

Protein Thermal Shift™ Assay using Applied Biosystems' Real-Time PCR Instruments

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ABSTRACT

The Protein Thermal Shift™ (PTS) Assay is an excellent high-throughput screening method which enables researchers to rapidly monitor protein thermo-stability and to identify optimal conditions that favor protein stability, including the investigation of protein-ligand interactions and mutations in protein sequences. Protein Thermal Shift is based on temperature-induced protein denaturation, monitored using Applied Biosystems' Protein Thermal Shift™ Dye. This assay does not require any prior knowledge of protein function or ligand activity. Applied Biosystems' Real-time PCR Systems plot the fluorescence data throughout the thermal melt, generating a fluorescence profile specific to the protein of interest within the test buffer environment or in the presence of a test ligand. As a result, we developed the Protein Thermal Shift™ software v1.0 which utilizes the Boltzmann equation to calculate the T_m of the protein from the fluorescence melt plot. Comparisons can then be made between T_m values obtained using a range of buffer conditions, addition of different ligands, or protein sequence mutations that alter protein folding and stability. In this study, we demonstrate the utility of this assay in buffer and ligand screening for T4 DNA ligase, as its T_m shifts in the presence of ATP, making Protein Thermal Shift™ Assay an important new screening tool for X-ray crystallographers looking for conditions that promote the stability of their protein of interest. In addition, we demonstrate the utility of this PTS Assay to discriminate between point mutation variants of M-MLV, SuperScript®II and SuperScript®III Reverse-Transcriptase. Finally, we demonstrate that this PTS can effectively detect the binding of antibodies to its target protein.

INTRODUCTION

The difficulty and expense often involved in isolating or obtaining a protein of interest demands that care be taken with subsequent handling or storage in order to maximize the utility and longevity of the protein and to ensure data quality is not effected by degradation or aggregation events. Conditions that favor long-term stability are a common requirement for almost all research or applied techniques involving proteins. There are many factors that may affect protein stability, including salt concentrations, pH or the use of specific ligands that can interact with proteins in different ways. Given the large number of possible combinations one could test in order to determine the environmental conditions that would favor maximum stability, it is highly desirable to adopt a technique that can simplify and streamline the investigation process. The PTS Assay on AB® Real Time PCR Systems is a fast and convenient screening method for detecting changes in protein thermal stability.

MATERIALS AND METHODS

The use of Applied Biosystems' Protein Thermal Shift™ Dye alleviates any requirement for prior knowledge of protein function or ligand activity. In the presence of a native protein, the dye is naturally quenched. When the protein of interest starts to denature in response to an increase in temperature, the hydrophobic core of the protein is exposed. The dye will react to this change in environment and will start to fluoresce (see Figure 1).

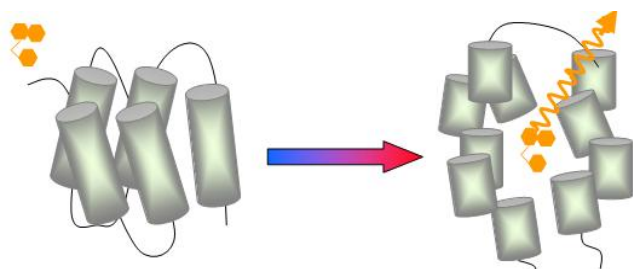


Figure 1. Schematic of the predicted action of PTS Dye. The naturally quenched dye will only fluoresce when exposed to the hydrophobic regions of a denaturing protein.

RESULTS

Applications of Protein Thermal Shift™:

- Screening buffer conditions for optimized protein stability
- Screening for ligands that bind and enhance protein stability
- Screening through point mutations for enhanced stability
- Screening for conditions that enhance stability for protein crystallization.

Figure 2. Effect of Ligand Interaction on Protein Stability

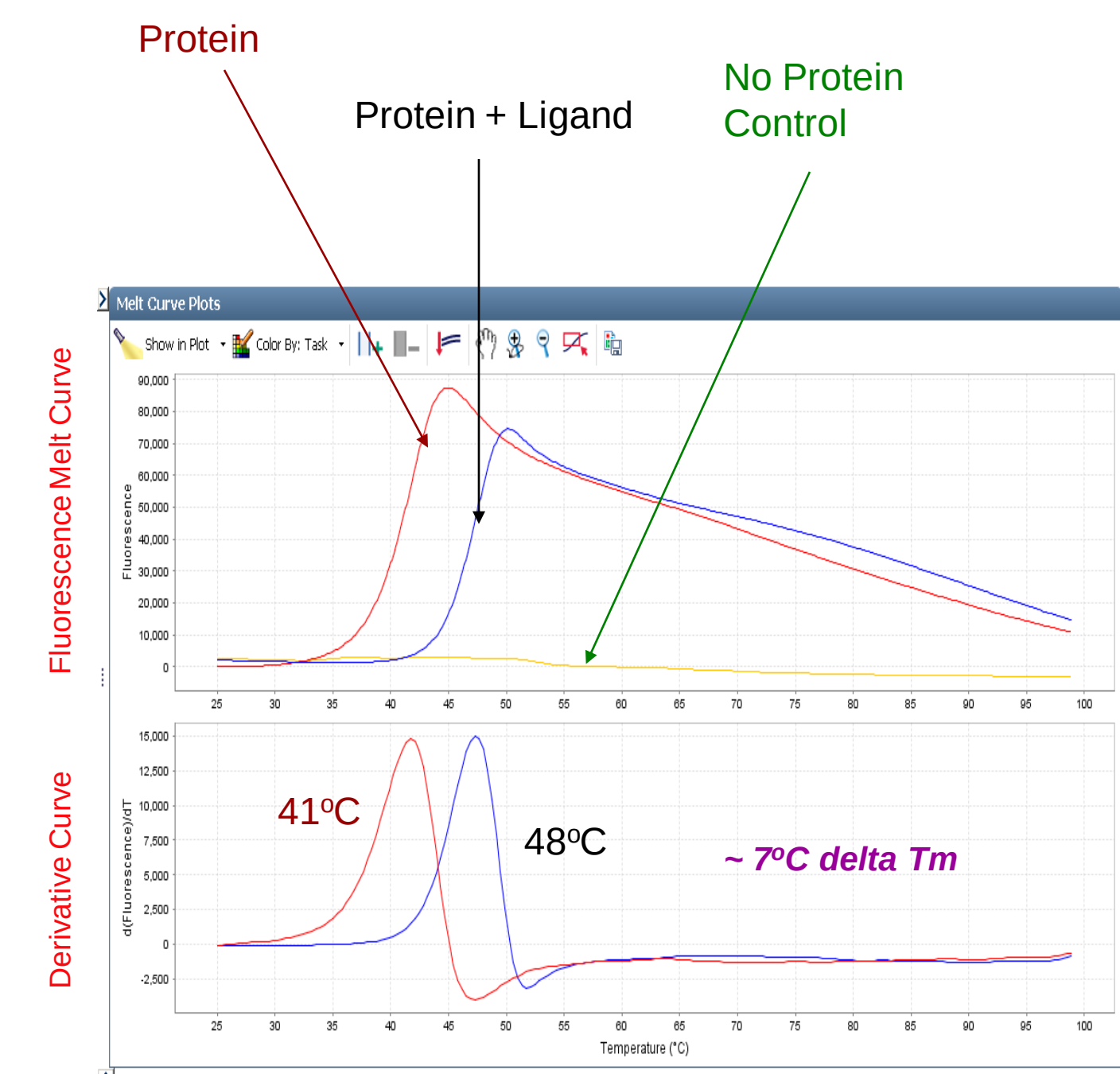


Figure 2. Melt Curve data generated on a Viia™7 Real Time PCR System showing the effect of a specific ligand (ATP) on protein stability (T4 DNA Ligase). The T_m of the curve increases over 7°C in the presence of the ligand. Figure 2 depicts quadruplicate reactions of a specific protein-ligand TSA. The melt curves are shown on the left graph and the derivative of the melt curves are plotted on the right. The red curves representing T4 DNA Ligase in the absence of no ligand, the blue curves represent T4 DNA Ligase in the presence of ATP and the green curves are controls. T4 DNA Ligase has a T_m of around 41°C, which is shifted when ATP is added to the reactions, resulting in a shift of approximately 7°C higher. This shift is a clear demonstration of the increased stability afforded by the addition of ATP.

Figure 3. Applied Biosystems' Protein Thermal Shift™ software v1.0 calculates the T_m

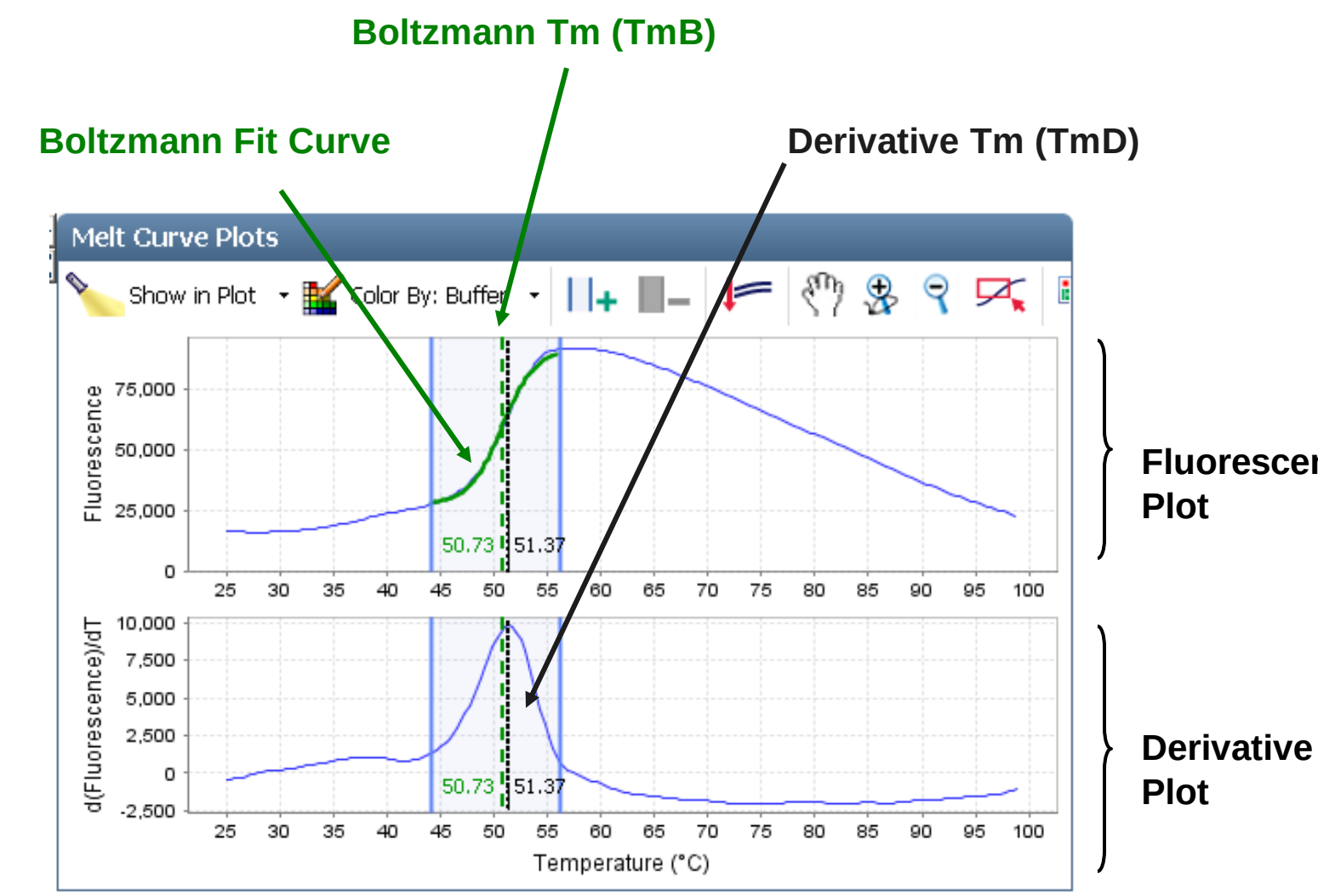
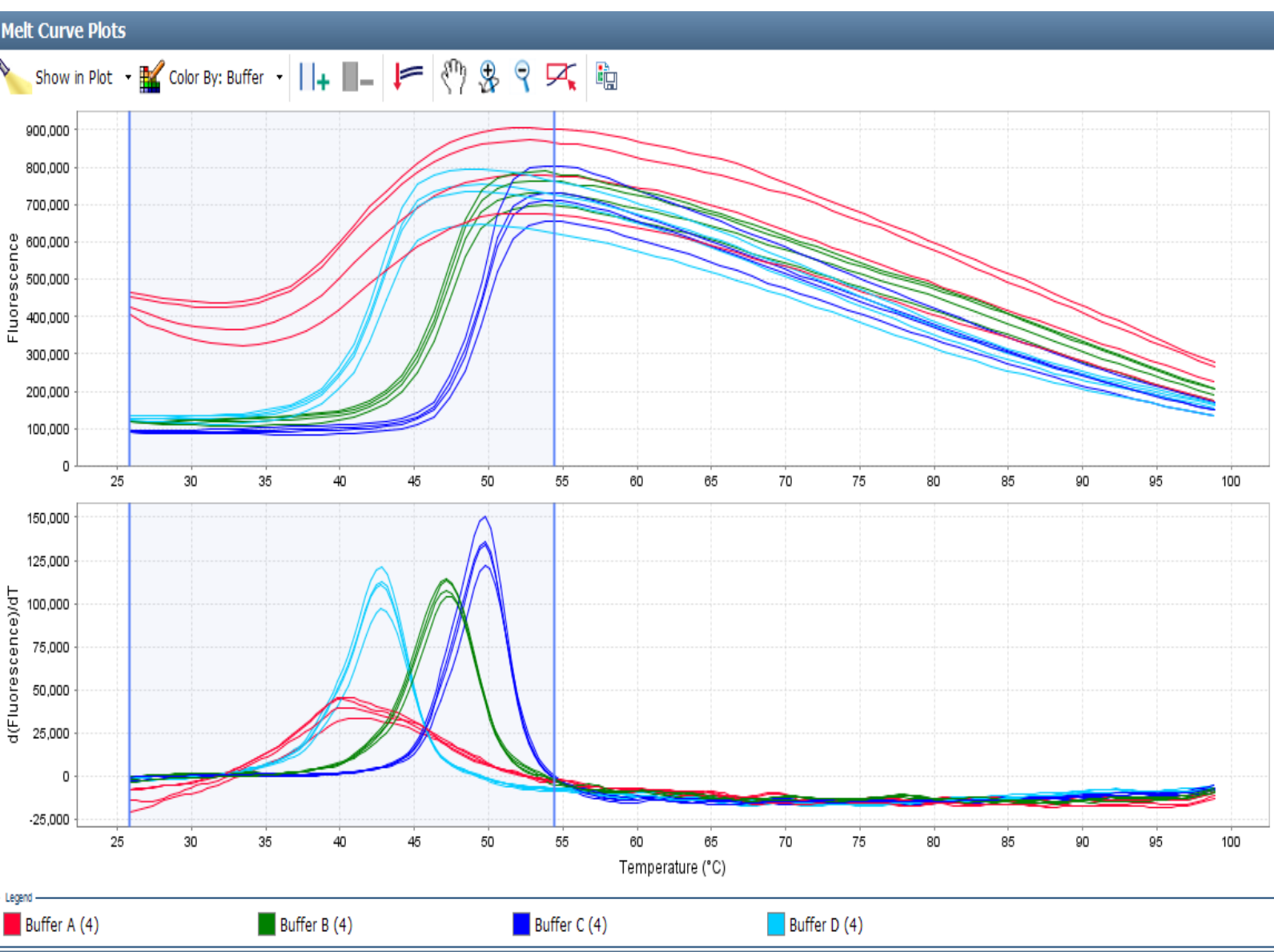


Figure 3. The Protein Thermal Shift™ software v1.0 calculates the T_m of the protein from the shape of fluorescence plot. Fit region of interest to the Boltzmann equation.

Applied Biosystems' Protein Thermal Shift™ software v1.0 provides:

- Protein T_m calculated via Boltzmann curve-fitting and Derivative peaks
- Delta T_m values against reference curve(s)
- Plot, tabular, and export results
- Multi-plate analysis with replicate statistics
- Diamond plots for quick Delta T_m visualization

Figure 4. Salt Concentration and pH affect Protein Thermal Shift™



Buffer Test Panel (A1-D4):

- A (100 mM Sodium Citrate, pH 5.5, 0-150 mM NaCl)
- B (100 mM Potassium Phosphate, pH 6.0, 0-150 mM NaCl)
- C (100 mM Potassium Phosphate, pH 7.0, 0-150 mM NaCl)
- D (100 mM Hepes, pH 7.5, 0-150 mM NaCl)

Figure 4. The derivative melt curves from a PTS experiment investigating the effect of salt concentration and pH on the stability of T4 DNA Ligase on the on the 7500 Fast Real-Time PCR Instrument (non-expert mode). T_m differences across the plot range from 39.5°C (A3) to 45°C (C4), demonstrating the effect of buffer conditions on protein thermal stability. Salts within a storage or handling buffer interact with the charged amino acids of a protein, ultimately affecting the stability of the resulting protein tertiary structure.

Figure 5. Effect of Point Mutations on PTS

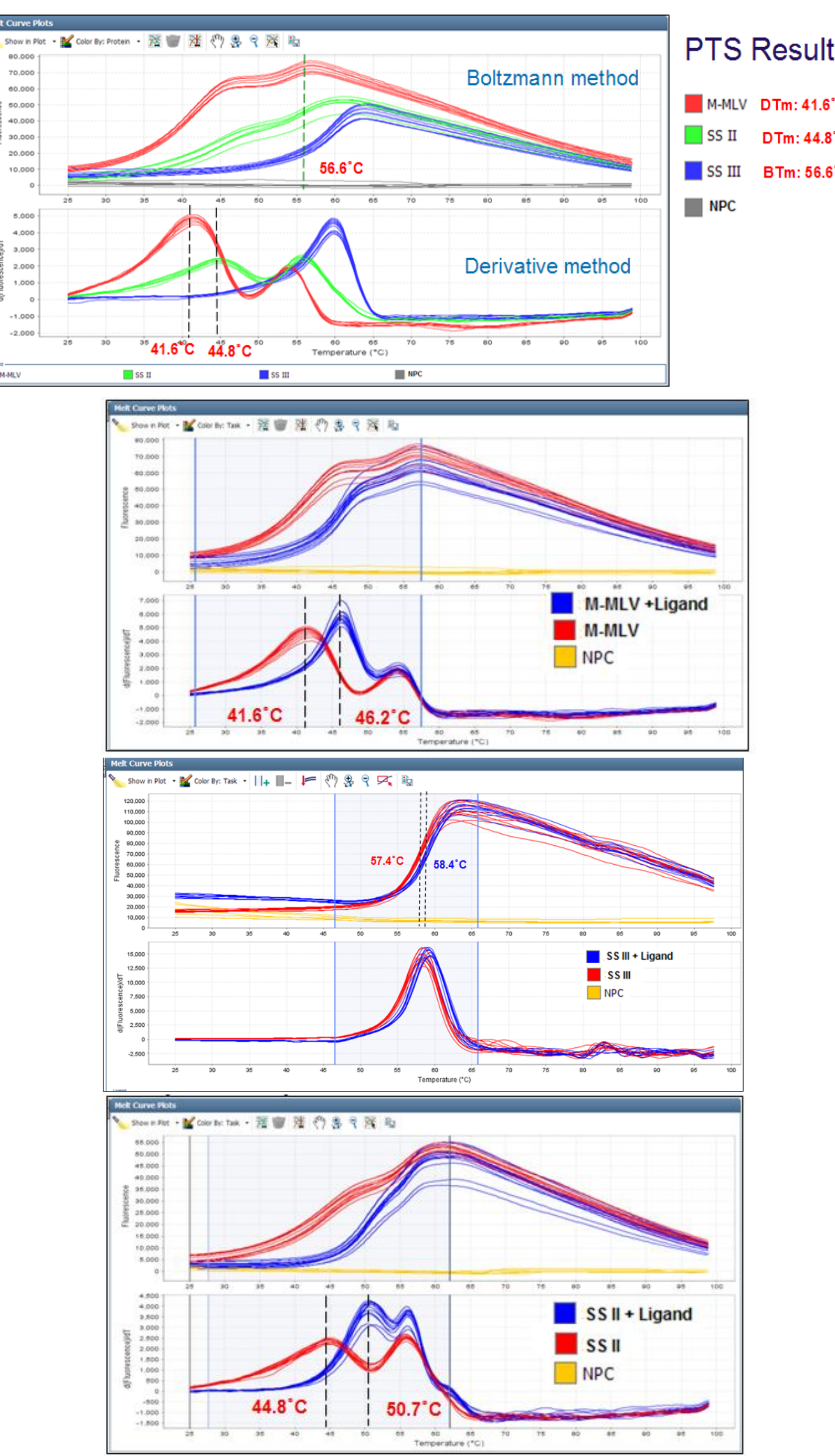


Figure 5. PTS Assay can differentiate between Point Mutations in a protein that affect the protein's Thermal Stability. PTS data generated on a Viia™7 instrument showing the Fluorescence and Derivative Melt profiles from experimental MMLV, SuperScript®II and SuperScript®III (top) and each individual enzyme thermally shifted by incubating with ligand (bottom).

Figure 6. Antibody Binding increases Protein Thermal Stability

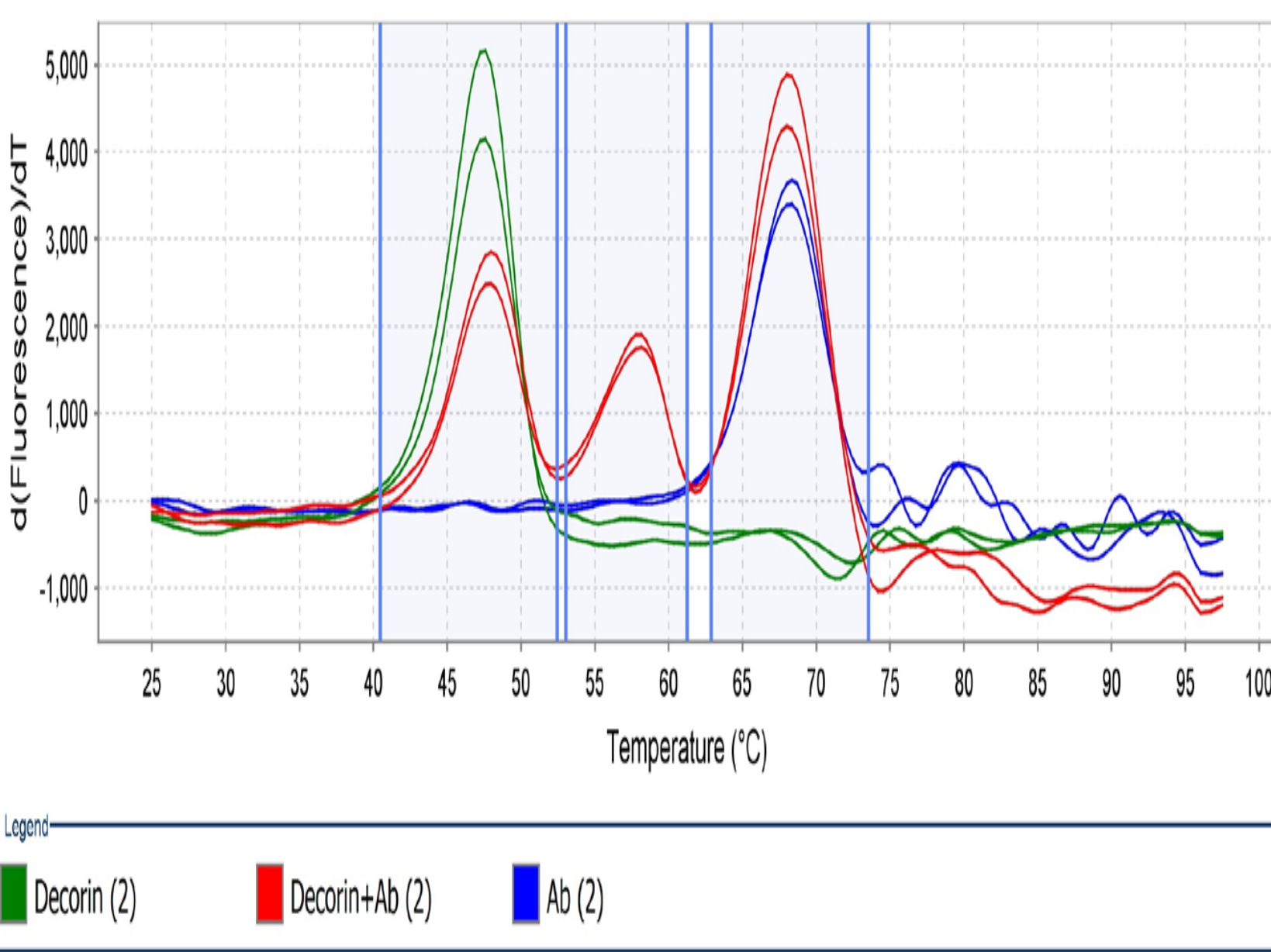


Figure 6. Binding of the Monoclonal Ab (anti-human Decorin protein) to the human Decorin protein results in a PTS of greater than 10°C, demonstrating effective detection of Ab binding to the protein. This PTS experiment was performed on the 7500 Fast Real-Time PCR Instrument (non-expert mode).

CONCLUSIONS

Protein Thermal Shift™ is a rapid, inexpensive and straight forward tool for screening of suitable conditions that maximize protein stability. The use of AB Real Time PCR Instruments offers a range of benefits to assist the Protein Thermal Shift™ testing workflow. In addition, the flexibility of the operational software allows for the collection of fluorescent data across a wide temperature range at any desired ramp speed, with data easily viewed within the system software, or exported for further analysis offline. These data have been obtained from the whole range of Applied Biosystems' Real Time PCR Systems, including the 7500, 7500 Fast, StepOne™, StepOnePlus™, and Viia™7 Real Time PCR Instruments, demonstrating the versatility of these systems. The benefits of performing a Protein Thermal Shift™ assay with an AB Real Time PCR System and Protein Thermal Shift™ software v1.0 include the automatic generation of a T_m, with the added flexibility of run-method programs and the ability of the user to reset the temperature range in the cases of more complex melt profiles, all in small reaction volumes, providing fast and accurate results with only a few µg of protein.

To learn more about Protein Thermal Shift™ products please visit www.appliedbiosystems.com/proteinmelt

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TRADEMARKS/ LICENSING

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