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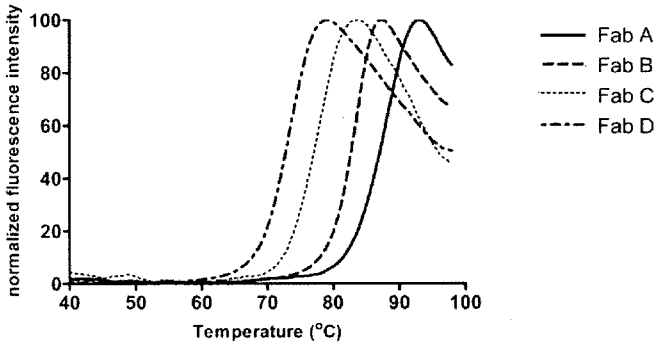
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(54) Title: THERMOFLUOR METHOD

Figure 4



(57) Abstract: The present invention relates to an analytical method of determining thermal stability comprising the steps: a) raising the temperature of two or more distinct protein/polypeptide samples, at a rate suitable for measuring fluorescence therefrom, until substantially all the protein in each of the samples is unfolded, wherein each sample comprises a buffer, a fluorescent dye and a protein or polypeptide for analysis, and b) after excitation at an appropriated wavelength measuring fluorescence as a function of temperature, and c) optionally calculating the Tm of each sample, wherein steps a) and b) may be effected concomitantly.

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Thermofluor Method

The present invention relates to a method, for example for high-throughput stability analysis of samples containing different active ingredients, in particular a method comparing distinct proteins to establish which entity is most thermally stable.

Currently the most widely used method for establishing and comparing the thermal stability is DSC (i.e differential scanning calorimetry). DSC is generally recognized to be the 'gold standard' for analyzing thermal stability of proteins. It is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference material are measured as a function of temperature. Both the sample and reference material are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample temperature increases linearly as a function of time. A reference sample is usually chosen to have a well-defined heat capacity over the range of temperatures to be scanned.

The main application of DSC is in studying phase transitions and thus it can be used to identify the temperature at which proteins unfold and the temperature at which they become denatured. However, the method is not particularly amenable to high-throughput analysis. Typically employing DSC to analyze one sample takes about 2 hours and thus this known method is not amenable to rapid high-throughput analysis.

Thermofluor techniques have been used to establish how components of a given formulation influence the stability of a particular protein, for example as disclosed in Ericsson *et al*, Analytical Biochemistry 357 (2006) 289-298.

The thermofluor method was originally developed to allow rapid identification of ligands of target proteins from compound libraries and is based on distinguishing folded from unfolded proteins, by measurement of a hydrophobic fluorescent dye bound to the protein.

When the protein is folded, for example when it is in an aqueous solution, the hydrophobic areas and/or residues are buried in the folded structure and thus inaccessible to the hydrophobic dye. The dye is therefore unable to bind these residues and ultimately is unable to fluoresce after excitement by light of the required wavelength. In contrast as the protein is heated and starts to unfold then these hydrophobic areas/residues become

accessible to the dye which binds the same and with appropriate excitation the amount of fluorescence from the bound dye can be measured as a function of temperature.

Thus the principle of the thermofluor method is a protein sample heated in the presence of a fluorescent dye. As the protein melts, the hydrophobic core of the protein (which is normally buried within the folded structure of the protein) becomes solvent-exposed leading to a measurable increase in the fluorescence of the dye.

The temperature at which 50% of the protein is unfolded is designated the *Tm*. Experimentally, the *Tm* is determined as the inflection point of the fluorescence-vs.-temperature curve (i.e. the temperature where the fluorescence-vs.-temperature curve is the steepest). This is represented diagrammatically in Figure 1.

For some proteins, a single transition from folded to unfolded is observed, characterized by a single *Tm* (see left panel in Figure 1).

For complex, multi-domain proteins, individual portions of the protein can melt independently giving rise to multiple *Tm*'s (See right panel in Figure 1).

Thus the thermofluor curve produced may be analyzed for the rate of change of fluorescence. The *Tm* is when the rate of change of fluorescence is at its highest i.e. at the point of inflection.

The present inventors believe that whilst the thermofluor method has been used to identify an optimised formulation which stabilises a given protein (i.e. the side by side comparison of different formulations of a given protein to identify which parameters provide best stabilization) it has never been suggested to use thermofluor techniques to compare the stability of different proteins/polypeptides, under the same conditions, for example in the same buffer, in an high-through-put method.

Thus there is provided a method of determining the thermal stability of at least two protein samples comprising the steps:

a) providing two or more samples of distinct proteins at an appropriate dilution and comparable concentration,
b) adding a suitable amount of fluorescent dye to each sample,
c) raising the temperature of the samples at a rate suitable for measuring the fluorescence emitted therefrom until substantially all the protein in each of the samples is unfolded, and

d) after excitation at an appropriated wavelength measuring fluorescence as a function of temperature, and
e) optionally calculating the *Tm* of each sample.

Brief Description of the Drawings

Figure 1 Shows examples of typical thermofluor curves

Figure 2 Shows a thermofluor analysis of an antibody and its corresponding Fab fragment, measured under the same buffer conditions

Figure 3 Shows two superimposed thermofluor curves for two antibodies, measured under the same buffer conditions. In this instance, the antibodies had different constant regions namely IgG1 (Ab1) and IgG4 (Ab2).

Figure 4 Shows four superimposed thermofluor curves for four distinct antibodies Fab fragments, measured under the same buffer conditions

Figure 5 Shows five superimposed thermofluor curves for a set of five bispecific antibodies wherein the linker length has been varied, and the analysis was performed in the same buffer

Figure 6 Shows a thermofluor analysis of two bispecific fusion proteins with different variable regions

Figure 7 Shows a thermofluor for a purified Fab superimposed on a thermofluor for a Fab in the presence of soluble aggregate.

Figure 8 Shows a comparison of results obtained employing thermofluor analysis and DSC analysis

Advantageously the method of the present invention allows the selection of proteins with optimized stability profiles, which can be an important feature in fields, such as biological pharmaceuticals. This method is potentially a very powerful tool, for analysis of large numbers of samples and has many applications.

The unfolding of the protein/polypeptide occurs gradually with rising temperature, first exposing some hydrophobic residues, possibly followed by some aggregation, followed by unfolding and ultimately denaturing.

In one embodiment the method comprises the further step of identifying the most thermally stable samples, for example samples with the highest *Tm*.

Thus there is provided a method of determining the thermal stability of at least two protein/polypeptide samples comprising the steps:

a) providing two or more samples of distinct proteins at an appropriate dilution and comparable concentration,
b) adding a suitable amount of fluorescent dye to each sample,
c) raising the temperature of the samples at a rate suitable for measuring the fluorescence emitted therefrom until substantially all the protein/polypeptide in each of the samples is unfolded, and
d) after excitation at an appropriated wavelength measuring fluorescence as a function of temperature, and
e) calculating the *Tm* of each sample.

Of course steps a) and b) above may be performed in any order.

In one embodiment the method comprises:

a) raising the temperature of two or more distinct protein/polypeptide samples, at a rate suitable for measuring fluorescence therefrom, until substantially all the protein in each of the samples is unfolded, wherein each sample comprises a buffer, a fluorescent dye and a protein or polypeptide for analysis, and
b) after excitation at an appropriated wavelength measuring fluorescence as a function of temperature, and
c) optionally calculating the *Tm* of each sample,

wherein steps a) and b) may be effected concomitantly.

Of course step a) and b) in the method directly above and steps c) and d) in the methods further above will generally be performed concomitantly.

There is further provide a method according to the present invention where the sample or samples with the highest *Tm* is/are identified, for example the samples with *Tm* values in the highest 10% are identified.

In one embodiment the method comprises the further step of selecting a stable sample, for example a sample with the highest *Tm* or 1 or more such as 1, 2, or 3 or all samples with a *Tm* value in the highest 10%.

In one embodiment the method of the present invention is a high-throughput method. High-throughput as employed herein is intended to refer to the concomitant analysis of 10 or more samples.

Concomitant analysis as employed herein means the same analysis-run.

"Substantially unfolded" as employed herein is sufficiently unfolded to calculate a *Tm* value.

Distinct proteins as employed herein is intended to refer to proteins with different primary structures i.e. different amino acid sequences and proteins with the same amino acid sequence but different physical characteristics, for example those expressed by different hosts and which therefore have different physical characteristics. In one embodiment the different/distinct proteins have different amino acid structures. The term "distinct proteins" does not refer to the same protein presented in different formulations.

Appropriate dilution as employed herein is intended to refer to a dilution at which the analysis can be performed.

Comparable concentration as employed herein is intended to refer to concentrations which allow a meaningful comparison of the thermofluor analytical results of the different samples.

In one embodiment the samples are analysed in microplates, which allows analysis of up to 96 samples or up to 384 samples in the same analysis run. The analysis of 1024 well plates may also be possible. In one embodiment the samples are analyzed in the same run. The number of samples that can be analysed by the method according to the invention can be increased if the instrument is employed with robotic sample handling equipment.

The same analysis run as used herein is intended to refer to the number of the samples that can be accommodated and analyzed autonomously by the instrument, without human intervention.

Employing the method of the present invention the analysis time for these "larger" numbers of samples is generally quite short, for example it may take 2 hours to analyse 384 samples, in comparison to DSC which typically requires about 2 hours to analyse one sample. Thus using DSC it would take 32 days to analyse 384 samples. What is more data generated by thermofluor analysis correlates well with data generated using DSC, see for example Figure 6.

In one embodiment, at least one, for example all of the samples, is/are duplicated, or triplicated or quadrupled in a sample tray for analysis according to the invention.

Fluorescent dyes suitable for use in thermofluor analysis are well known and include: sypro orange, thioinosine, and N-ethenoadenosine, formycin, dansyl derivatives, fluorescein derivatives, 6-propionyl-2-(dimethylamino)-napthalene (PRODAN), 2-anilinonapthalene, and N-arylamino-naphthalene sulfonate derivatives such as 1-anilinonaphthalene-8-sulfonate (1,8-ANS), 2-anilinonaphthalene-6-sulfonate (2,6-ANS), 2-aminonaphthalene-6-sulfonate, N,N-dimethyl-2-aminonaphthalene-6-sulfonat e, N-phenyl-2-aminonaphthalene, N-cyclohexyl-2-aminonaphthalene-6-sulfonate, N-phenyl-2-aminonaphthalene-6 -sulfonate, N-phenyl-N-methyl-2-aminonaph-thalene-6-sulfonate, N-(o-toluyl)-2-aminonaphthalene-6-sulfonate, N-(m-toluyl)-2-aminonaphthalene-6-sulfonate, N-(p-toluyl)-2-aminonaphthalene-6-sulfonate, 2-(p-toluidinyl)-naphthalene-6-sulfonic acid (2,6-TNS), 4-(dicyanovinyl)julolidine (DCVJ), 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)methyl)amino)naphthalenechl oride (PATMAN), nile red, N-phenyl-1-naphthylamine, 1,1-dicyano-2-[6-(dimethylamino)naphthalen-2-yl]propene (DDNP), 4,4'-dianilino-1,1-binaphthyl-5,5-disulfonic acid (bis-ANS), and DAPOXYL derivatives (Molecular Probes, Eugene, Oreg.).

In one embodiment the dye is Sypro orange available from Molecular Probes Inc., for example the material described by the manufacturer as '30x'.

In one embodiment the fluorescence is measured at a wavelength 530nm.

Suitable instruments for measuring the emitted light include any real-time PCR system, such as a 7900HT fast real-time PCR system available from Applied Biosystems. Other systems include Bio-Rad Opticon.

The temperature for a typical run will be approximately: from 20 °C to 99 °C using a temperature gradient of about 0.02°C/sec, for example based on 384 well block, but may be from 40 to 100 °C, if desired. In the case of the 7900HT system, the minimum ramp rate may, for example be 0.02°C/sec (1% power) based on 384 well block. The maximum ramp rate on this system may, for example be 2°C/sec (100% power) based on 384 well block. Thus a suitable temperature ramp range is about 0.02 to about 2°C/sec.

In one embodiment 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 94, 96, 100, 150, 200, 250, 300, 350 or more such as 384 or 1024 protein samples are analysed concomitantly. The instruments can be employed in combination with robotic equipment to automate the process further and increase the number of samples that can be processed without human intervention.

In one embodiment when one, two or three samples are murine IgG1 antibodies then at least four distinct proteins will be analysed concomitantly employing a method according to the present invention.

In one embodiment the sample is a liquid. In one embodiment the total volume of the sample is in the range about 1 to 15 μL, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 μL, in particular 10 μL. The liquid may, for example be in the well of a 96 or 384 well plate.

In one embodiment the total sample volume comprises about 1 μL of dye. The amount of dye may vary depending on the exact dye employed. However, a skilled person is well able to titrate the dye to establish a suitable value wherein the signal to noise ratio is above the base line.

In one embodiment the total sample volume comprises about 8 μL of buffer (such as PBS or citrate buffer).

In one embodiment the sample (for example the total sample) comprises about 0.2 μg to about 10 μg, such as 1, 2, 3, 4, 5, 6, 7, 8, or 9 μg, in particular 1 μg of protein or polypeptide for analysis, for example the sample is a 10 μL volume comprising 8 μL of buffer, 1 μL of dye and 1 μL of protein at a concentration of 1 mg/mL.

The method is suitable for analysis of a polypeptide with some level of folding, structure, such as polypeptide with at least 20 amino acid residues. Polypeptide as employed herein is intended to refer to an amino acid sequence with at least a secondary structure. Protein as used herein is intended to refer to an amino acid sequence with a tertiary structure and/or quaternary structure.

In one embodiment the protein is a complete antibody molecule having full length heavy and light chains or a fragment thereof, such as a domain antibody e.g. VH, VL, VHH; Fab, modified Fab, Fab', $F(ab')_2$, Fv or scFv fragment. In one embodiment the antibody or fragment is human, humanized or chimeric.

In one embodiment the protein is a fusion protein comprising parts derived from heterologous proteins. A fusion protein as employed herein extends to bispecific antibodies comprising two or more, such as 2, 3 or 4 binding domains.

In one alternative aspect at least two complexes of proteins/ligands are analysed to establish the thermal stability of the same.

The method according to the disclosure allows the *Tm*, final degradation, the temperature at which dissociation occurs, and co-operativity of the proteins/polypeptides to be compared.

Co-operativity in the context of the present specification is intended to refer to where the structural folding of the protein/peptide or complex is contributed to by "elements"/components of the protein such that the interaction is synergistic and not a mere admixture. Lack of co-operativity may be indicated by unfolding which can be divided distinct phases. The distinct phases may be indicated by two or more inflections in the plot of fluorescence as a function of temperature. The distinct phases may, have distinct gradients in the curves. Lack of co-operativity may be present where proteins have distinct domains.

The method of the disclosure may, for example be used to analyse the thermal stability of substances, in particular pharmaceutical substances, such as batches of proteins/polypeptides on "long term" stability studies. Long term stability studies as employed herein is intended to refer when samples are stored under controlled conditions, i.e. controlled temperature, humidity and/or UV light conditions, for predetermined periods and subsequently analysed to assess if the storage has resulted in chemical or physical instability such as degradation and/or aggregation. The stability may, for example be assessed by looking for a change in *Tm* or a change in the temperature at which unfolding starts. In particular a drop in the *Tm* may indicate some instability in the sample. Conversely, a rise in the *Tm* may be an indicator that changes of a chemical nature have occurred and thus there is some degradation.

The method of the present invention allows the analysis of protein/polypeptide without extensive purification. See for example Figure 7 wherein the protein is distinguishable from the aggregate present in the sample. What of course is important in deciding if a sample requires purification for meaningful analysis is whether the signal from the protein/polypeptide of interest is masked/hidden by the signal produced by the impurities or other components present.

In one embodiment of the method the protein or polypeptide of interested is analysed in the presence of an aggregate protein or polypeptide. The latter aggregate may comprise the same or different proteins to that which is of interest.

The method herein is also suitable for analyzing protein and/or polypeptide samples for soluble aggregates. The detection of soluble aggregates by prior art techniques can be involved, for example they may be analysed by dynamic light scattering techniques,

which is not quantitative and for 96 samples takes many hours. Alternatively size exclusion chromatography can be employed. However, the present disclosure provides a rapid high-throughput method of analyzing samples for the presence of soluble aggregates, comprising the steps of measuring the change of fluorescence as a function of temperature. The aggregates may be homogeneous or non-homogeneous. In some instance the protein of interest may be analysed in the presence of cell lysate.

Aggregated proteins/polypeptides tend to be less stable than the non-aggregated protein and thus they can be differentiated using thermofluor analysis. Figure 7 shows the aggregate, essentially as a high base line. Thus the presence of aggregates is characterized by fluorescence at a temperature below that at which the principal transition occurs. In the example shown in Figure 7 the temperature at which the principal transition can be considered to start occurring is approximately 70 °C.

Thus in one independent aspect there is provided a method of detecting soluble aggregate comprising performing a thermofluor analysis on a sample to establish if there is fluorescence at a temperature below that at which the principal transition starts occurs.

Whilst the method of the present disclosure is highly amenable to high-throughput analysis of samples, it is also highly reproducible and has a variance of typically 0.5°C *inter* assay variation, such as 0.4°C *intra* assay variation thereby allowing results of samples analyzed at different times using the same parameters to be compared. Thus the method of the present invention is also suitable for precision low throughput analysis.

Thus the method of the present disclosure is highly amenable to being used as a tool in the quality control/assurance settings, for example in the pharmaceutical and/or food/beverages industry.

When a large number of proteins are analysed in parallel then it becomes difficult to handle the data generated therefrom to obtaining information and insights into the properties of said proteins. However, simple computer programs, based on the required mathematical laws, can be written to accumulate and process the data.

In one embodiment the method includes the further step of collecting the data employing computer software and comparing the results for each protein to identify the protein with the desired profile, such as an optimized stability profile.

In one embodiment the method includes the further step of presenting the data visually as a plot and/or as a table. The data in the table(s) may be arranged such that the protein

with the highest *Tm* (or greatest stability) is highlighted, for example the table may be ranked to provide *Tm* in descending or ascending order. Of course a parameter other than *Tm* can be chosen, as required.

An optimized stability profile may, for example include a high *Tm* and/or a high temperature at which degradation/denaturing occurs.

In one embodiment there is provided use of an algorithm disclosed herein for calculating the point or points of inflection of a curve. This has broad application to any situation where it is useful to calculate the point or points of inflection in curve. Whilst methods of calculating a single point of inflection are known in the art, the inventors believe that that commercial tools are not available for calculating multiple points of inflection of a curve.

Thus in one aspect there is provide a method of calculating one or more (such as multiple) point(s) of inflection in a curve employing an algorithm disclosed herein. In one embodiment the method employs software, which may be saved on any suitable medium including a CD-rom, a hard drive, a portable memory device such as a memory stick or the like. The software or a recording thereof forms a further aspect of the invention.

T_m Determination – principle

Starting from a set of fluorescence values (F_t) recorded at different temperature points (t), the first derivative (F_t') is calculated by using pairs of fluorescence values (F_a and F_b; where a and b represent neighboring temperature points and $b>a$) as follows:

$$F_t' = \Delta F/\Delta t = (F_b-F_a)/(b-a)$$

In this calculation, Δt (the difference between temperature points b and a) is typically between 0.1°C and 1°C, depending on the set-up of the thermofluor experiment and the equipment used.

As discussed above, the T_m is a temperature at which the rate of protein (or peptide) unfolding is maximal and at which 50% of the protein (or peptide) can be considered to be unfolded. The T_m can also be defined as the temperatures at which the slope of F_t (i.e. the first derivative F_t') is maximal.

Note that proteins (or peptides) can have one or more such T_m's, depending on their structure. See Figure 1 which shows an example of thermograms (F_t plotted against t).

Left: Thermogram with single T_m. Right: Thermogram with two T_m's. In both cases, the slope of F_t at the different T_m's.

Determining T_m values from experimental data – description of algorithm

- To reduce the noise in the experimental thermofluor data, an unweighted sliding-average smoothing function is applied.
  Typically five data points are smoothed: e.g. for every temperature t
  $$F_t(smoothed) = (F_{t-2} + F_{t-1} + F_t + F_{t+1} + F_{t+2})/5$$
- Using $F_t(smoothed)$ values, F_t' is calculated as described above.
- T_m values are identified by comparing the value of the first derivative F_t' [calculated as $F_t' = \Delta F/\Delta t = (F_b-F_a)/(b-a)$] for a given temperature t to the F_t' values of neighboring temperatures (typically, F_t' values of ±2°C around t are examined). If the F_t' value of a given temperature t is higher than the F_t' values of the nearby temperatures, t is classed as a T_m.

EXAMPLES

Example 1

Figure 2 shows a thermofluor analysis comparing an antibody and its Fab fragment.

(A) Fab fragment of the antibody giving a single transition [T_m = 77.8°C]

(B) Full IgG antibody giving two transitions:

- one transition corresponding to the Fab portion of the antibody [T_m1= 77.8°C; same as for the isolated Fab fragment shown in panel (A)]
- another transition [T_m 2=69.3°C] corresponding to the Fc portion of the antibody

Example 2

Figure 3 shows thermofluor analysis comparing two IgG antibodies, Ab1 and Ab2.
In the case of Ab1, the Fab and Fc portions of the antibody melt at different temperatures giving rise to two distinct transitions (each with a unique *Tm*).
In the case of Ab2, the Fab and Fc portions of the antibody melt at similar temperatures. Therefore only one temperature transition (with a single *Tm*) is observed.

Example 3

Figure 4 shows thermofluor analysis of a set of four different antibody Fab fragments, measured in the same buffer condition. Each Fab fragment has a distinct profile and Tm, reflecting its stability..
Fab A shows the highest *Tm* and is therefore the most stable of the set.

Example 4

Figure 5 shows thermofluor analysis of a set of bispecific antibodies, in which two antibody components are linked by a stretch of amino acids containing repeats of a glycine-serine (G4S) motif.

Depending on how many repeats of the G4S motif are used in the linker, different thermostability of the bispecific antibody is observed. The bispecific antibody with the longest linker (5xG4s; containing 5 repeats of the G4S motif), shows the highest *Tm* and is therefore the most stable of the set.

Example 5

Figure 6 shows the thermofluor analysis of two antibody fusion proteins. For both these proteins, each component heavy chain of the Fab is joined by a linker GGGGSGGGGS to a variable domain (each Fab is joined to two variable domains).

Samples were run in quadruplicate using the method described in this invention.

For both fusion proteins, two Tm's were observed:

- For FabB-645Fv, *Tm*1 was 81.9°C ± 0.6°C, and Tm2 was 68.5°C ± 0.5°C
- For FabB-648Fv, *Tm*1 was 82.4°C ± 0.2°C, and Tm2 was 70.6°C ± 0.8°C

More specifically the constructs of the present example are Fab proteins (FabB) wherein each component heavy chain and light chain of the Fab are each joined by a linker GGGGSGGGGS to a variable domain (thus each Fab is joined to two variable domains). In "dsFv" constructs a disulfide bond is present between the variable domains appended to the Fab fragment.

In FabΔB no hinge region is present and thus the Fab does not have the normal interchain disulfide between the heavy and the light chain.

Samples (1µl of sample at ~1mg/ml, 8µl of PBS and 1µl of 30x stock of Sypro orange fluorescent dye) were run in quadruplicate in 384 well plates. The plate is heated from 20-99°C using a 7900HT fast real-time PCR system and the fluorescence (excitation at 490nm, emission at 530nm) measured. For each of the proteins two *Tm*s were observed (see Table 1 and 2 below)

The introduction of an interchain disulphide bond into the Fv part of a Fab-Fv of either a 645Fv or 648Fv increased the thermal stability of the Fv therein compared with the Fab-Fv in which the Fv did not have an interchain disulphide. The removal of the natural interchain disulphide bond from the Fab part of a Fab-Fv decreased the thermal stability of the Fab part of the Fab-Fv

Table 1

	Tm °C (Fab)	Tm °C (Fv)
FabB-645Fv	**81.9 ± 0.6**	**68.5 ± 0.5**
FabB-645dsFv	83.6 ± 0.3	71.6 ± 0.3
FabΔB-645dsFv	79.5 ± 0.1	70.8 ± 0.6
FabB-648Fv	**82.4 ± 0.2**	**70.6 ± 0.8**
FabB-648dsFv	82.8 ± 0.3	75.0 ± 0.6
FabΔB-648dsFv	n.d.	73.6 ± 0.8

n.d. = not determined. The analysis software was unable to resolve this inflection point.
The data for FabB-645Fv (FabB-didAb dAbL1) and FabB-648Fv (FabB-didAb dAbL2) is also represented below in **Table 2.**

Table 2

	Tm °C (Fab)	Tm °C (Fv)
FabB-didAb, -dAbL1(CK-G_4Sx2) & -dAbL1(CH1-G_4Sx2)	81.9 ± 0.6	68.5 ± 0.5
FabB-didAb, -dAbL2(CK-G_4Sx2) & -dAbL2(CH1-G_4Sx2)	82.4 ± 0.2	70.6 ± 0.8

The data in Table 2 is also represented in Figure 6.

Example 6

Figure 7 shows thermfluor analysis of an antibody Fab fragment in the presence and absence of soluble aggregate (to remove aggregate, the sample was ultra-centrifuged at 100000g)

For both the aggregate-containing sample and the aggregate-free sample, a similar *Tm* was observed.
However in the presence of soluble aggregate, the thermogram shows a distinct slope or gradient at below-transition temperatures.
In contrast, the thermogram of the aggregate-free sample shows little or no slope at below-transition temperatures.

Example 7

Figure 8 shows a comparison of the *Tm* determined using the thermofluor method (plotted on the Y-axis) and the *Tm* determined using the Differential Scanning Calorimetry method (DSC; plotted on the X-axis).

In this experiment, 10 distinct proteins (3 full antibodies and 7 antibody Fab fragments) were analyzed by Thermofluor and DSC. For those samples giving multiple transitions, only the transition with the lowest Tm was included.

For the 10 samples tested, the *Tm* values measured by Thermofluor and DSC are highly correlated (R^2 value =0.97).

Claims:

1. An analytical method of determining thermal stability comprising the steps:
   a) raising the temperature of two or more distinct protein/polypeptide samples, at a rate suitable for measuring fluorescence therefrom, until substantially all the protein in each of the samples is unfolded, wherein each sample comprises a buffer, a fluorescent dye and a protein or polypeptide for analysis, and
   b) after excitation at an appropriated wavelength measuring fluorescence as a function of temperature, and
   c) optionally calculating the *Tm* of each sample,

wherein steps a) and b) may be effected concomitantly.

2. A method according to claim 1, wherein the protein is an antibody or a fragment thereof.

3. A method according to claim 2, wherein the antibody or fragment thereof is humanized or chimeric.

4. A method according to any one of claims 1 to 3, wherein the buffer in each of the samples is the same constitution.

5. A method according to any one of claims wherein the protein/polypeptide in the sample is present at an amount in the range 0.2 μg to 10 μg.

6. A method according to any one of claims 1 to 5, wherein about 1 μL of dye is present.

7. A method according to any one of claims 1 to 6, wherein about 8 μL of buffer is employed.

8. A method according to any one of claims 1 to 7, wherein the total volume of the sample is about 10 μL.

9 A method according to any one of claims 1 to 8 wherein the *Tm* of each sample is calculated.

10. A method according to any one of claims 1 to 9, wherein the sample or samples with the highest *Tm* is/are identified.

11. A method according to claims 9 or 10, wherein the *Tm* in each sample is compared and represented in visually a table or a plot.

12. A method according to any one of claims 1 to 11, wherein the data generated is analysed by software, for example employing an algorithm disclosed herein

13. A method according to claim 12, wherein the data points generated are smoothed.

14. A method according to claim 12 or 13, wherein the data is organized to identify the samples with the most desirable properties.

Figure 1

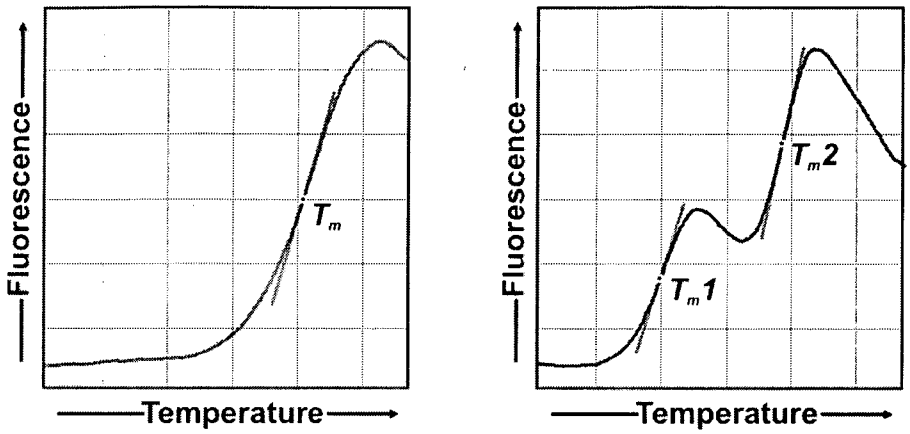

Fluorescence
Temperature
T_m
Fluorescence
Temperature
T_m1
T_m2

Figure 2

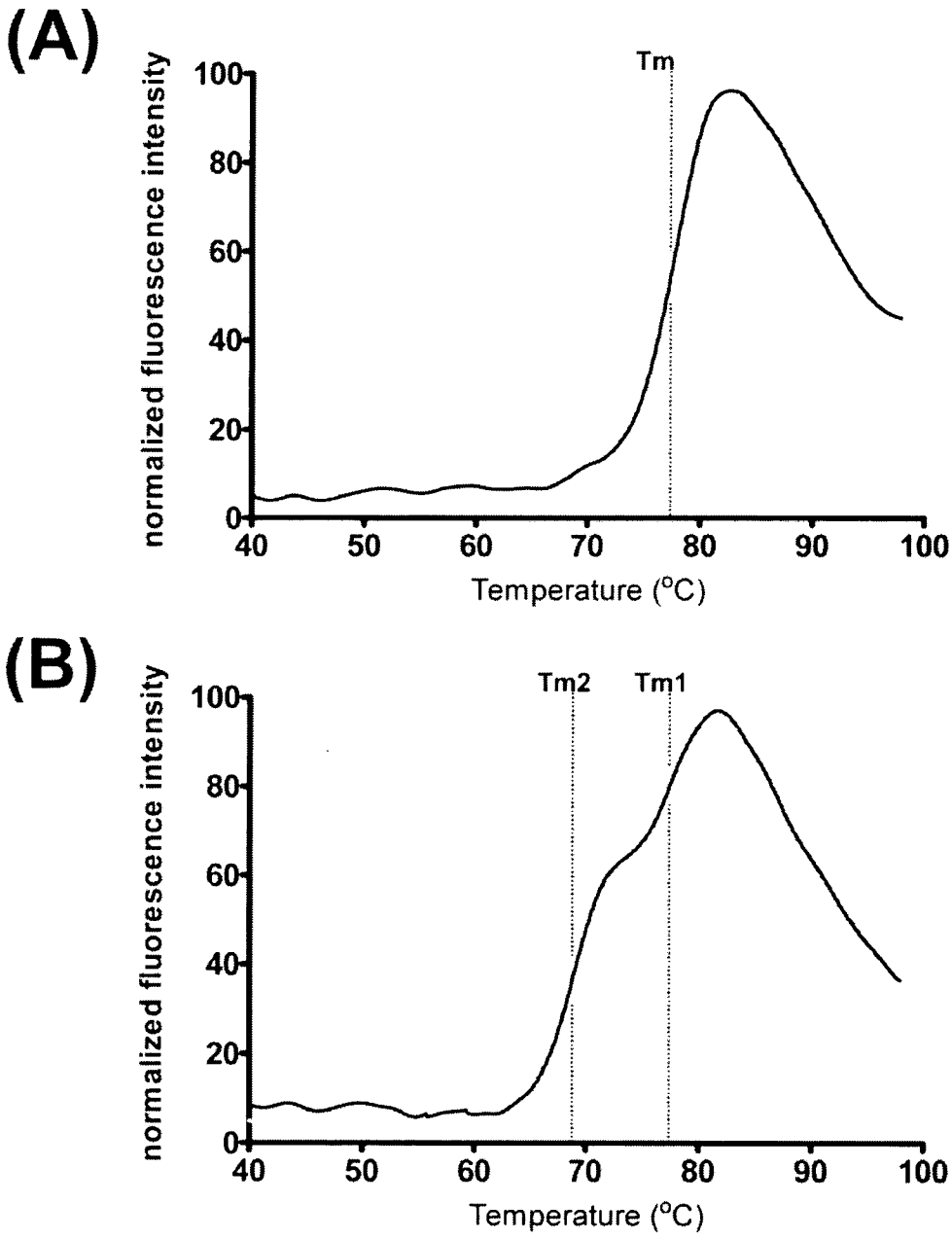

(A)
normalized fluorescence intensity
100
80
60
40
20
0
Tm
40
50
60
70
80
90
100
Temperature (°C)
(B)
normalized fluorescence intensity
100
80
60
40
20
0
Tm2
Tm1
40
50
60
70
80
90
100
Temperature (°C)

Figure 3

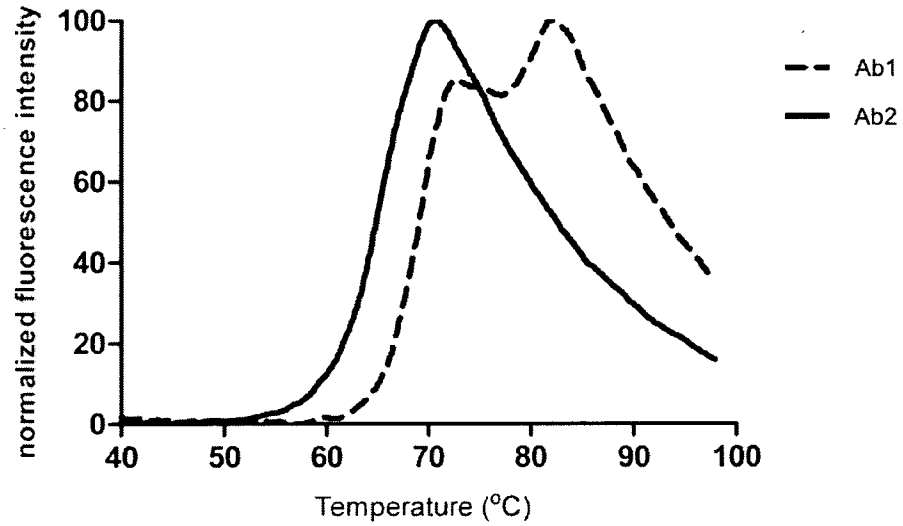


Figure 4

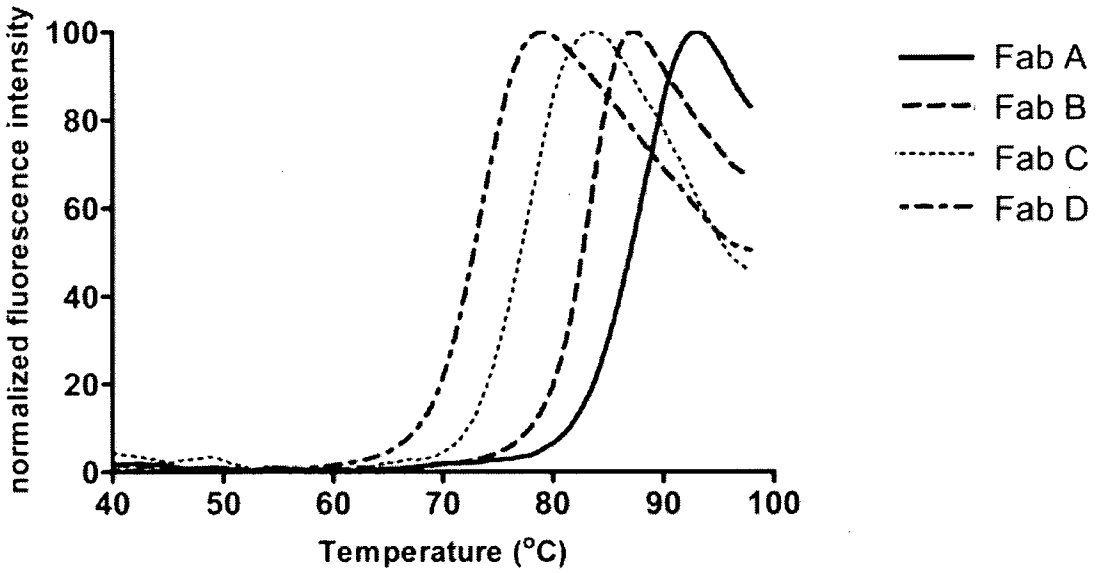


Figure 5

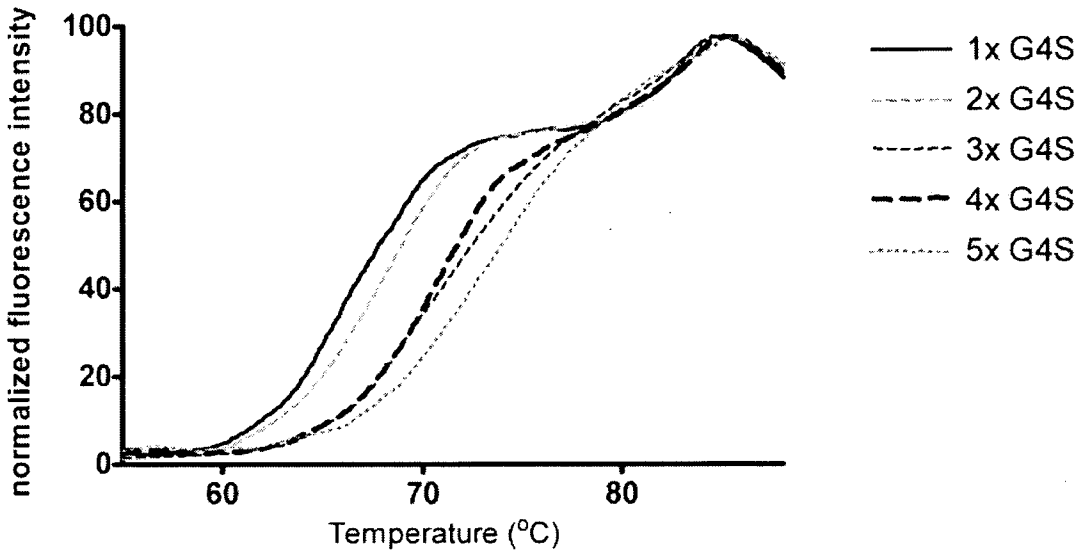


Figure 6

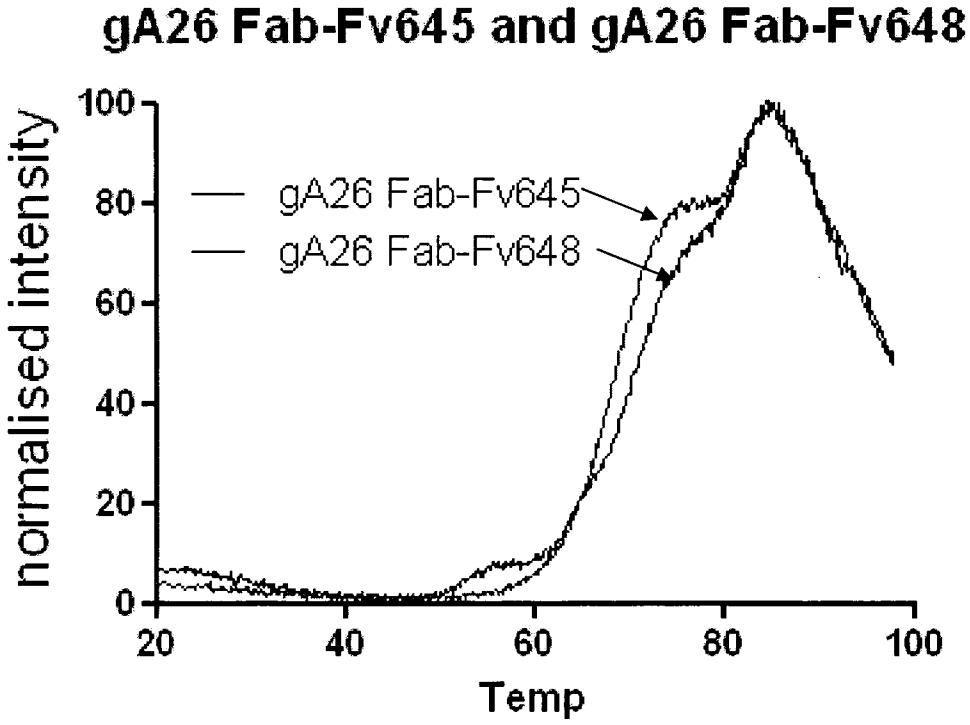


Figure 7

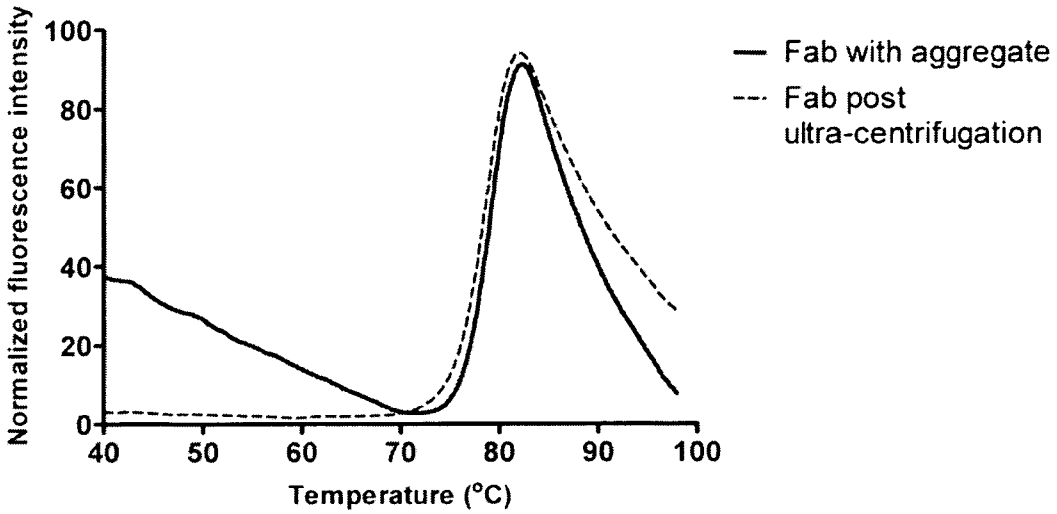
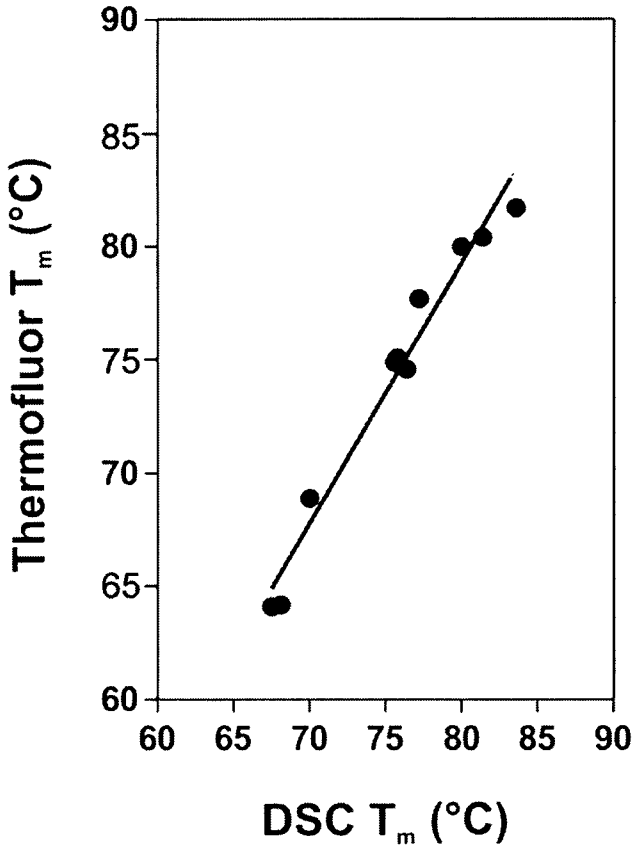

Normalized fluorescence intensity
100
80
60
40
20
0
40
50
60
70
80
90
100
Temperature (°C)
Fab with aggregate
Fab post ultra-centrifugation

Figure 8


Thermofluor T_m (°C)
90
85
80
75
70
65
60
60
65
70
75
80
85
90
DSC T_m (°C)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/000578

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MEZZASALMA TARA M ET AL: "Enhancing recombinant protein quality and yield by protein stability profiling" JOURNAL OF BIOMOLECULAR SCREENING, LARCHMONT, NY, US LNKD- DOI:10.1177/1087057106297984, vol. 12, no. 3, 1 April 2007 (2007-04-01), pages 418-428, XP008124176 ISSN: 1087-0571	1,4-14
Y	the whole document in particular: * abstract paragraph: "Thermofluor(R) assay plate assembly"; page 420, left-hand column table 1 figures 1,2,4	2,3

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2010	22/07/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Tuynman, Antonin

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/000578

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	the whole document in particular: * abstract paragraph: "Thermal shift assay"; page 290 - page 291 paragraph: "Buffer screens"; page 292, right-hand column - page 294, left-hand column figures 1,2	2,3,10, 14
X	PARTI RAJESH ET AL: "Evaluation of thermal stability of monoclonal antibody formulations by fluorescence spectroscopy" PHARMACEUTICAL RESEARCH (NEW YORK), vol. 10, no. 10 SUPPL., 1993, page S84, XP008124178 & AAPS (AMERICAN ASSOCIATION OF PHARMACEUTICAL SCIENTISTS) EIGHTH ANNUAL MEETING AND EXPOSITION; ORLANDO, FLORIDA, USA; NOVEMBER 14-18, 1993 ISSN: 0724-8741	1,2
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Y	WO 99/24050 A1 (DIMENSIONAL PHARM INC [US]) 20 May 1999 (1999-05-20) * abstract page 38, line 28 - page 39, line 11	9-14
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PCT/GB2010/000578

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