# Chemical and Thermal Unfolding

Application Note NT-PR-009

# Chemical and Thermal Stability Screening of an IgG1-Antibody

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## Abstract

**The main purpose in formulation development of biologicals, such as monoclonal antibodies, is to establish optimal conditions for long-term stability of the protein.**

**Here we used the Prometheus NT.48 instrument by NanoTemper Technologies to perform a buffer screening for an IgG1-Antibody. This instrument uses nanoDSF technology to measure thermal and chemical stability as indicators to predict the best buffer conditions for the protein.**

**The results show that thermal and chemical unfolding, together with short-term stability studies, provides a complementary screening tool for finding optimal buffer conditions.**

## Introduction

Biopharmaceuticals, especially monoclonal antibodies, have reached an increasingly important position on the drug market over the last years. To screen for a formulation with a long shelf-life, analyses focusing on the unfolding of the protein are often used as optimization tool, since unfolding may lead to aggregation and therefore needs to be avoided. There are two common procedures used to induce protein unfolding, i.e. by constantly increasing temperature (thermal unfolding) and by titrating a denaturing agent such as guanidine hydrochloride into the protein sample (chemical unfolding). The Prometheus NT.48 instrument can be used for both experimental approaches. It measures changes in intrinsic fluorescence of the protein. Once the unfolding process begins, induced either by thermal or chemical stress, the tryptophan and tyrosine groups expose themselves to the solvent. This causes a shift in fluorescence emission especially of the tryptophan groups, which is plotted as the ratio between 350 and 330 nm. The inflection point of the resulting sigmoidal curve provides the temperature ($T_m$) or the concentration of the denaturing agent ($C_m$), at which half of the protein amount is unfolded. [1]

The Prometheus NT.48 is well suited for buffer screening because of its possibility to measure up to 48 samples simultaneously as well as its low consumption of sample (10 µL per capillary). The needlessness of an additional fluorescence dye and the easy handling are further advantages.

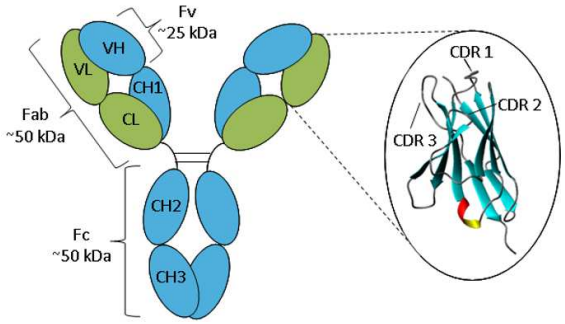


**Figure 1**: Structure of an Antibody: $F_{ab}$, $C_H2$, $C_H3$ domains highlighted. The enlargement of the variable domain shows a ribbon representation of the Beta-sheet framework and CDR loops.

# Material and Methods

### Preparation of protein formulations

By ultrafiltration/diafiltration (UF/DF), nine formulations containing an IgG1-Antibody at a protein concentration of 25 mg/mL were prepared. All formulations were used both for thermal and chemical unfolding experiments.

**Table 1:** overview of the buffers and their pH-values used in the screening

| Buffer | pH-value | | |
|---|---|---|---|
| 20mM $Na_2HPO_4 \cdot 2H_2O$ | 5.5 | 6.5 | 7.5 |
| 10mM/10mM Histidine/Glycine | 5.5 | 6.5 | 7.5 |
| 50mM TRIS-buffer | 6.5 | 7.5 | 8.5 |

### Thermal unfolding experiments

The capillaries were filled with the different antibody formulations and placed onto the capillary tray of the Prometheus NT.48. Start and end temperature as well as heating rate were defined (1 °C/min, from 20 °C to 95 °C).

### Chemical unfolding experiments

Chemical unfolding of the IgG1-Antibody was induced by constantly increasing the concentration of the denaturant, while keeping the protein concentration and pH constant. This was done by combining three solutions as presented in Table 2:

i. a fixed amount of protein stock solution (concentration 25 mg/mL)
ii. varying amounts of 8 M guanidine hydrochloride stock solution (denaturant)
iii. buffer stock solution

Before data are recorded, it is important that the folding/unfolding reaction reaches an equilibrium state. Therefore, the twenty experimental solutions were given an equilibration time of 24 hours. The solutions were measured using the Prometheus NT.48 employing the same instrument settings as already mentioned (1 °C/min, from 20 °C to 95 °C). The changes in the fluorescence ratio (F350/F330) of every experimental solution were recorded versus increasing temperature. However, for this Application Note the fluorescence ratio at a fixed temperature of 20 °C was used for detecting the chemical stability ($C_m$) to have only the denaturant concentration as varying parameter.

**Table 2:** experimental solutions were prepared volumetrically from GdmCl, buffer and protein stock solutions following this schema

| Number | GdmCl stock solution [mL] | Buffer stock solution [mL] | Protein stock solution [mL] | GdmCl experimental solution [mol/L] |
|---|---|---|---|---|
| 1 | 0.1 | 1.9 | 0.5 | 0.32 |
| 2 | 0.2 | 1.8 | 0.5 | 0.64 |
| 3 | 0.3 | 1.7 | 0.5 | 0.96 |
| 4 | 0.4 | 1.6 | 0.5 | 1.28 |
| 5 | 0.5 | 1.5 | 0.5 | 1.60 |
| 6 | 0.6 | 1.4 | 0.5 | 1.92 |
| 7 | 0.7 | 1.3 | 0.5 | 2.24 |
| 8 | 0.8 | 1.2 | 0.5 | 2.56 |
| 9 | 0.9 | 1.1 | 0.5 | 2.88 |
| 10 | 1.0 | 1.0 | 0.5 | 3.20 |
| 11 | 1.1 | 0.9 | 0.5 | 3.52 |
| 12 | 1.2 | 0.8 | 0.5 | 3.84 |
| 13 | 1.3 | 0.7 | 0.5 | 4.16 |
| 14 | 1.4 | 0.6 | 0.5 | 4.48 |
| 15 | 1.5 | 0.5 | 0.5 | 4.80 |
| 16 | 1.6 | 0.4 | 0.5 | 5.12 |
| 17 | 1.7 | 0.3 | 0.5 | 5.44 |
| 18 | 1.8 | 0.2 | 0.5 | 5.76 |
| 19 | 1.9 | 0.1 | 0.5 | 6.08 |
| 20 | 2.0 | 0.0 | 0.5 | 6.40 |

### Stability upon 6 weeks storage

Furthermore the Prometheus NT.48 was used for a stability experiment to generate more data on the stability of the IgG1-Antibody in the different buffers. For this, all formulations were stored at 2 °C-8 °C as well as in a heating chamber at 40 °C. Thermal unfolding curves were then measured at $T_0$ and after 7, 14, 21 and 42 days of storage.

# Results and Discussion

Measurements of the chemical and thermal unfolding with the Prometheus NT.48 reveal typical sigmoidal curves with a left plateau representing the fully native state and a right plateau representing the fully denatured state of the protein. The inflection points, which are plotted between these plateaus, correspond to the maximum value of the first derivative. They indicate the temperature or GdmCl-concentration, at which the amount of folded and unfolded molecules are equal. Due to

the fact that antibodies are multi-domain proteins there is more than one inflection point: every domain of an antibody ($F_{ab}$ fragment, $C_H2$ domain, $C_H3$ domain) can unfold independently. Temperature or pH value may have different effects on the $T_m$ or $C_m$ of the individual domains.[2]

In the past, DSC- and DSF diagrams of individual fragments and the intact antibody were compared to understand the individual transitions of a multi-domain antibody .[3,4,5] In his DSF analyses, Menzen identified the first melting transition of a monoclonal antibody as the $C_H2$ domain and the second one as the $F_{ab}$ fragment, whereas the unfolding of the $C_H3$ domain could not be detected.[3] Thies compared the stability of $C_H3$ and $C_H2$: The $C_H2$ domain proved to be less stable than $C_H3$ concerning thermal and chemical denaturation.[5] He showed that the large hydrophobic surfaces of the $C_H2$ domain are strongly exposed to the solvent, which means loss of stability. In contrast, these hydrophobic regions of $C_H3$ are hidden because of the association of monomeric $C_H3$ domains, which leads to entropic stabilizations.[5]
These assumptions have been used to analyze the unfolding curves provided by the nanoDSF technology of the Prometheus NT.48.

The denaturation curves show that the IgG1-Antibody produces two peaks deriving from at least two domains, which denature under distinct conditions (Figure 1). The first transition point correlates with the $C_H2$ domain and the second one with the $F_{ab}$ fragment. A transition point of the $C_H3$ domain could either not be detected by the Prometheus NT.48 or may be overlaid by the $F_{ab}$ peak, since the latter peak seems to be quite broad.

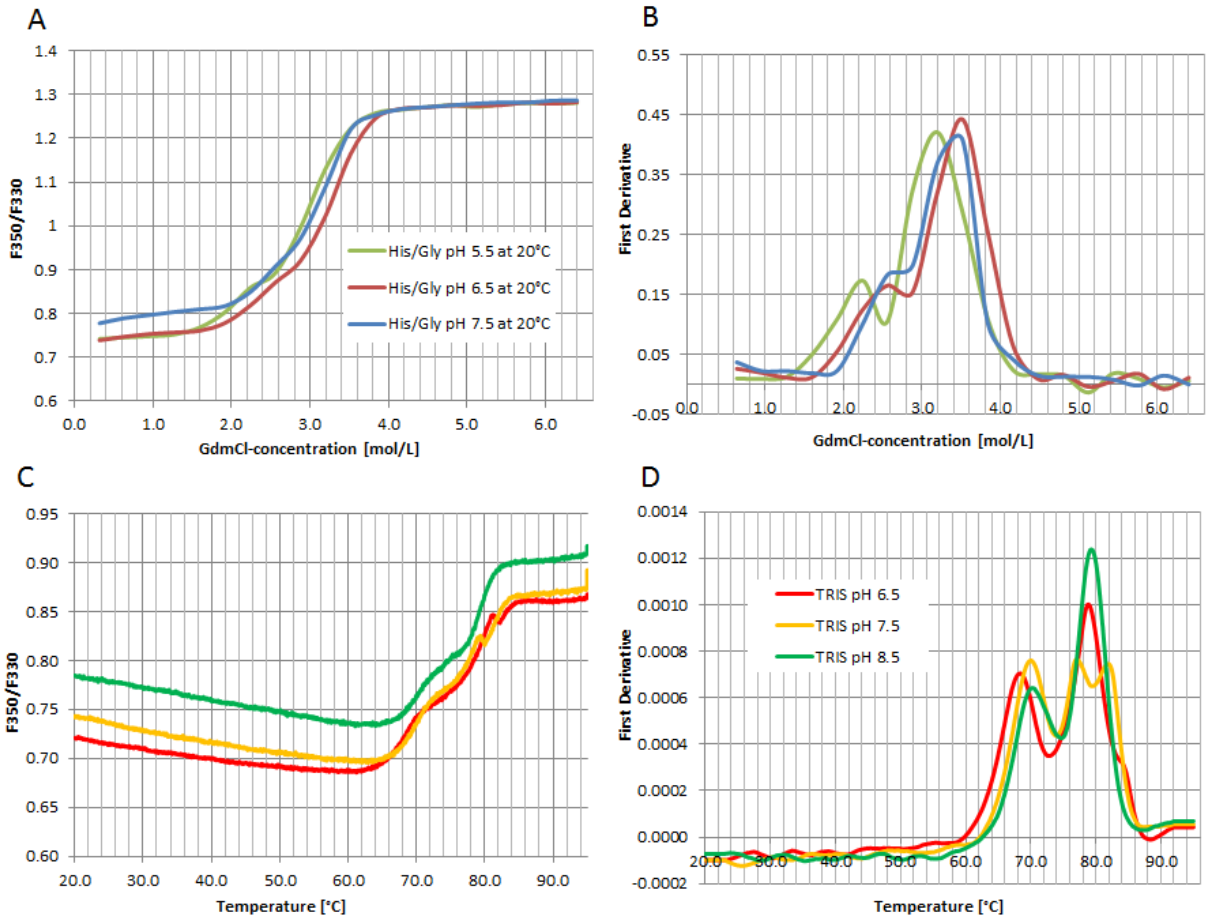


**Figure 1 Formulation screening of an IgG1-Antibody as a tool to find optimal conditions for increased protein stability**
(A) Chemical unfolding curves in His/Gly buffer at different pH values at 20 ℃ (B) Corresponding fir st derivative indicating the denaturation midpoints $C_m1$ and $C_m2$ (C) Thermal unfolding curves in TRIS buffer at different pH values (D) Corresponding first derivative indicating transition midpoints $T_m1$ and $T_m2$

However, there are certain buffer and pH conditions e.g. TRIS buffer at pH 7.5 (Figure 1D) and $Na_2HPO_4 \cdot 2H_2O$ buffer at pH 6.5 that cause a splitting of the last peak. This could either be induced by $C_H3$ or by the presence of partly denatured intermediates during the transition of the $F_{ab}$ fragment, which has been described in the literature.[6,7] In order to determine a $T_m$-value of the $F_{ab}$ fragment in those systems the average was used.

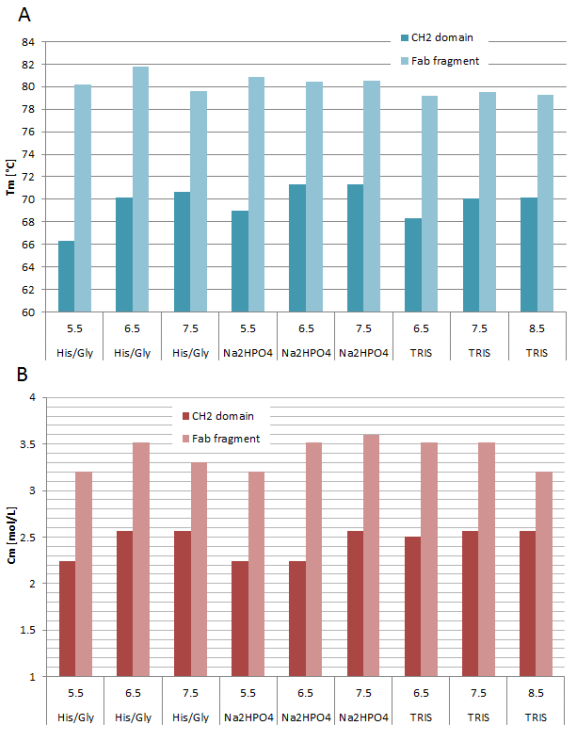


**Figure 2 Buffer and pH screening of nine formulations** (A) Summary of $T_m$ values of different domains of an IgG1-Antibody in nine formulations (B) Summary of $C_m$ values of different domains of an IgG1-Antibody in nine formulations

Figure 2A reveals that the His/Gly buffer at pH 6.5 is the most stable formulation for the protein because of high $T_m$-values, particularly of the $F_{ab}$ fragment (70.2 °C for $C_H2$ and 81.8 °C for $F_{ab}$). It has already been described in literature that the temperature of the $F_{ab}$ transition point may be a better indication of protein stability than the $T_m$ of the first transition point.[8]

Measurements of the chemical stability can serve as a supporting element: here the His/Gly buffer system at pH 6.5 produces high $C_m$-values for both domains as well (Figure 2B). Having a look at the other buffer types, $Na_2HPO_4 \cdot 2H_2O$ at pH 7.5 and TRIS at pH 7.5 are the best options. Nevertheless, one should note that the differences in $C_m$- and $T_m$-values between the nine formulations are quite small.

Comparison of Figure 2A and 2B shows that the transition points of the domains have different sensitivities to pH changes: Lowering pH leads both to smaller $C_m$- and $T_m$-values of the $C_H2$ domain, whereas the $F_{ab}$ fragment does not seem to be sensitive to pH changes. This trend is in good agreement with some other studies on denaturation of immunoglobulins.[3,9]

Results of the stability experiment upon six weeks storage are presented in Figure 3 using His/Gly- and TRIS buffer as examples. The $T_m$-values of $C_H2$ did not change significantly; therefore these values are not shown in the figure. Despite of a short storage period of 42 days $T_m$-values of the $F_{ab}$ fragment decreased continuously in particular systems, being His/Gly pH 7.5, TRIS pH 7.5 and TRIS pH 8.5. These systems seem to be sensitive to thermal stress, on the contrary His/Gly at pH 5.5 and 6.5 as well as TRIS at pH 6.5 appear to be more robust.

Figure 3A reveals that the His/Gly buffer at pH 6.5 provides the most stable environment for the protein. This is in good agreement with the data in Figure 2A, from which this system was selected as the best option out of the nine tested systems.

Figure 3B demonstrates that the antibody tested favors pH 6.5 as the best TRIS buffer option, whereas the data depicted in Figure 2 do not show a clear difference between the three TRIS systems. Consequently, a single $T_m$ measurement as indication of protein stability might not be sufficient. Instead, it could be helpful to monitor thermal stability over a period of storage at increased temperature. Such an experiment can serve as an additional tool to identify the most stable formulation, especially if $T_m2$ values at $T_0$ are very similar, as can be observed in Figure 3B.

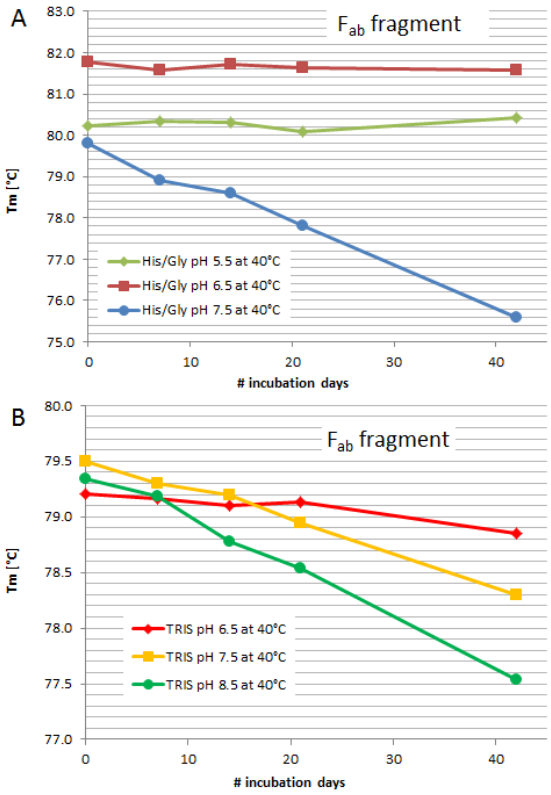


**Figure 3 Changes in thermal unfolding upon six weeks storage of an IgG1-Antibody in His/Gly- and TRIS buffer:** formulations were incubated at 2 ℃-8 ℃ and 40 ℃ and $T_m$ values were measured over a period of 42 days. Here $T_m$ values of the $F_{ab}$ fragment are shown.

## Summary

In this Application Note, different applications of the nanoDSF technology as a buffer screening tool have been tested. With the high resolution provided by nanoDSF in thermal unfolding experiments we could resolve unfolding of single antibody domains and showed that the stability of the individual domains is dependent on pH and temperature: the $C_H2$ domain seems to be most sensitive to lowering pH, in contrast the $F_{ab}$ fragment seems to be most sensitive to temperature stress. These findings show good agreement with already existing literature. The combination of thermal and chemical unfolding studies with short-term stability studies at elevated temperature, show to be a promising tool in finding optimal buffer conditions. In conclusion, the Prometheus NT.48 is well suited for a variety of buffer screening experiments.

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