Research Article

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Qualification of a differential scanning calorimetry method for biophysical characterization of monoclonal antibodies and protein vaccine antigens

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Background: Analytical method qualification consists of a simplified evaluation of a subset of validation characteristics with a goal to demonstrate that an analytical method is scientifically sound and suitable for its intended use. In contrast to validation, analytical method qualification is performed without predefined acceptability criteria. Qualification may be performed as a prerequisite to method validation, or when an assay for product knowledge has not yet been established as a test for a critical product quality attribute. **Methodology:** The focus of this study was the qualification of a differential scanning calorimetry method that is used to examine thermal stability and tertiary structure of protein vaccine antigens and monoclonal antibody products. The analytical parameter evaluated was precision, through assessment of the characteristics of repeatability and intermediate precision. **Conclusion:** The results demonstrated that the assay met the desired performance characteristics, and is suitable for its intended use, namely to measure the thermal transitions of different vaccine antigen proteins and monoclonal antibodies in solution.

Introduction

Analytical method qualification

Analytical methods are laboratory methods used to determine the concentration, purity, identity, and potency of a **protein antigen** or monoclonal antibody, as well as its higher order structure and function. Normally, a combination of biochemical, biophysical, immunochemical, and biological/functional methods is used to build an analytical package for a product of interest.

Analytical method qualification is defined as a process, including a series of laboratory experiments, designed to document analytical method performance, and to demonstrate that the method is fit for its intended purpose [1–3]. As a prerequisite to method qualification, an analytical procedure should be developed, and should be documented to describe, for example, the objective of the analysis, scientific principle of the method, types of products to be analyzed, list of equipment and reagents, operating conditions, analysis of results, and assay performance or assay validity criteria.

Analytical method qualification is not specifically defined by regulatory guidelines and may be referred to differently in different organizations [4]. Nevertheless, qualification can be defined as above and can further be distinguished from method validation [4] in that it consists of a simplified evaluation of a subset of validation characteristics, and that there are no predefined acceptability criteria for evaluation of method performance. Thus, a method cannot 'fail' qualification; however, in some cases the results of a qualification study (e.g., the method did not achieve the desired performance characteristics), could lead to a decision to re-develop or re-optimize the method before proceeding with routine product testing.

The execution of an assay qualification plan or protocol demonstrates the performance of the assay with respect to the parameters being evaluated. Method qualification can be applied to tests used for product knowledge, product comparability studies [5], assessment of impurities, process validation studies, product release, in-process con-

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Key terms

Analytical methods: Used to determine the concentration, purity, identity, and potency of a protein antigen, as well as its higher order structure and function. Normally, a combination of biochemical, biophysical and immunochemical methods is used to build an analytical package for a protein antigen of interest.

Protein antigen: A protein that induces an immune response in the body (e.g. the production of antibodies).

Analytical method qualification: The process of determining the reliability of the method in order to verify the applicability of the method for its intended use.

trol, and/or stability studies [2–3,6]. For products in an early developmental stage (e.g., preclinical, Phase I/II), a qualification study is desirable in order to demonstrate and document analytical method performance. For an analytical method which will be validated at a later date (e.g., prior to Phase III), the results from the qualification study may be used to establish the acceptability criteria to be defined in the subsequent validation protocol.

The selection of qualification characteristics (e.g., specificity, precision, linearity) should be scientifically driven and defined case by case based on the type of test, the technology, and its intended use. An experimental design based on these qualification characteristics should be established prior to execution of the method qualification, and can be captured in a qualification plan or protocol.

It is important to note that qualification corresponds only to an evaluation of the method performance. The results must always be evaluated based on the objective of the study that uses the analytical method.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) is an experimental method to measure the heat energy uptake and release that takes place in a sample during a controlled increase or decrease in temperature [7,8]. However, the scope of the DSC method application in this study is heat absorption. With high instrument sensitivity, the characteristic thermal transitions of macromolecules such as proteins in solution or suspension can be measured.

DSC may be used to determine the thermal transition temperature, also referred as 'melting' temperature for the samples in solution, solid, and mixed phases. The advantage of DSC is that it can directly measure intrinsic thermal properties of the protein, such as protein unfolding (i.e., loss of tertiary structure as a function of temperature) without any pretreatment of the sample using chemical modification or extrinsic probes. In addition, because DSC is a calorimetric method that does not rely on optics or interactions with a chromatographic medium, it is often amenable to analysis of complex mixtures such as suspensions. This is important in particular for analysis of vaccines, which may consist of, for example, aluminum adjuvant suspensions and/or complex antigens such as virus-like particles (VLPs) [9,10]. Present day DSC instruments equipped with user-friendly software are sensitive enough to detect even a subtle difference in conformation of the proteins. Protein antigen or monoclonal antibody conformation may be impacted by changes either in the upstream fermentation, or purification, formulation of drug substance and drug product, or other factors. Thus, DSC is useful in product lot comparability assessments, stability studies, and other investigations.

DSC offers a variety of applications in the evaluation of protein stability. In particular, it is possible to determine the most suitable conditions for stabilizing liquid formulations of proteins [11]. In DSC studies, the temperature at the maximum of the C_p curve is defined as the mid-point transition temperature (T_m), is diagnostic of the macromolecule's stability [11–15]. Through the first calorimetric studies of the temperature-induced unfolding of compact globular proteins, it has been noticed that the thermal denaturation profile depends on the solution conditions, for example, pH. As a readout for protein tertiary structure conformation, DSC can be performed as part of a product characterization package that would also normally include tests for content, purity, identity, potency, and other attributes. In the context of vaccine antigens, tertiary structure can be informative as an indicator of process consistency (i.e., ability of the expression, purification, and formulation processes to generate a protein of consistent and uniform folded structure). In turn, a consistent folded structure can be considered as predictive of correct presentation of epitopes on the antigen molecule surface, with implications for immunogenicity of the vaccine. DSC can thus provide such information about product and process consistency, even in the absence of detailed knowledge of the protective epitopes of an antigen, which may frequently be the case for early-stage vaccine products. While DSC does not have the power of techniques such as x-ray diffraction and NMR to determine high-resolution 3D protein structures, the availability of highly sensitive instruments and techniques enables measurement of the thermal transitions associated with both intra- and inter-macromolecular structures [8]. As a result, DSC is one of the most frequently used techniques to determine thermal stability of proteins [11] and to measure the thermodynamic parameters of protein unfolding [11,16–18].

In our experience, DSC can provide qualitative and quantitative information about product variants,

including product-related impurities (i.e., variants with different biological activities than the active substance) and/or product degradants (e.g., resulting from forced-degradation studies). For example, samples with different ratios of product to product variant(s) may display differences in DSC parameters such as transition enthalpy, as well as in overall thermal stability profiles. Certain product variants with different biological activities may not be detectable by methods commonly used to assess product purity and impurities, such as SDS-PAGE, capillary electrophoresis, and mass spectrometry. An obvious example of this would be misfolded proteins. In addition, DSC in our hands has proven to be a useful tool to assess the completeness of conjugation and other chemical modification (e.g., inactivation) of the protein.

DSC methods have been developed and assessed in our laboratory for several protein vaccine antigen products. During method development and optimization, two key parameters typically examined were scanning rate and concentration. For proteins that are prone to aggregation during the DSC experiment (due to the sample heating), the scanning rates of 90°C/h and 120°C/h yielded the highest response. We observed that protein antigens that were expressed as insoluble inclusion bodies, and subjected to denaturation and refolding during the purification process, fell into that category (data not shown). However, the majority of the protein vaccine product candidates examined are assessed using a scanning rate of 60°C/h, and produce consistent results. For the products presented in this paper, we have tested them at different concentrations. However, a decrease in concentration did not result in Tm shift, indicating that there were no intermediate stages during thermal unfolding, and the two state model was applicable.

Qualification of a DSC method for antibody products has been previously described by Wen *et al.* [19]. In this work, we additionally describe the novel qualification of a DSC method for characterization protein vaccine antigens.

Qualification of the DSC method

To demonstrate that the DSC method is suitable for its intended application, that is, to measure the thermal transitions of vaccine antigen proteins and monoclonal antibodies in solution, assay precision was assessed. The precision of the DSC method was evaluated through a multi-analyst and multi-day study, where the same data collection and analysis method were used. Mid-point transition temperatures, Tm and enthalpies of transition, ΔH were collected and analyzed statistically. The precision of DSC analysis from this study can be used to define and assess desired method performance parameters.

Purpose

The purpose of the qualification study was to determine the suitability of the DSC method to measure Tm, and ΔH) of the protein vaccine antigens 1 and 2, and a monoclonal antibody at two different manufacturing stages.

Scope

The scope of the qualification study was assessment of the intermediate precision and repeatability for the DSC parameters, Tm and ΔH collected for the monoclonal antibodies and protein vaccine antigens. Samples representative of different steps in the respective manufacturing processes were used in the qualification study. The samples included monoclonal antibody intermediate, conjugated antibody drug substance, and two different chemically inactivated protein vaccine antigens.

In this study, DSC thermograms were collected for chemically modified protein vaccine antigens and recombinant monoclonal antibody products. These materials are manufactured in Sanofi Pasteur and stored in aqueous buffer of neutral pH. The monoclonal antibody does not contain any excipients, whereas the chemically modified protein antigen contains one excipient.

Analytical procedure

As mentioned above, DSC measures the heat capacity of macromolecules during temperature-induced transitions. During the DSC experiment, two parameters are calculated: the temperature midpoint, Tm of the conformational transition, and the enthalpy ΔH, also referred to as ΔH_{cal}, which is the energy that maintains the folded tertiary structure at constant pressure and volume. These parameters provide a measurement of the strength of the interactions required to maintain the tertiary structure of the molecule.

For a protein vaccine antigen and monoclonal antibody product, the molar heat capacity C_p is measured as a function of temperature. The C_p of the solution containing a macromolecule is measured with respect to the C_p of buffer, contained in the reference cell [19]. The sample could be a protein, a protein-lipid complex, a protein-DNA complex, formulated suspension. In this paper, we focus on DSC applications for protein vaccine antigens and monoclonal antibody products.

The C_p at constant pressure is a temperature derivative of the enthalpy function [$C_p = (\Delta H/\Delta T)_p$], and thus, the enthalpy function can be measured through integration of the C_p [$H(T) = \int T/T0\, C_p(T)dT + H(T_0)$] [20].

Van't Hoff enthalpy (ΔH_{VH}) is an independent measurement of the transitional enthalpy according to the model of the experiment [20,21]. ΔH_{VH} is determined

Key terms

Calorimetric enthalpy ΔHcal or ΔH: The total integrated zone below the thermogram peak, which indicates total heat energy uptake by the sample after baseline correction.

through the shape analysis of an experimental graph of C_p versus T [16,19–20]. The state of the transition is evaluated by comparing ΔH_{VH} with ΔH_{cal} [19–22]. If ΔH_{VH} is equal to ΔH_{cal}, the transition occurs in a two-state mode. When ΔH_{VH} is more than ΔH_{cal}, inter-molecular cooperation occurs, for example self-association or aggregation. A ratio of $\Delta H_{VH}/\Delta H_{cal}$ corresponds to a portion of the structure that had melted as a thermodynamic value. This portion of the structure is also referred to as the size of the cooperative unit [23].

Calorimetric measurements were performed using a Capillary VP-DSC microcalorimeter with autosampler (GE Healthcare/Microcal, Northampton, MA, USA). The heat capacity versus temperature curves were recorded using the VP Viewer 2000 DSC, version 1.03 software. The melting temperature and enthalpy of transitions were determined using the Microcal Origin™, version 7.0 software. The scan rate was 1°C/min, and pressure was 40 p.s.i.

Study design

The purified product samples for the qualification study were aliquotted freshly each day of the experiment by adding an aliquot of 400 µl into the 96-well plate. Each sample of protein vaccine antigen 1 and 2 was analyzed by two analysts over 3 days with six replicates each during the same experiment. The experiment that was conducted by one analyst in one day is called one 'run' of the qualification study. Therefore, the study was designed with three runs and six replicates within each run. The factor 'run' was taken into account when assessing the precision of the method ('Statistical analysis' section). Each sample of monoclonal antibody was analyzed by two analysts over 3 days with four replicates each day using the same equipment, but with independent reference buffer preparation. Test articles for the protein vaccine antigens were analyzed at 0.5–1 mg/ml without dilution, whereas monoclonal antibody intermediate and drug substance samples were prepared by dilution of each stock solution to a final concentration of 2 mg/ml. No changes to the qualification plan were made during its execution. Data analysis for all sets of samples was performed following the experiments. The data was then sent for the statistical analysis. The predefined system suitability criteria were met for each assay run performed.

Data analysis

DSC measures enthalpy (ΔH) of unfolding as a result of heat denaturation. The transition midpoint T_m is considered as the temperature, where 50% of the protein owns its native conformation, and the rest remains denatured. Higher T_m values would be representative of a more thermally stable molecule. **Calorimetric enthalpy ΔH** means the total integrated area under the thermogram peak, which indicates total heat energy uptake by the sample after suitable baseline correction affecting the transition [21]. The Microcal Origin™ software provides two options for baseline fit: linear or cubic. In most cases, including for the two products discussed in this study, linear baseline is used. Cubic baseline is selected for profiles that show curvature on the peak flanks, which is typically the case for proteins with multiple transition peaks (i.e., ≥3).

Figure 1 shows an example of the DSC profiles obtained for protein vaccine antigens 1 and 2, whereas Figure 2 shows the DSC profiles for the monoclonal antibody intermediate and conjugated drug substance. The black trace represents the buffer-subtracted, baseline-corrected thermogram, whereas the red traces rep-

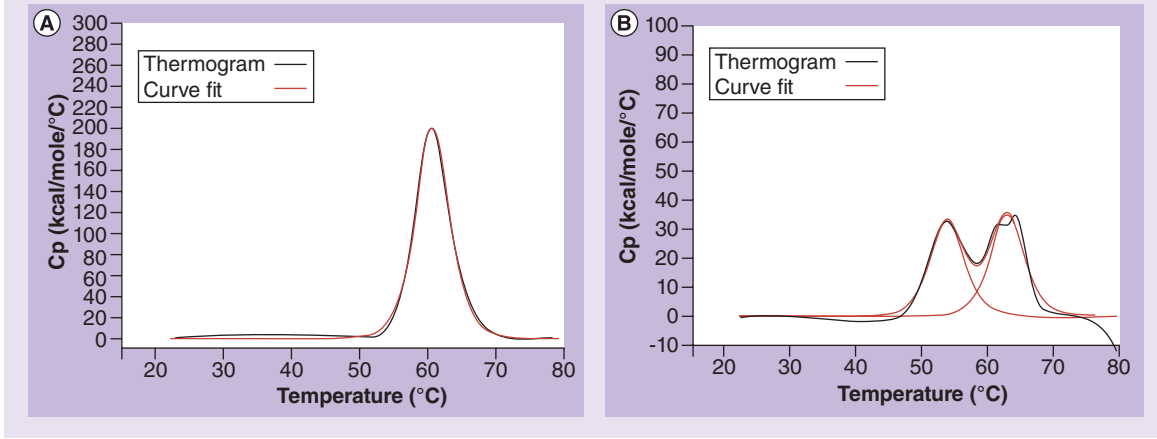

Figure 1. Differential scanning calorimetry thermograms. (A) Protein vaccine antigens 1 and **(B)** 2.

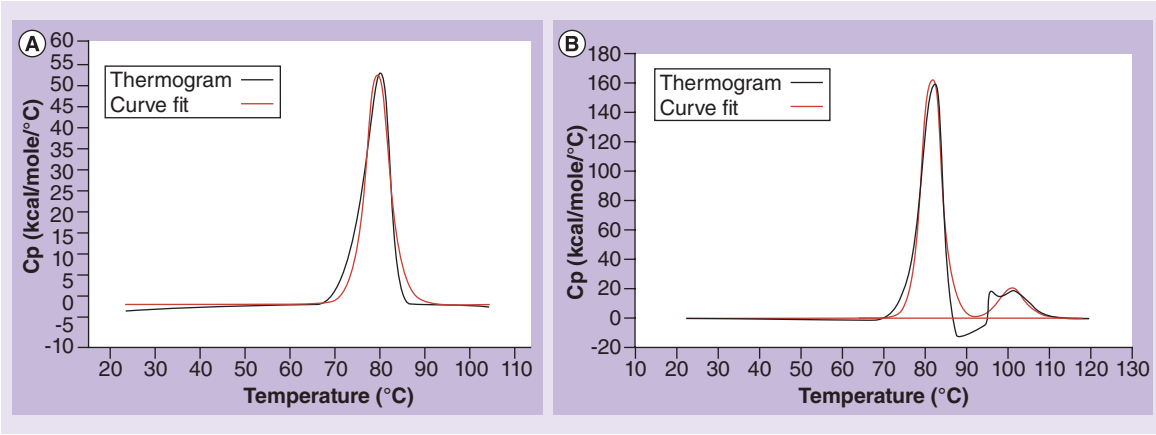

Figure 2. Differential scanning calorimetry thermograms. (A) Monoclonal antibody intermediate and **(B)** conjugated drug substance.

resent the curve fits generated by the Origin software. Transition midpoint temperature (Tm) and molar enthalpy of transition (ΔH) values for protein vaccine antigen and monoclonal antibody are summarized in Table 1. The thermal stability of both the protein vaccine antigens (Tm ~53–63°C) and monoclonal antibody (~80–81°C) is relatively high, indicating overall stable conformations. An interesting feature of the thermogram of the monoclonal antibody drug substance is the presence of a secondary, higher-temperature transition (Figure 2, right panel). As the shape and Tm of the primary transition of the drug substance is similar to that of the intermediate (Figure 2, left panel), it is likely that this secondary transition arises from the chemical conjugation.

As mentioned in Section 2.4, a total of 18 measurements of Tm and ΔH were collected for protein vaccine antigens 1 and 2, and a set of 12 measurements was collected for the monoclonal antibody intermediate and conjugated drug substance. The data were subjected to statistical analysis as described below.

Statistical analysis

Precision was assessed using the reportable values calculated as discussed above. When the data are evident of normality with homogenous variances across different days (defined as the factor 'run'), their precisions were assessed with variance component model using all the data obtained from different runs, with factor 'run' considered as random. An overall repeatability was then presented.

Variance component models were built as the following:

Repeatability: $y_{ij} = \mu + \varepsilon_{ij}$

Intermediate precision: $y_{ij} = \mu + R_i + \varepsilon_{ij}$

Where y_{ij} is the i^{th} run ($i = 1,\ldots, I$) and the j^{th} replicate ($j = 1,\ldots, J$) result. μ is the overall mean and R_i ($=\mu_i - \mu$) is the deviation from the mean of the i^{th} run mean. ε_{ij} is the deviation from the run mean of the ij^{th} result. R_i are normally distributed with mean 0 and variance σ^2_{br}; ε_{ij} are normally distributed with mean 0 and variance σ^2_{wr} and R_i are independent of the ε_{ij}

The estimation of the variance components was conducted using the SAS MIXED procedure with Restricted Maximum Likelihood estimation method considering 'Run' as a random effect. The total random error (intermediate precision) was calculated as $\sigma_{IP}^2 = \sigma_{br}^2 + \sigma_{wr}^2$

Both repeatability and intermediate precision were calculated using the formula below:

$$\%CV = 100\% \times \left(\frac{\sqrt{\sigma^2}}{mean}\right)$$

Where $\sigma^2 = \sigma_{wr}^2$ for repeatability and $\sigma^2 = \sigma_{IP}^2$ for intermediate precision.

Table 1. Differential scanning calorimetry parameters for protein vaccine antigens and monoclonal antibody.

Material	T_m (°C)	ΔH (kcal/mol)
Protein vaccine antigen 1	60.8	13.5×10^5
Protein vaccine antigen 2	53.8, 62.9	2.28×10^5, 2.43×10^5
Monoclonal antibody intermediate	79.5	3.72×10^5
Conjugated drug substance	81.5, 100.8	10.1×10^5, 1.71×10^5

In cases where data are too sparse and the normality cannot be demonstrated (Shapiro–Wilk W Test p <0.01), the standard deviation was calculated using the range method [24] as described below:

$R = \max(x_i) - \min(x_i)$,

Where $x_1, x_2, \ldots x_i \ldots x_n$ are random samples of n observations from a normal distribution with mean μ and variance σ^2.

The standard deviation is estimated as

$$\hat{\sigma} = \frac{R}{d^2}$$

Where d_2 is a constant used to estimate standard deviation and the value varies with different sample size n, and are available in [24].

The repeatability values for each run were expressed as %CVs and the average of the three repeatability values were further calculated (Table 2).

The intermediate precision was expressed as %CV and calculated using the entire data across three runs.

Qualification results

Qualification results for the repeatability and intermediate precision determinations are summarized in Table 2, for the two protein vaccine antigens and the monoclonal antibody. The desirable performance values were defined based on previous in-house experience with the DSC method and the number of lots tested for each of the products.

The repeatability %CV for the T_m of protein vaccine antigens 1 and 2 ranged from 0.1 to 0.2% and for ΔH ranged from 2.6 to 5.1%. The intermediate precision for the T_m of protein vaccine antigen 1 and 2 ranged from 0.1 to 0.3% and for ΔH ranged from 2.6 to 6.8% CV. These data indicate that both the repeatability and intermediate precision of the assay met the desired performance levels of <2% and <15% CV for T_m and for ΔH respectively (Table 2).

The repeatability %CV of T_m for the monoclonal antibody intermediate ranged from 0.2 to 0.3% and for ΔH ranged from 0.5 to 0.6%. The intermediate precision of T_m for the monoclonal antibody intermediate was 0.23% and for ΔH was 0.59% CV. The repeatability %CV of T_{m1} and T_{m2} for the conjugated drug substance ranged from 0.2 to 0.3%, the %CV for ΔH_1 was 0.59%, whereas for ΔH_2 it was 2.65%. The intermediate precision of T_{m1} and T_{m2} for the monoclonal antibody intermediate was 0.17 and 0.19% respectively, whereas %CV for ΔH_1 was 0.59% and for ΔH_2 was 2.65%. These data indicate that both the repeatability and intermediate precision of the assay met the desired performance levels of <2% and <15% CV for T_m and ΔH, respectively (Table 2). For both the protein antigens and monoclonal antibody products, the variability of ΔH was observed to be higher than that of Tm and is highly dependent on the product. This observation was consistent with our in-house experience with the DSC assay and was

Table 2. Qualification study results for protein vaccine antigens 1 and 2, and monoclonal antibody.

Characteristic	Design	Desirable performance	Analyte	Results	
				ΔH%CV	T_m%CV
Repeatability	Repeatability was calculated from 6 independent determinations for each day of analysis (N = 6 on each day)	<15% CV for ΔH, <2% CV for T_m	Protein vaccine antigen 1	2.6	0.2
			Protein vaccine antigen 2	5.1/4.7†	0.2/0.1†
	Repeatability was calculated from 4 independent determinations for each day of analysis (N = 4 on each day)		Monoclonal antibody intermediate	0.6	0.2
			Conjugate drug substance	0.6/2.7†	0.3/0.2†
Intermediate precision	Intermediate precision was calculated from all determinations across all days and all analysts (N = 18)	<15% CV for ΔH, <2% CV for T_m	Protein vaccine antigen 1	2.6	0.3
			Protein vaccine antigen 2	6.8/6.5†	0.2/0.1†
	Intermediate precision was calculated from all determinations across all days and all analysts (N = 12)		Monoclonal antibody intermediate	0.6	0.3
			Conjugate drug substance	0.6/2.7†	0.2/0.2†

†Two peaks were identified for Protein vaccine antigen 2 and monoclonal antibody conjugated drug substance. The %CV are representative of peak 1 and peak two, respectively.

taken into account when establishing the desirable performance values.

Conclusion & recommendations

The qualification experiments showed that the DSC method for the protein vaccine antigens 1 and 2, and monoclonal antibody intermediate and drug substance met the desired performance levels for repeatability and intermediate precision, for both T_m and ΔH. It is concluded that this method is suitable for its intended use; namely to assess the thermal stability of protein vaccine antigens, and monoclonal antibody products at different stages of manufacturing. The information obtained during the qualification experiments will be useful in setting acceptability criteria for future validation studies, if required for the respective products at the appropriate developmental stage.

Future perspective

Overall, a careful choice of relevant analytical methods will facilitate product development by, for example, accumulating knowledge of various product attributes, assessing the impact of process changes, assessing lot to lot comparability, measuring stability, and serving as the basis of a product release test package, where specific tests have been identified as critical product quality attributes. A well thought out qualification study design and execution will ensure that the specific method achieves the desired performance characteristics and will be suitable for its intended purpose. Qualification should be documented appropriately, for example by means of a written qualification plan and qualification report. DSC is an instrument-based biophysical method which does not require specific reagents (e.g., antibodies), modifications to the sample (e.g., chemical conjugation or use of dyes), and does not employ media such as chromatography columns or capillaries. In addition, DSC can be performed in complex sample matrices and formulations, such as adjuvants. Thus, as a part of a test package for investigational products, DSC offers considerable advantages as a conformational readout.

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Executive summary

Background

- A differential scanning calorimetry method is described, with specific focus on qualification as a product characterization method for monoclonal antibodies and vaccine protein antigens

Qualification study design

- Aspects to consider in qualification study: purpose and scope of the analytical procedure, product type(s), qualification experimental design, and data analysis including the use of statistical methods.
- Aspects of method qualification are discussed, including rationale, reporting values, desirable performance, and qualification characteristics (in this case, repeatability and intermediate precision).

Qualification results & interpretation

- DSC method qualification examples are presented for the following products – a monoclonal antibody intermediate and conjugated drug substance, and two protein vaccine antigens.

Conclusion & recommendations

- Successful completion of the qualification study provides scientific evidence that the method is suitable for its intended use. The qualification results can also provide guidance and useful information for the eventual method validation, where required.

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