**Optim® 1000: Fluorescence data analysis methods**

*Technical note*

Fluorescence in proteins
A protein is a complicated molecule which can occupy a unique, functional, three-dimensional native conformation or one of an ensemble of unfolded or partially unfolded, non-functional conformations. Changing the thermodynamic or chemical conditions can cause a protein to unfold and this process can be followed by monitoring changes in the intrinsic protein fluorescence spectrum. When small proteins or small parts of larger proteins unfold, they do so cooperatively which leads to the transitions seen in the fluorescence data having a mid-point that can be used to describe the stability of the protein. The higher the value of the mid-point, the more stable the protein is against the particular stress being applied. If the protein is subjected to heating for example, then the thermal stability is characterised by a melting temperature, $T_m$, which is the mid-point temperature of the transition from a folded to an unfolded ensemble. A similar argument applies to other stresses; the addition of chaotropic compounds for example. In this case the mid-point is one of concentration rather than temperature.

This technical note aims to demonstrate the capabilities of the Optim 1000 as a high throughput, low sample volume fluorimeter to follow protein unfolding.

Optim 1000 as a fluorimeter
To demonstrate the performance of the Optim 1000 as a fluorimeter, consider the spectra in Figure 1(a). As can be seen, the fluorescence emission measured on the Optim 1000 overlay those acquired on a leading conventional instrument using hundreds of microlitres of sample. The Optim 1000 has the significant advantage that it uses significantly lower sample volumes and it acquires and analyses the data in a fully automated, intelligent fashion from up to 48 samples at a time.

Primary analysis
The software for the Optim 1000 provides two levels of analysis of acquired spectra. Primary analysis extracts information about the fluorescence from the acquired spectrum. The parameters extracted are integrated fluorescence intensity, peak emission wavelength, expectation wavelength and an intensity ratio (set as default to be the ratio of the fluorescence emission at 350 and 330 nm where tryptophan fluoresces in its unfolded and folded states). These parameters each have a different utility summarised in Table 1. The optimum parameter to use to monitor unfolding is somewhat protein dependant. For example cytochrome c exhibits little observable change in peak position on unfolding but has a large increase in fluorescence, whereas IgGs typically shows a clear peak shift when they unfold. An example of the results of a primary analysis is given in Figure 1(b), which shows the peak position variation of 48 identical polyclonal IgG samples as a function of temperature showing a clear transition mid-point, $T_m$, at 52 °C.

Secondary analysis
The secondary level of analysis performed extracts $T_m$ values from the primary analysis (in this case the peak position vs temperature curves) by finding the point of maximum gradient using the second differential. The data is smoothed and a linear regression curve is fitted locally to the root to reduce the effect of noise in the data. Figure 1(c) demonstrates the output from the secondary analyses for 48 identical samples ($T_m = 51.7±0.9$ °C).

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1 For a discussion on protein folding and the $T_m$ parameter as a measure of thermal stability see the Optim 1000 technical notes, “An introduction to proteins, protein folding and aggregation” and “Comparison of Optim performance with standard methods to determine Tm”.

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**ISOGEN**

**L I F E S C I E N C E**
Figure 1. (a) Fluorescence emission spectra of an IgG sample measured on the Optim 1000 at 25 °C and 90 °C (blue and red) and the same sample measured on a Shimadzu RF5301PC (purple and green). (b) The peak position of the fluorescence emission of all 48 identical polyclonal IgG samples recorded in the Optim with the position of $T_m$ marked. (c) Reproducibility of the Optim 1000 data collection and analysis is demonstrated by the $T_m$ data for each of the 48 samples ($T_m = 51.7 \pm 0.9 \, ^\circ\text{C}$).

Table 1. A summary of the different primary analysis techniques available, demonstrating the method used to obtain the parameters, advantages for each method and an example of an appropriate protein where applicable.

<table>
<thead>
<tr>
<th>Primary analyses</th>
<th>Calculation method</th>
<th>Advantages</th>
<th>Example protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak fit</td>
<td>Fits a lognormal function to a peak between 300 and 360 nm. (The wavelength limits can be user defined.)</td>
<td>• Clear physical interpretation relating to polarity of aromatic amino acids.</td>
<td>BSA</td>
</tr>
<tr>
<td>Integrated fluorescence intensity</td>
<td>Integrates under the fluorescence curve to give a measure of total intensity between 280 and 450 nm.</td>
<td>• Signal proportional to population of microstates in protein (assuming no temperature dependence etc.).</td>
<td>Cytochrome c, BSA</td>
</tr>
<tr>
<td>Intensity ratio</td>
<td>Takes the ratio between the fluorescence intensities at 350 and 330 nm.</td>
<td>• Less susceptible to noise than other methods.</td>
<td>Most IgGs</td>
</tr>
<tr>
<td>Expectation wavelength</td>
<td>Calculates a barycentric mean of the fluorescence emission between 280 – 450 nm.</td>
<td>• Easy to interpret like peak position.</td>
<td>Lysozyme</td>
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</tbody>
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