



**Abstract:** The selection of buffer conditions that maximise stability is central to the formulation of protein drugs. In this study the effect of buffer pH on the thermal stability of an example protein was determined by dynamic multimode spectroscopy (DMS) using the Chirascan™ 6-Cell Peltier Cell Holder to allow near-synchronous data collection across 6 conditions.

- ▶ Greatest thermal stability occurs in the range pH 4.0 to 6.0
- ▶ High experimental reproducibility
- ▶ Up to six samples analysed in a single experiment
- ▶ Unattended operation – overnight measurement

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## CHIRASCAN SERIES APPLICATION NOTE

# Chirascan™ 6-Cell Peltier Cell Holder: Rapid Optimisation of Buffer Conditions for Stabilising Protein Therapeutics

## INTRODUCTION

Circular dichroism is a well-established method for protein secondary structure determination and allows slight changes in protein structure to be detected and quantified. Dynamic Multimode Spectroscopy, a technique in which CD and other spectral information, e.g. fluorescence, are gathered over the course of a temperature ramp, allows the thermodynamic parameters of thermal protein unfolding to be determined and thus an assessment of the protein stability to be made.

For the formulations scientist seeking optimal conditions for stabilising the native conformation of a protein drug, CD and DMS spectroscopies are valuable tools. The desirability of screening many conditions, for example pH, ionic strength, detergent/excipient combinations, favours the use of automated systems such as the Chirascan™-plus ACD. Alternatively, the Chirascan™ 6-Cell Peltier Cell Holder allows users to access many of the advantages of the Chirascan™-plus ACD for this application in a reduced cost system. Briefly, the 6-Cell Peltier Cell Holder incorporates peltier temperature control, integrated N<sub>2</sub> purging and magnetic stirring at all sample positions. The device is compatible with all detection modes (CD, Absorbance, and Fluorescence) and its control is fully integrated within the Chirascan™ software.

## KEYWORDS

▶ Chirascan	▶ Quantitative Results
▶ Circular Dichroism	▶ Dynamic Multimode Spectroscopy
▶ Automated	▶ Formulation
▶ Thermal Stability	▶ Protein therapeutics

In this study, lysozyme was selected to simulate a protein drug and CD spectra at pH values of 2, 3, 4, 5, 6 and 7 were acquired during continuous temperature ramps to assess the effect of pH on thermal stability. The acquired data, which was recorded simultaneously with absorbance data (not shown), was analysed using Global 3 software to yield melting temperatures ( $T_m$ ) and van't Hoff enthalpies at each pH. The experiment was also carried out using identical lysozyme samples prepared at pH 7.0 as a control.

The control data set shows the high reproducibility of the system and yields an average melting temperature across all 6 cell positions at pH 7.0 of 72.7°C; the standard deviation on this measurement is 0.1°C. The variable pH data set shows that the melting temperature of lysozyme is pH-dependent and that the native fold is most stable at pH 5, consistent with the optimal pH for lysozyme stability reported in the literature<sup>(1,2)</sup>.

## MATERIALS AND METHODS

Lysozyme was purchased from Sigma Aldrich (Product Number L4919) and dissolved to a concentration of 3mg/ml in water. This solution served as a 10x concentration stock for dilution into phosphate/citrate buffer prepared from 50mM disodium phosphate and 25mM citric acid. The conditions screened were pH values ranging from pH 2.0 to pH 7.0 in single pH unit steps. The pH 2.0 condition lies slightly outside the conventional buffering range of phosphate/citrate, though the first pKa of phosphate is pH 2.15, and was achieved by adding a small amount of perchloric acid.

Buffers and samples were analysed in six 0.5 mm pathlength non-demountable 'bottle type' cuvettes (approx. volume = 135µL) which were presented to the Chirascan measuring beam sequentially by the 6-Cell Peltier Cell Holder. All six samples were heated continuously at 0.23°C per minute from 25°C to 95°C while absorbance and CD spectra were acquired simultaneously from 260nm to 195nm in 1nm steps with a sampling time of 0.75 seconds per point. Buffer absorbance contributions exceeded 2.0AU below 195nm and thus CD data collection was limited to wavelengths > 195nm. Each set of six spectra took approximately 8 minutes to measure allowing a spectrum at each turret position to be collected every 2°C rise in temperature producing a total of 35 far-UV spectra per denaturation.

Parameter	Value
Spectrometer	Chirascan™
Detector	Photomultiplier tube (PMT)
Temperature controlled cuvette holder	6-cell Peltier Cell Holder with Quantum Northwest TC-1 temperature controller
Pathlength	0.5 mm (non-demountable cells, bottle type)
Wavelength range	195-260 nm
Wavelength step	1 nm
Bandwidth	1 nm
Time per point	0.75 seconds
Temperature range	25°C to 95 °C (continuous ramp)
Temperature interval	2 °C
Temperature ramp rate	0.23 °C/minute

All thermal data analysis was carried out by the Applied Photophysics' Global 3 analysis package.

## RESULTS

### Proof of principle: Six replicate DMS analyses of lysozyme at pH 7.0

Six samples were analysed by DMS at pH 7.0 in order to confirm the reproducibility of the system (see figure 1). The CD and absorbance (not shown) data were highly reproducible. To account for minor variances in the six 0.5 mm pathlength cuvettes the data in figure 1, which is presented as 'raw' unsmoothed non-baseline corrected spectra, was set to zero mDeg. at 260 nm. Thermal denaturation profiles were modelled to a single transition with independent baseline correction allowed before and after the transition (see figure 2). The average  $T_m$  for lysozyme at pH 7.0 was 72.7°C with a standard deviation of 0.1°C and the average van't Hoff enthalpy was 381 kJ/mol with a standard deviation of 23 kJ/mol (see table 1). These results confirm that differences between turret positions, in terms of temperature control and data acquisition, are insignificant and they provide a proof of principle to support the variable pH data set (see below).

Figure 1. DMS CD spectra of lysozyme at each temperature, six replicates analysed simultaneously at pH 7.0.

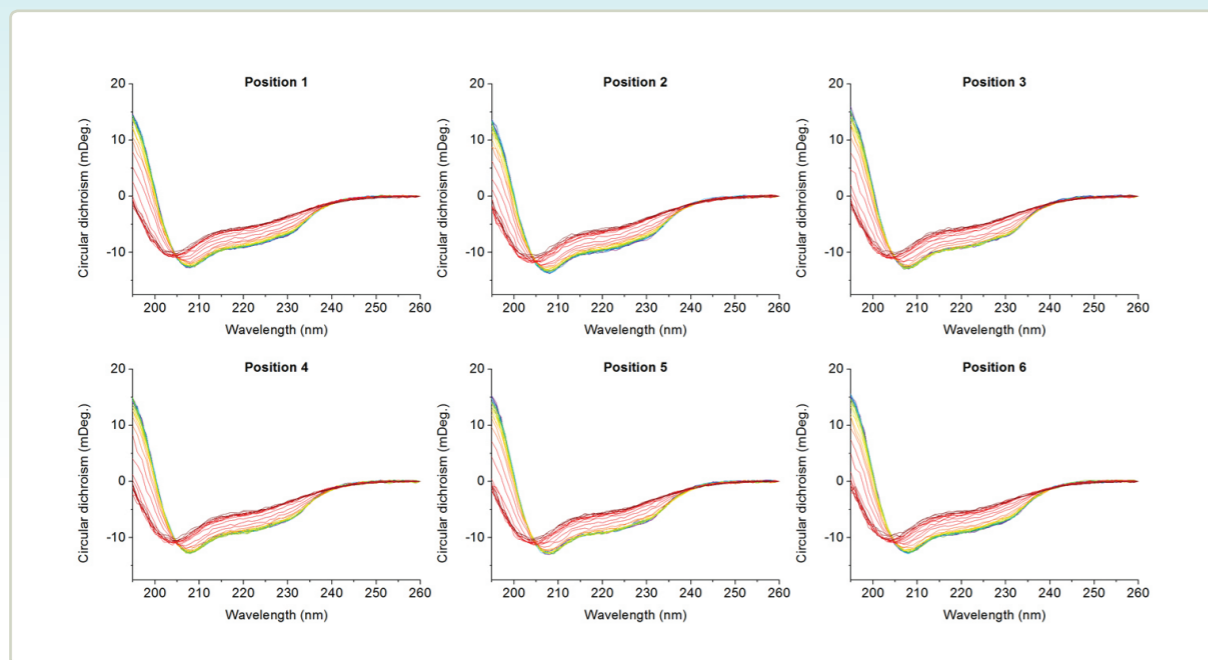
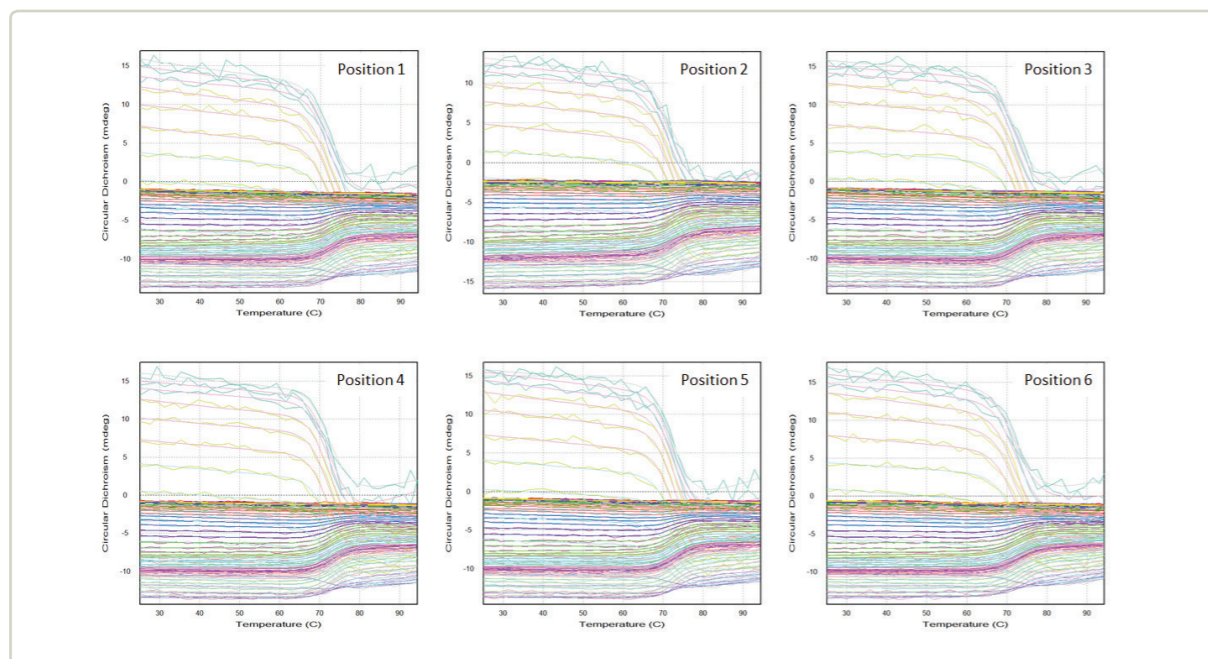


Figure 2. CD vs. Temperature for all wavelengths for lysozyme, six replicates analysed simultaneously at pH 7.0 with the fitted model overlaid.



pH screening experiment: Six DMS analyses of lysozyme at various pH values

The effect of pH on the stability of the protein was investigated by monitoring thermal denaturation using CD spectroscopy with temperature ramping at pH values from 2.0 to 7.0, in one pH unit steps. The acquired CD spectra and temperature vs. CD plots (see Figures 3 and 4) show that the thermal stability of the protein is dramatically affected by pH. The model derived  $T_m$  values (see table 2) range from 55.4°C at pH 2.0 to 76.9°C at pH 5.0 representing the conditions where the native fold is least and most stable, respectively. The trend in the derived  $T_m$  values is shown in figure 5 and demonstrates that the stability of the native fold of the protein is relatively insensitive to pH at values greater than approx. 3.0.

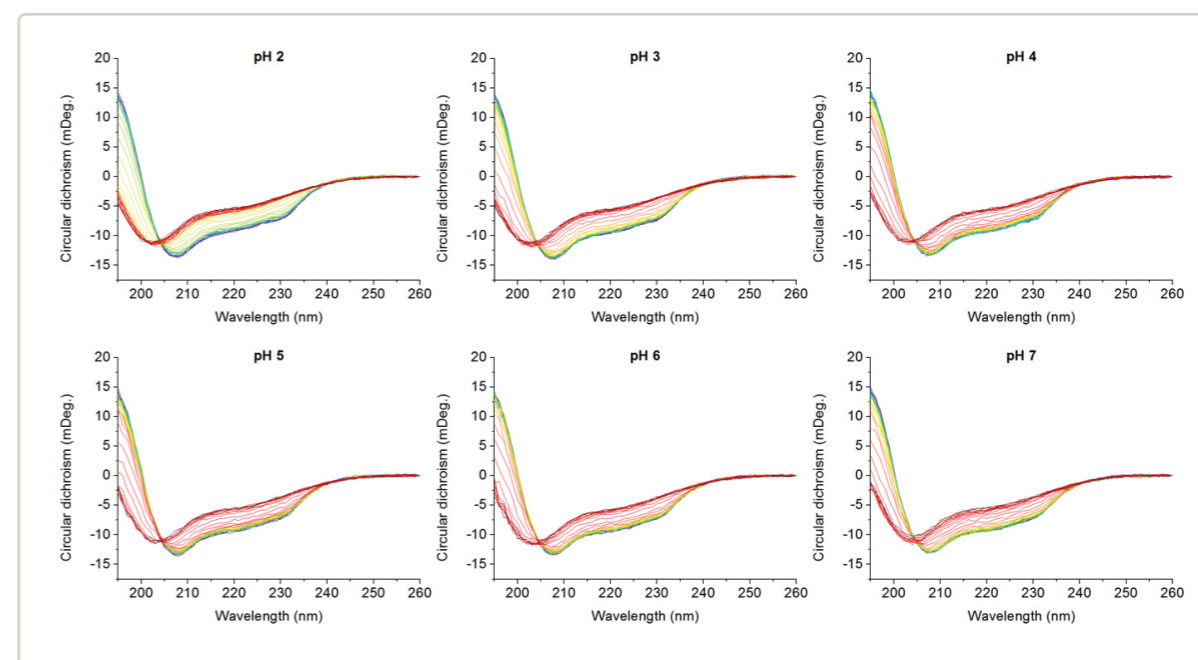


Figure 3. DMS CD spectra of lysozyme at each temperature, six samples at pH values ranging from 2.0 to 7.0.

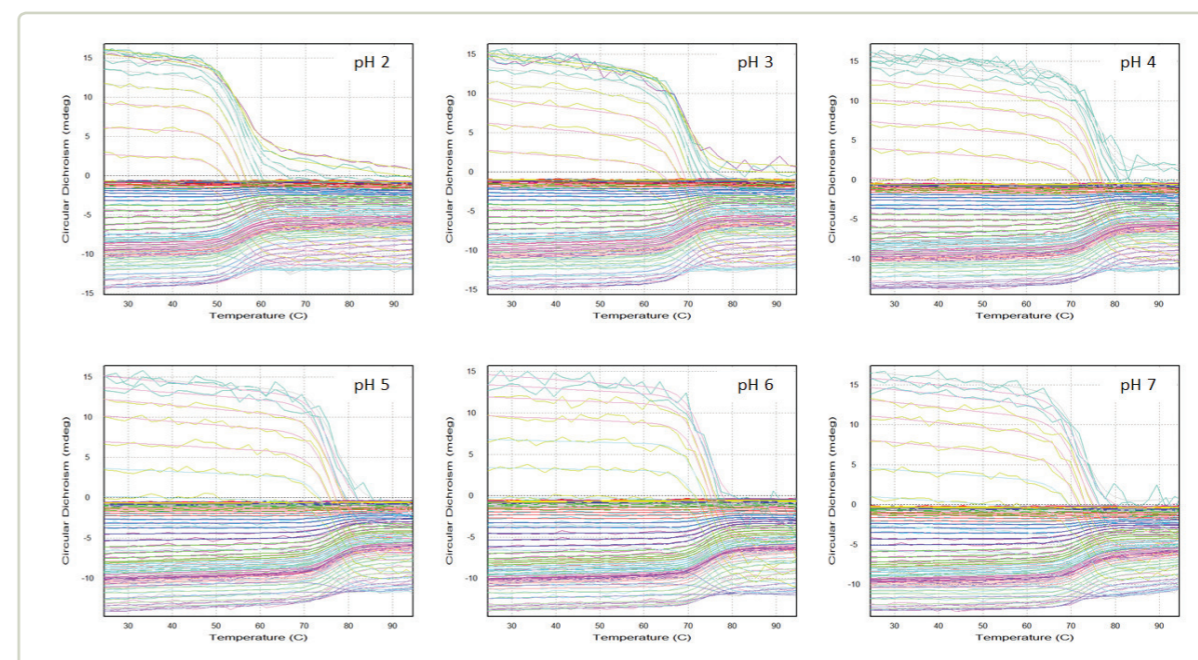


Figure 4. CD vs. Temperature for all wavelengths for lysozyme, six samples at pH values ranging from 2.0 to 7.0 analysed simultaneously with the fitted model overlaid.

Table 1. Control dataset

Cell/Turret position	pH	Melting temperature (°C)	van't Hoff enthalpy (kJ/mol)
1	7	72.5	379
2	7	72.6	374
3	7	72.7	367
4	7	72.7	404
5	7	72.8	417
6	7	72.7	347
Average		72.7	381
Standard deviation		0.1	23

Table 2. pH scan dataset

Cell/Turret position	pH	Melting temperature (°C)	van't Hoff enthalpy (kJ/mol)
1	2	55.4	354
2	3	69.4	385
3	4	75.8	380
4	5	76.9	400
5	6	74.2	423
6	7	72.7	367

## CONCLUSION

The Chirascan™ with 6-Cell Peltier Cell Holder allows rapid investigation of the effect of buffer conditions on stability of protein therapeutics. In the presented study, experiments were of 5 hours duration and were performed without user intervention after initial set up.

The results demonstrate that the native fold of lysozyme is most stable in buffers with pH 3 – 7 and is destabilised in buffers with pH < 3; conclusions which are consistent with the published literature on lysozyme<sup>(1,2)</sup>. Absorbance data collected at 260 nm, a wavelength at which the protein does not absorb, allows the extent of aggregation to be assessed based on light scattering. A comparable and minor amount of aggregation was detected at all pH conditions (data not shown). The control data set (six replicates at pH 7) and the reproducibility of the pH scan data sets give the formulations scientist confidence in the results.

This approach can easily be applied to other parameters, for example, ionic strength, detergent/excipient screening. Furthermore, the 6-Cell Peltier Cell Holder is compatible with fluorescence mode measurements allowing additional information on the sample to be obtained.

## REFERENCES

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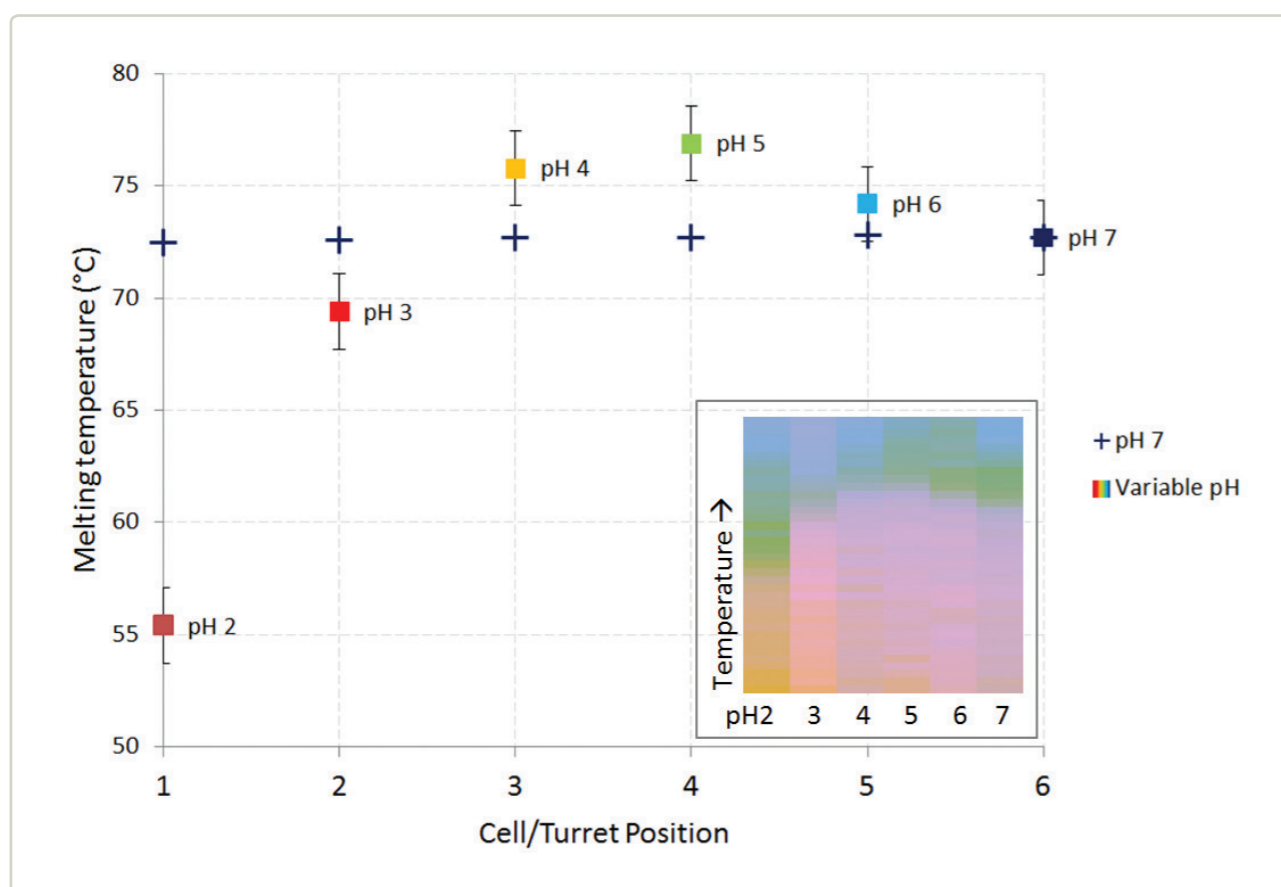


Figure 5. Plot of model derived  $T_m$  value against cell/turret position annotated with corresponding pH values. pH 7 control dataset  $T_m$  values are shown as blue crosses. Variable pH data set  $T_m$  values are shown as coloured squares. Error bars are plus-minus 3 x standard deviation of three repeats carried out on different days with independently prepared buffer and lysozyme solutions; they represent 99% confidence limits. Inset: data represented as an empirical phase diagram (see reference 3, or the applied photophysics blog: [http://bit.ly/APL\\_EPD](http://bit.ly/APL_EPD)).

## ORDERING INFORMATION



Clockwise from top left: 6-Cell Peltier Cell Holder, Quantum Northwest TC1 temperature controller, two 10x10mm cuvettes, a cuvette holder with a 0.5 mm non-demountable cuvette.

The carousel design enables fully automated measurement of up to six samples, as well as simultaneous measurement of CD, absorbance and fluorescence. A sealed light path design ensures that the nitrogen environment is not compromised when the sample chamber is opened to replace sample cuvettes. This increases productivity and ensures that the absorbance measurements, acquired simultaneously with the CD measurement, are of the highest possible accuracy. Temperature ramping experiments between  $-20^{\circ}\text{C}$  and  $+110^{\circ}\text{C}$  can now be performed on up to 6 samples in a single experiment.

Part Number	Description
CS/PC6	High performance 6-cell autochanger for 10x10mm cuvettes
CS/SP6	Spacers kit for CS/PC6 high performance 6-cell autochanger

4207Q153 v.1.00

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