Challenge

Investigation of thermal protein stability in high troughput.

Solution

Application of thermal shift Assay using SYPRO® Orange in 96 well format.

Thermal Shift Assay using SYPRO® Orange to Detect Protein Melting Temperatures

Introduction

The stability of proteins depends on ligand interactions, buffer conditions or changes in conformation, which is traditionally investigated by time-consuming Circular Dichroism (CD) Spectroscopy. In addition, the thermal shift assay is based on temperature-induced denaturation and can be monitored using SYPRO® Orange. This fluorescence dye is a naturally quenched dye and interacts with the hydrophobic core of proteins which is exposed after denaturation.

Through this interaction, the SYPRO® Orange dye releases a fluorescence signal. The performance of a melting curve by using the qTOWER³ real-time PCR thermal cycler thus allows an easy and fast determination of protein melting temperatures. The midpoint or melt peak of the generated melting curve corresponds to the melting temperature (Tm value) of the protein under current conditions. Protein thermal shift assays are sensitive and rapid tools to examine protein thermal stability, helping to evaluate proteinligand binding and thus to find optimal buffer conditions or analyze protein variations. In this experiment, the Tm values of α-Chymotrypsinogen A in TBS with three different NaCl concentrations were determined.

analytikjena
An Endress+Hauser Company

Materials and Methods

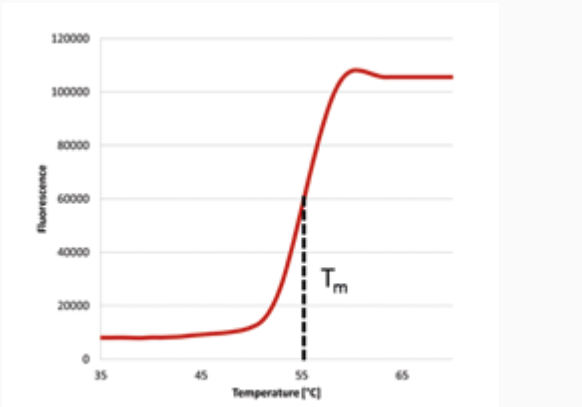
Chemicals

- NaCl stock solution (5 M and 0.2 M in TBS buffer)
- 10 mg/mL
- SYPRO® Orange (1:200)
- TBS buffer (10 mM)

Instuments

The qTOWER³ including the Protein 1 – SYPRO® Orange Color module (490 nm / 580 nm) was used for the measurements.

Each sample was measured in triplicate with 20 µL per reaction. Additionally, a negative control was used for reference.



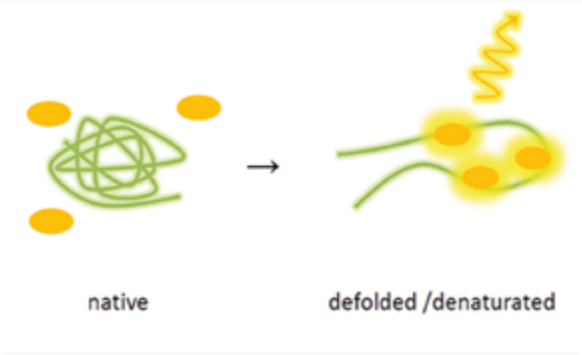


Fig. 1: Melting curve of a Protein detected by using qTOWER³ in combination with SYPRO® Orange

This naturally quenched dye interacts with the hydrophobic core of proteins which are exposed after denaturation. The quenching effect initiated by water is reduced and the increasing fluorescence signal can be measured. Therefore, the temperature in the middle of the thermal denaturation process is defined as melting temperature Tm. Shifts of the Tm provide an indication of a change in protein stability.

Table 1: Temperature and time protocol

Profile	Temp.	Holding	Ramp. rate
Equilibration	25 °C	10 sec	max.
Melting curve*	25 – 90 °C and 6 sec with ΔT = 1 °C		

* Data acquisition: Color Module Protein 1 (490 – 580 nm) and Gain 5

Table 2: NaCl dilutions in TBS containing 1 mg/ml α-Chymotrypsinogen A

Component	20 mM NaCl	500 mM NaCl	2 M NaCl
α- Chymotrypsinogen A	1 µL	1 µL	1 µL
TBS buffer	59 µL	59 µL	38 µL
NaCl solution (5 M)	-	7 µL	28 µL
NaCl solution (0.2 M)	7 µL	-	-
SYPRO® Orange	3 µL	3 µL	3 µL
Final Volume	70 µL	70 µL	70 µL

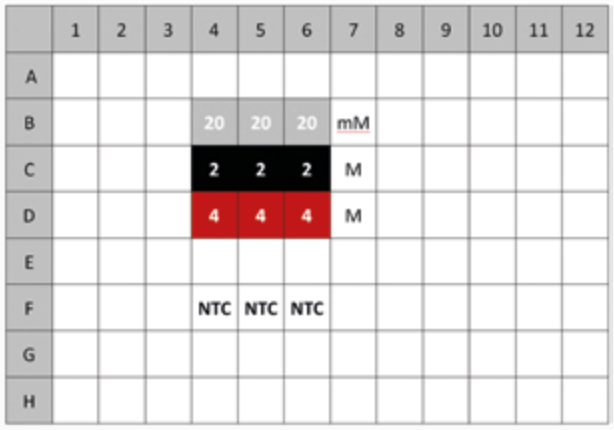


Fig. 2: Plate layout for melting curve analysis

Results and Discussion

Figures 3 and 4 show the melting curve of α-Chymotrypsinogen A and accordant analysis. The calculation and display of first derivatives of the melting curves is completed automatically by the qTOWER³'s qPCRsoft control and analysis software. The shift in Tm shows a clear influence of the different NaCl concentrations to the thermal stability of the protein.

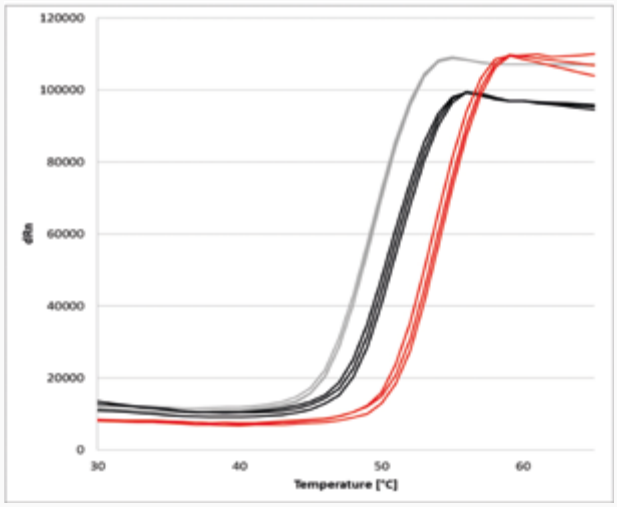


Fig. 3: Melting curves of α-Chymotrypsinogen A under influence of different NaCl concentrations: 20 mM (grey), 500 mM (black) and 2 M (red)

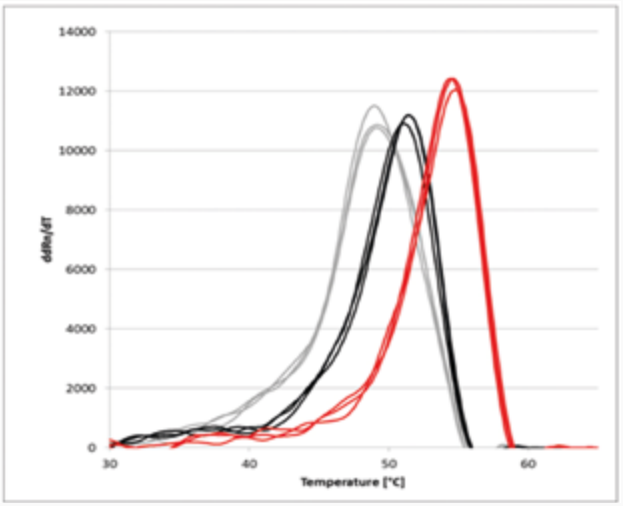


Fig. 4 : Melting curve derivatives of the thermal shift assay using SYPRO® Orange and qTOWER³ ; NaCl concentrations: 20 mM (grey), 500 mM (black) and 2 M (red)

Table 3: Melting points of α-Chymotrypsinogen A

NaCl	Tm	Mean Tm
20 mM	49.1 °C	49.1 °C
20 mM	49.2 °C	
20 mM	49.0 °C	
500 mM	51.4 °C	51.3 °C
500 mM	51.1 °C	
500 mM	51.4 °C	
2 M	51.4 °C	54.6 °C
2 M	51.6 °C	
2 M	51.7 °C	

When NaCl concentrations rise, the thermal stability of α-Chymotrypsinogen A rises as well. Higher salt concentrations lead to the formation of hydrationshells surrounding the proteins, thus stabilizing α-Chymotrypsinogen A. Therefore, the melting point Tm is shifted by nearly 6 degrees Celsius from approx. 49 degrees Celsius at 20 mM NaCl to approx. 55 degrees Celsius at 2 M NaCl.

Conclusion

In contrast to a traditional CD Spectroscopy assay which takes approximately one hour per sample, the current experiment was performed in ten minutes for nine samples with excellent resolution of the investigated melting point. Even in case of using maximum number of 96 samples the experimental time remains the same, which is one big advantage of using the qTOWER^3 technology in thermal shift assays. The high reproducibility is due to simple procedures, the high precision of qTOWER^3 qPCR system, and the striking sensitivity of SYPRO® Orange.

Headquarters
Analytik Jena GmbH
Konrad-Zuse-Strasse 1
07745 Jena · Germany

Phone +49 3641 77 70
Fax +49 3641 77 9279

info@analytik-jena.com
www.analytik-jena.com

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