

CIRCULAR DICHROISM

Theory and Applications

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Seminar Overview

- I. Circular dichroism theory basics
- II. Instrument design and components
- III. Biological applications
- IV. CD accessories

Second part of this webinar series will be at a later date and will discuss measurement parameter optimization, sample considerations, and how to acquire good CD data.

What is Circular Dichroism?

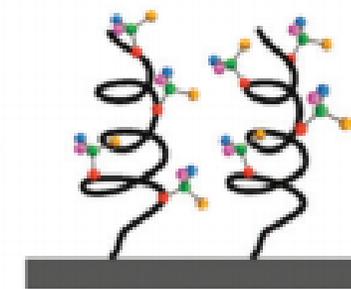
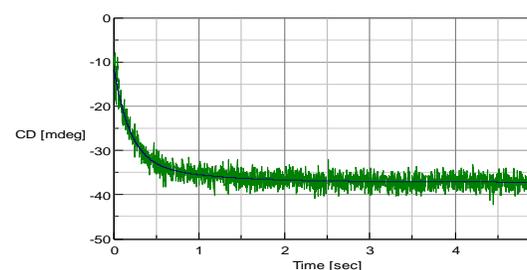
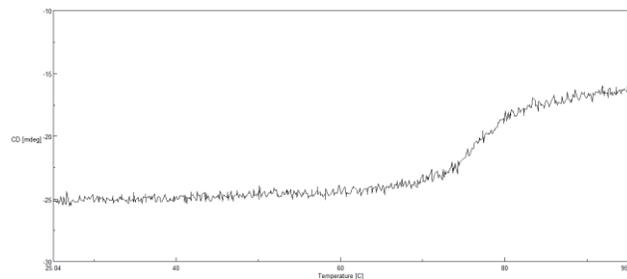
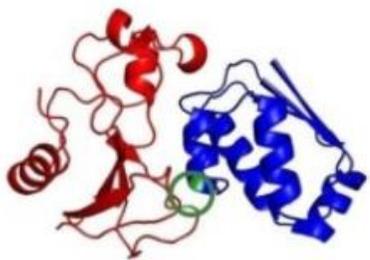
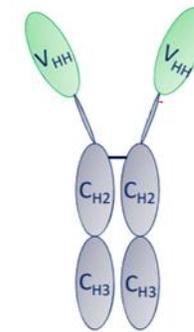
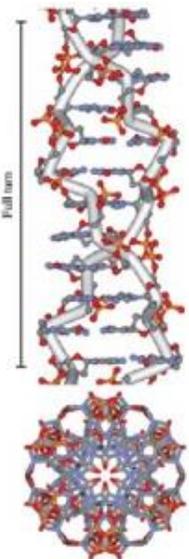
Difference in absorption of left and right circularly polarized light

CD signal observed when a chromophore is chiral (optically active)

- Intrinsically chiral
- Covalently linked to chiral center
- Placed in asymmetric environment

Can I use CD for my application?

- Protein structure
- Antibody structure
- DNA/RNA structure
- Protein - Protein interactions
- Protein - Nucleic Acid interactions
- Ligand binding (Induced CD)
- Carbohydrate structure
- Chemical stability studies
- Thermal stability studies
- Kinetic studies
- Stereochemistry
- Materials characterization
- Chiral Discrimination/Absolute configuration

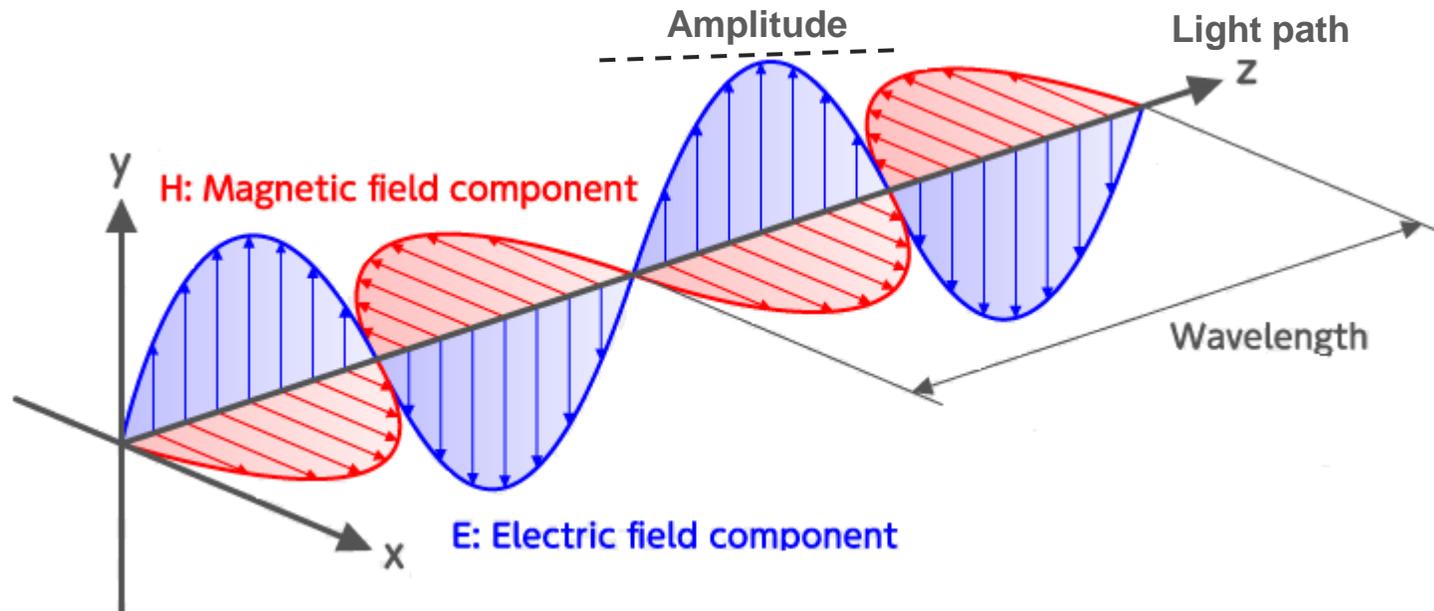


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Why use CD?

1. Uniquely sensitive to asymmetry.
2. Information on molecular and electronic structure.
3. Experiments are relatively quick and easy to perform.
4. Non-destructive (can recover most samples).
5. Solution phase.
 - Crystallation process could change molecular structure.
6. Low concentrations (0.1 mg/mL).
 - Doesn't require a concentration which could change the system being studied.

Electromagnetic wave

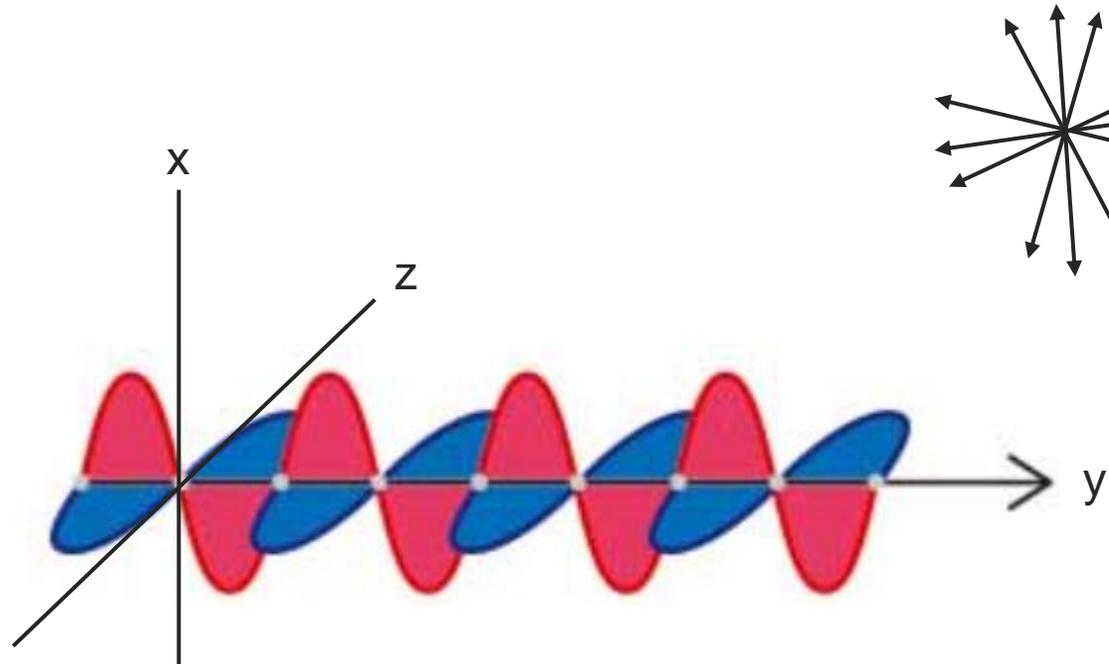


Amplitude: intensity of wave from tip of crest to central axis

Wavelength: distance between two consecutive crests

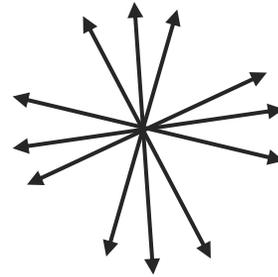
Polarization: directionality of electric and magnetic fields

Types of polarization

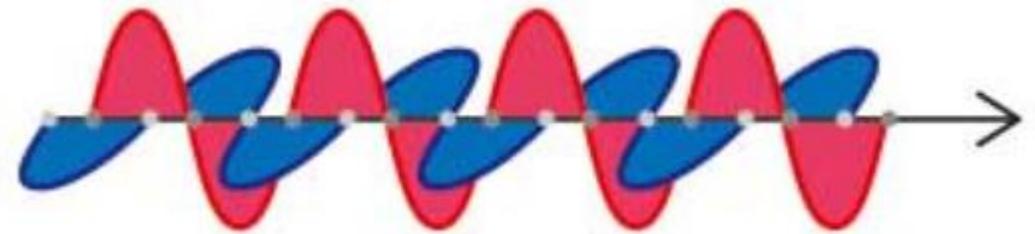


Linearly polarized light: electric field components confined to a single plane (x,y and y,z)

- Perpendicular, equal in amplitude



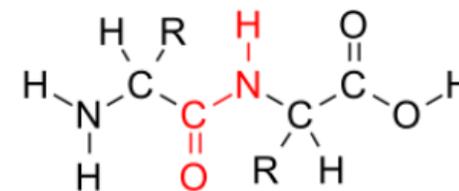
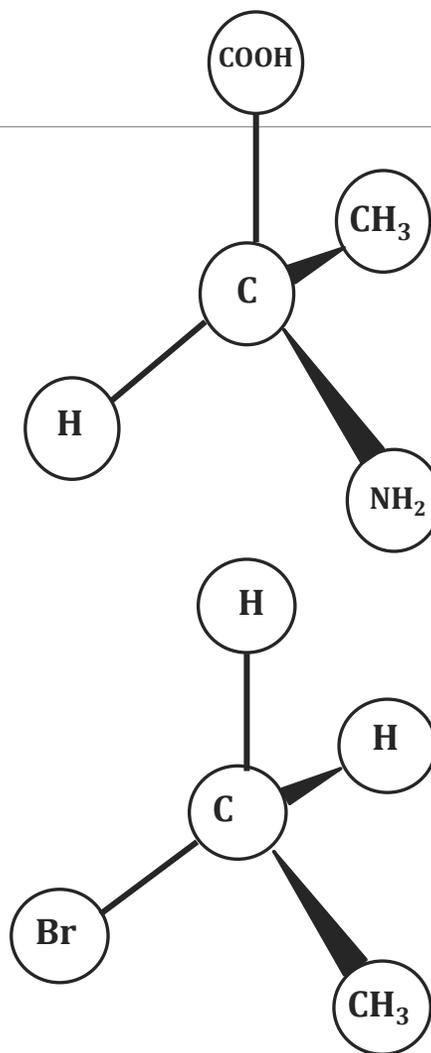
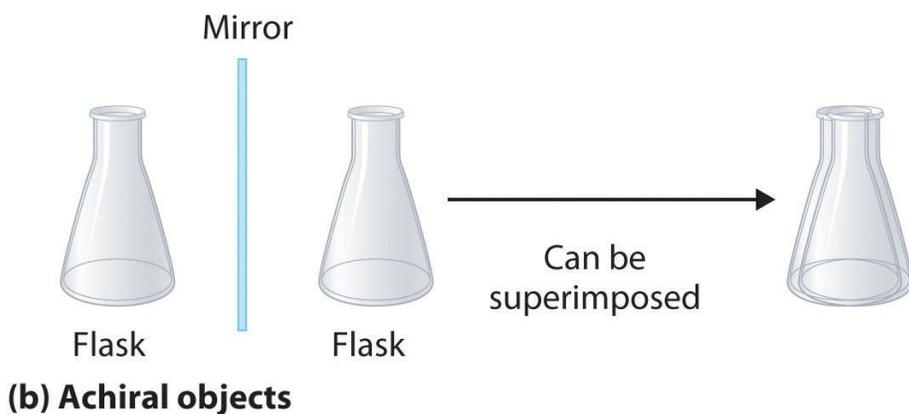
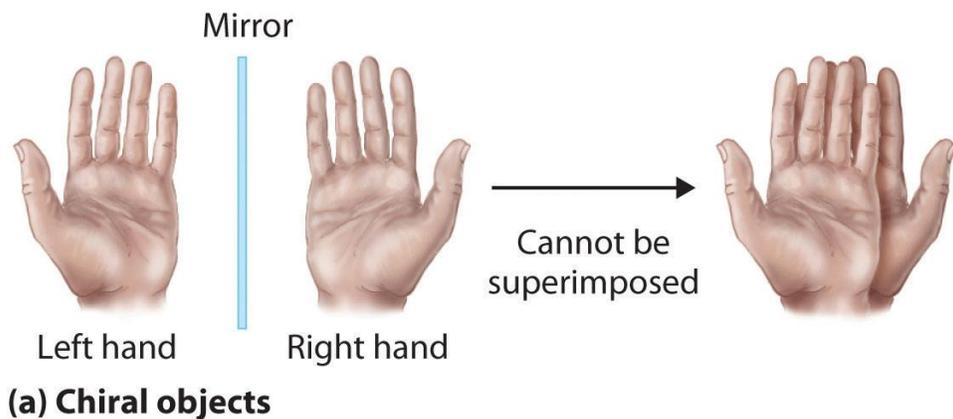
Unpolarized light



Circularly polarized light: electric field components rotate along beam propagation

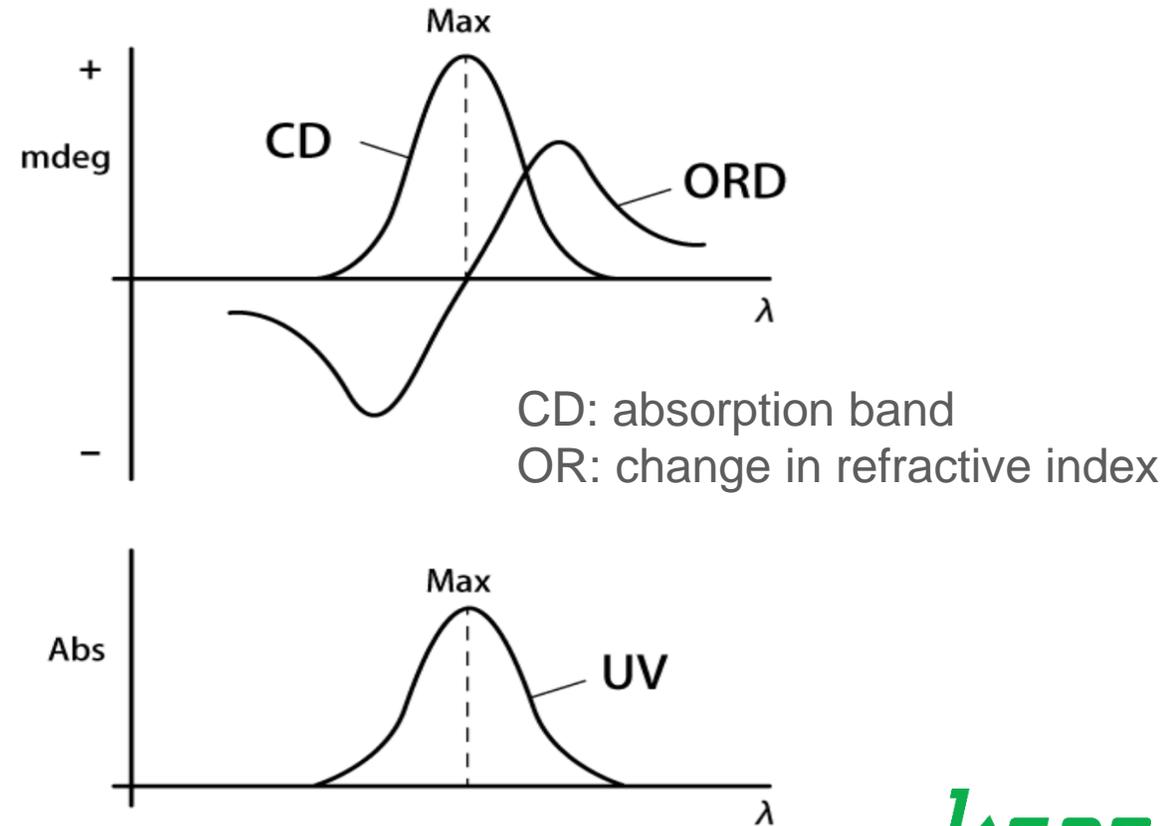
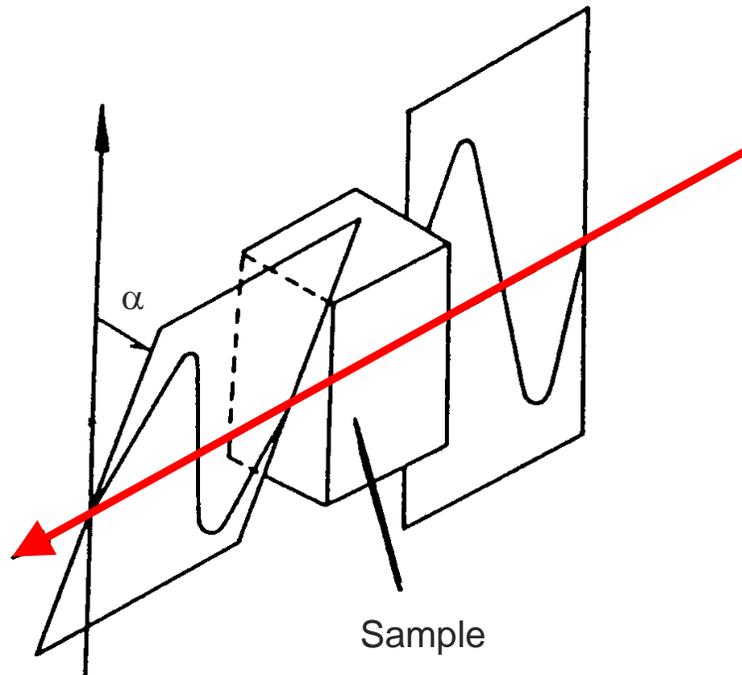
- Perpendicular, equal in amplitude, 90° phase difference

Chiral molecules



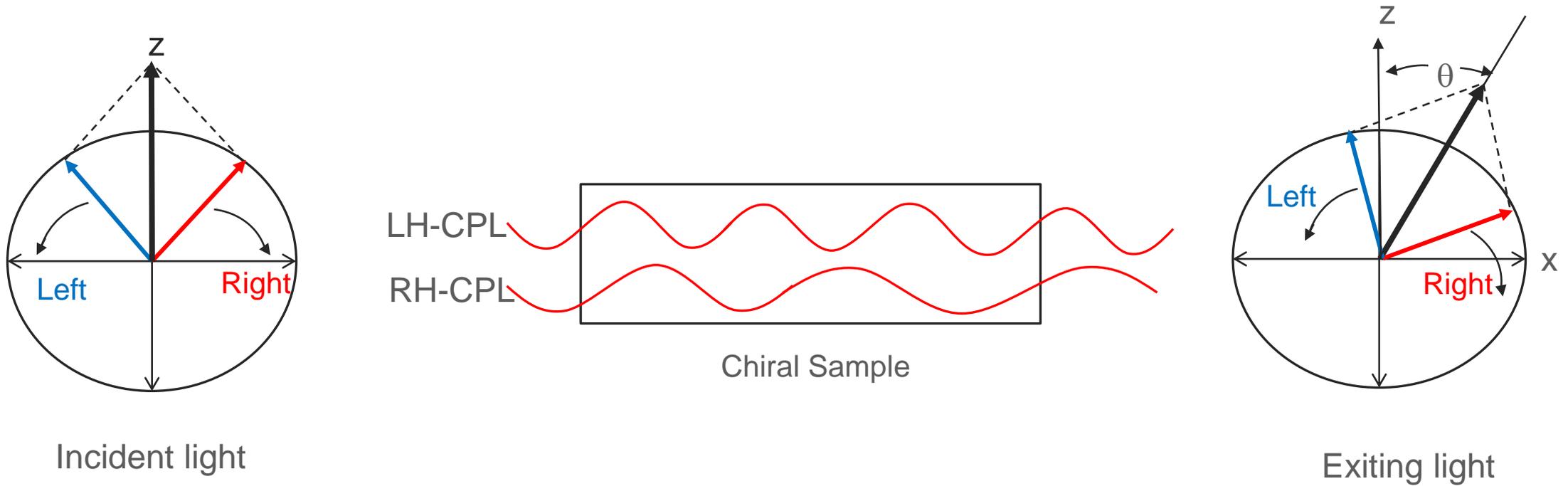
Optical Rotation and the Cotton Effect

Optical rotation (a): rotation of plane of polarized light



Circular birefringence

Circular Birefringence: velocity of the light passing through an optically active medium will differ depending on the medium's refractive index

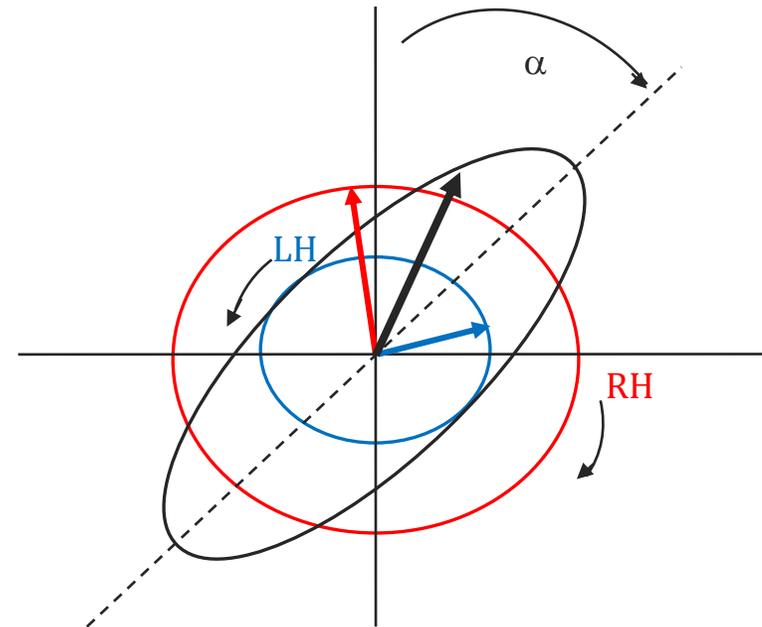


Elliptically polarized light

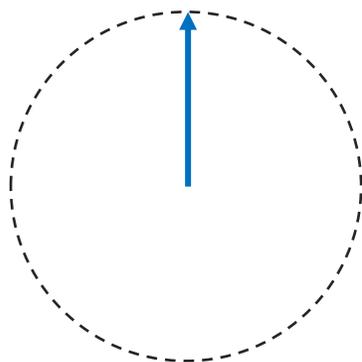
Circularly polarized light: electric field components rotate along beam propagation, perpendicular, *equal in amplitude*, 90° phase difference

Elliptically polarized light: electric field components rotate along beam propagation, perpendicular, *unequal in amplitude*, 90° phase difference

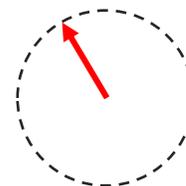
$$\Delta A = A_L - A_R$$



Time = 0



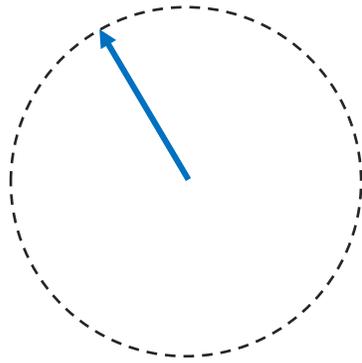
Left Circularly
Polarized Light



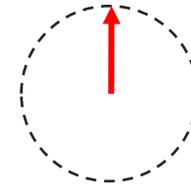
Right Circularly
Polarized Light

*Retarded and absorbed
by Chiral Compound*

Time = 1

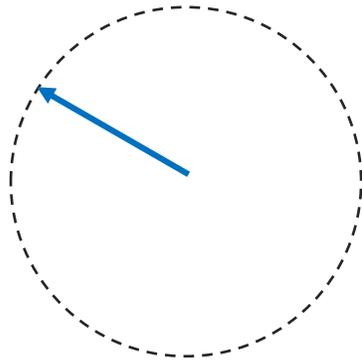


Left Circularly
Polarized Light

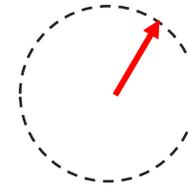


Right Circularly
Polarized Light

Time = 2

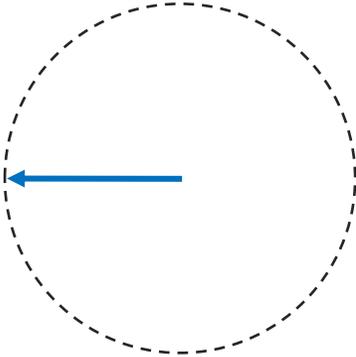


Left Circularly
Polarized Light

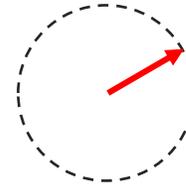


Right Circularly
Polarized Light

Time = 3

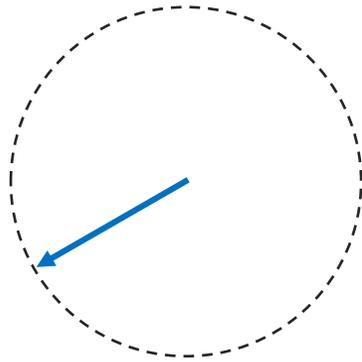


Left Circularly
Polarized Light

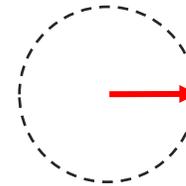


Right Circularly
Polarized Light

Time = 4

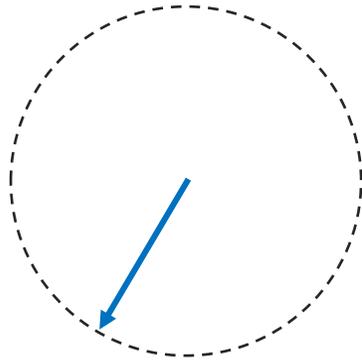


Left Circularly
Polarized Light

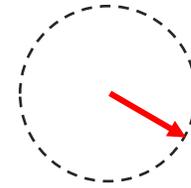


Right Circularly
Polarized Light

Time = 5

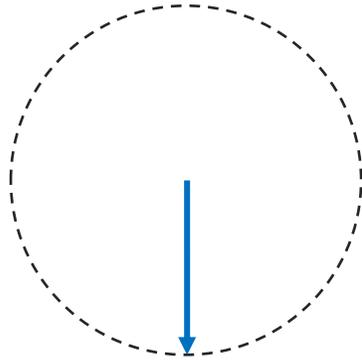


Left Circularly
Polarized Light

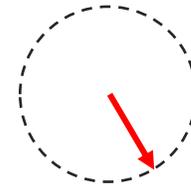


Right Circularly
Polarized Light

Time = 6

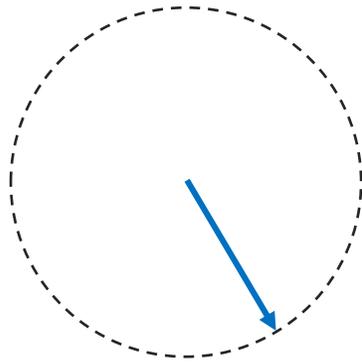


Left Circularly
Polarized Light

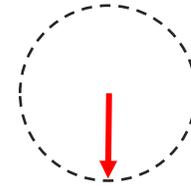


Right Circularly
Polarized Light

Time = 7

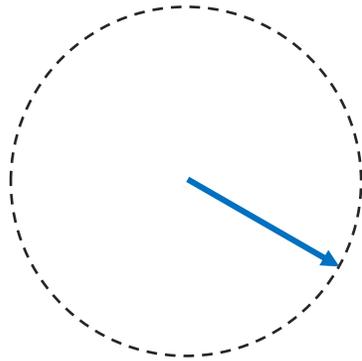


Left Circularly
Polarized Light

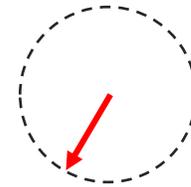


Right Circularly
Polarized Light

Time = 8

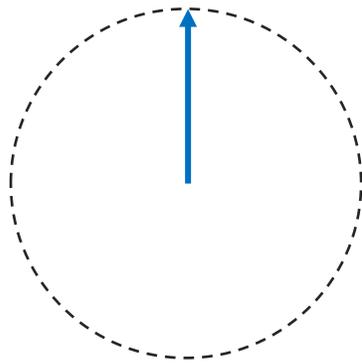


Left Circularly
Polarized Light

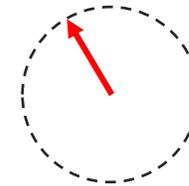
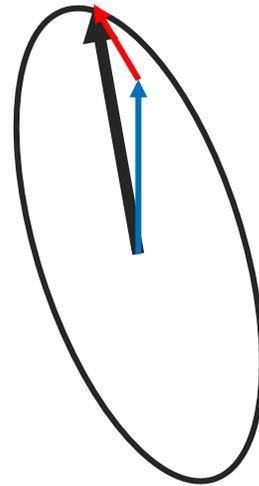


Right Circularly
Polarized Light

Time = 0



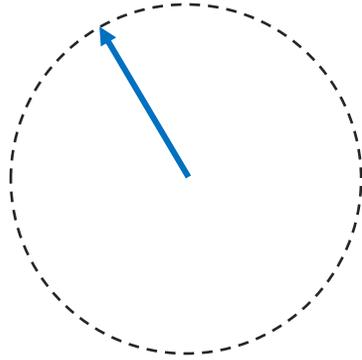
Left Circularly
Polarized Light



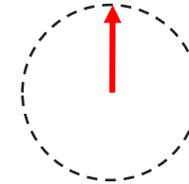
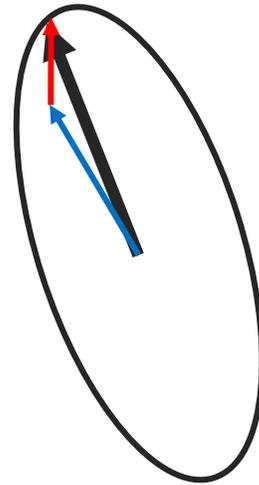
Right Circularly
Polarized Light

*Retarded and absorbed
by Chiral Compound*

Time = 1

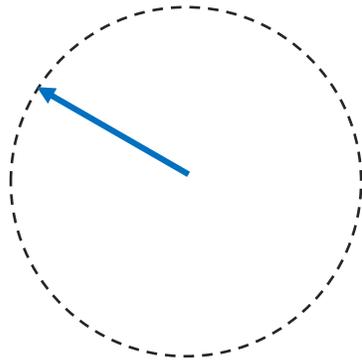


Left Circularly
Polarized Light

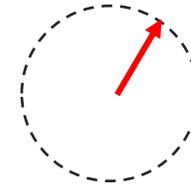
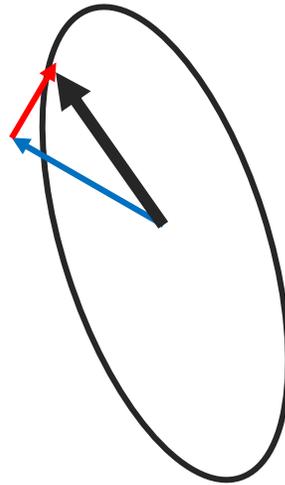


Right Circularly
Polarized Light

Time = 2

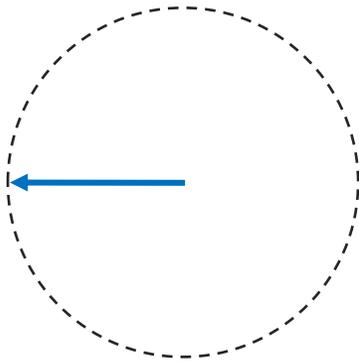


Left Circularly
Polarized Light

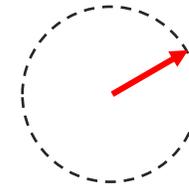
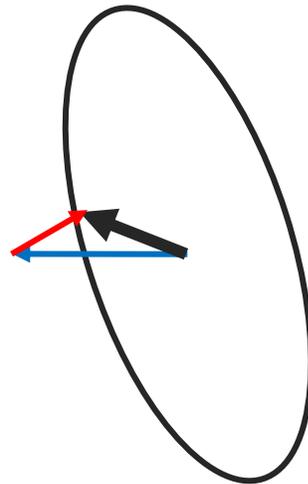


Right Circularly
Polarized Light

Time = 3

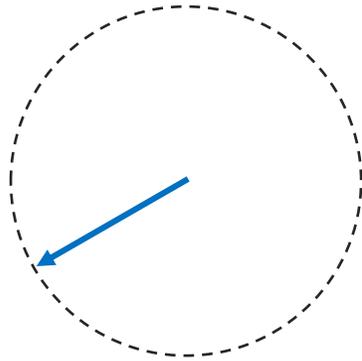


Left Circularly
Polarized Light

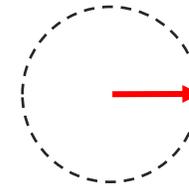
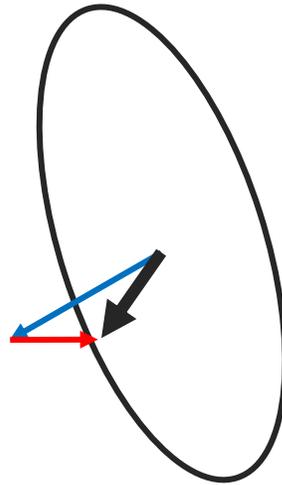


Right Circularly
Polarized Light

Time = 4

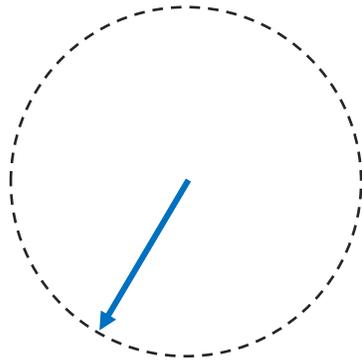


Left Circularly Polarized Light

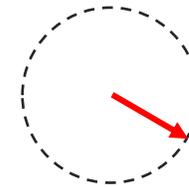
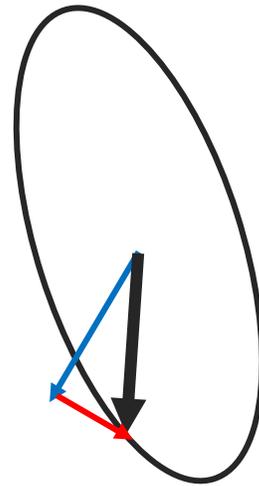


Right Circularly Polarized Light

Time = 5

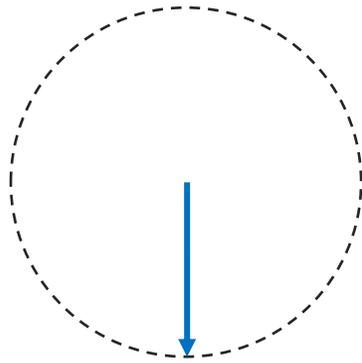


Left Circularly Polarized Light

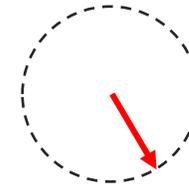
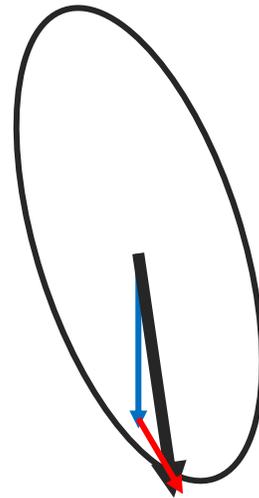


Right Circularly Polarized Light

Time = 6

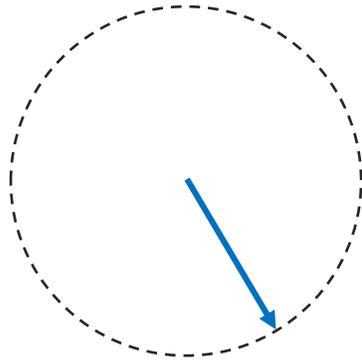


Left Circularly Polarized Light

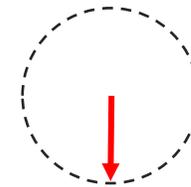
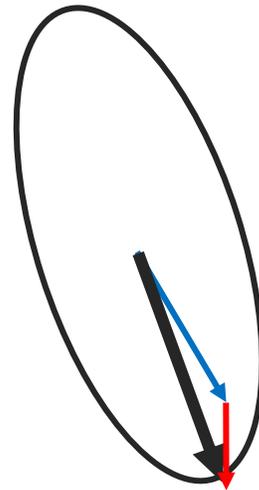


Right Circularly Polarized Light

Time = 7

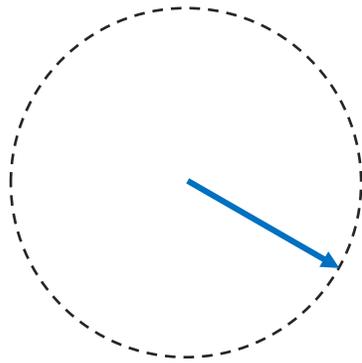


Left Circularly Polarized Light

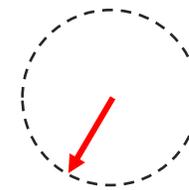
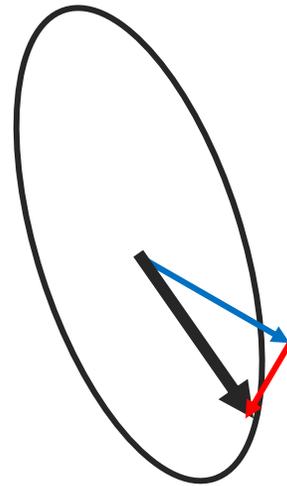


Right Circularly Polarized Light

Time = 8



Left Circularly Polarized Light

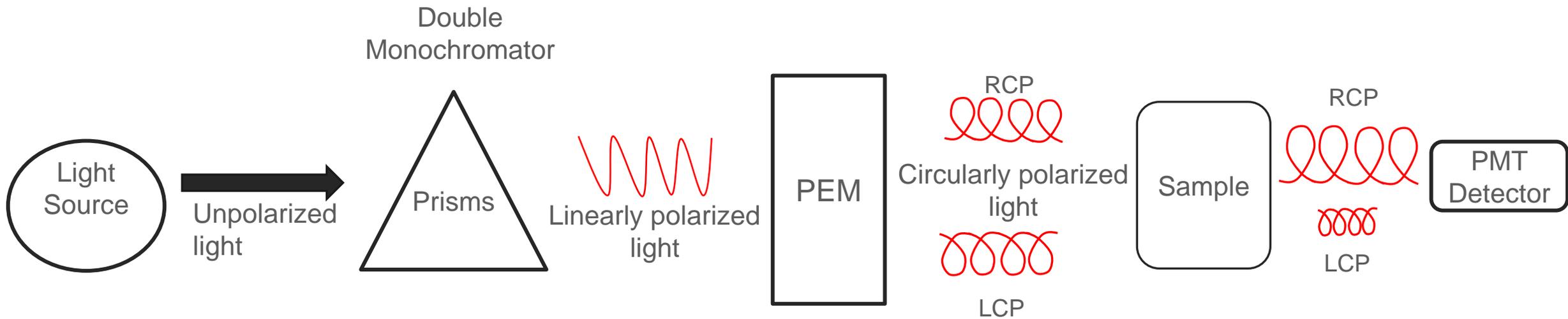


Right Circularly Polarized Light

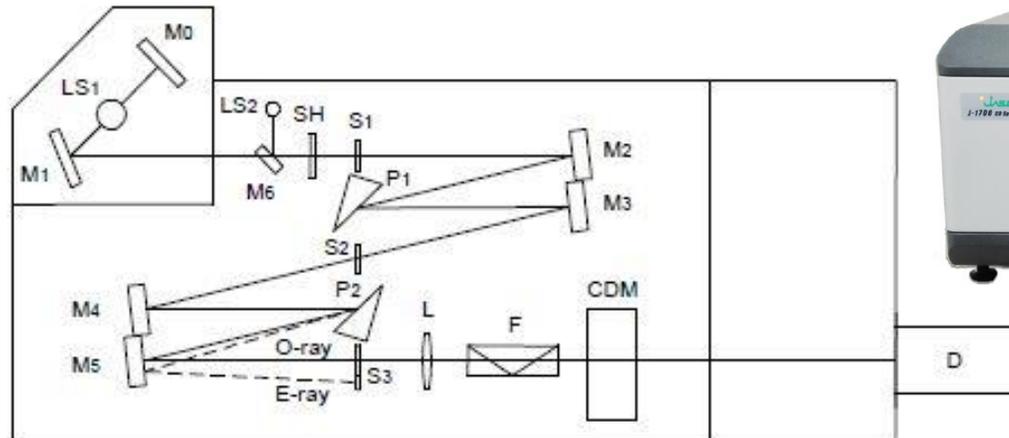


Instrumentation

Principle of Measurement



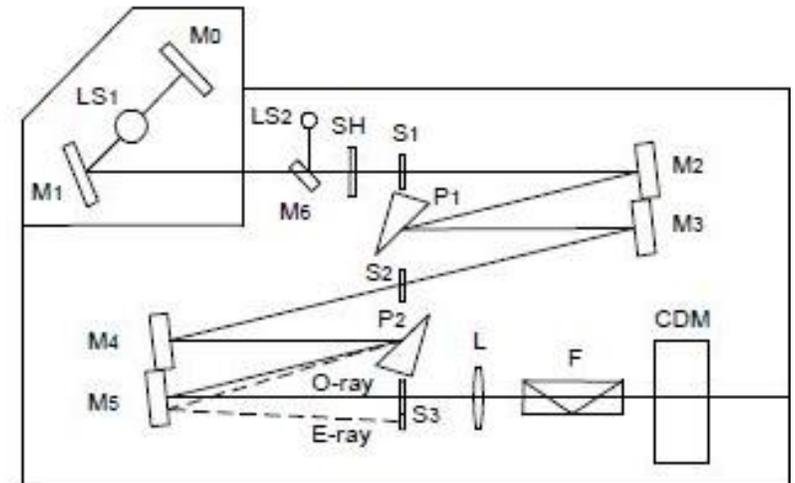
Optical Diagram of J-1500



- | | | | |
|----------|--|-------|---------------------|
| M0 ~ M6: | Mirrors | O-ray | : Ordinary ray |
| LS1 | : Xenon lamp (for sample measurement) | E-ray | : Extraordinary ray |
| LS2 | : Mercury lamp (for the instrument inspection) | L | : Lens |
| SH | : Shutter | F | : Filter |
| S1 ~ S3 | : Slits | CDM | : Modulator (PEM) |
| P1 | : First prism (horizontal optical axis) | D | : PMT |
| P2 | : Second prism (vertical optical axis) | | |

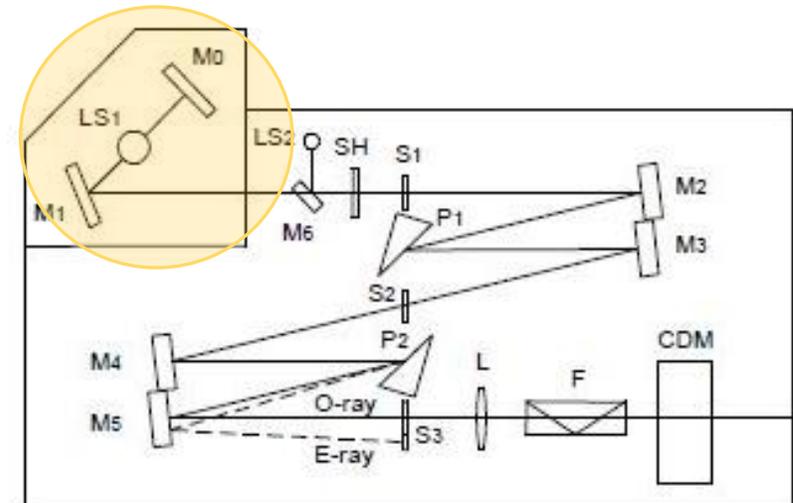
Double Monochromator and Stray Light

- CD is difference in absorption → very small signal
- Stray light: any light that does not fall under Gaussian distribution at a specific wavelength.
- Allow you to measure higher optical densities
- $3 \text{ Abs} = 0.001\%T$ → Less light transmitted through sample, more stray light effects



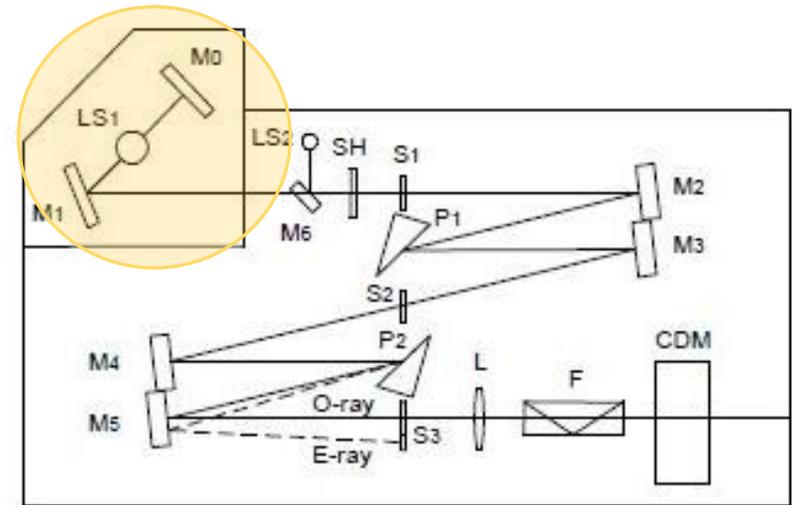
Light Source

- 150 W Xenon arc lamp (high pressure ~8 atm)
 - Broad spectral output (160-2000 nm)
 - Lasts ~ 1000 hours



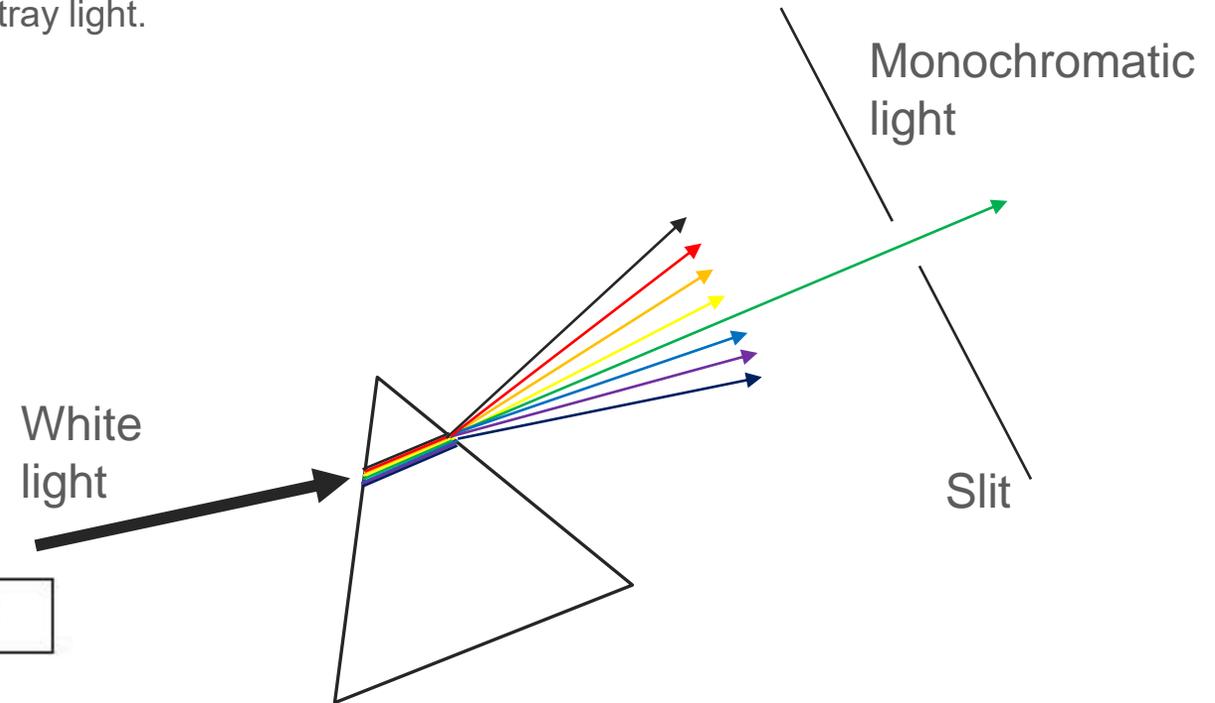
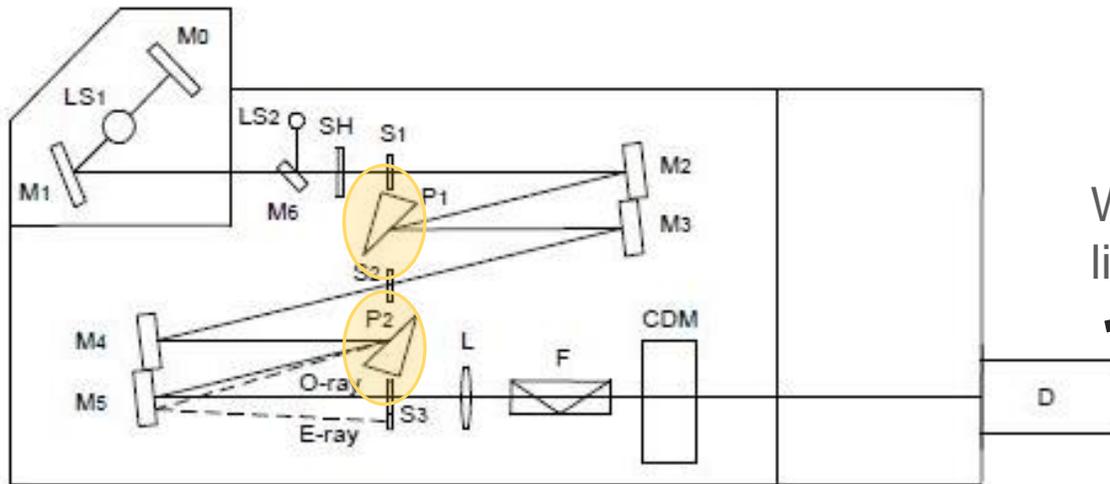
Nitrogen Purge

- Lamp creates substantial amount of UV radiation in far-UV
 - When UV radiation strikes O_2 molecule, O_3 created.
 - O_3 oxidizes mirrors (lose reflectivity and S/N gets worse).
- Push out O_2 with N_2 so O_2 doesn't absorb in far-UV.
- Flowrates: shorter wavelengths, higher flowrate
 - >185 nm: 2 L/min
 - <180 nm: 5 L/min



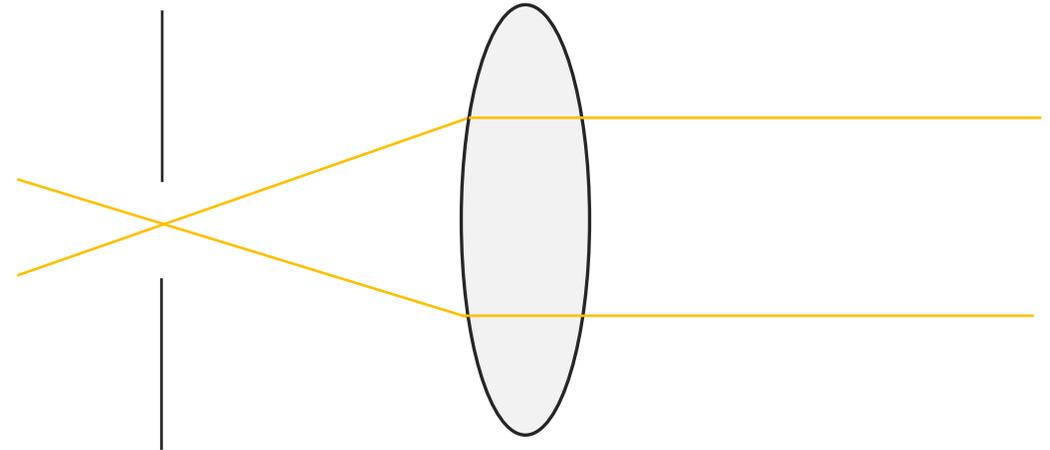
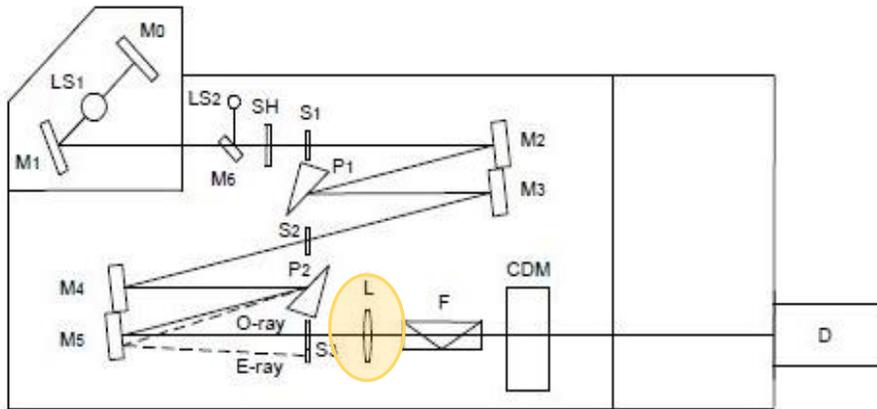
Prisms

- Dispersive element used for wavelength selection
 - Doesn't produce second order effect, which is a source of stray light.
- Creates linearly polarized light.



Lens

- Collimates light to constant width.
- PMT will collect more light if there are two parallel beams.



Filter

- Unpolarized light incident on birefringent material splits into two rays: O- and E-ray.
 - Filters out any E-ray that gets through the slit.
- In far-UV, slit is more open (more dispersion of light, need more light throughput).

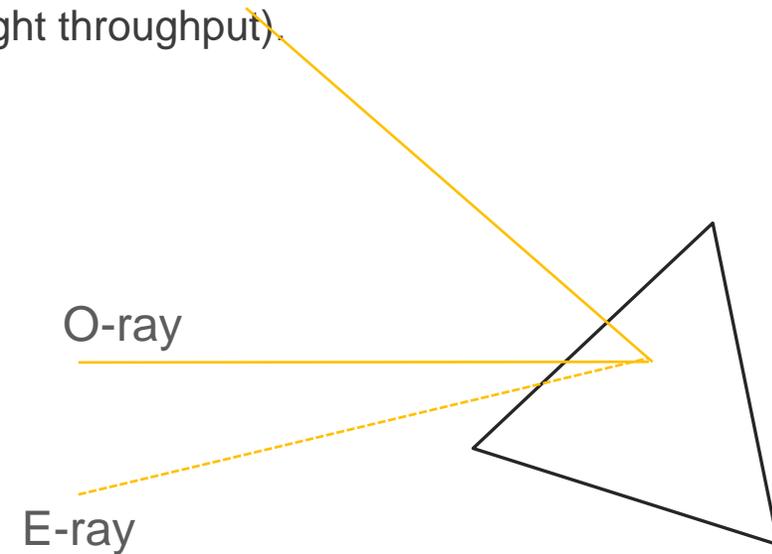
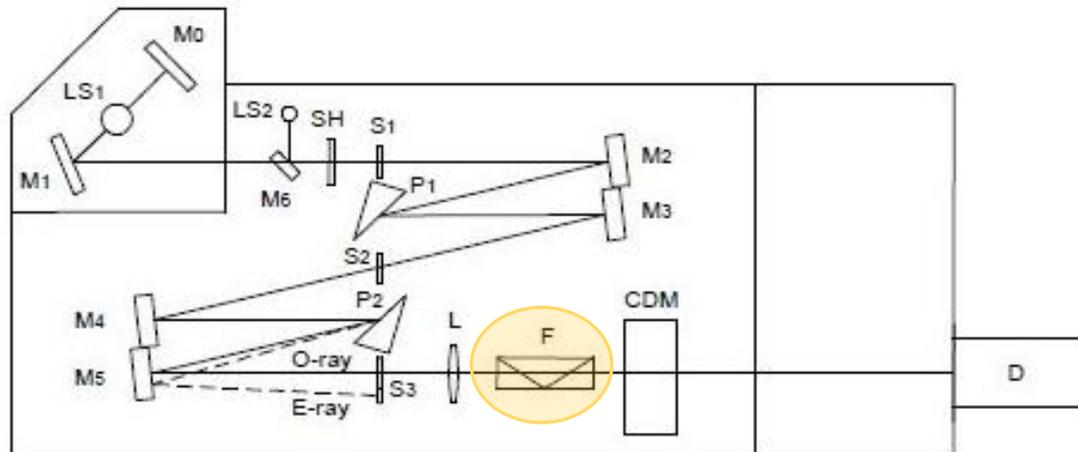
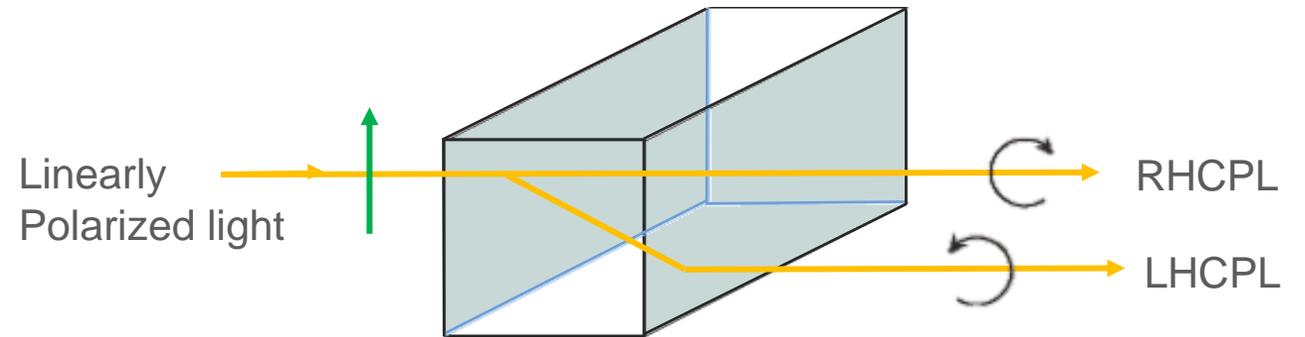
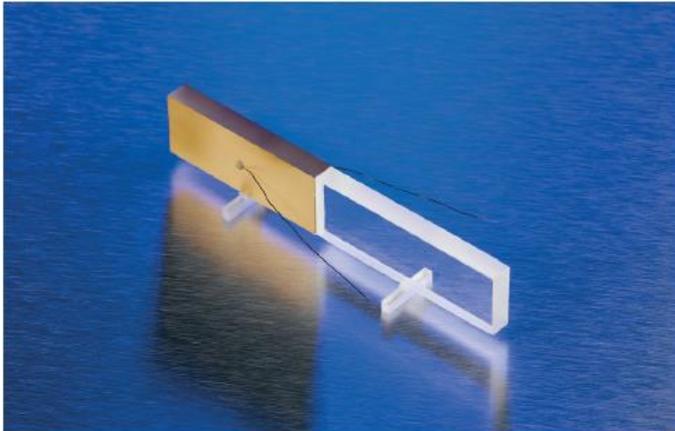


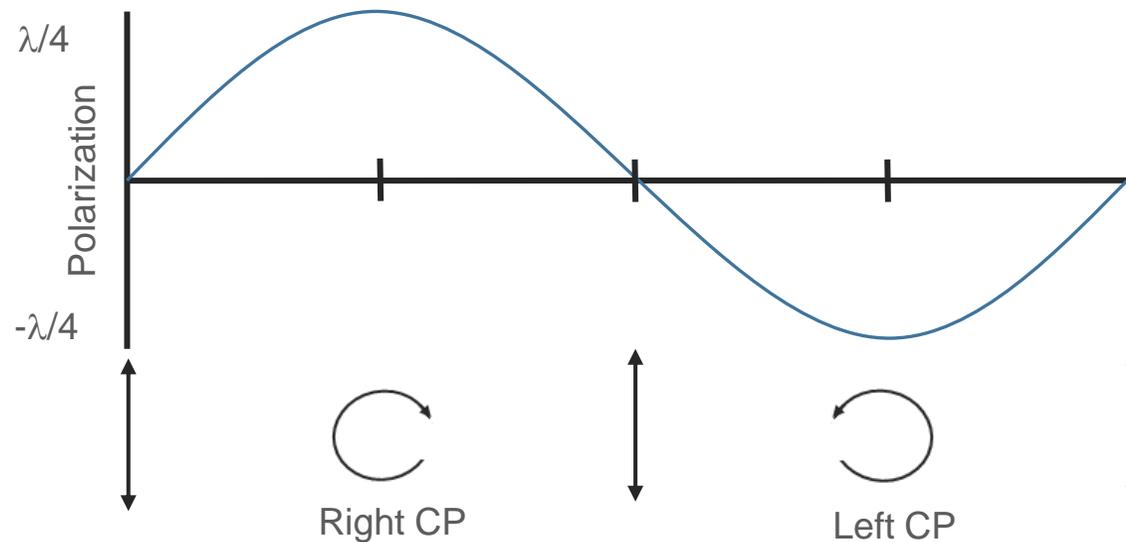
Photo-elastic Modulator (PEM)



- Converts linearly polarized light to circularly polarized light.
- Voltage is applied to vibrate piezoelectric element at resonance frequency (~50 kHz).
- Stresses and bends quartz attached to element, which induces birefringence.
- Linearly polarized light components travel through the birefringent quartz piece at different speeds.

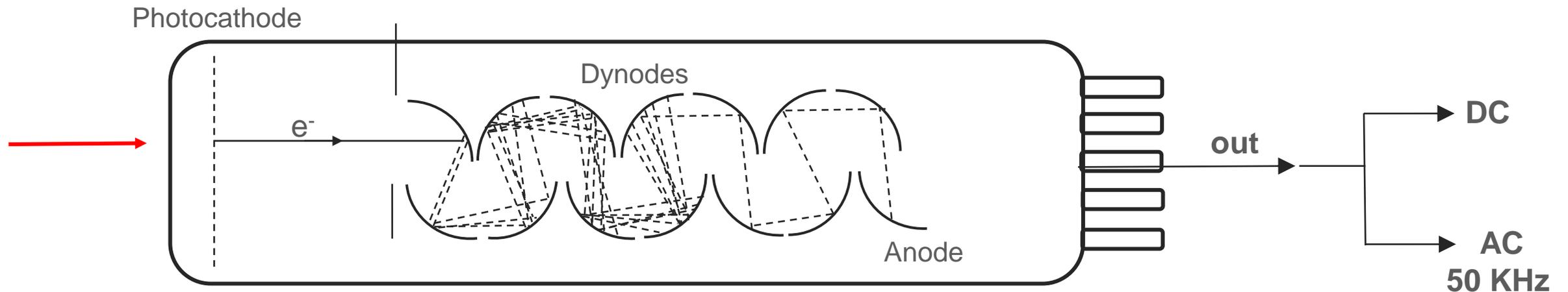
Photo-elastic Modulator (PEM)

- When quartz compressed, polarization parallel to modulation axis travels faster than vertical component.
- When quartz stretched, parallel component lags behind vertical component.
- Modulates between left- and right-handed CPL at different times.



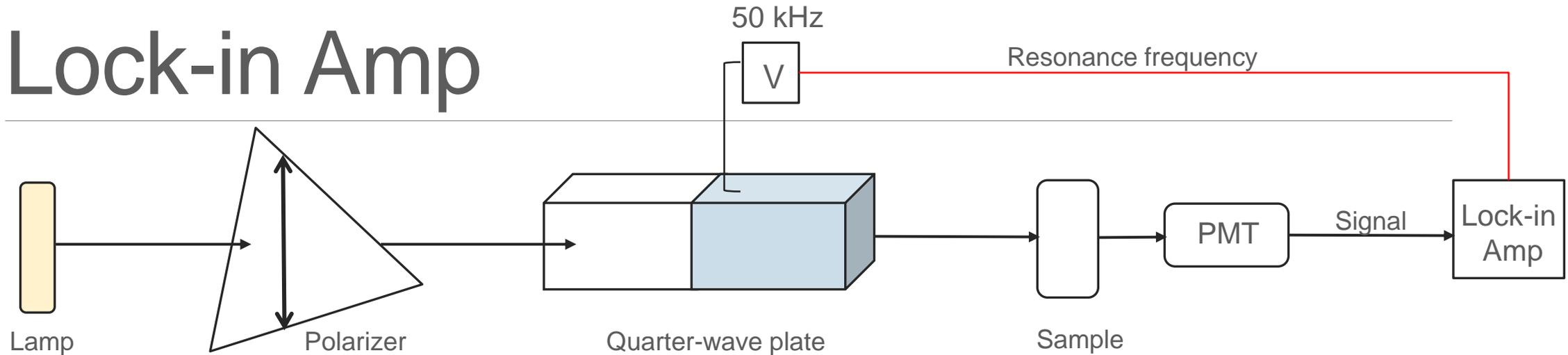
Photomultiplier Tube Detector

Absorption of photon \rightarrow emission of electron



Every time electron hits plates, more electrons fall off, amplifying the number of electrons

Lock-in Amp



- Since CD is the *difference* absorption in left- and –right CPL, the signal is very small
 - Detector measures difference ($A_L - A_R$)
- Lock-in amplifier is tuned to the resonance frequency of the PEM (i.e. the voltage applied to the crystal to create CPL)
- CD signal can now be detected from an extremely noisy environment

Circular Dichroism Applications

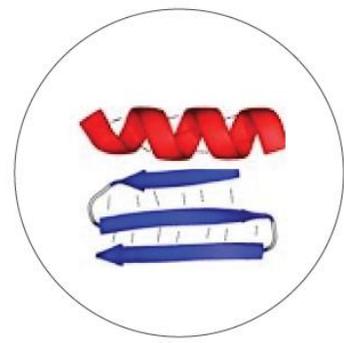
Circular Dichroism Applications

- I. **Structural Characterization of proteins**
- II. Antibody stability evaluation
- III. Thermal stability study
- IV. DNA and G-quadruplex structure
- V. Stopped flow folding study
- VI. Microassay methods

Structural Information Obtained from CD Measurements

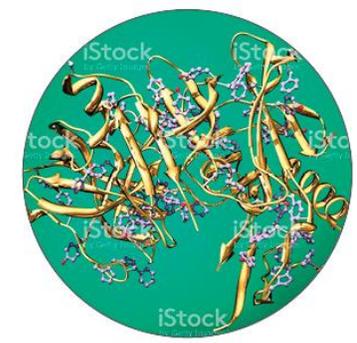


**Far-UV (< 260 nm)
Secondary Structure**



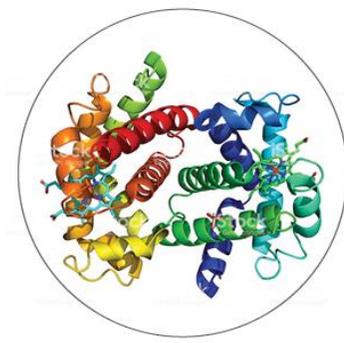
Chromophore: Peptide Bond
Sensitive to changes in the protein backbone bond angles and can be used to estimate secondary structure components.

**Near-UV (< 340 nm)
Tertiary Structure**



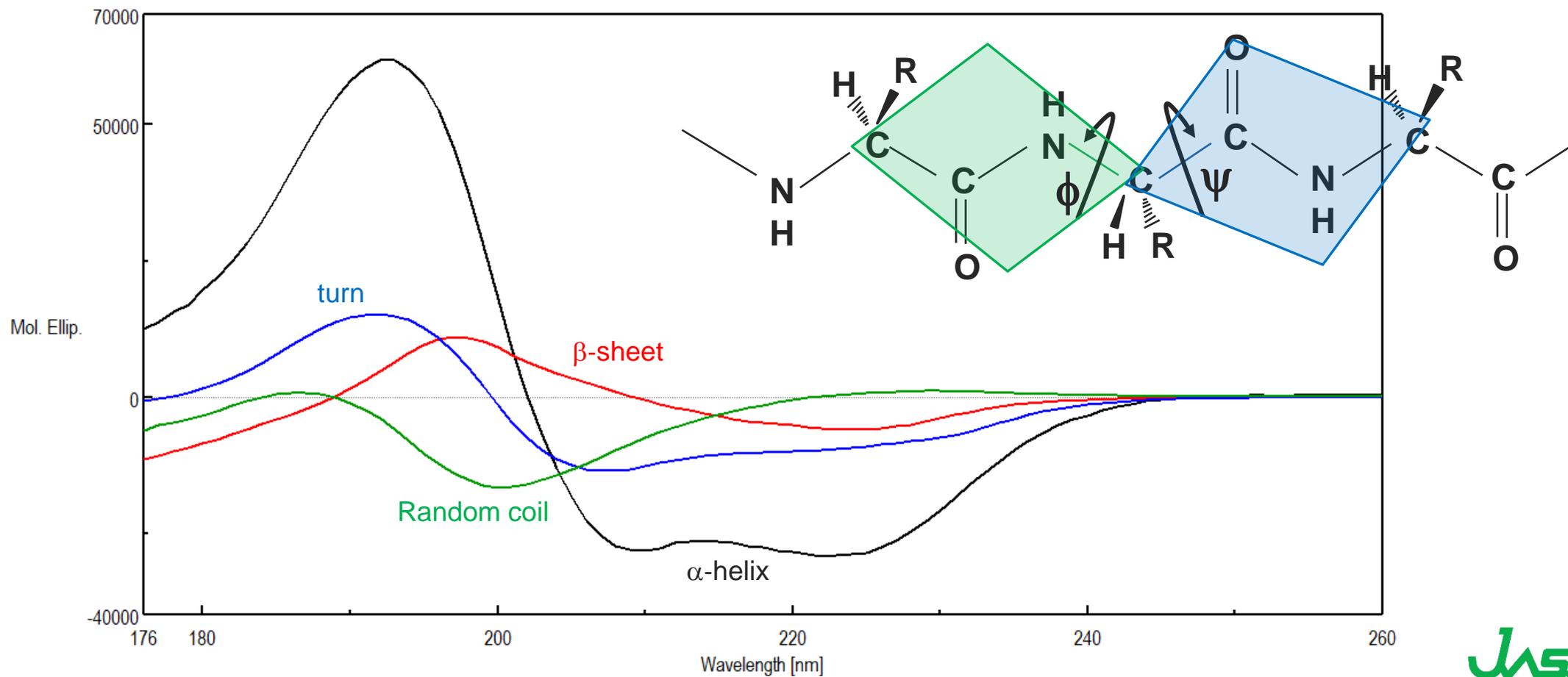
Chromophore: Aromatic Amino Acid Residues
Probes solvent environment and interactions of aromatic acid side chains, as well as the disulfide bonds.

UV-Visible

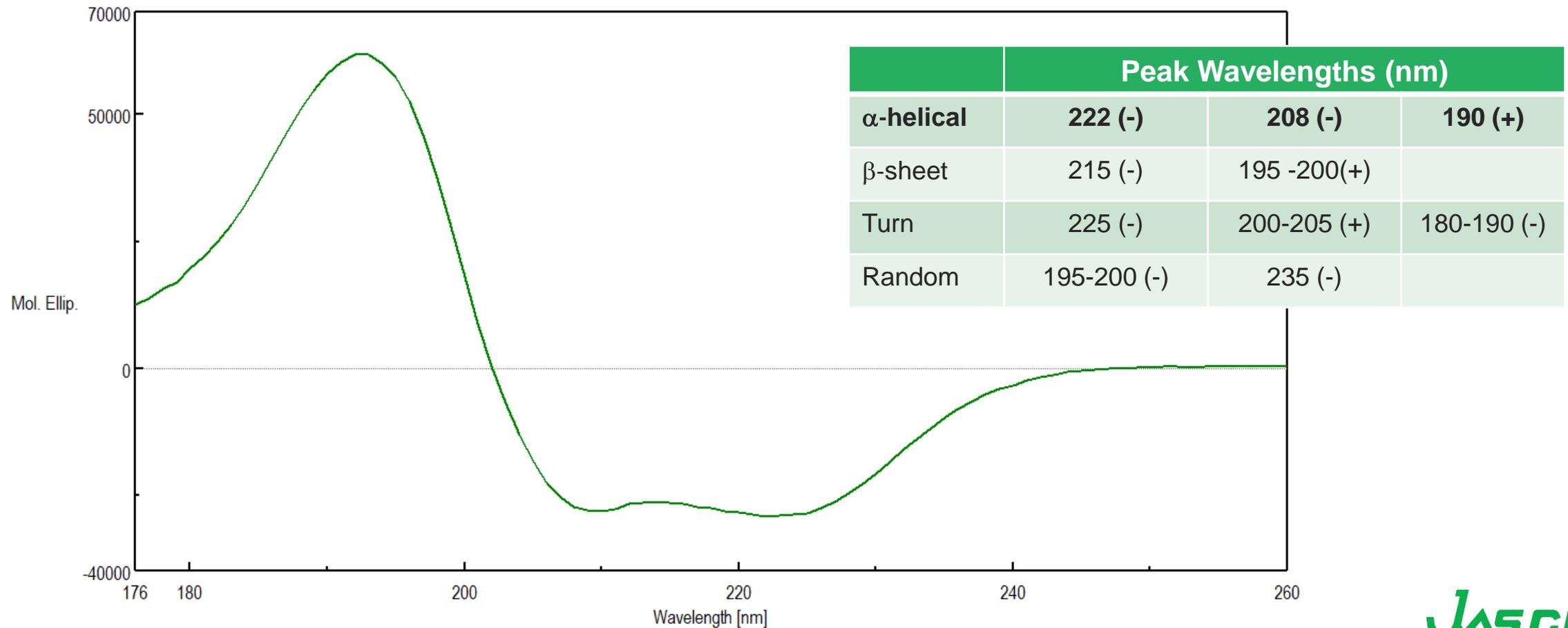


Chromophore: Prosthetic Group At visible wavelengths, structural information can be obtained for prosthetic groups such as heme in hemoglobin, although these are not strictly proteins.

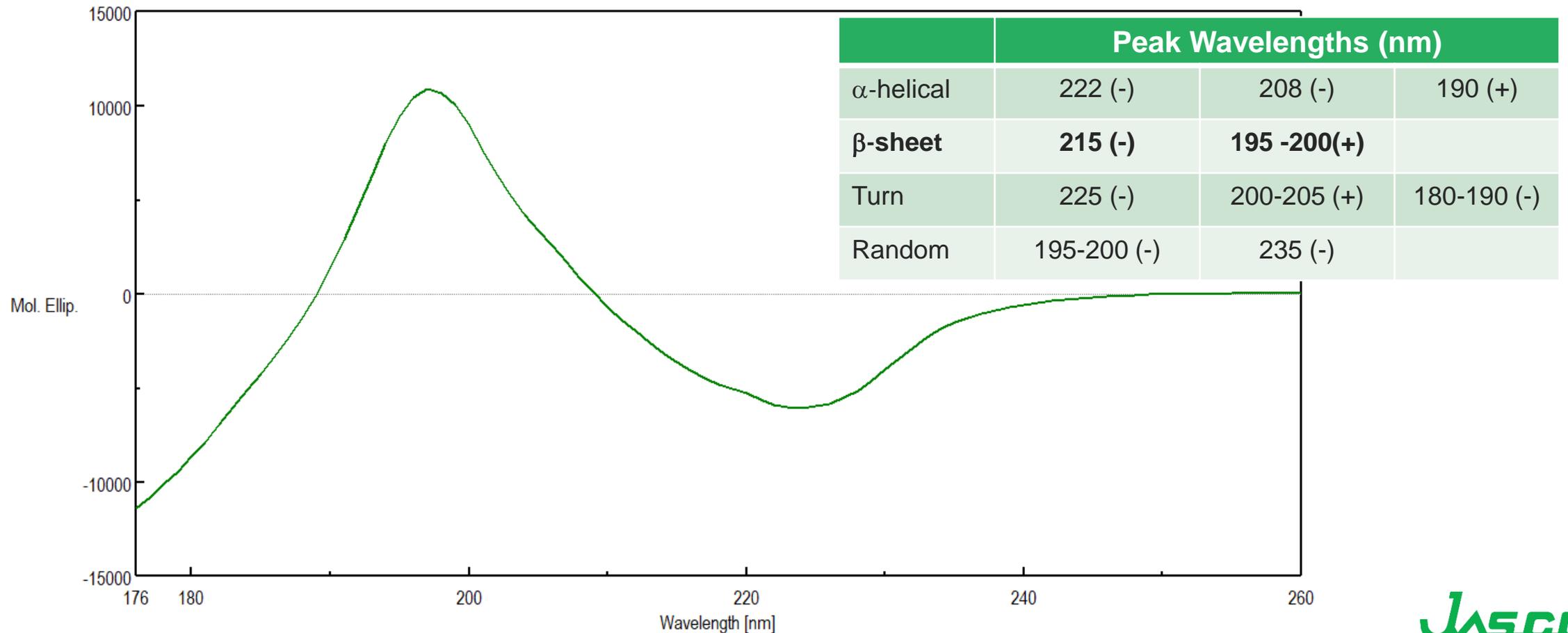
Far-UV CD spectra of protein solutions



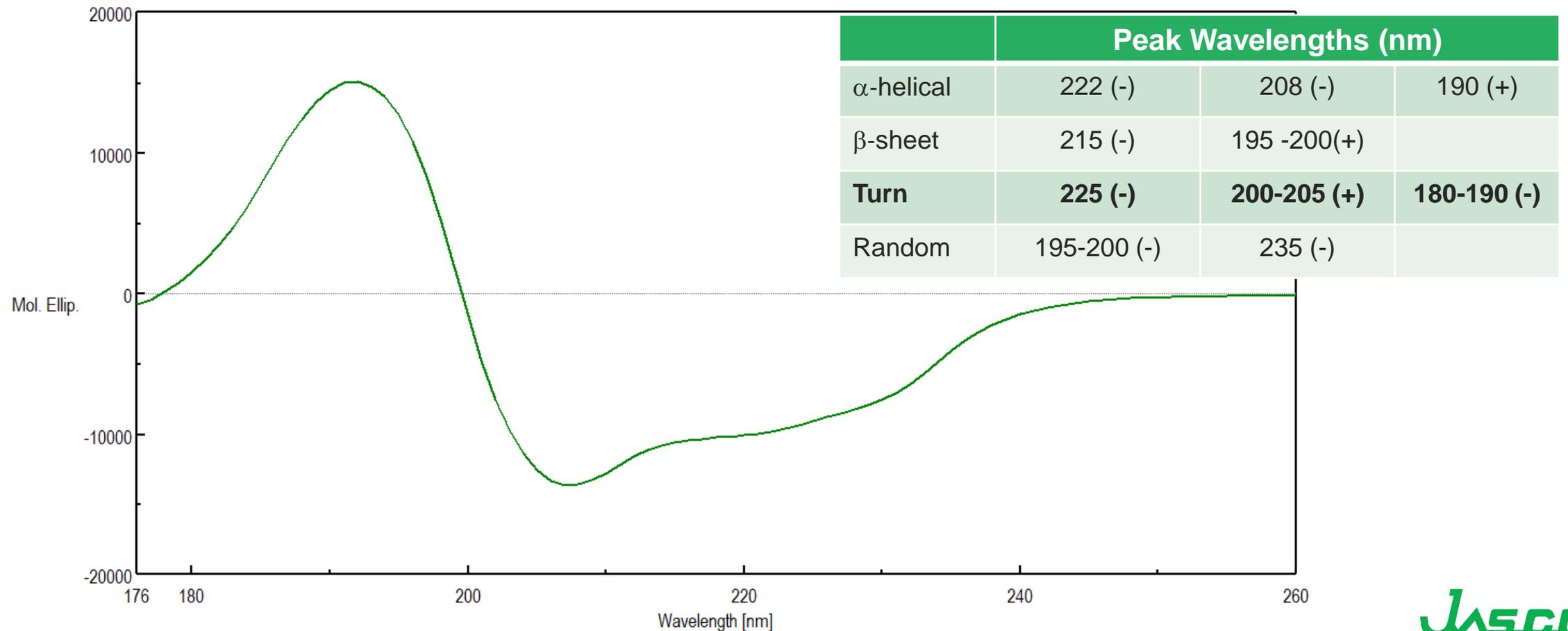
Far-UV CD spectra of protein solutions



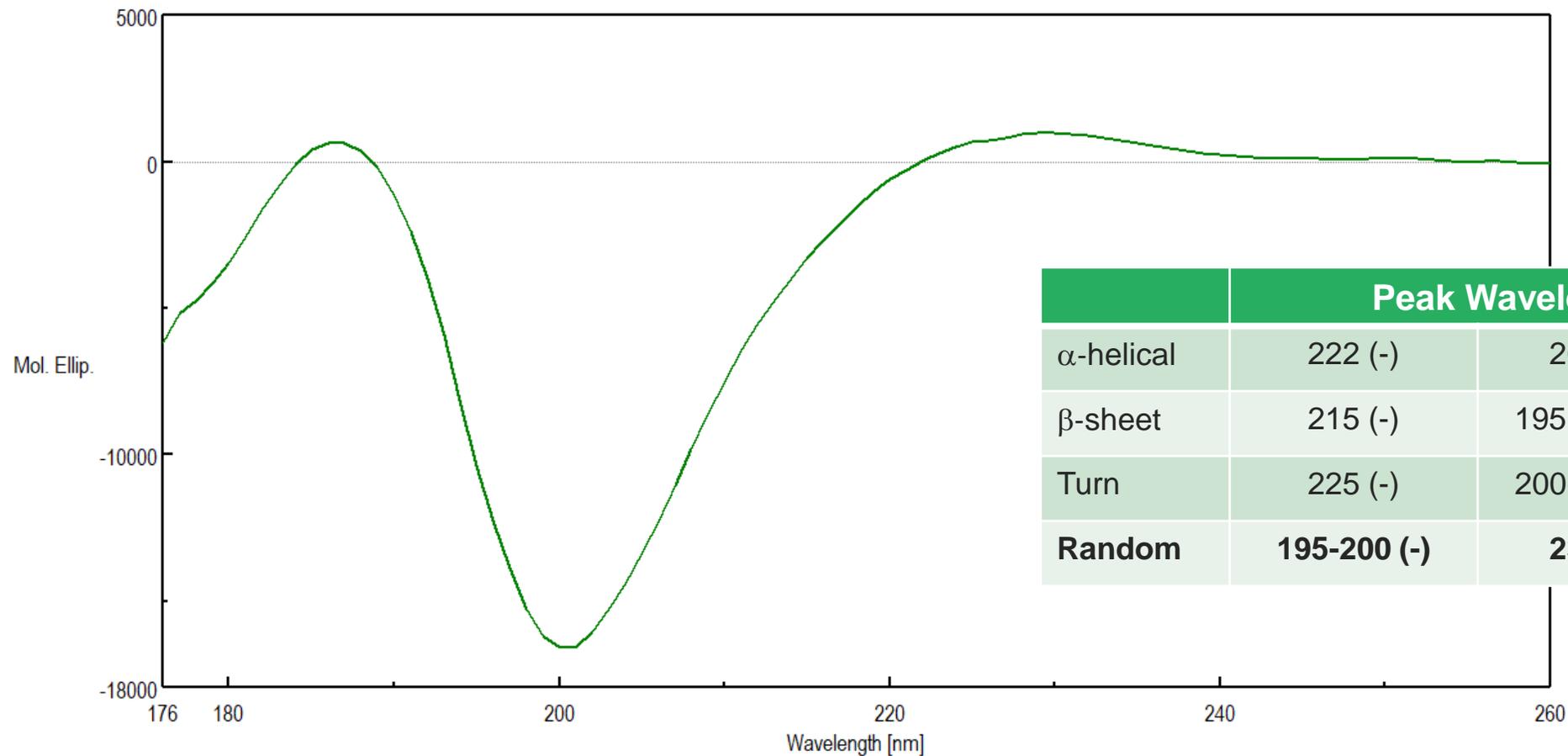
Far-UV CD spectra of protein solutions



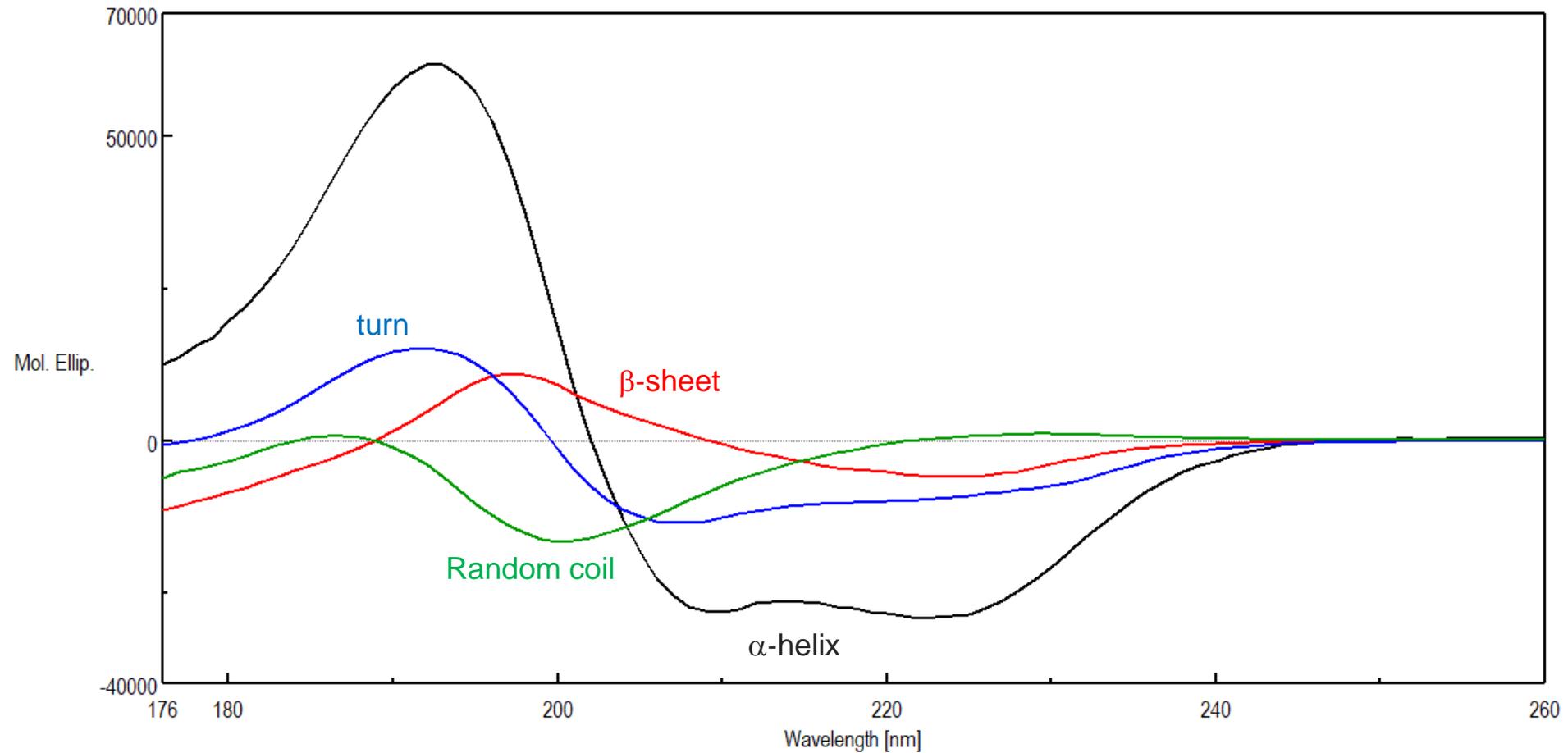
Far-UV CD spectra of protein solutions



Far-UV CD spectra of protein solutions



Far-UV CD spectra of protein solutions



Protein Secondary Structure Estimation

The CD spectrum of a protein can be expressed as the sum total of its CD spectra component (α -helix, β -sheet, turn, and random coil), multiplied by their respective abundance ratios.

$$[\theta]_{\lambda} = f_{\alpha}[\theta]_{\alpha} + f_{\beta}[\theta]_{\beta} + f_t[\theta]_t + f_u[\theta]_u$$

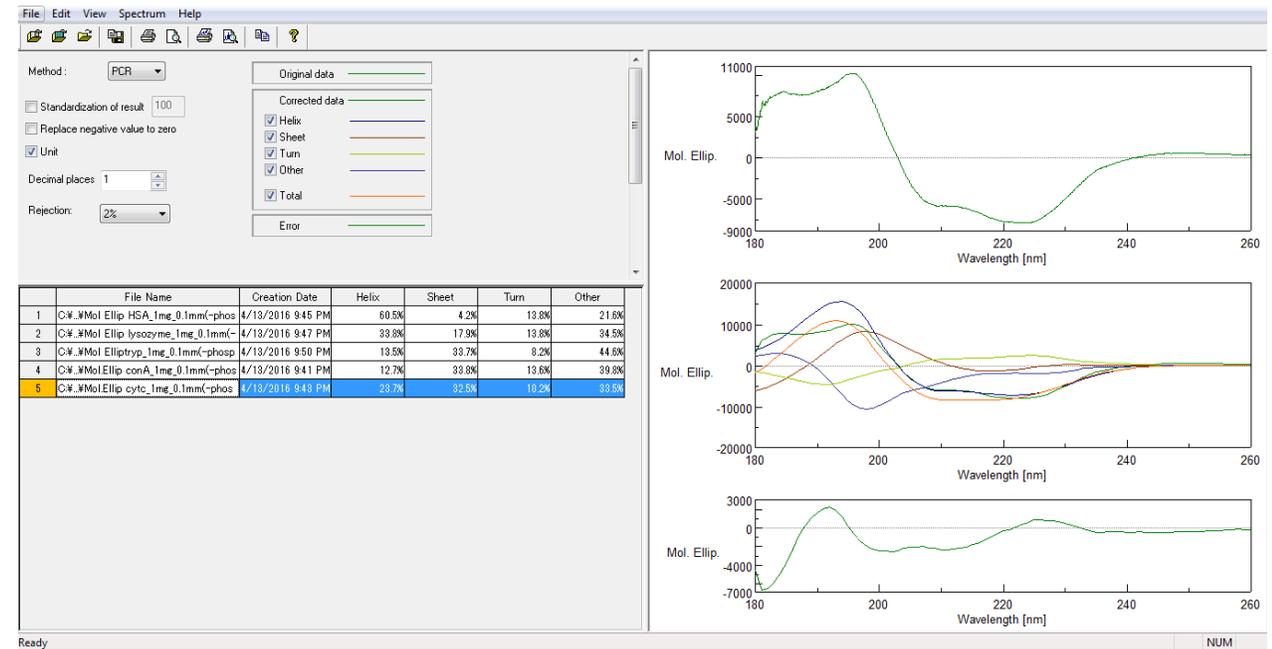
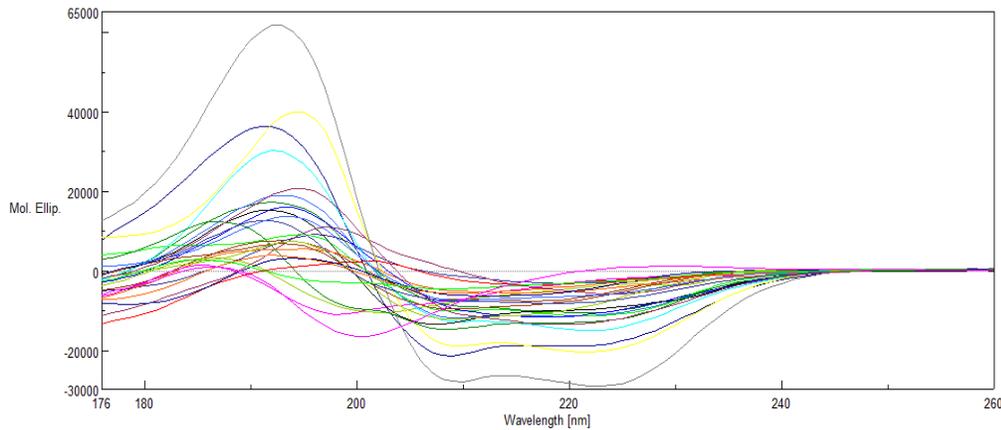
$\theta_{\lambda,i}$: ellipticity at each wavelength of each i th secondary structure component (CD Spectra)

f_i : fraction of each secondary structure

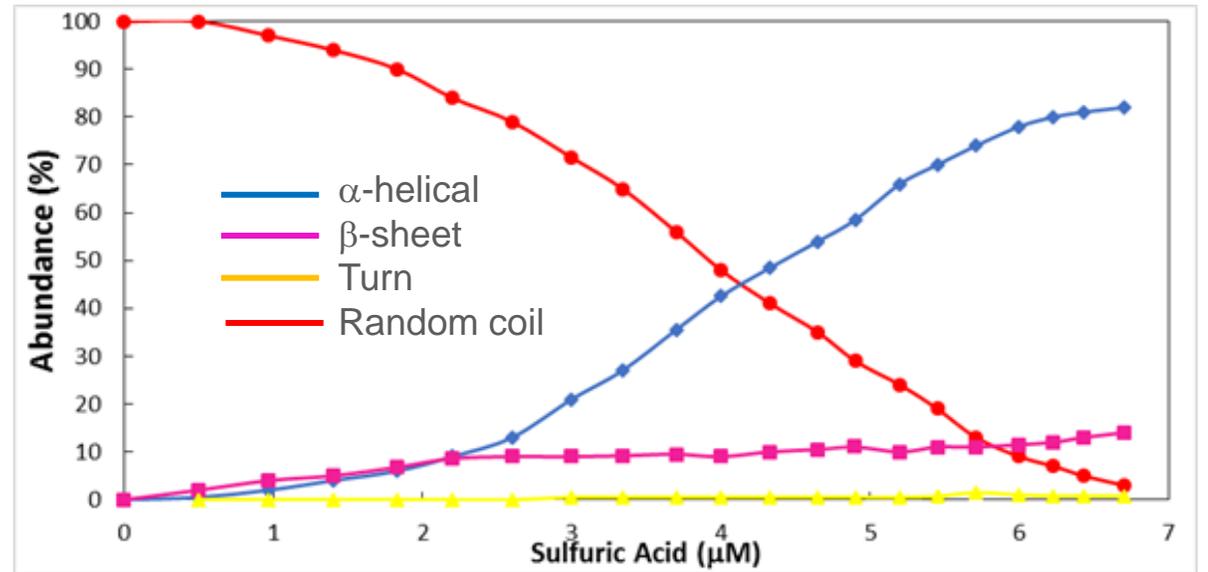
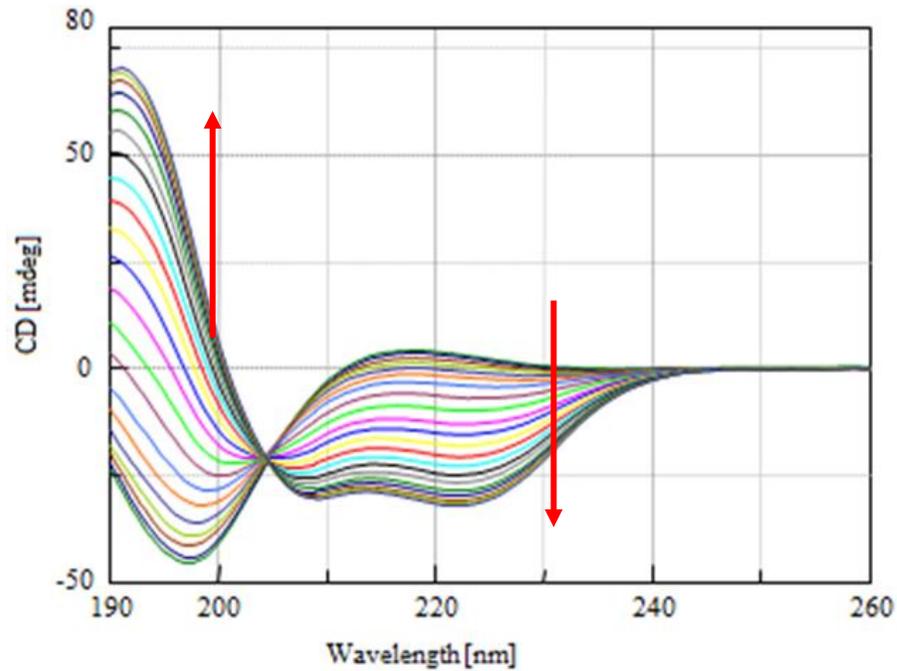
Secondary structure abundance ratios are determined using different component analysis and regression techniques to ensure the minimum distance between measured and calculated spectra.

CD Multivariate SSE Program

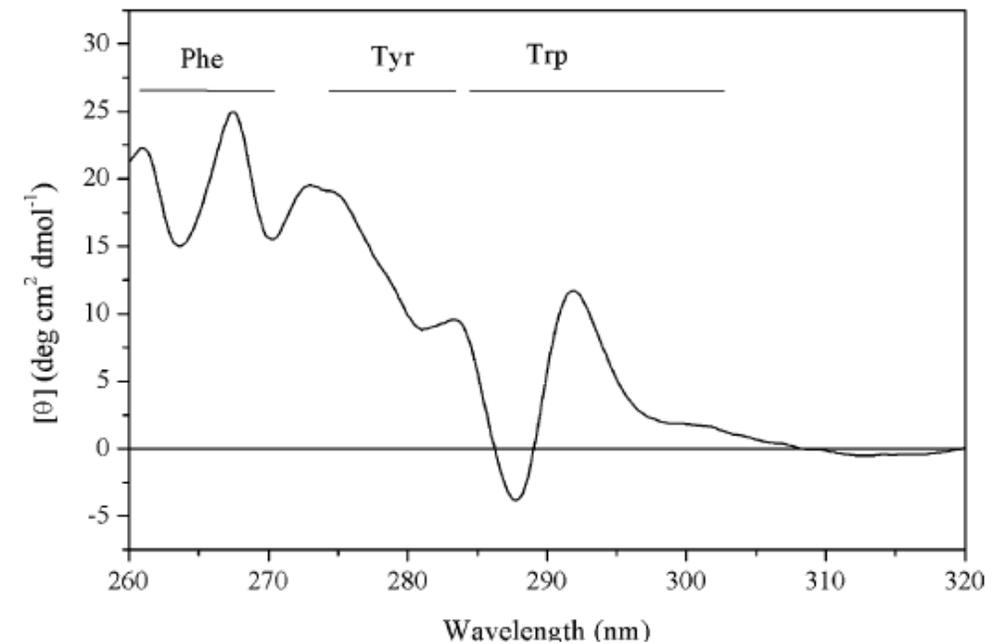
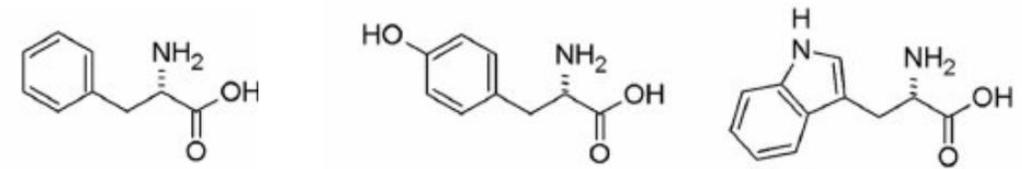
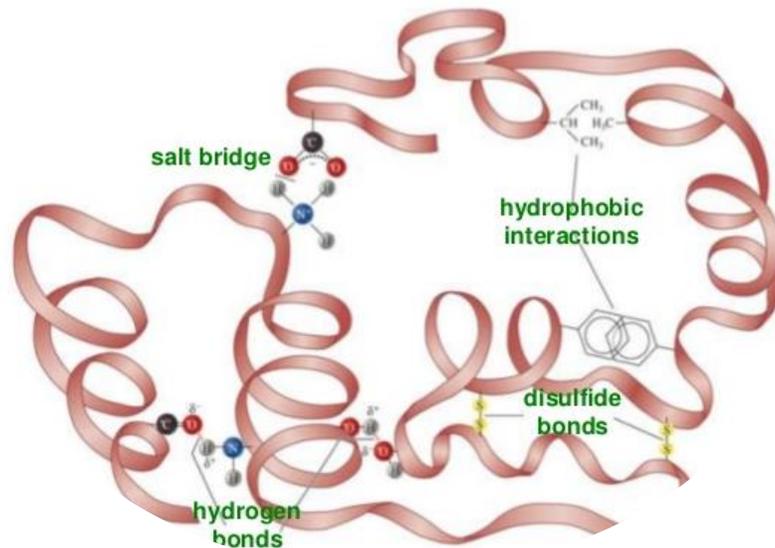
A calibration model for secondary structure abundance ratios obtained by X-ray crystallography and CD is produced using PCR or PLS, which is then used to estimate the unknown secondary structure of proteins.



Peptide titration with dilute sulfuric acid



Near-UV CD spectra of protein solutions

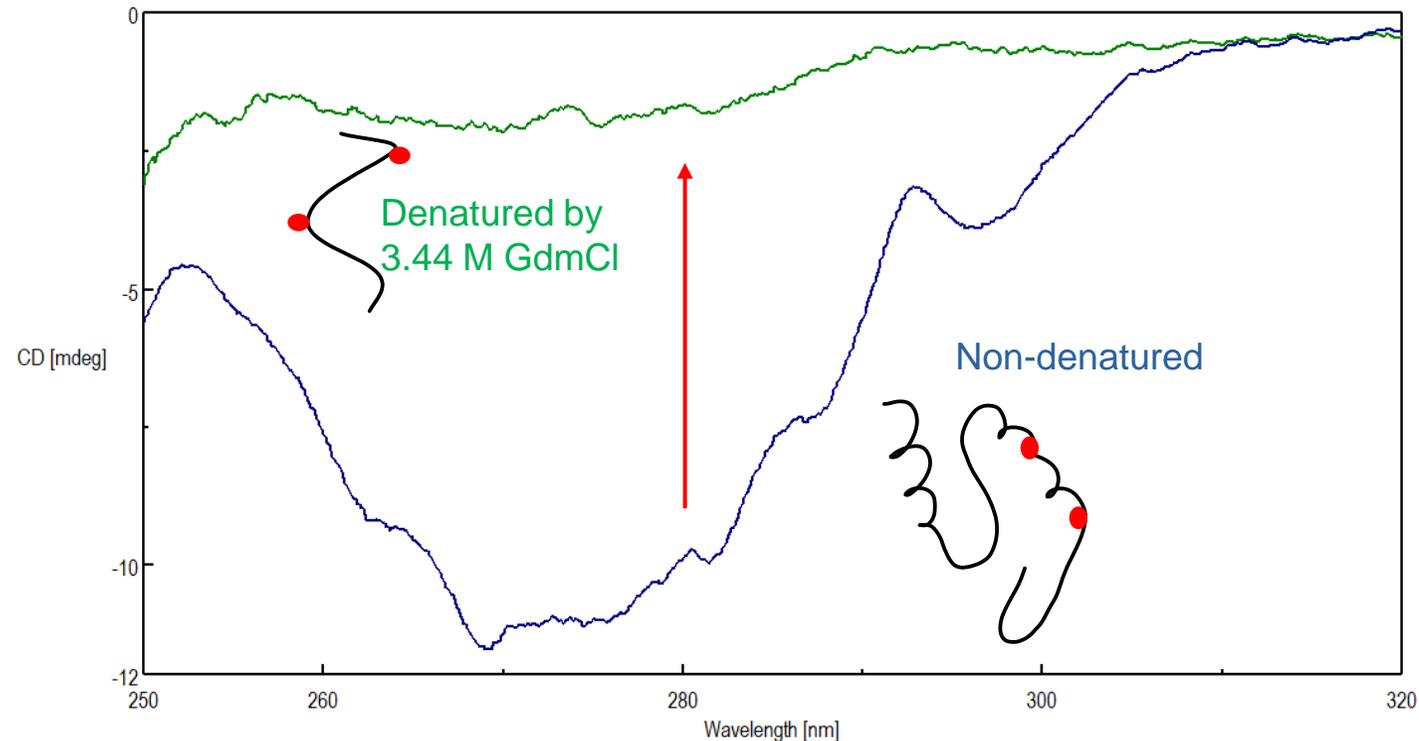


Factors that influence Near-UV Spectra

- Solvent environment (hydrogen bonding, polar groups)
- Interactions with aromatic amino acid residues (distance)
- Rigidity of protein
- Number of aromatic amino acid residues in protein

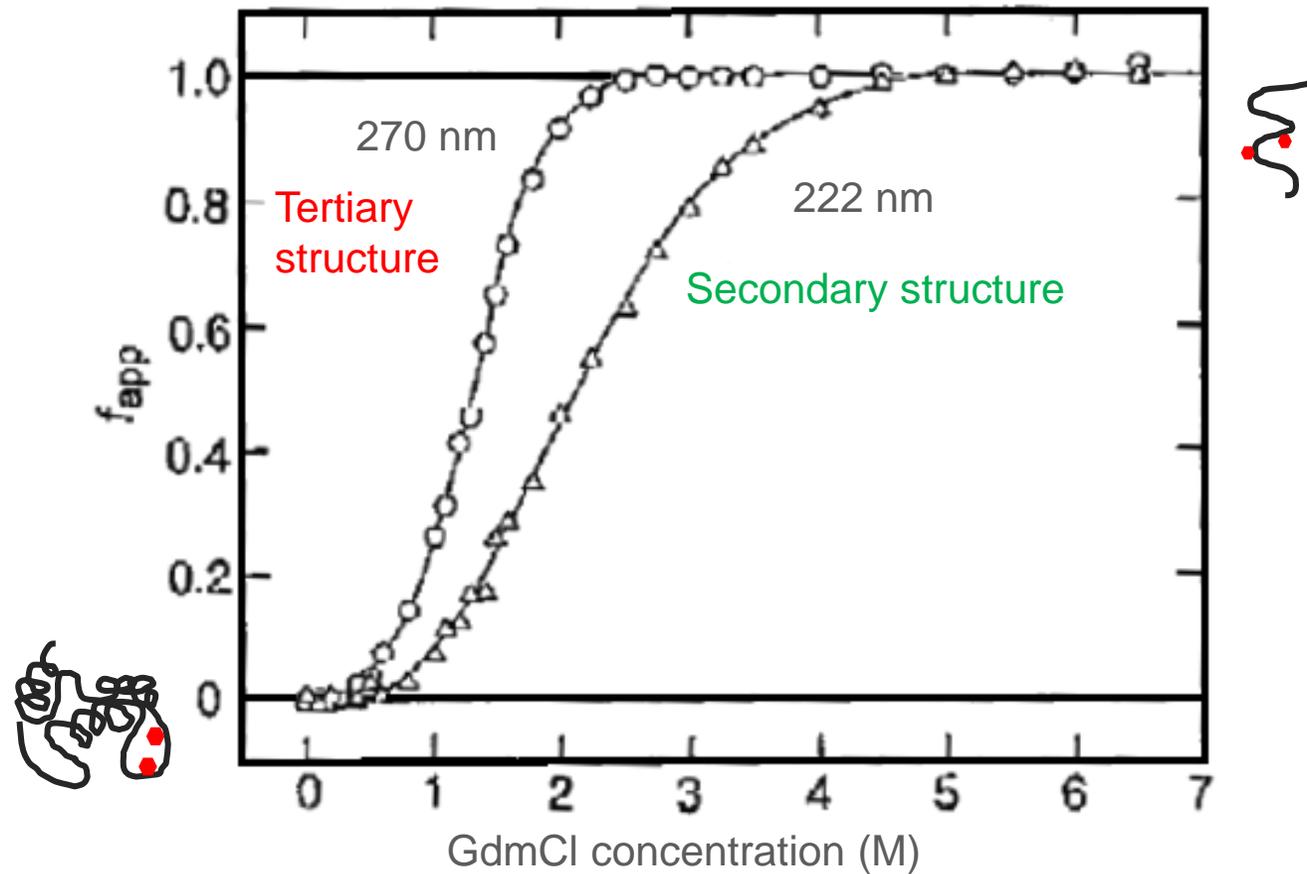
Chemical denaturation of apo- α -lactalbumin

The spectral changes suggest that the aromatic amino acids in the interior of the protein were exposed as a result of protein unfolding under denaturant conditions



●: Aromatic amino acid

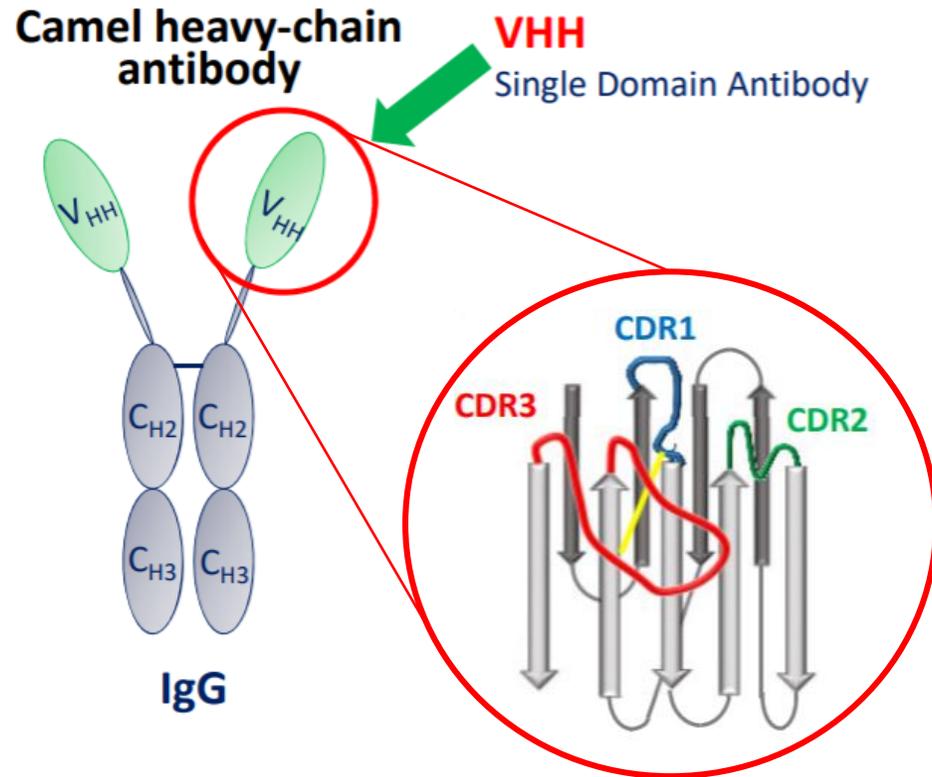
Chemical denaturation of apo- α -lactalbumin



Circular Dichroism Applications

- I. Structural Characterization of proteins
- II. Antibody stability evaluation**
- III. Thermal stability study
- IV. DNA and G-quadruplex structure
- V. Stopped flow folding study
- VI. Microassay methods

VHH Antibodies



- Highly stable with respect to heat, pH, and denaturing agents
- High affinity and specificity due to long CDR3
- Low molecular weight (~15 kDa) increases mass production efficiency
- Unique binding capacity to small cavities
- High solubility (good imaging agents)
- Easily modified

The effects of ambient environmental factors such as temperature, pH, salt concentration may cause antibody drugs such as VHH and IgG to undergo a change in their higher order structure, so that they lose their activity and function.

Evaluation of VHH structural changes due to pH and salt concentration

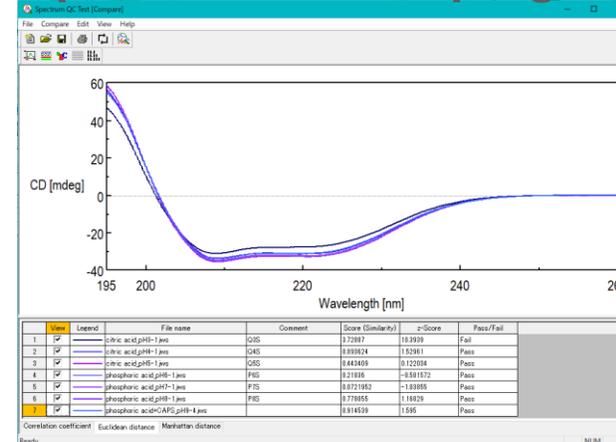
High Throughput CD System



Automatic measurements of multiple samples

- Sample aspirating, measurement, and flow path washing and drying are fully automated
- Automatic measurement of up to 192 samples
- Runs all night for high operational efficiency

Spectrum QC Test program

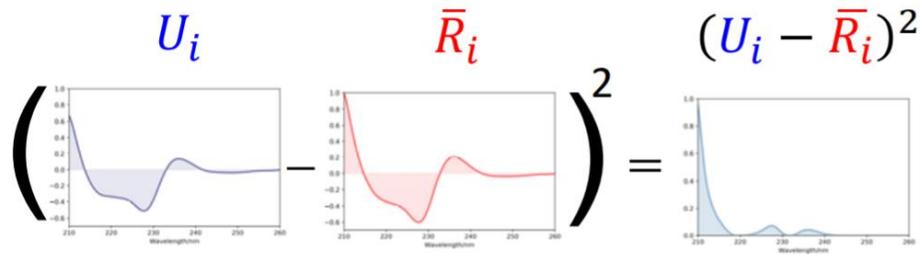


Quantification and judgement of changes in spectra

- Automated judgement whether or not there have been changes in spectra based on statistical methods
- Evaluation of stability and structural comparability of antibodies, peptides, and nucleic acid drugs

Quantifying Spectral Differences with the Spectrum QC Test program

Step 1: Square the difference spectrum

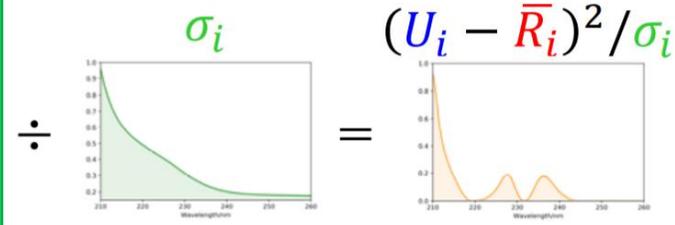


U_i : Denatured CD spectrum

\bar{R}_i : Average of native spectra

σ_i : Standard deviation of noise for unknown spectrum

Step 2: Weighting



Step 3: Quantitation

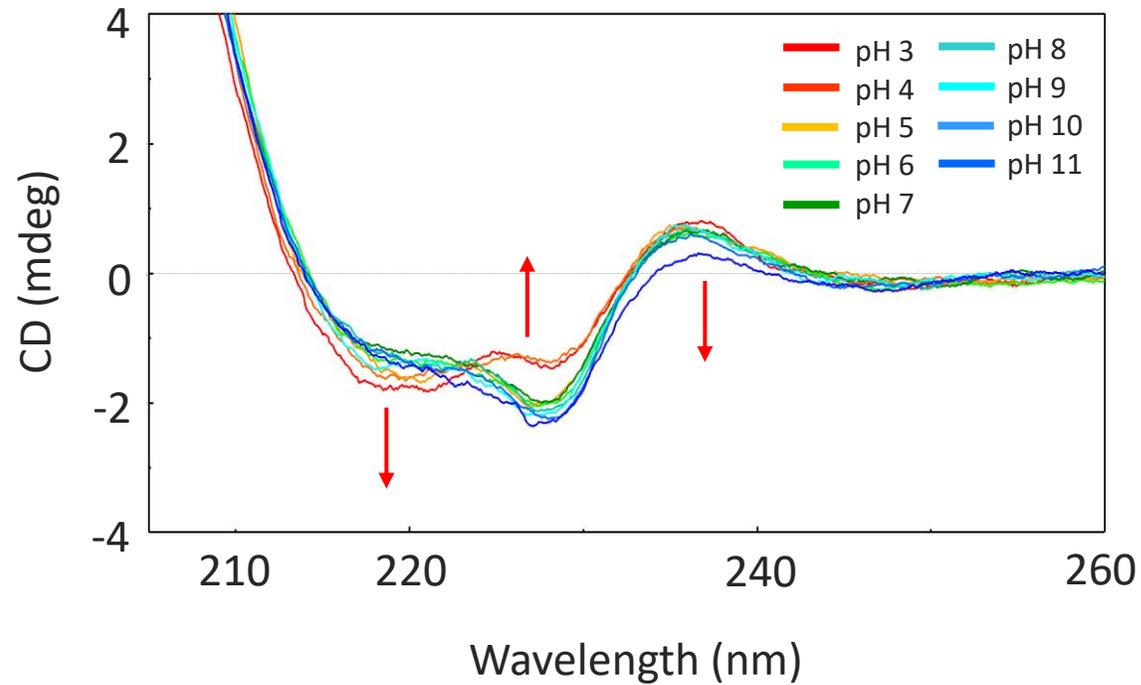
$$I = \sqrt{\frac{1}{n} \sum_{i=\lambda_1}^{\lambda_n} (U_i - \bar{R}_i)^2 / \sigma_i}$$

Instrument noise is larger at shorter wavelengths where there is less light throughput so the effects of noise must be reduced to detect slight changes.

- Change is evaluated as small where noise is larger, and large where effects of noise are smaller..
- Slight changes in sample differences are detected with high sensitivity.

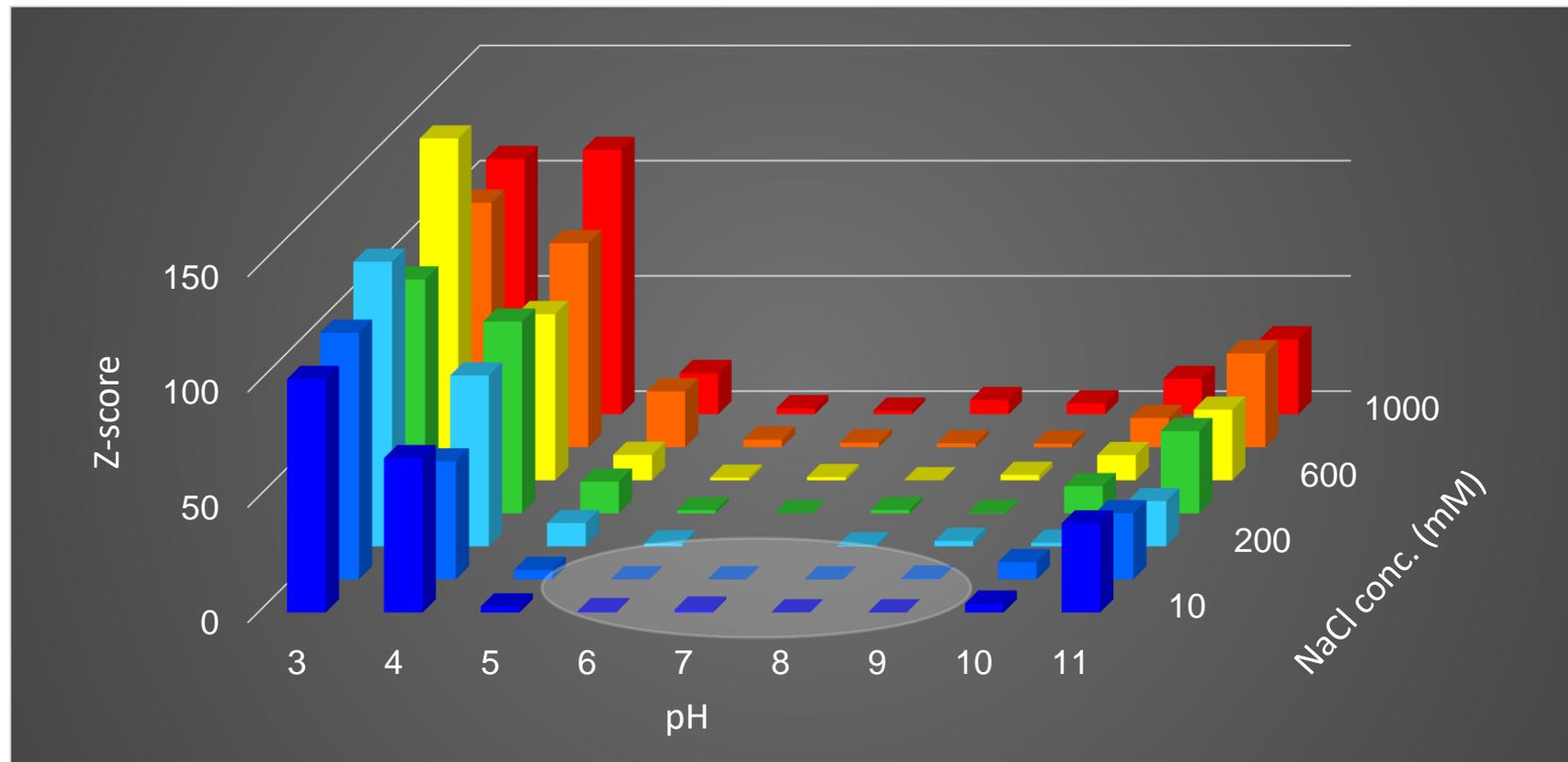
Measurement conditions and CD spectra

The CD spectra of VHH at various pH and salt concentrations were measured. Differences in the spectra from the native spectra were quantified.



Quantifying changes in the CD spectra

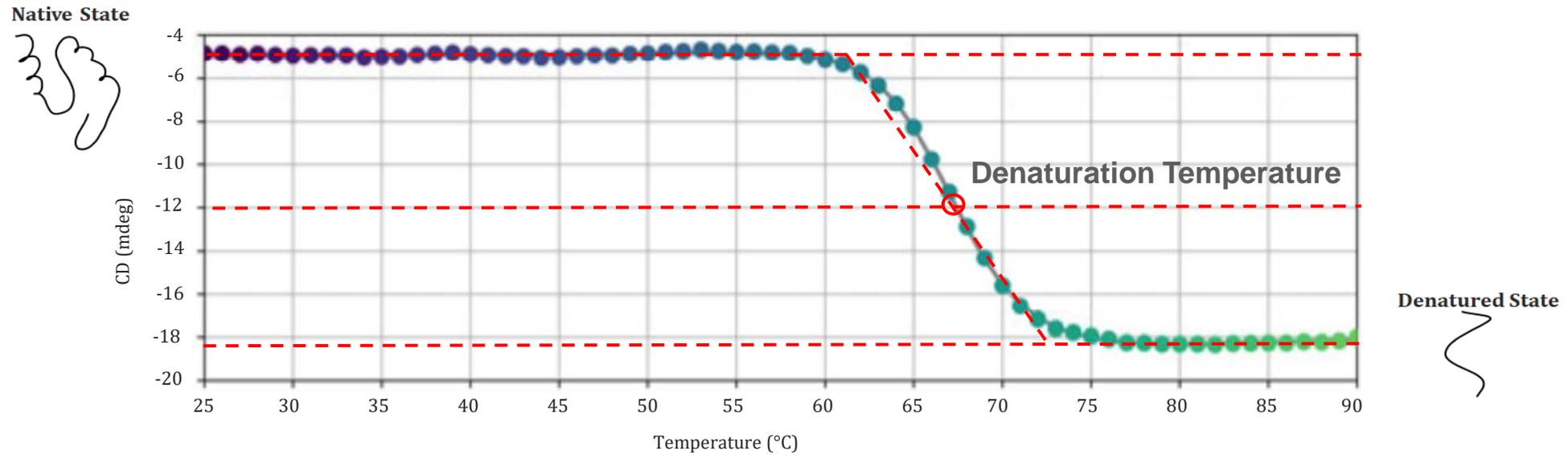
The larger the Z-score, the greater the distance or difference between the native and measured spectra.



Circular Dichroism Applications

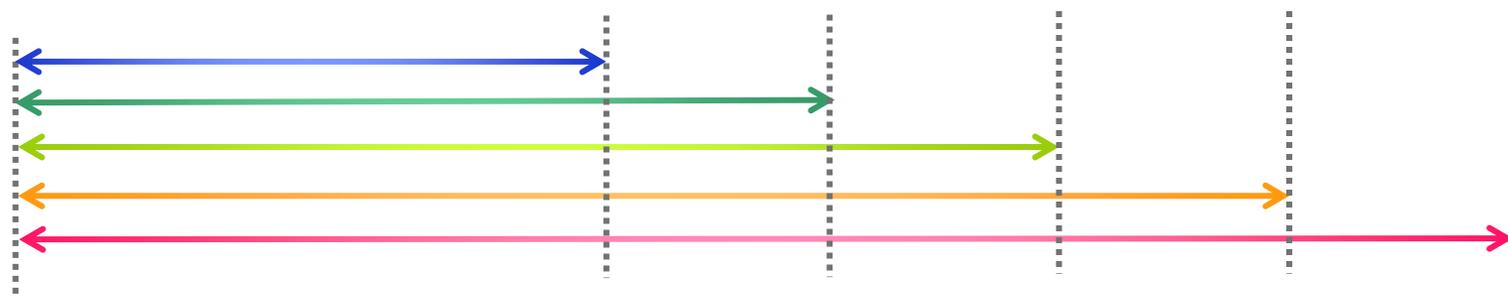
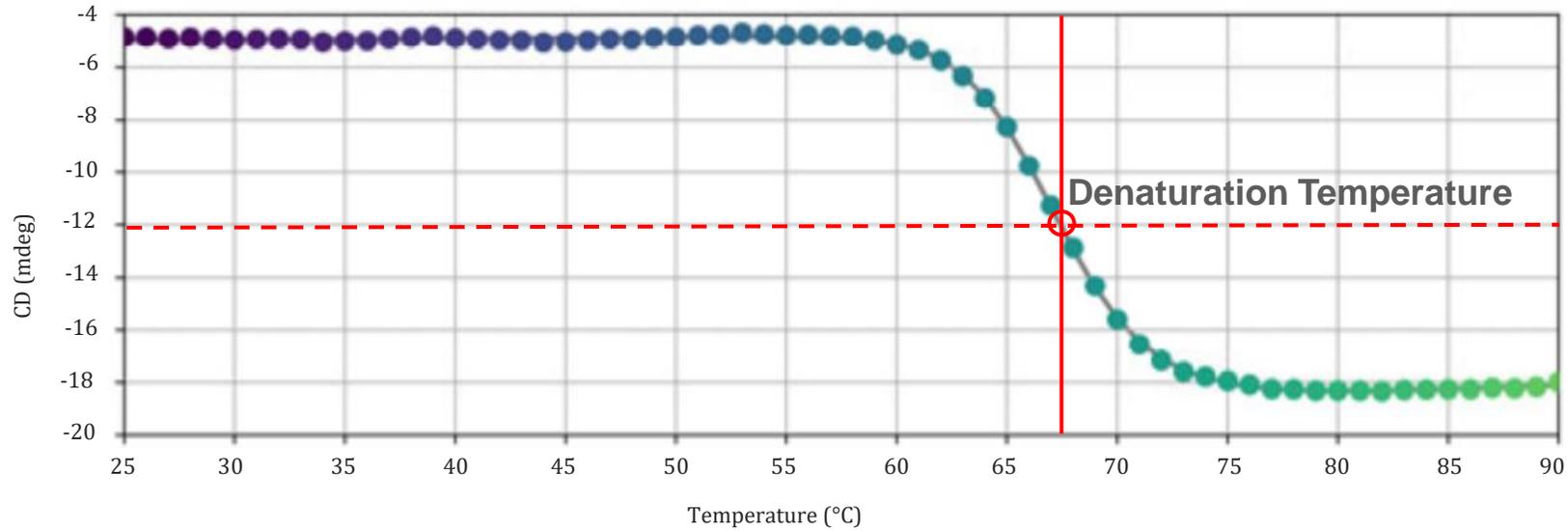
- I. Structural Characterization of proteins
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Method for evaluating the thermal stability of proteins

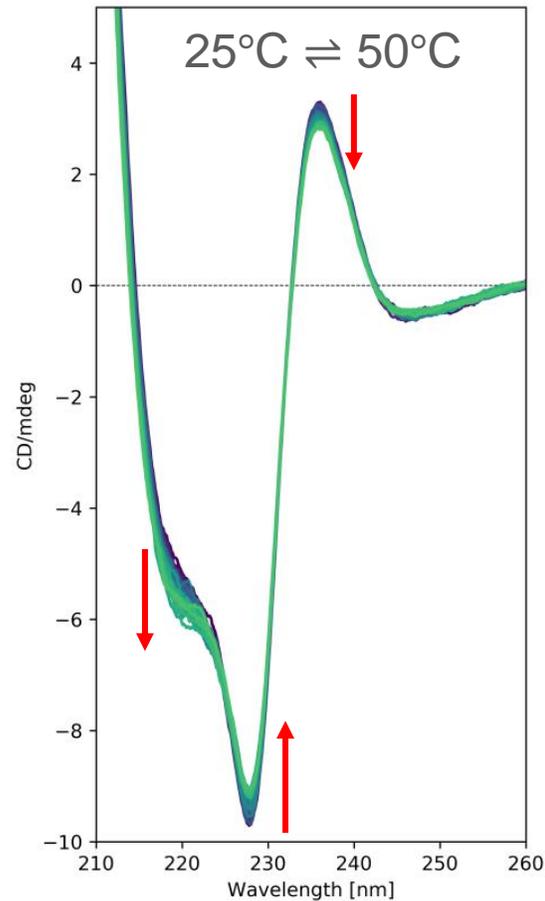


	T_m (°C)	ΔH (kJ/mol)	ΔS (J/mol·K)
Sample 1	47.40 ± 0.067629	820.558 ± 44.389	2559.83 ± 138.477
Sample 2	47.61 ± 0.065995	782.463 ± 40.3946	2439.37 ± 125.932

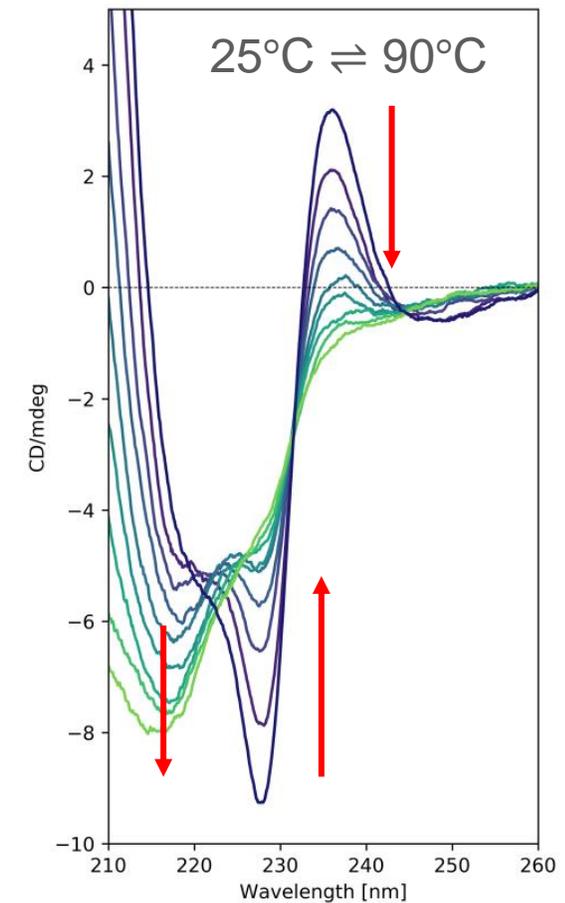
Thermal stability and reversibility of VHH



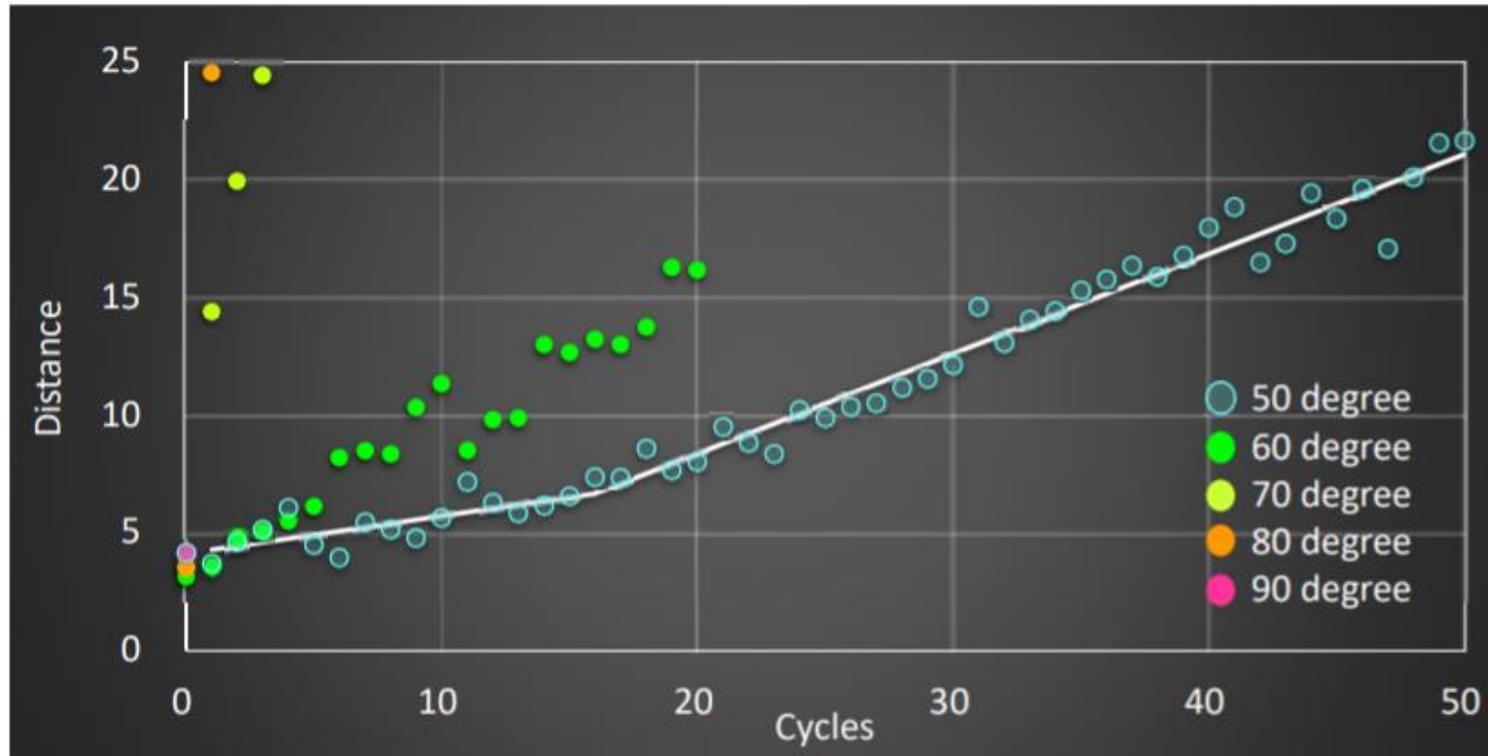
Thermal stability of VHH



- At 50°C, there is a slight change in the CD spectrum.
- At 90°C, the spectrum changes greatly after the temperature is raised and lowered once, indicating that the secondary structure has changed.



Thermal reversibility



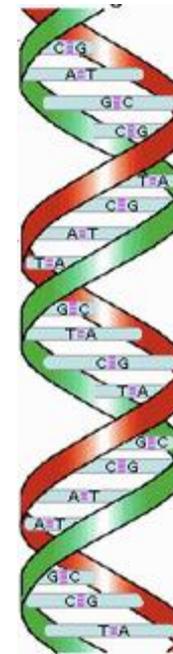
At 50°C, the slope increases sharply from the 16th cycle, indicating that the structure changed as a result of repeated increases and decreases in temperature, even though 50°C was lower than the denaturation temperature.

Circular Dichroism Applications

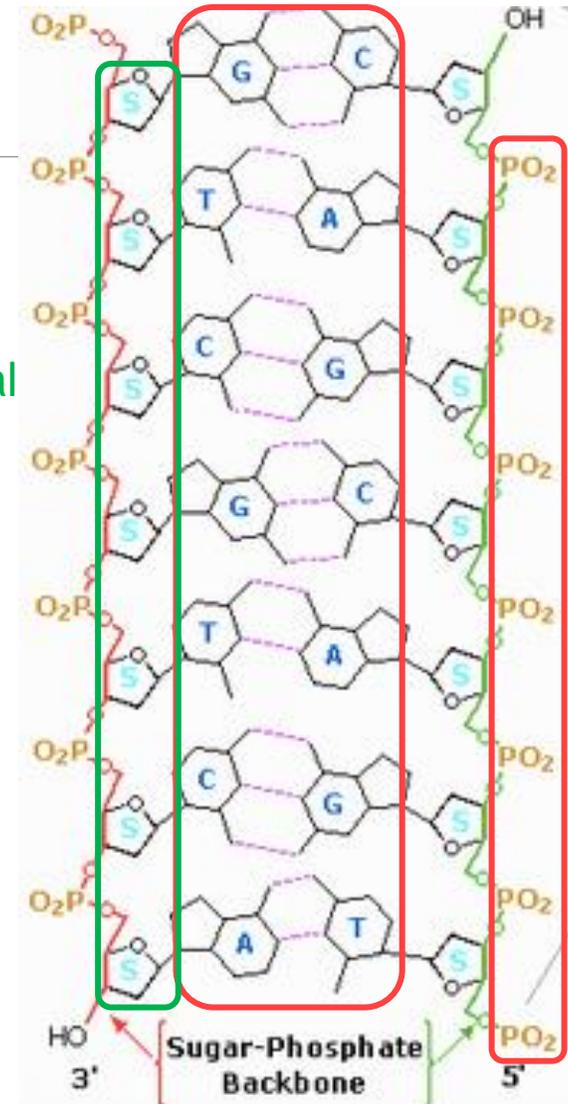
- I. Structural Characterization of proteins
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Inducing chirality into an electronic transition: DNA

- CD observed because chiral sugar units bonded to base and phosphate groups.
- Measure CD induced into transition of bases as a result of coupling with backbone transitions.
- Spectrum arises from $\pi \rightarrow \pi^*$ transitions of stacked bases (200-300 nm).



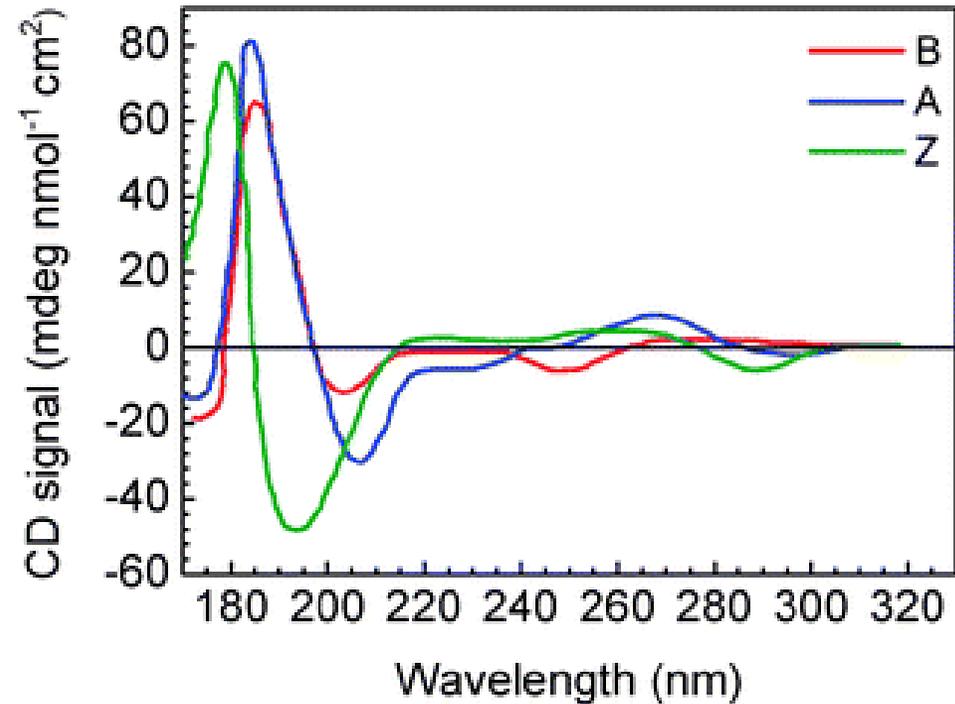
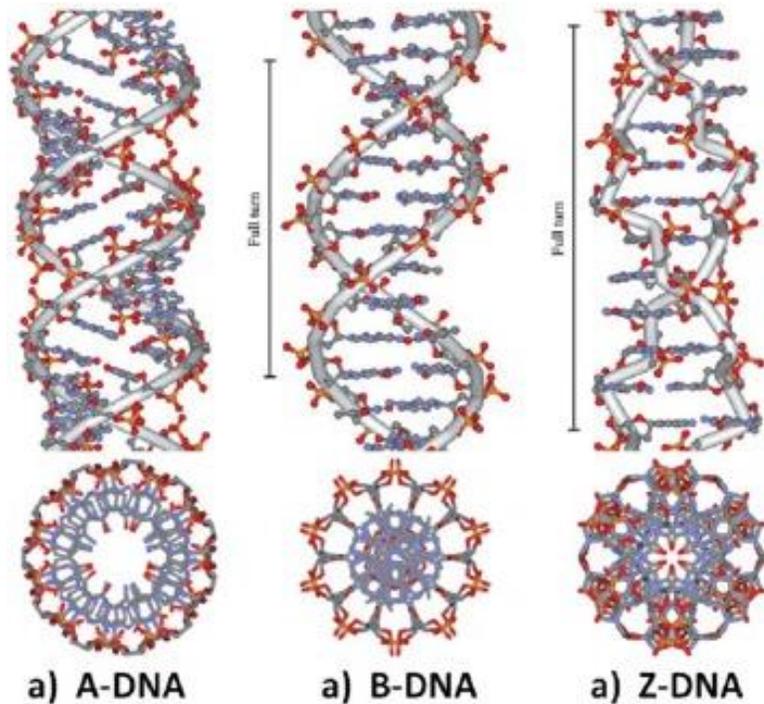
Chiral



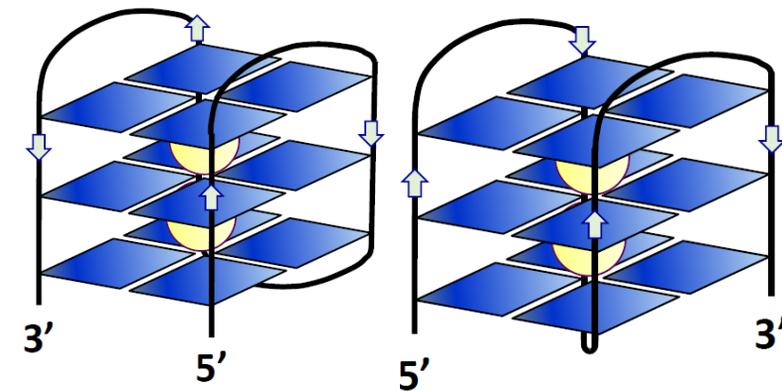
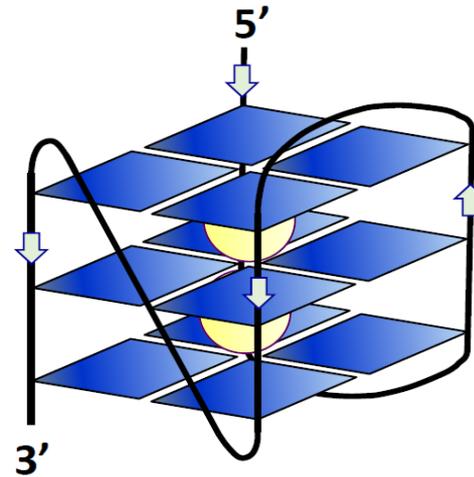
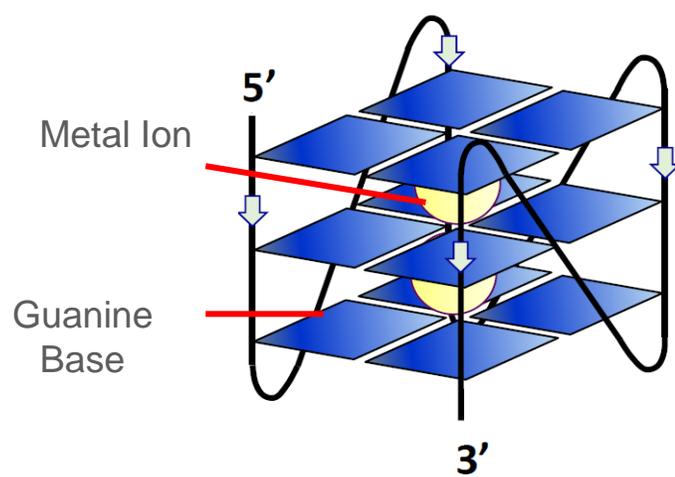
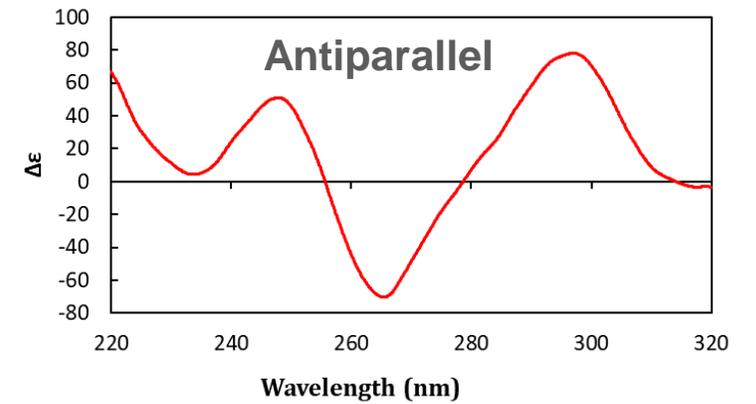
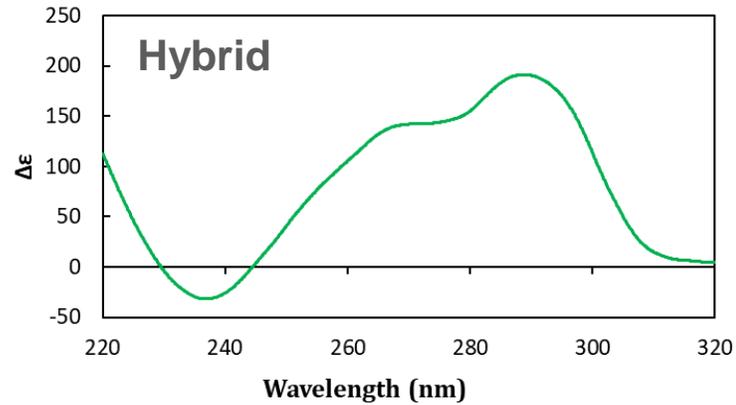
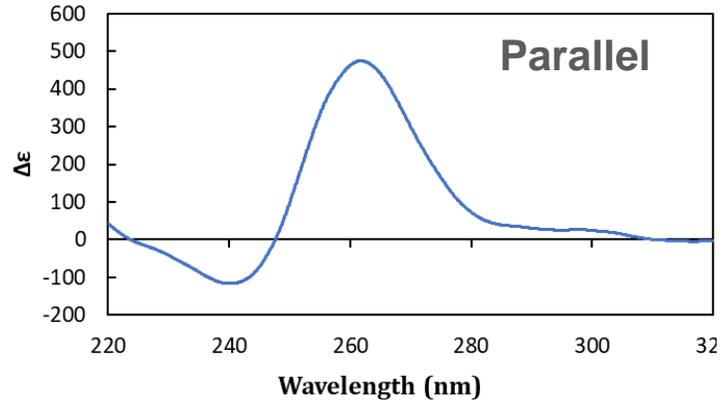
Achiral

JASCO

DNA Structure Studies



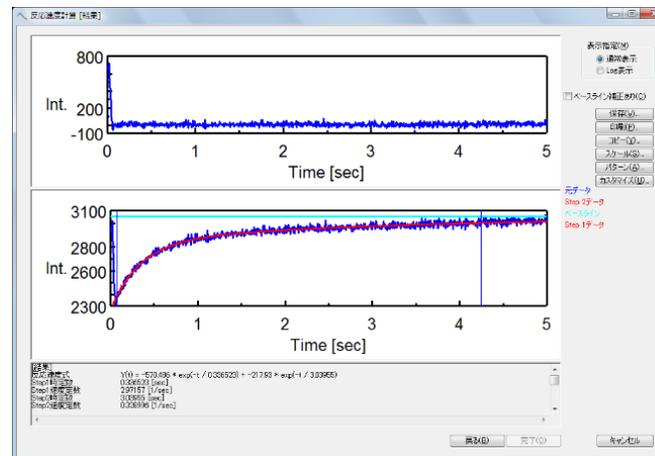
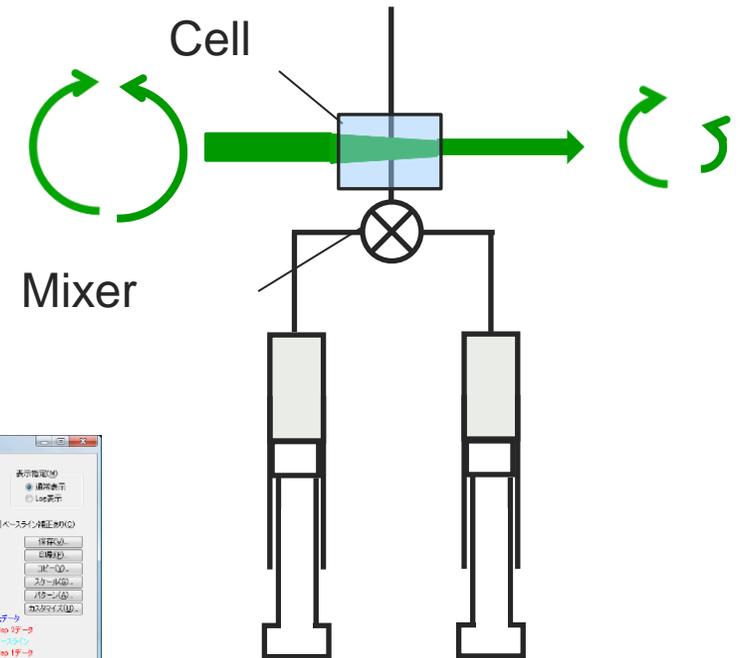
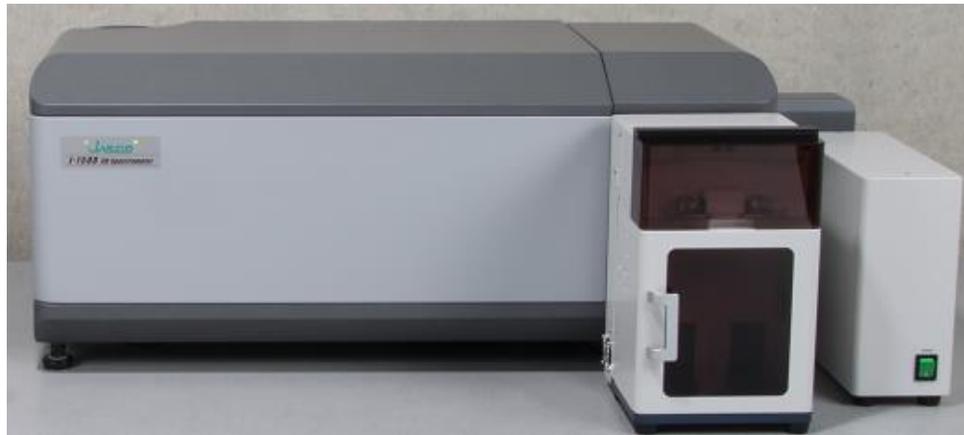
G-quadruplex topology



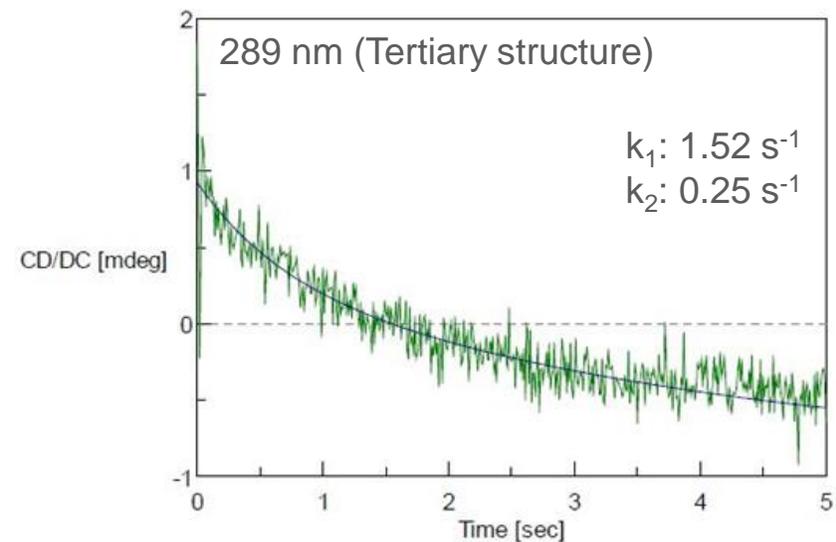
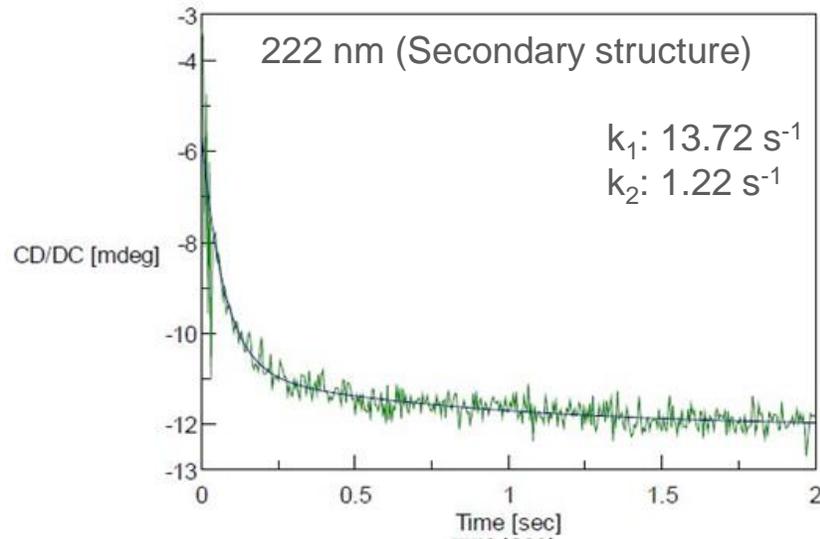
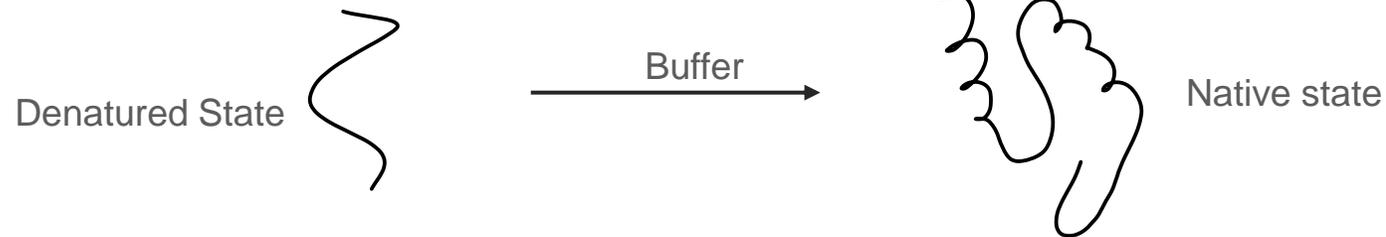
Circular Dichroism Applications

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Stopped flow method



Tracking the refolding of cytochrome c



The rate constant is smaller for the near-UV than for the far-UV, indicating the tertiary structure of the protein refolds slower than the secondary structure.

Circular Dichroism Applications

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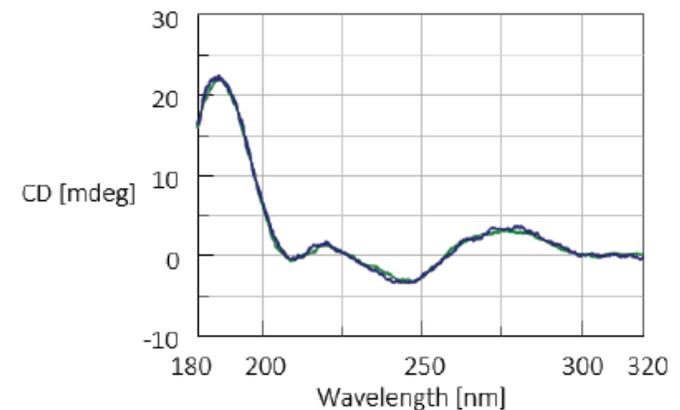
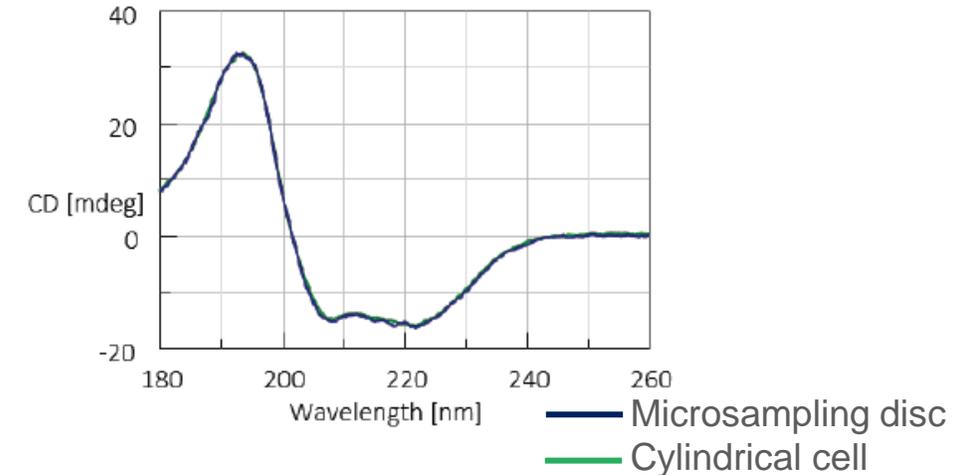
Structural characterization using a microsampling disc



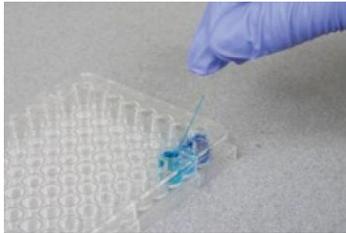
2 μL : pathlength 0.2 mm
10 μL : pathlength 1 mm \rightarrow 1 $\mu\text{g}/\text{sample}$



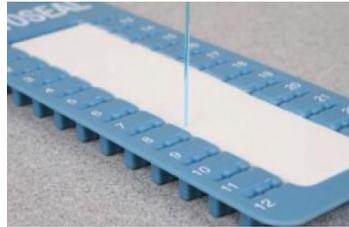
1. Drop the sample on the disk with micropipette
2. Put the cover in place
3. Place the disk in the sample compartment



Thermal denaturation measurement using a capillary cell



1. Sample is drawn into a capillary



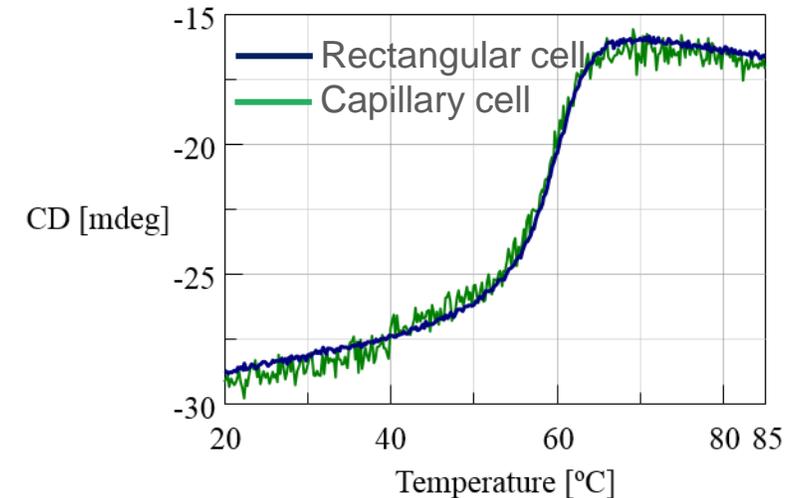
2. The end of the capillary is closed with sealant



3. The capillary is placed in a cell jacket and into the Peltier cell holder.

Approximately 10 μL (0.5 mm pathlength) \rightarrow ~ 2 μg /sample

S/N is lower than rectangular cell because smaller aperture but the melting temperature can be obtained from the melting curve.



JASCO Educational Resources

Upcoming Webinars:

- Vibrational Circular Dichroism
- FTIR Theory, Instrumentation, and Techniques
- FTIR Microscopy
- Circular Dichroism Measurement Optimization
- Raman Microscopy and Imaging
- SFC Theory and Applications

E-books and Tips and Tricks Posters

- Raman
- Fluorescence
- FTIR
- CD

KnowledgeBase

**NEXT WEBINAR WILL BE ON
VCD THEORY AND APPLICATIONS**

**DR. CARLOS MORILLO
TUESDAY APRIL 21TH AT 2:00 PM EDT**

*Thank you for attending our CD
Webinar Part 1!*

ANY QUESTIONS?

Jasco