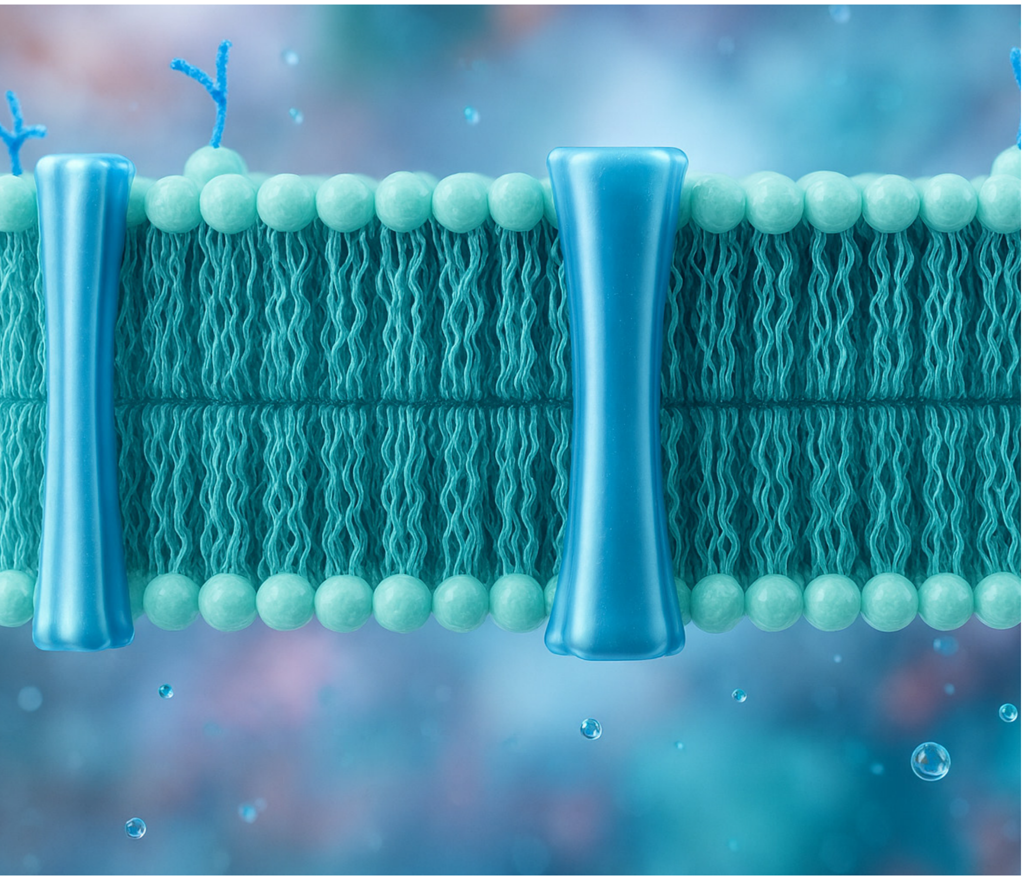


Challenge
Efficiently assessing the thermal stability of hydrophobic proteins under medium- and high-throughput conditions can be challenging.

Solution
Thermal shift assays performed on the qTOWERiris qPCR Thermal cycler (96- or 384-well) using fluorescent dyes such as 1,8-ANS or CPM enable reliable, high-throughput detection of hydrophobic proteins.

Intended audience
Researchers working in drug discovery, pharmaceutical research, protein stability, membrane protein studies, or related life science fields

High-Throughput UVA-Based Thermal Shift Assays for Hydrophobic Proteins Using the qTOWERiris

Introduction

Determining the melting temperature (T_m) is an established method for analyzing the thermal stability of proteins. Fluorescent dyes such as SYPRO® Orange and GloMelt ™ enable sensitive detection of protein unfolding by binding to hydrophobic regions that become exposed upon thermal denaturation. This approach is particularly suitable for soluble proteins, as their unfolding typically results in a pronounced increase in accessible hydrophobic surface area and, consequently, a strong fluorescence signal. SYPRO Orange is widely used for this purpose and provides reliable signal increases, whereas GloMelt, a novel dye with the same basic mechanism, offers higher signal intensity and lower background fluorescence. Both dyes enable precise and reproducible determination of the T_m of soluble proteins in high-throughput formats using a Real-Time PCR thermocycler [1].

However, this method is only of limited use for membrane proteins. These proteins inherently possess extensive hydrophobic surfaces in their native state, which interact with lipid membranes or detergents and therefore do not produce the characteristic fluorescence changes observed for soluble proteins. Yet, the thermal stability of membrane proteins is a crucial parameter for functional characterization, structural elucidation, and pharmaceutical development. Conventional dyes often reach their limits in this context due to the intrinsic hydrophobicity of membrane proteins and the need for detergent-containing buffers [2]. Alternative fluorescent dyes, such as CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin) and 1,8-ANS (1-anilinonaphthalene-8-sulfonate), offer specific advantages for the thermal analysis of this protein class. Their spectral properties allow reliable detection of conformational changes in membrane proteins under near-native conditions, facilitating the optimization of stability parameters, the selection of suitable detergents, and the preparation of samples for structural studies [2,3].

Assays based on CPM and 1,8-ANS require an optical system that provides excitation in the UV-A range while detecting their emission in the visible spectrum. The qTOWERiris UV-ready configuration is a Real-Time PCR thermocycler equipped with a dedicated UV excitation module (375 ± 15 nm) and a matching emission channel (475 ± 15 nm). In combination with the instrument's precise temperature control, this optical setup enables sensitive and reliable thermal-shift analysis of membrane proteins using the respective fluorescence dyes.

In this study, the fluorescent dyes CPM and 1,8-ANS were applied in thermal shift protein assays (TSA) using the real-time PCR devices qTOWERiris and qTOWERiris 384, both equipped with innovative UV modules. The model proteins alpha-chymotrypsinogen and beta-lactoglobulin were examined under varying protein concentrations, pH levels, and salt conditions in the buffer. Additionally, the qTOWERiris 384 demonstrated its capability to accommodate different assay volumes, highlighting its versatility and suitability for high-throughput analyses.

Materials and Methods

Samples and reagents

- Samples
  - Stock: 10 mg/mL α-chymotrypsinogen
  - Stock: 10 mg/mL β-lactoglobulin
- Buffer conditions
  - Phosphate buffered saline (PBS)
  - pH 5 / 6 / 7 / 8 / 9
- Sodium chloride (NaCl) stock solutions in PBS, each:
  - 0.2 M
  - 2.0 M
- Fluorophores
  - 1,8-ANS (1-Anilinonaphthalene-8-Sulfonic Acid) protein dye
  - CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin)

Instrumentation

- qTOWERiris UV with color modules 1 and 7 (Analytik Jena)
- qTOWERiris 384 UV with color modules 1 and 7 (Analytik Jena)

Sample preparation

All measurements were performed using two distinct fluorophores: 1,8-ANS (1-anilinonaphthalene-8-sulfonic acid) and CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin). Alpha-chymotrypsinogen solutions at a final concentration of 0.5 mg/mL were prepared for detection with 1,8-ANS. The solutions differed in NaCl concentration (50, 100, 250, 500 mM) and pH value (5, 7, 9). All conditions were prepared in triplicate. Reaction volumes were 20 µL in the 96-well format and 10 µL in the 384-well format.

For CPM detection, β-lactoglobulin solutions at a final concentration of 0.5 mg/mL were prepared. These solutions differed in NaCl concentration (50, 100, 250, 500 mM) and pH value (5, 6, 7) and were likewise prepared in triplicate. Reaction volumes were 20 µL in the 96-well format and 10 µL in the 384-well format.

Table 1: Preparation of samples with 1,8-ANS

Parameter / condition		**Final conc. NaCl [mM]**				**PBS pH**		
	0	50	100	250	500	5	7	9
NaCl [M] stock	-	0.2	2	2	2	-	-	-
NaCl [µL]	-	17.5	3.5	8.8	17.5	-	-	-
Buffer (PBS) [µL]	63	45.5	59.5	54.2	45.5	63	63	63
α-chymotrypsinogen (10 mg/mL) [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
1,8-ANS [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Total volume [µL]	**70**	**70**	**70**	**70**	**70**	**70**	**70**	**70**

Table 2: Preparation of samples with CPM

Parameter / condition	**Final conc. NaCl [mM]**				**PBS pH**		
	50	100	250	500	5	6	7
NaCl [M] stock	0.2	2	2	2	-	-	-
NaCl [µL]	17.5	3.5	8.8	17.5	-	-	-
Buffer (PBS) [µL]	45.5	59.5	54.2	45.5	63	63	63
β-Lactoglobulin (10 mg/mL) [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
CPM [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Total volume [µL]	**70**	**70**	**70**	**70**	**70**	**70**	**70**

Instrument settings for detection of 1,8-ANS

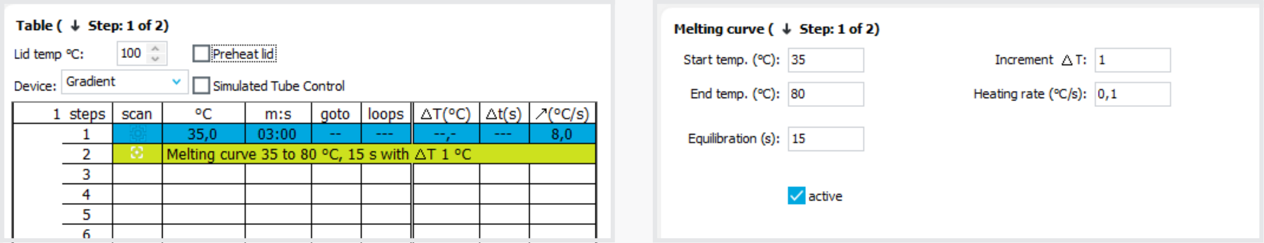


Table (↓ Step: 1 of 2)

Lid temp °C: 100 ☐ Preheat lid

Device: Gradient ☐ Simulated Tube Control

1 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
1		35,0	03:00	--	---	--,-	---	8,0
2		Melting curve 35 to 80 °C, 15 s with ΔT 1 °C						
3								
4								
5								
6								

Melting curve (↓ Step: 1 of 2)

Start temp. (°C): 35

End temp. (°C): 80

Equilibration (s): 15

Increment ΔT: 1

Heating rate (°C/s): 0,1

☑ active

Figure 1: Temperature-time protocol for 1,8-ANS thermal shift assay. Fluorescence was recorded using color module 7 (Ex 375 ± 15 nm / Em 475 ± 15 nm, LED gain 5).

Instrument settings for detection of CPM

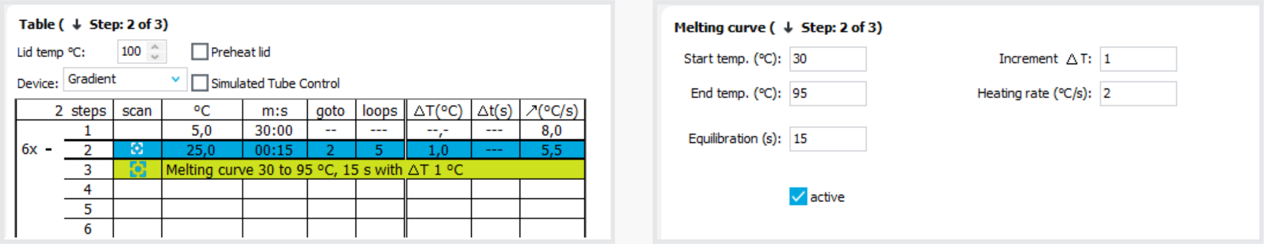


Table (↓ Step: 2 of 3)

Lid temp °C: 100 ☐ Preheat lid

Device: Gradient ☐ Simulated Tube Control

	2 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		5,0	30:00	--	---	--,-	---	8,0
6x -	2		25,0	00:15	2	5	1,0	---	5,5
	3		Melting curve 30 to 95 °C, 15 s with ΔT 1 °C						
	4								
	5								
	6								

Melting curve (↓ Step: 2 of 3)

Start temp. (°C): 30

End temp. (°C): 95

Equilibration (s): 15

Increment ΔT: 1

Heating rate (°C/s): 2

☑ active

Figure 2: Temperature-time protocol for CPM thermal shift assay. Fluorescence was recorded using color module 7 (Ex 375 ± 15 nm / Em 475 ± 15 nm, LED gain 5).

Results and Discussion

96-well format: 1,8-ANS and α-chymotrypsinogen in thermal shift assays

The thermal stability of α-chymotrypsinogen was assessed using temperature-dependent fluorescence shifts of 1,8-ANS in the 96-well format on the qTOWERiris. Buffer pH and NaCl concentration were systematically varied to evaluate their influence on the melting temperature (T_m). Fluorescence data were detected using color module 7 (Ex 375 ± 15 nm / Em 475 ± 15 nm) and analyzed with qPCRsoft.

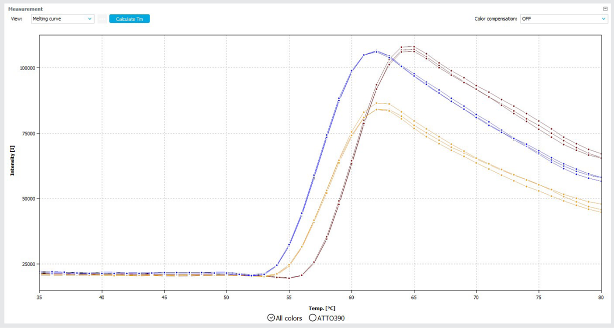

Measurement
View: Melting curve
Color compensation: OFF

Figure 3: Raw data of 1,8-ANS detection of α-chymotrypsinogen at different buffered pH values (5,7,9) using color module 7 on qTOWERiris.

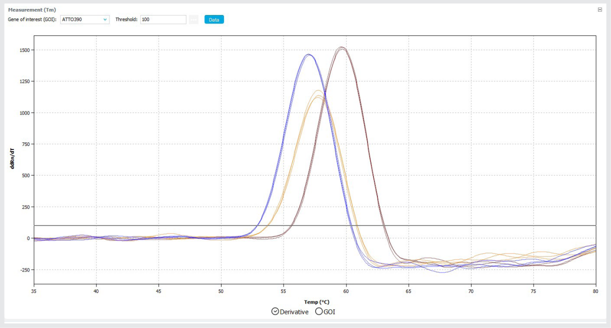

Measurement (Tm)
Gene of interest (GOI): ATTO390
Threshold: 100

Figure 4: Calculated melting temperatures of α-chymotrypsinogen detected with 1,8-ANS at different buffered pH values (5,7,9) using color module 7 on qTOWERiris.

Table 3: pH-dependent shift in melting temperature of α-chymotrypsinogen, determined from temperature-dependent fluorescence changes of 1,8-ANS.

	1,8-ANS	
	PBS	
pH	T_m	Mean T_m
5	59,7	59,67
	59,7	
	59,6	
7	57,8	57,77
	57,8	
	57,7	
9	57,1	57,03
	57,0	
	57,0	

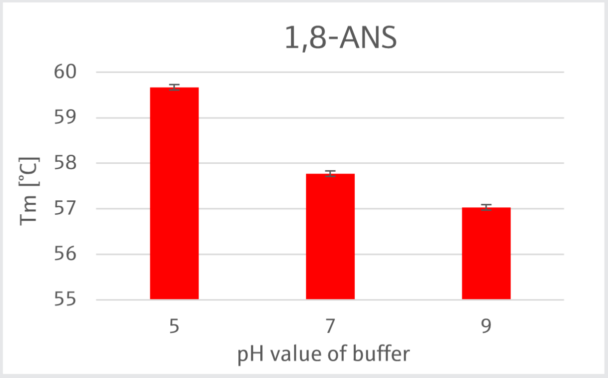


Figure 5: pH-dependent shift in the melting temperature of α-chymotrypsinogen in PBS buffers.

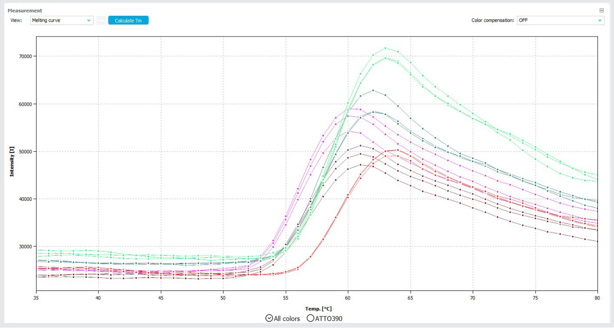

Measurement
View: Melting curve
Color compensation: OFF

Figure 6: Raw data of 1,8-ANS detection of α-chymotrypsinogen at different NaCl concentrations using color Module 7 on qTOWERiris; 96-well format.

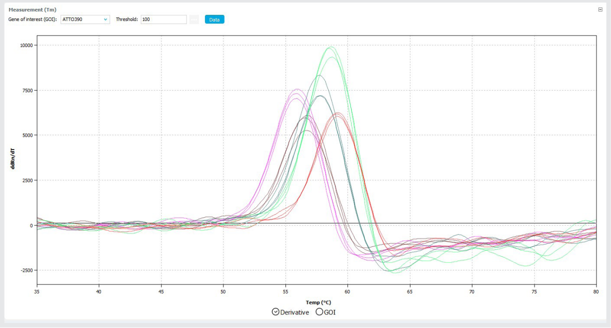

Measurement (Tm)
Gene of interest (GOI): ATTO390
Threshold: 100

Figure 7: Calculated melting temperatures of α-chymotrypsinogen detected with 1,8-ANS at different NaCl concentrations using color module 7 on qTOWERiris.

Table 4: NaCl concentration dependent shift in melting temperature of α-chymotrypsinogen, determined from temperature-dependent fluorescence changes of 1,8-ANS.

	1,8-ANS	
	PBS	
NaCl [mM]	T_m	Mean T_m
500	59,2	59,17
	59,1	
	59,2	
250	58,7	58,63
	58,7	
	58,5	
100	57,8	57,73
	57,7	
	57,7	
50	56,7	56,70
	56,6	
	56,8	
0	55,9	55,90
	55,9	
	55,9	

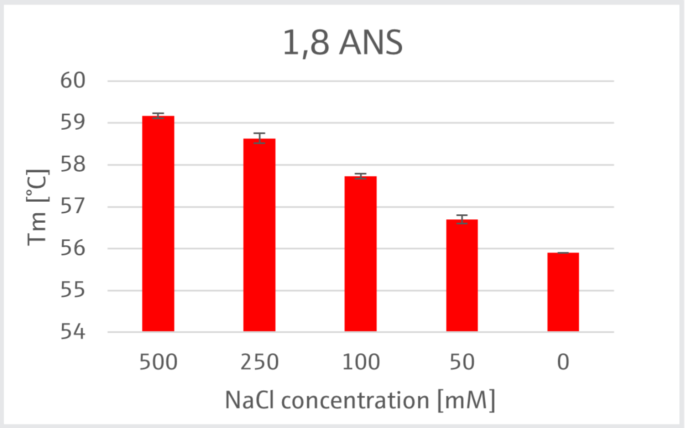
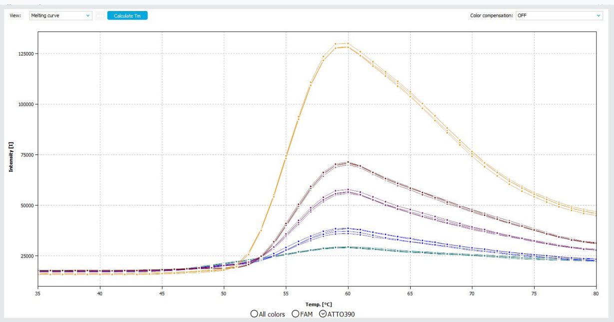


Figure 8: NaCl-dependent shift in the melting temperature of α-chymotrypsinogen in PBS buffer, determined via temperature-dependent fluorescence changes of 1,8-ANS.

384-well format: 1,8-ANS and α-chymotrypsinogen in thermal shift assays

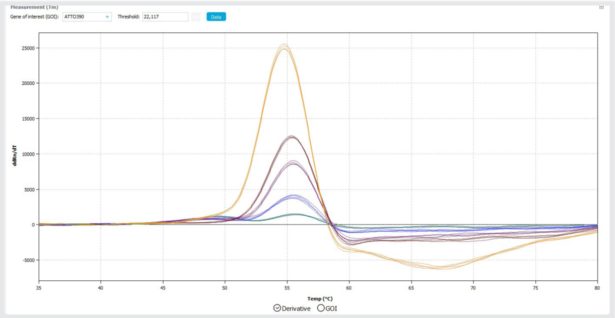
Using the 384-well format, the NaCl concentration dependency of the thermal stability of α-chymotrypsinogen was investigated as a proof of principle, using an experimental setup identical to that applied in the 96-well format.



View: Melting curve

Color compensation: OFF

Figure 9: Raw data of 1,8-ANS detection of α-chymotrypsinogen at different salt concentrations using color module 7 on qTOWERiris 384.



Measurement (Tm)

Gene of interest (GOI): ATTO390

Threshold: 22,117

Figure 10: Calculated melting temperatures of 1,8-ANS detection of α-chymotrypsinogen in different salt concentrations using color module 7 on qTOWERiris 384.

96 well format: CPM and β-lactoglobulin in thermal shift assays

The thermal stability of β-lactoglobulin was assessed using temperature-dependent fluorescence shifts of CPM in a 96-well format on the qTOWERiris. Buffer pH and NaCl concentration were systematically varied to evaluate their influence on the melting temperature (T_m). Fluorescence data were recorded using qTOWERiris with color module 7 (Ex 375 ± 15 nm / Em 475 ± 15 nm) and analyzed with qPCRsoft.

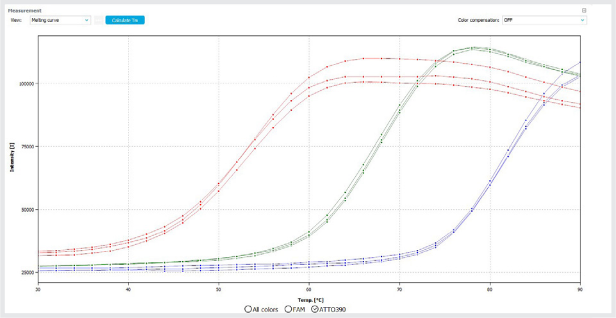


Figure 11: Raw data of CPM detection of β-lactoglobulin in different buffered pH values (5, 6, 7) using color module 7 on qTOWERiris.

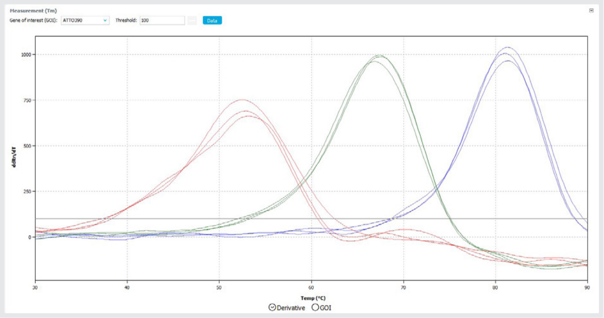


Figure 12: Calculated melting temperatures of CPM detection of β-lactoglobulin in different buffered pH values (5, 6, 7) using color module 7 on qTOWERiris.

Table 5: pH-dependent melting temperature shifts of β-lactoglobulin detected via CPM on the qTOWERiris.

	CPM	
	PBS	
pH	**T_m**	**Mean T_m**
7	52,4	52,80
	53,2	
	52,8	
6	67,6	67,27
	67,4	
	66,8	
5	81,4	81,27
	81,0	
	81,4	

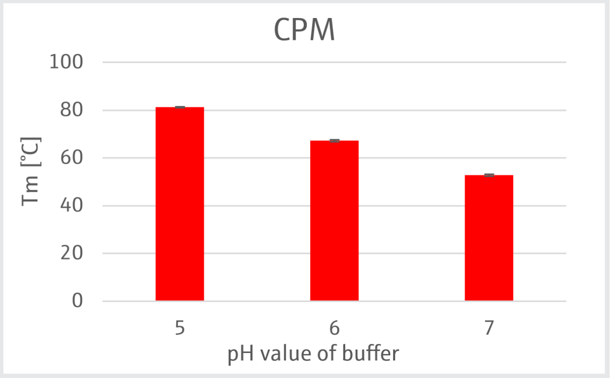


Figure 13: pH-dependent shift in the melting temperature of α-chymotrypsinogen in PBS buffers detected via temperature-dependent fluorescence shifts of CPM.

384 well format: CPM and β-lactoglobulin in thermal shift assays

Thermal shift assays of β-lactoglobulin were performed in a 384-well format using CPM to assess the influence of pH and NaCl concentration on protein stability. Fluorescence data were recorded using qTOWERiris 384 with color module 7 (Ex 375 ± 15 nm / Em 475 ± 15 nm) and analyzed with qPCRsoft.

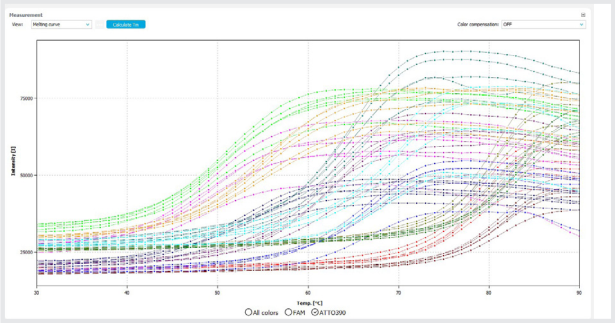


Figure 14: Raw data of CPM detection of β-lactoglobulin in different buffered pH values (5, 6, 7) as well as NaCl concentrations using color module 7 on qTOWERiris 384.

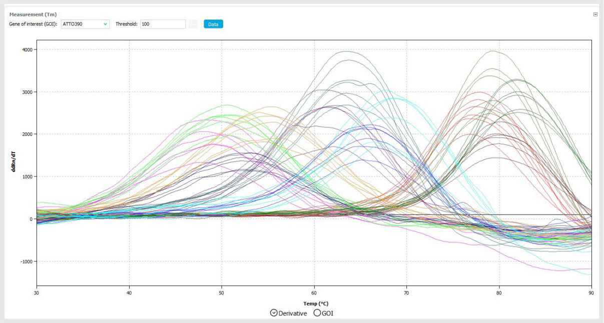


Figure 15: Calculated melting temperatures of CPM detection of β-lactoglobulin in different buffered pH values (5, 6, 7) using color module 7 on qTOWERiris 384.

Table 6: pH- and NaCl-dependent melting temperature shifts of β-lactoglobulin detected via temperature-dependent fluorescence shifts of CPM. Fluorescence data was measured and analyzed using qTOWERiris 384 in high throughput.

		CPM				CPM				CPM	
		PBS				PBS				PBS	
pH	**NaCl**	**T_m**	**Mean T_m**	**pH**	**NaCl**	**T_m**	**Mean T_m**	**pH**	**NaCl**	**T_m**	**Mean T_m**
5	50	77,9	77,73	6	50	62,4	61,63	7	50	48,4	48,67
		77,8				60,8				48,0	
		77,5				61,7				49,6	
5	100	79,3	79,20	6	100	63,5	63,53	7	100	51,0	50,60
		79,3				63,7				50,6	
		79,0				63,4				50,2	
5	250	80,2	79,93	6	250	66,1	65,77	7	250	52,2	52,60
		79,7				65,9				52,4	
		79,9				65,3				53,2	
5	500	81,9	82,00	6	500	68,6	68,23	7	500	54,1	54,83
		82,0				68,4				54,8	
		82,1				67,7				55,6	

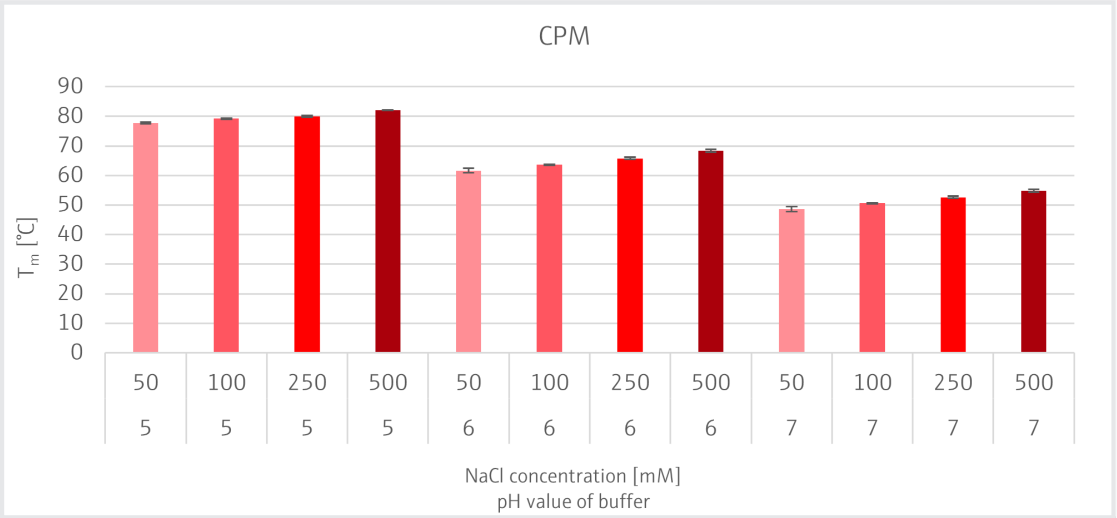


Figure 16: pH- and NaCl-dependent melting temperature shifts of β-lactoglobulin detected via CPM fluorescence in a 384-well format.

Summary

Real-time PCR thermal cyclers of the qTOWERiris series, in combination with color module 7, enable rapid and precise assessment of protein thermal stability in both 96- and 384-well formats, including for membrane proteins. This allows efficient screening of storage conditions to maximize protein stability and evaluation of the effects of small compounds, ligands, or cofactors on hydrophobic proteins.

The excitation and emission wavelengths of the dyes are perfectly matched to color module 7, allowing selection of different fluorophores such as 1,8-ANS or CPM depending on the protein. The high-performance optical system detects minute changes in fluorescence, while the accurate and homogeneous temperature control of the thermal block enables high-resolution melting curves to reliably determine melting temperatures. Melting curve analysis in qPCRsoft further facilitates data interpretation by computing T_m values directly from the derivative of fluorescence data. 1,8-ANS binds to hydrophobic surfaces that become exposed during unfolding. Compared to SYPRO Orange, it exhibits lower nonspecific binding to detergents and provides clearer signals upon thermal unfolding, making it particularly suitable for studying membrane proteins in near-native environments. In contrast, CPM is a thiol-reactive dye that selectively reacts with free cysteine residues, which are often buried in the native state and only accessible upon denaturation. CPM provides sensitive detection of unfolding without interference from detergents and has been successfully applied in stability studies of ABC transporters and other membrane-bound proteins. UV dyes are sensitive to light, temperature, and environmental conditions. To ensure reproducible and reliable results, it is essential to:

- Pipette rapidly and precisely to minimize exposure and prevent degradation.
- Work on cooling blocks or ice to maintain stability during handling.
- Perform experiments in a darkened area to protect dyes from photochemical degradation.
- Avoid direct sunlight during preparation and storage.

Strict adherence to these handling conditions is crucial for high-quality, reproducible data, as even small deviations can significantly affect fluorescence intensity and result comparability.

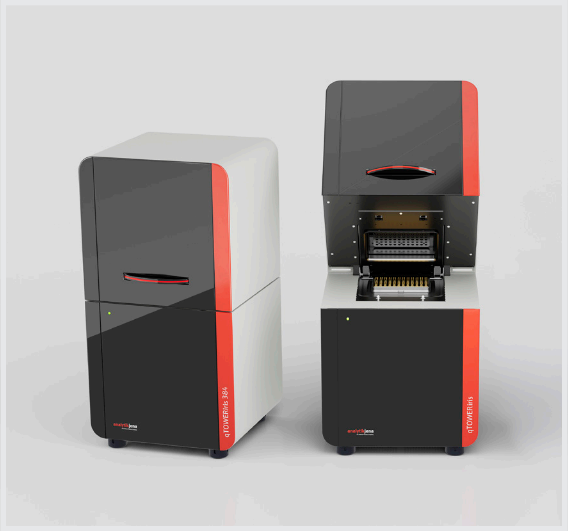


Figure 17: qTOWERiris und qTOWERiris 384

Recommended device configuration

Table 11: Overview of required equipment and recommended accessories and consumables.

Item	Order number	Description
qTOWERiris, UV-ready, incl. color module 1	844-00854-x*	Real-time PCR system designed in the 96-well format, operable via PC, customizable with up to 6 color modules.
qTOWERiris 384, UV-ready, incl. color module 1	844-00859-x*	Designed for high-throughput real-time PCR applications in 384-well format.
Color module 7 for qTOWERiris series	844-00826-0	Color module for the excitation and emission of fluorescence dyes such as ATTO390, ATTO425.

References

[1] Pantoliano, M., et al. (2001) High-Density Miniaturized Thermal Shift Assay as a general strategy for drug discovery, J Biomol Screen J Biomol Screen. 2001 Dec;6(6):429-40.

[2] Alexandrov, A. I. et al. (2008) Microscale Fluorescent Thermal Stability Assay for Membrane Proteins Ways & Means Ways & Means Volume 16, Issue 3, 11 March 2008, Pages 351-359.

[3] Kazlauskas, E., et al. (2020) Standard operating procedure for fluorescent thermal shift assay (FTSA) for determination of protein-ligand binding and protein stability Eur Biophys J. 2021 May;50(3-4):373-379.

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