

**Abstract** *The relative stabilities of the 9th-10th type III domain pair of fibronectin (FIII9-10) and two mutants thereof, all of which have immunoglobulin-like beta-sandwich domains, were investigated using dynamic multimode spectroscopy (DMS) with automated circular dichroism (ACD). It is shown that the mutants are significantly more stable than the wild-type, that the folded and unfolded protein conformations for all variants are similar, and that aggregation accompanies unfolding.*

- ▶ Thermal stability of the mutants is superior to that of wild-type (WT)
- ▶ Pre-transition WT conformation is maintained in mutants
- ▶ Unfolding pathway is conformationally similar for all variants
- ▶ Quantitative results with errors are easily calculated
- ▶ Aggregation accompanies unfolding in all variants

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## CHIRASCAN SERIES APPLICATION NOTE

# Automated Circular Dichroism with Dynamic Multimode Spectroscopy: a powerful tool for establishing conformational stability of cloned proteins

## INTRODUCTION

Recombinant proteins represent one of the strongest areas of growth in the biotechnology and biopharmaceutical industries. There is currently a great deal of effort being directed into expanding the function of these proteins through improvements in their physical properties, the canonical example being the development of monoclonal antibodies and antibody-like scaffolds.

One of the most critical physical properties is the conformational stability of the protein fold. Increased stability leads to improved storage longevity and tolerance to environmental factors like heat and mechanical stress, while reducing adverse behaviour like aggregation (Chi et al. 2003; Wang 2005). These attributes are central to protein bioprocessing and protein formulation.

Protein conformation defines the function of the protein. Therefore, during a protein engineering programme with the aim to improve the conformational stability, it is important that the native fold of the protein is maintained. Circular dichroism (CD) provides very sensitive analytical characterization of protein conformation (Fasman 1996; Kelly et al. 2005), and can be measured with the protein under a number of different stresses. CD is particularly useful for measuring changes in protein stability while verifying that there is no conformational difference between samples.

## KEYWORDS

▶ Chirascan	▶ Protein
▶ Circular Dichroism	▶ Fibronectin
▶ Thermal Stability	▶ Quantitative results
▶ Recombinant	▶ Dynamic Multimode Spectroscopy

The Chirascan™-plus Automated Circular Dichroism (ACD) system is a very powerful tool to leverage circular dichroism measurements, allowing unattended measurements of multiple samples presented in a microplate format. When the ACD is combined with the Dynamic Multimode Spectroscopy (DMS) method of measuring protein conformation stability, it becomes possible to perform sophisticated measurements of thermal stability on a wide variety of proteins, or on one protein under a large number of conditions.

As an example of an engineered protein, in this note we will investigate the stability of the 9th-10th type III domain pair of fibronectin (FIII9-10) and two mutants thereof, all of which have immunoglobulin-like beta-sandwich domains. Using ACD-DMS, not only can we show that the mutants are significantly more stable than the WT, but also that the folded and unfolded protein conformations are similar for all three.

## EXPERIMENTAL

Three FIII9-10 samples (WT and two mutants) were prepared as 0.6mg/mL solutions in buffer. Sufficient sample to make three independent measurements of each was loaded into a 96-well microtitre plate. Samples were transferred from the plate to a 0.2mm spectroscopic flow-cell via an injection port, using a fixed-probe x, y, z robot.

Each sample was heated continuously at 1°C per minute from 20°C to 95°C while measuring far-UV spectra from 250nm to 190nm in 1nm steps with a sampling time of 0.7s per point. Each spectrum took approximately 1 minute to measure, giving a spectrum for every 1°C rise in temperature and thus a total of 75 far-UV spectra per denaturation. After each denaturation experiment, a measurement of the buffer was made that could be used to baseline-correct the sample spectra, and check for potential carry-over. Three independent measurements of the denaturation of each sample were made.

Protein concentration	0.6mg/mL
Buffer	10mM NaH <sub>2</sub> PO <sub>4</sub> / 50mM NaCl, pH6.0
Volume used per denaturation	60µl
Protein used	<40µg
Cell path-length	0.2mm
T-ramp range and rate	20°C - 95°C @ 1°C/minute
Duration of measurement	75 minutes
Optical properties measured	CD and absorption
Wavelength range measured	250nm - 190nm
Bandwidth	1nm
Step size / time per point	1nm / 0.7s

Table 1. Summary of experimental parameters.

At the end of a thermal ramp to 95°C, the sample holder temperature was returned to the start temperature in preparation for the next sample. In the time required to equilibrate the cell, an extensive cleaning routine was performed to ensure there was no carry-over of denatured or aggregated protein.

All thermal data analysis was carried out using the Applied Photophysics' Global3 analysis package. The fibronectin data could be fitted to a single transition denaturation model, with pre- and post-transition slopes to account for baseline shifts.

## RESULTS

The wild-type and two mutant proteins of FIII9-10 were shown to have very similar thermal denaturation profiles, and were easily fitted using a single transition model. Figure 1 shows a raw data set for the WT and each of the mutant proteins, highlighting the progression of the thermal denaturation, as well as the overlay of the fit of the single transition model to each data-set.

The data were very easily modelled with a single transition, with the high reproducibility indicated by the precision of the fitted parameters from the three replicate measurements for each sample. The most important results show a significant stability improvement with mutant 1 having a 9.2°C increase in  $T_m$  and mutant 2 showing an increase in  $T_m$  of 11.8°C over the WT protein. The van't Hoff enthalpies indicate that the transition is sharper in the mutants than it is in the WT.

	WT	Mutant 1	Mutant 2
T <sub>m</sub> °C 1	47.9	56.8	59.4
T <sub>m</sub> °C 2	47.5	56.7	59.3
T <sub>m</sub> °C 3	47.7	57.3	59.8
T <sub>m</sub> °C mean	47.7	56.9	59.5
T <sub>m</sub> °C SD	0.2	0.3	0.3
van't Hoff enthalpy (kJ/mol) 1	289	397	462
van't Hoff enthalpy (kJ/mol) 2	314	411	414
van't Hoff enthalpy (kJ/mol) 3	292	451	457
van't Hoff enthalpy (kJ/mol) mean	298	419	444
van't Hoff enthalpy (kJ/mol) SD	14	29	27

Table 2. Fit parameters for the single transition thermal denaturation data of FIII9-10 mutants.

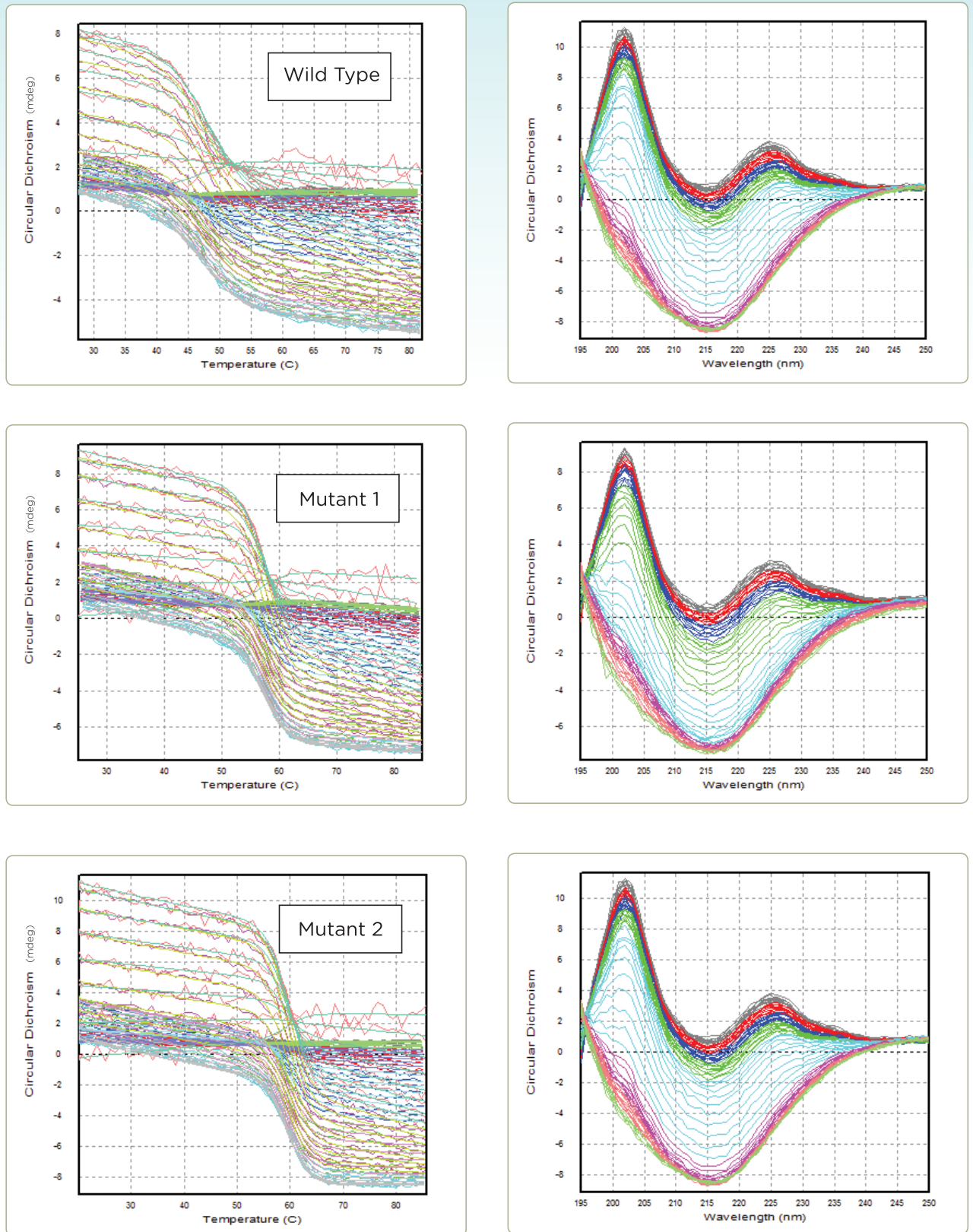
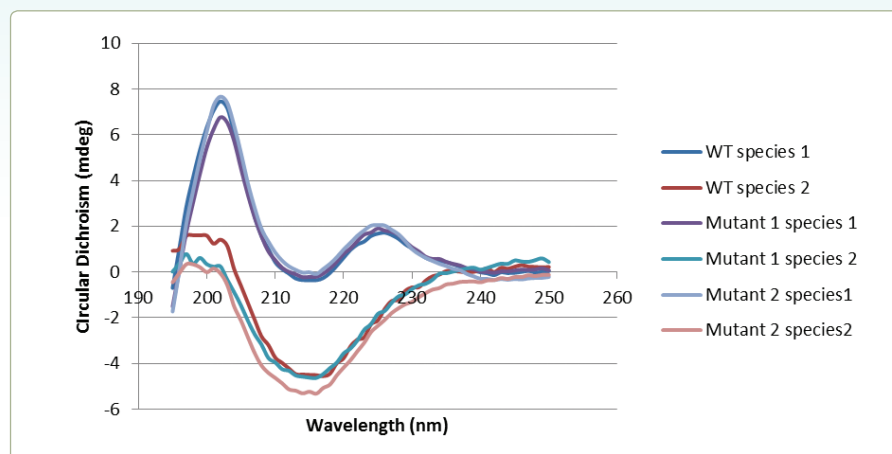


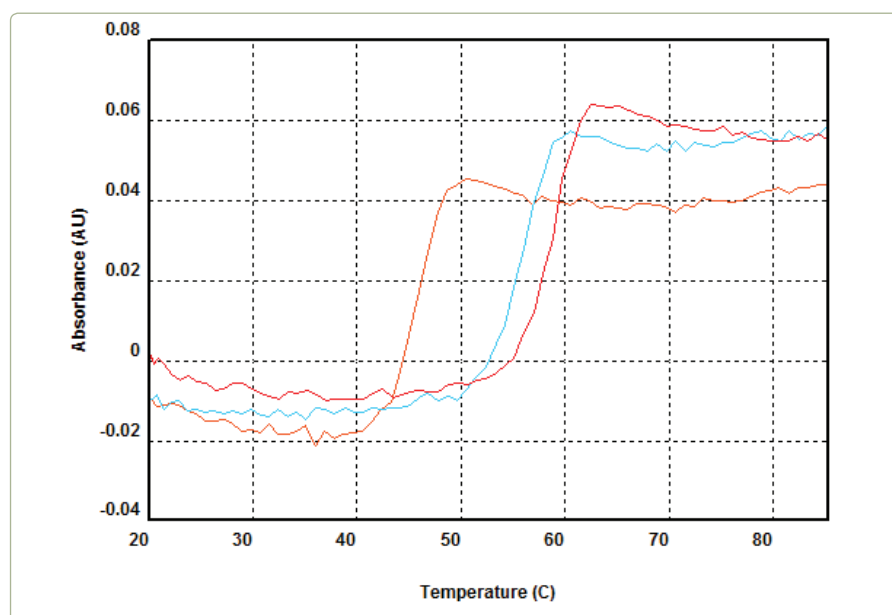
Figure 1. Example DMS CD Thermographs of the various mutant forms of FIII9-10. Left are temperature vs CD plots for all wavelengths and the fitted model in blue. Right are the spectra for each temperature.

The significant increase in  $T_m$  indicates that there has been a significant increase in the stability of the folded structure. The CD spectra, derived from the global fit of the data, are very similar for both the folded and unfolded forms of all the protein variants (Figure 2), a good indication that the unfolding pathway has stayed the same, but the stability of the folded form has increased.



**Figure 2.** The resultant derived species folded (species 1) and unfolded (species 2) spectra for the F119-10 variants. These have been baseline corrected and normalised for concentration by the absorbance at 205 nm.

Absorbance spectra were measured simultaneously with the CD spectra to monitor aggregation of the protein. There is very little absorbance at 250nm (Figure 3) due to the absence of any strongly absorbing chromophore and any apparent change in absorption will be due to light scattering by insoluble aggregates. It is apparent that all samples aggregate to a similar extent after unfolding of the protein.



**Figure 3.** Absorbance thermograms of F119-10 forms at 250nm showing aggregation. Orange: WT, blue: mutant 1, red: mutant 2.

## CONCLUSION

The data from a single overnight ACD-DMS run showed a statistically significant improvement in thermal stability of the mutants relative to the WT protein. The CD spectra showed similar structures were present before and after the unfolding event in each case, demonstrating that the folded structure and unfolding pathway are retained for the mutants.

DMS is an information-rich, label-free technique that gives access to thermodynamic, structural and aggregation data in a single experiment. The combination of DMS and automation gives rise to a very powerful tool for understanding the thermal denaturation pathway of proteins, generating quantitative results from small samples with little effort.

The automation of DMS experiments using Chirascan™-plus ACD dramatically reduces the work required to collect these data. The experiment described here took less than 30 minutes to set up and was run overnight without further user intervention.

## REFERENCES

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## NEW APPLICATIONS FOR CD SPECTROSCOPY

### **Optimising biotherapeutic formulations**

Used as a label free stability-indicating assay, Chirascan™-plus automated circular dichroism (ACD) can identify good formulation candidates earlier for further downstream processing. By culling formulations that are likely to fail early and focusing on those that are more viable for real time and accelerated stability studies, users can make savings in both time and money.

Combining the label-free and information-rich technique of dynamic multimode spectroscopy (DMS) with the productivity of automation gives a whole new approach to establishing conformational stability under different formulation conditions. The conformational integrity of biotherapeutics as a function of more than one stress condition (e.g. Temperature, pH, ionic strength) is readily determined in unattended operation.

### **Statistical comparison of similar proteins (Biosimilarity)**

Research into biosimilar pharmaceutical products has grown exponentially over the last few years turning it into a multi-billion pound business. Structure, biological activity and stability are just a few of the complex studies required and, traditionally, these use multiple techniques which are very time consuming and labour intensive.

Automation lends itself to measuring samples repeatedly and thus to generating statistical comparisons. To answer the question: 'Are these two CD spectra the same?' is no longer a matter of guesswork – a statistical significance can be associated with the measurements and a quantitative judgment about similarity or otherwise can be made.

### **Drug Discovery**

Fast determination of protein characteristics is key to any drug discovery department in pharmaceutical research. Applied Photophysics offers a unique solution providing simultaneous circular dichroism, absorbance and fluorescence measurements in a single, easy to use, automated experiment.

The Chirascan™-plus ACD spectrometer can provide structural, functional, thermodynamic and aggregation data. By automating our system we provide unparalleled productivity, low sample volumes and no human error reducing the pressure on analytical labs and enabling them to focus on discovery.

### **Protein Engineering**

Monoclonal antibodies, antibody-like proteins, and other biotherapeutics represent a large and growing number of molecular entities entering human clinical trials in virtually all disease indications. The long-term stability of these potential therapeutics is of crucial importance for their development to drug products.

The Chirascan™-plus ACD spectrometer provides rapid, accurate, and easy to perform measurement of the thermal melting ( $T_m$ ) points, which has proven to be an exceptionally good indicator of the relative stability of engineered proteins.

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