. Click the **Background** button on the **Background** panel.
3. **Acquire a buffer CD spectrum** (Section 2.3.5). This gives you the absorbance of the buffer and provides a baseline CD spectrum, i.e. the spectrum of the buffer that you will later subtract form the sample spectrum.
    a. Load the cuvette containing buffer only into the Single Cell Peltier Holder (Section 2.3.4).
    b. Set the file name for the buffer.
    c. Click the **Acquire** button on the **Spectrum** panel.
4. **Acquire a sample CD spectrum** (Section 2.3.6).
    a. Remove and clean the cuvette and fill with the sample, reload the cuvette into the Single Cell Peltier Holder (Section 2.3.4)
    b. Set the file name for the sample.
    c. Acquire the CD spectrum of the sample by clicking the **Acquire** button on the **Spectrum** panel.

2.3.2 Adjusting the measurement settings

The settings for spectral acquisition are set up on the Pro-Data Chirascan Spectrometer Control Panel (SCP), Figure 2.3. The Pro-Data SCP is divided into a number of panels: **Signal** (with subpanels **Options** and **Background), Temperature Control Unit**, **Sample Handling Unit (ESHU)**, **Monochromator**, **Sampling**, **Sequencer,** and **Progress and Status**, which group the parameters relevant for the control or management of each panel's function. In addition, there is an unlabeled panel for controlling shutter function and detector high voltage (Chirascan) or detector emulated high voltage (Chirascan V100) in the upper right section of the SCP.

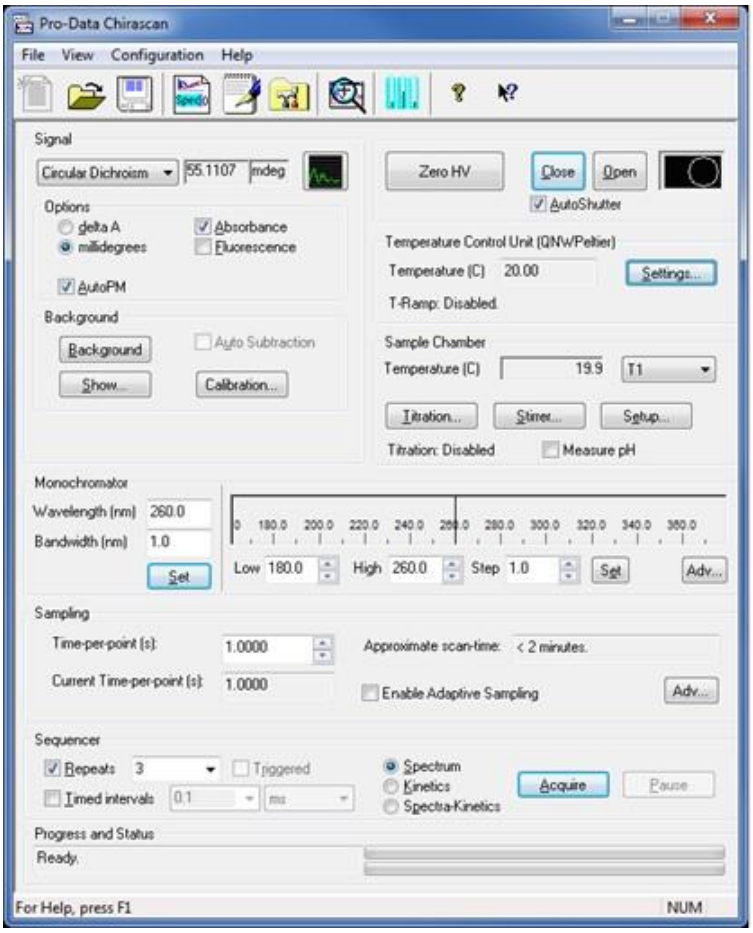

Figure 2.3: The Pro-Data SCP

A configuration of acquisition settings can be opened or saved by choosing **File > Open…** or **File > Save as…** in the menu, respectively. The acquisition settings that need to be configured are summarized in Table 2.1. The example values provided are typical for proteins, although proteins containing cofactors such as haems may have wider wavelength ranges. For more detailed information about how to choose suitable acquisition settings, please refer to Section 6. For more information about how to use the SCP, please refer to Section 6.

In addition to measurement settings, optional information can be provided by the user as described in Section 4.7.

Table 2.1: Summary of typical parameter values for measuring CD spectra of proteins

Parameter	Setting
Wavelength range	For proteins, the usual ranges are the far-UV, from 260 nm to as low as possible, and the near-UV, from 350 to 250 nm.
Step size	1 nm is usually OK, although sometimes 0.5 nm is used in the near-UV, where the spectra are more detailed than in the far-UV.
Bandwidth	1 nm is typical, although if you choose to use multiple repeats, this can be reduced to avoid sample photolysis.
Time-per-point	1 second is typical.
Repeats	3 is convenient although more are sometimes used to give an indication of data spread. 5 is typically used if statistical analysis of data is required.
Temperature	As required.

2.3.3 Acquiring a background

Every CD spectrometer has a CD background, caused by the interaction of CPL with the slight birefringence found in the detector window and to a lesser extent in the cuvette. To see the true CD spectrum of your sample, this background must be measured and subtracted from the CD spectrum of the sample.

Meaningful CD data can only be obtained if the total absorbance is within a reasonable range. To evaluate if this requirement is fulfilled, simultaneous acquisition of CD and absorbance is recommended. An absorbance reference is required to obtain total absorbance. As water absorbs strongly at low wavelengths, this reference should be obtained with an empty cuvette holder.

Set the parameters for spectral acquisition of the background:

1. In the **Monochromator** panel, choose a wavelength range (Low and High), set the step size (e.g. 1 nm), and click **Set** to confirm (see Section 4.3.1 for more details).
2. In the Monochromator panel, set the bandwidth (e.g. 1 nm) and click **Set** to confirm (see Section 4.3.1 for more details).
3. In the **Sampling** panel, set the Time-per-Point (e.g. 1 s) (see Section 4.3.5 for more details).

Note that subsequent acquisitions can have a different wavelength range or step size if each data point has a corresponding value in the background. For example, if you plan to acquire sample spectra in both the near- and far-UV, acquire a background spectrum including both ranges. Also, subsequent acquisitions must have the same bandwidth.

4. To acquire an absorbance reference together with the CD background, make sure the **Absorbance** check box is ticked.
5. If necessary, install the correct cuvette holder for the cuvette to be used (see Section 3.6.3).
6. Make sure there is no cuvette in the cuvette holder and Click **Background** in the **Background** panel to acquire a background (Section 4.2.2).

The CD background is measured and a graphical display by Pro-Data Viewer automatically opens and displays the data. The data are stored in the current working directory as a .dsx file, the name of which is generated by a seed name (the default seed name for background data is ‘Base’) and a running number with leading zeros (e.g., ‘Base0001.dsx’). The **Auto Subtraction** check box becomes active at the conclusion of a background scan: if you wish to subtract the background automatically from subsequently measured CD spectra, tick the **Auto Subtraction** check box.

An example of a CD background is shown in Figure 2.4.

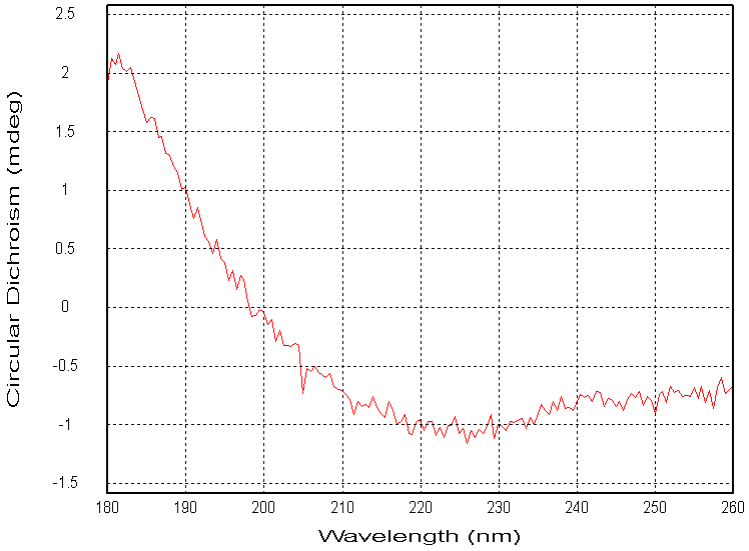

Figure 2.4: Background CD spectrum

2.3.4 Loading the buffer or sample

For information about availability and compatibility of cuvettes, please refer to Section 1.3.6.

To avoid spillages, the cuvette is best removed from the cuvette holder before loading. Most samples can be pipetted into the cuvette. Make sure that there is enough buffer or sample in the cuvette to cover the window in the cuvette holder, and that there are no bubbles. If the measurement is to be run at high temperatures, it may be necessary to remove dissolved gas before, to prevent bubble formation.

The cuvette should insert easily into the holder, and is sprung against the face of the holder to ensure reproducible positioning. Always record the orientation of the cuvette so that it can be replaced in the same orientation; usually, a mark on the cuvette indicating its type helps to distinguish the two sides.

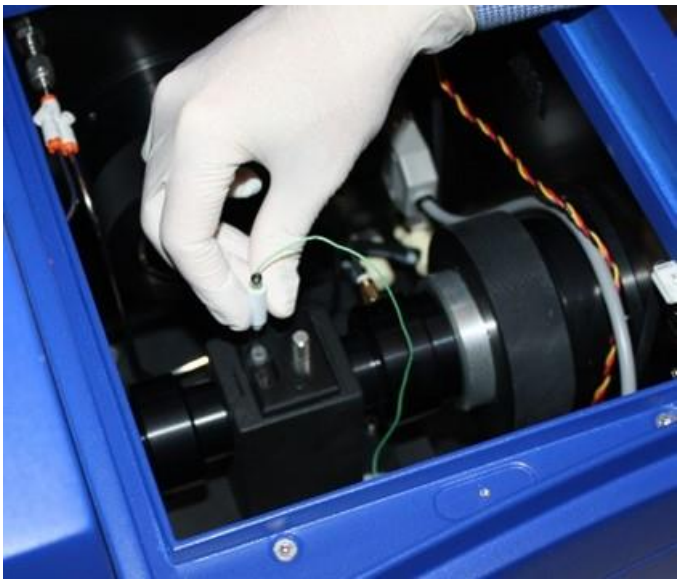

Figure 2.5: Inserting the temperature thermocouple

If using a temperature thermocouple (Figure 2.5), ensure that the thermocouple is immersed in the buffer or sample to a depth of a least 2 mm, but is not intruding into the light beam.

Finally, close the lid on the Sample Chamber.

2.3.5 Acquiring a buffer spectrum

To account for the CD signal of a buffer or other solvent, a baseline CD spectrum must be measured on the buffer without sample. The baseline spectrum should be acquired using the same conditions as are used for the sample spectrum, including using the same cuvette in the same orientation. Record the orientation of the cuvette in the holder, i.e. which side faces the lamp. Usually, a label on the cuvette helps to distinguish the two sides.

1. Load the cuvette with buffer, place the cuvette in the holder and insert the temperature thermocouple.
2. Set the measurement temperature: click **Settings…** in the **Temperature Control Unit** panel. Then adjust the temperature value, click **Set** to confirm and click **OK** (for details, refer to Section 4.4.2).
3. Tick the **Repeats** checkbox in the Sequencer panel. Set the repeats number (e.g. 3) and adjust other acquisition settings as required (for details, refer to Section 4.3.7).

It is recommended to always acquire repeat scans as these help in identifying baseline drift or sample photolysis. All remaining parameters, including wavelength range, step size, bandwidth, and time-per-point, should have already been set for the background.

4. Let the buffer equilibrate until the target temperature displayed in the Sample Handling Unit (ESHU) panel is reached (see Section 4.4.1).
5. Set the file name for the baseline spectrum in the Spectrum input field of the File Names dialog.
6. Click **Acquire** in the **Spectrum** panel to start acquisition of the baseline spectrum.

When any CD spectrum is acquired on the Chirascan or Chirascan V100, other properties are simultaneously recorded. These include the voltage applied to the detector, the temperature, and the absorbance spectrum (if selected on the **Options** panel). They can be viewed by right-clicking on the label of the y-axis in the graphical display and selecting the property of choice in the context menu.

7. Inspect the corresponding absorbance spectrum: right-click the label of the y-axis in the graphical display and select **Absorbance** in the context menu.
8. If the absorbance is too high, reduce the pathlength or change the buffer (see Sections 1.3 and 6.1.7 for more details on how to optimize absorbance).

For details on how to view and select spectra, refer to Section 5.2.

2.3.6 Acquiring a sample spectrum

Once the CD background and baseline have been measured, the sample spectrum can be acquired.

1. Empty and clean the cuvette. Then load it with the sample.
2. Place the cuvette with sample and temperature thermocouple into the cuvette holder in the same orientation as used for the baseline.
3. Let the sample equilibrate until the target temperature is reached.
4. Set the file name for the sample spectrum in the **Spectrum** input field of the File Names dialog.

As the parameters for the acquisition of the sample spectrum are identical to those for the baseline, there are no parameters that need to be adjusted at this point.

5. Click **Acquire** in the **Spectrum** panel to start acquisition of the sample spectrum.

The CD spectrum is recorded under the conditions applied to the baseline and stored in the current working directory as a file named as specified (Section 4.9.6). Again, Pro-Data Viewer automatically displays the data as it is collected; an example is given in Figure 2.6.

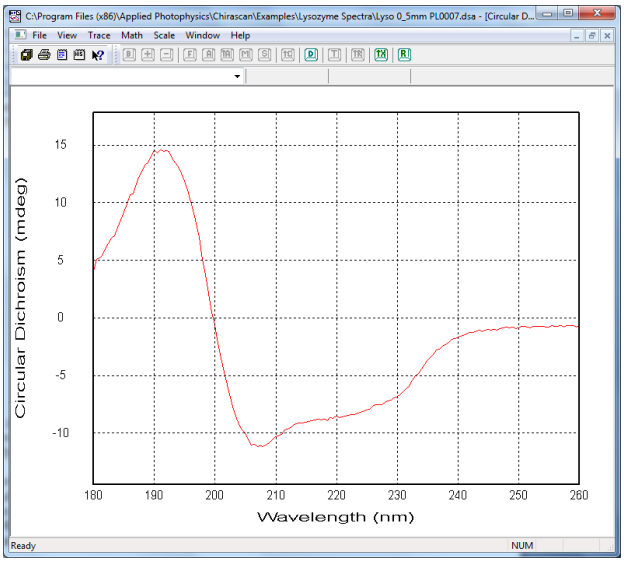

Figure 2.6: CD spectrum of lysozyme (raw data)

1. Ceck that repeat spectra overlay to make sure that the sample does not undergo photolysis.
2. Check the corresponding absorbance spectrum.
3. If the absorbance is above 2 AU, reduce the pathlength or sample concentration (see Sections 1.3.1 and 6.1.7 for more details on how to optimize absorbance).
4. On completion of the measurement, remove the cuvette from the holder for cleaning and re-use.

⚠CAUTION The cuvette, cuvette holder and Single Cell Peltier Holder may be very hot or cold, causing injury to the user when touched. Ensure that they have been allowed to reach a safe temperature before handling.

2.3.7 Basic data analysis

In the example below, there are six traces (3 repeat baselines and 3 repeat lysozyme spectra). The data used in this example can be found in the 'Examples\Lysozyme Spectra' folder which is installed with the Chirascan software (the path will depend on the operating system). The example demonstrates how to average the baseline spectra, average the sample spectra, subtract the average baseline spectrum from the average sample spectrum, smooth the resulting baseline-corrected sample spectrum and remove the original data. The end result will show the sample spectrum and its residual (smoothed minus unsmoothed spectrum).

1. With the the Pro-Data Viewer open, navigate in the usual way until you find the baseline measurement file and double-click on it.

Pro-Data Viewer displays the data contained in the Datastore in a graphical display.

2. Drag-and-drop the sample measurement file onto the graphical display with the baseline spectra.

Both baseline and sample spectra are shown in the same graphical display.

3. Click on  in the graphical display toolbar to call up the **Trace manipulation** dialog (Figure 2.7)

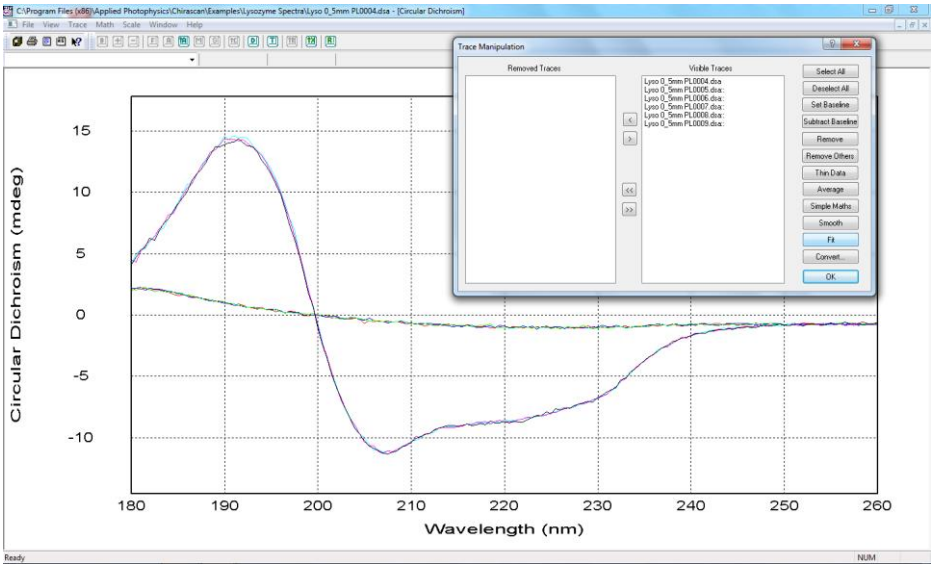

Figure 2.7: Raw data with Trace manipulation dialog called up

The first three list entries are baseline spectra; the second set of three are the lysozyme spectra.

4. Select the first group of three list entries and click **Average**.
5. Select the second group of three list entries and click **Average**.

Two list entries named Average: 0 and Average: 1 with running numbers will be added to the **Visible Traces** list and plotted in the display. The first of these will be the average of the baseline spectra, and the second the average of the sample spectra.

6. Select the first Average list entry and click **Set Baseline**.
7. Select the second Average list entry and click **Subtract Baseline**.

The list entry Subtracted: 0 (baseline-subtracted sample spectrum) will be added to the **Visible Traces** list and plotted in the display.

8. Click **Unset Baseline**
9. Select the Subtracted: 0 list entry and click **Remove Others**.

The **Trace Manipulation** dialog will appear as in Figure 2.8.

10. Click **OK** to close the dialog.

Note that even when the **Trace Manipulation** dialog is closed, any removed traces are remembered. Should you wish to carry out further manipulation, simply re-open the dialog and all the traces will be listed, whether visible or removed, enabling you to carry on where you left off.

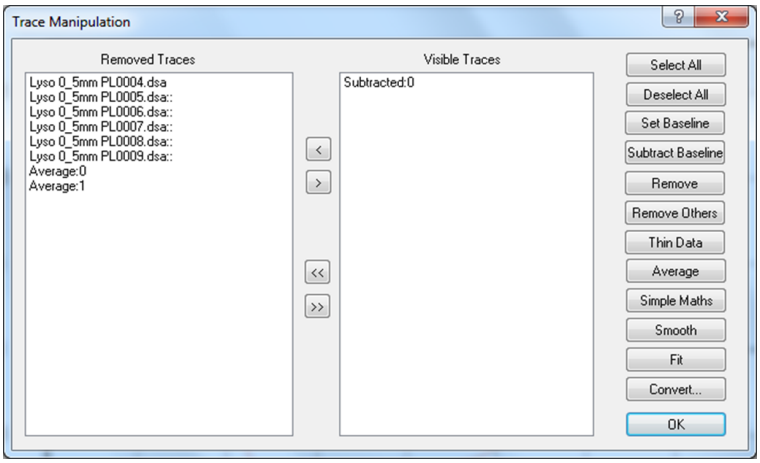

Figure 2.8: Trace manipulation dialog after spectrum manipulation

For final presentation, you might want to smooth spectra as described in Section 5.3.2. The result of the above manipulation and subsequent smoothing is shown in Figure 2.9, with the smoothed and baseline-corrected spectrum of lysozyme and the smoothing residuals plotted.

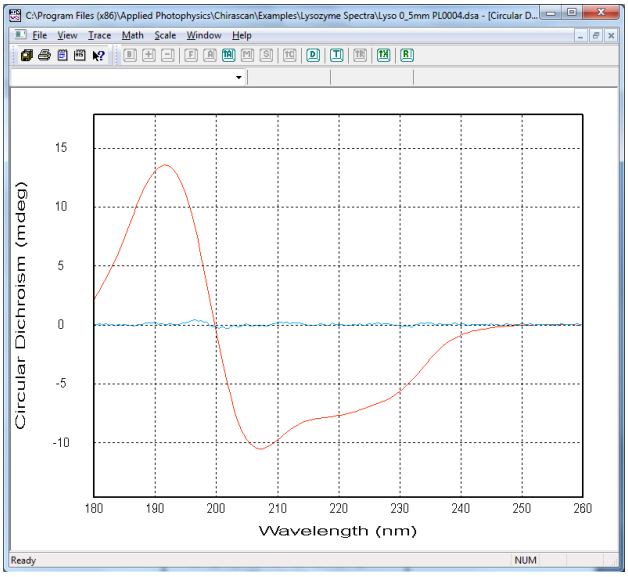

Figure 2.9: Smoothed and baseline-corrected spectrum of lysozyme (red) and smoothing residuals (cyan)

11. To save the result, go to **File > Save Current Plot…** or or **File > Save Selected Traces…** in the Pro-Data Viewer menu to call up the **Save As** dialog.

The results of any manipulation are stored in a new file when saved and a complete history of the manipulated traces is contained in the **History** of that file. The file history can be viewed on the **View** menu of Pro-Data Viewer.