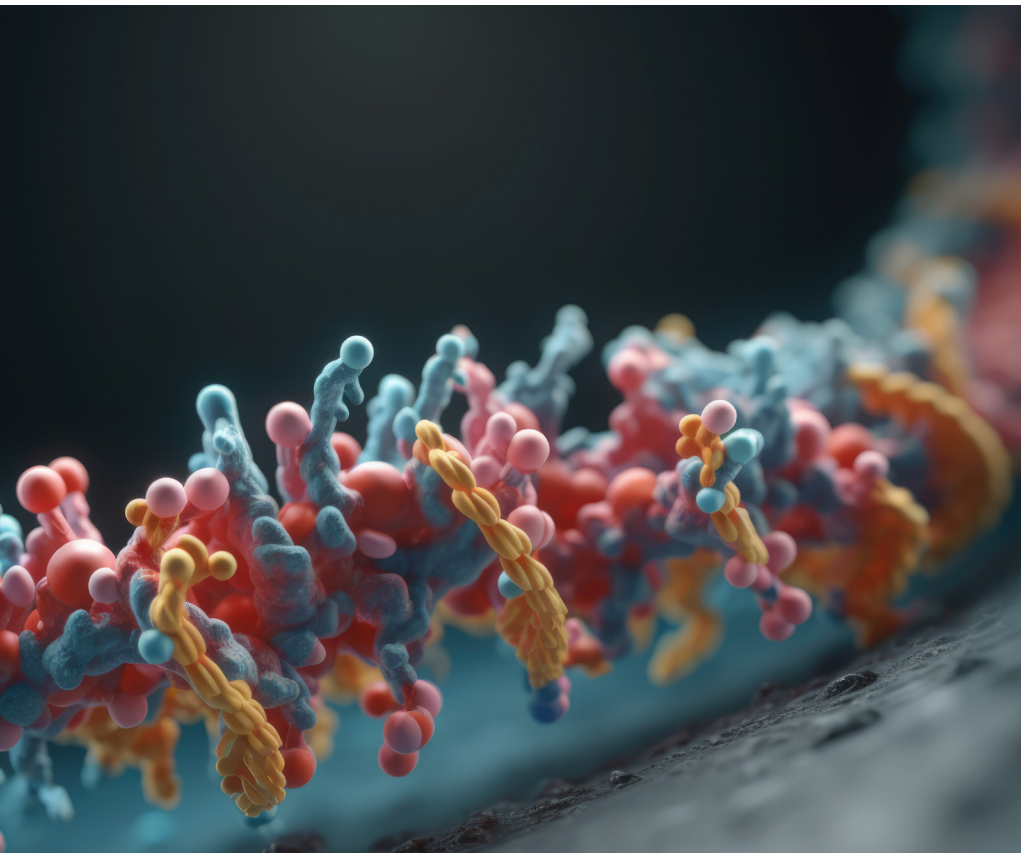

Challenge

Efficient and reliable evaluation of protein stability derived from their melting temperature, applicable in both medium and high-throughput settings.

Solution

Easily perform thermal shift assays with the qTOWERiris Series, utilizing various fluorophores. Working in either 96 or 384 well format allows screening multiple conditions simultaneously to efficiently optimize protein stability.

Intended audience

General qPCR users working in the field of molecular biology, genetics, or related areas.

Detecting Protein Melting Temperatures via Thermal Shift Assay with SYPRO® Orange Dye

Introduction

Most proteins have a well-defined three-dimensional structure in their native state. The thermal stability of this structure largely depends on intramolecular interactions of the protein's amino acids. These interactions - and thus the protein's thermal stability - can be affected by various factors such as buffer components, pH, chemical additives or the presence of cofactors or ligands. The composition of the protein itself also plays an important role and may be altered by mutations or transient modifications such as phosphorylations.

Traditionally, the thermal stability of proteins has been measured using circular dichroism or differential scanning calorimetry, both of which require a high input of protein while being time-consuming. With respect to both parameters, sample input and time, thermal shift assays employing fluorophores offer a significant advantage. The fluorophores are quenched in an aqueous environment and show an increase in fluorescence when bound to hydrophobic surfaces, such as hydrophobic regions of denatured proteins. Thus, the transition of a protein from its native to a denatured state is marked by an increase in fluorescence, which can be detected using qPCR thermal cyclers.

We have used two distinct fluorophores, SYPRO® Orange and GloMelt™ Dye, in combination with qTOWERiris and qTOWERiris 384 to monitor the denaturation of α-chymotrypsinogen and thus infer its melting temperature. Here, we have shown the impact of alterations in pH and salt concentrations on the stability of the protein. The sensitivity of the qTOWERiris family's optical system enables the reliable detection of shifts in fluorescence with minimal protein and dye input. The use of the 96 or 384 well format allows users to test a wide range of parameters in a single run. The precision of the qTOWERiris (384) block heated by Peltier elements can address minimal shifts in temperature, allowing the detection of their impact on the melting temperature. This permits optimization of buffer compositions to increase the thermal stability of proteins.

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Materials and Methods

Samples and reagents

Samples

- Stock: 10 mg/mL α-chymotrypsinogen

Buffer conditions

- Phosphate buffered saline (PBS)
  pH 5 / 7 / 9
- Tris-buffered saline (TBS)
  pH 5 / 7 /9
- Sodium chloride (NaCl) stock solutions in PBS and TBS, each:
  0,2 M
  5,0 M

Fluorophores

- GloMelt™ Dye (200x stock, 20x predilution)
- SYPRO® Orange (undiluted stock, 1:250 predilution)

Instrumentation

qTOWERiris or qTOWERiris 384 with:

- Color module 1 for detection of the GloMelt™ Dye
- Color module protein 1 for detection of SYPRO® Orange

Sample preparation

Various alpha-chymotrypsinogen solutions with a final concentration of 0.5 mg/mL were set up. Solutions differed with regards to the buffer reagent (PBS, TBS), salt concentration (20, 500, 2000, 4000 mM NaCl) and pH (5, 7, 9). All measurements were done with two distinct fluorophores (SYPRO® Orange, GloMelt™). SYPRO® Orange was diluted 1:5000 in the final reaction volume from a 1:250 predilution. GloMelt™ was diluted to a final concentration of 1x from a predilution of 20x. Solutions were set up to allow triplicate detection. In the 96-well format 20 µL were used per reaction. For the 384-well format 10 µL reaction volume was used.

Table 1: Preparation of samples. To check the impact of the salt concentration four different mixes were prepared (20 mM, 500mM, 2000 mM, and 4000 mM). To check the impact of the buffer pH three different mixes were prepared (pH 5, 7, and 9).
Fluorophore *a* - SYPRO® Orange (1:250 stock); Fluorophore *b* - GloMelt™ (20x stock)

Components	**Final concentration NaCl [mM]**				**Buffer pH**		
	20	500	2000	4000	5	7	9
NaCl stock, pH 5	0.2 M	5.0 M	5.0 M	5.0 M	-	-	-
NaCl [µL]	7	7	28	56	-	-	-
Buffer (PBS or TBS) [µL]	56	56	35	7	63	63	63
α-chymotrypsinogen (10mg/mL) [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Fluorophore (*a* or *b*) [µL]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Total [µL]	**70**	**70**	**70**	**70**	**70**	**70**	**70**

Instrument settings

The temperature-time protocol is programmed in qPCRsoft under the Settings > Thermal Cycler. Settings for the thermal shift assays using the respective fluorophores are shown in Figure 1 (GloMelt™) and Figure 2 (SYPRO® Orange).The preheat lid option is disabled so as not to exceed 70 °C temperature difference between block and lid. Setting a low heating rate in the “Melting curve” tab, increases the resolution of the melting curve and ensures that the lid is heating faster than the block to avoid condensation of the samples on the adhesive foil or lids of the reagent vessels.

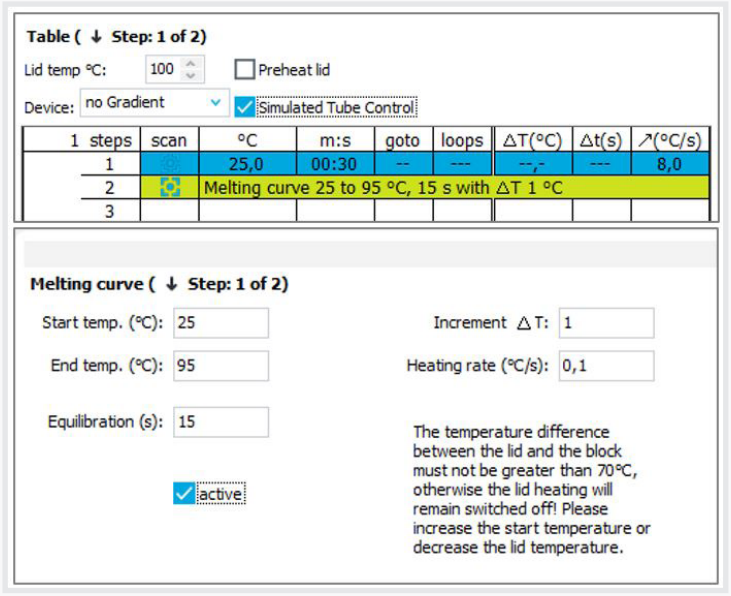

Fig. 1: qTOWERiris settings within qPCRsoft to run thermal shift assay using GloMelt™ dye.

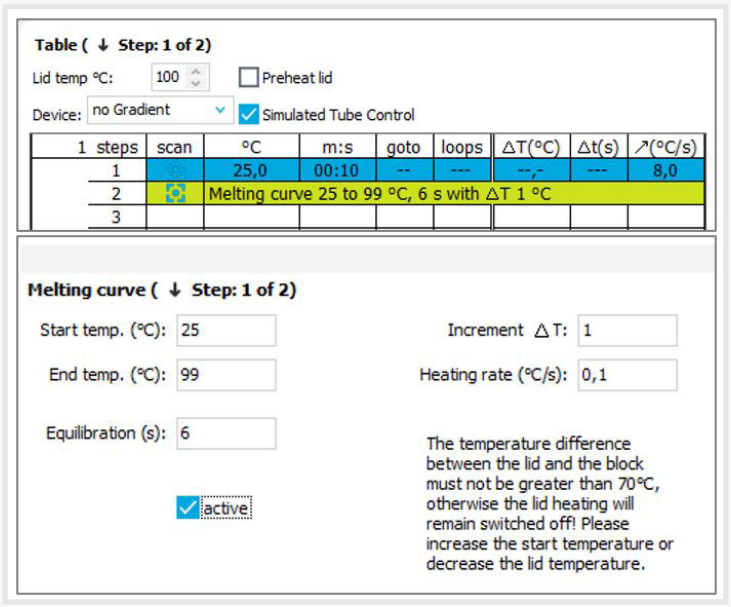

Fig. 2: qTOWERiris settings within qPCRsoft to run thermal shift assay using SYPRO® Orange dye.

Results and Discussion

Thermal shift assays in 96 well format

The melting temperature of alpha-chymotrypsinogen was analyzed in dependence on pH and salt concentration of the buffer solution.
The results of the analysis of pH-dependency in the 96 well format are shown in Table 2 and Figure 3. Alpha-chymotrypsinogen is most stable under acidic conditions (pH 5), with a melting temperature that is about 2 °C higher than at pH 7 and 4 °C higher than in alkaline conditions (pH 9). This is consistent with the fact that α-chymotrypsinogen is the precursor protein to chymotrypsin A and B, both of which are digestive enzymes present and active in the stomach. These enzymes are optimized to work in low pH conditions.

Two distinct thermal shift assays were employed to analyze the effect of the buffer conditions on the thermal stability of the protein. Both assays yielded comparable results with regards to the pH-dependency. Melting temperatures determined using GloMelt™ were only marginally higher than those using SYPRO® Orange (0.3 °C on average). As this shift is consistent across all measurements, the conclusions drawn with regards to the impact of buffer conditions on protein stability are unaffected by this deviation.
Irrespective of the fluorophore used, melting temperatures were also affected by the buffer system used. The protein is slightly more stable (higher melting temperature) in PBS than TBS buffer. This may be due to a higher temperature-dependent impact on the pKa value of TBS than that of PBS.

Table 2: pH-dependent shift in melting temperature of alpha-chymotrypsinogen in PBS and TBS buffers detected via temperature-dependent fluorescence shifts of SYPRO® Orange or GloMelt™, respectively. Fluorescence data was measured and analyzed using qTOWERiris in combination with qPCRsoft.

	SYPRO® Orange				GloMelt™			
	PBS		TBS		PBS		TBS	
pH	Tm	Mean Tm	Tm	Mean Tm	Tm	Mean Tm	Tm	Mean Tm
5	61.4	61.4	60.8	60.7	61.7	61.7	60.9	60.9
	61.3		60.6		61.7		61.0	
	61.4		60.7		61.6		60.9	
7	59.2	59.2	59.0	59.0	59.5	59.5	59.4	59.3
	59.1		58.9		59.5		59.3	
	59.2		59.0		59.4		59.3	
9	57.5	57.5	57.0	57.0	58.1	58.1	57.7	57.7
	57.5		57.0		58.1		57.7	
	57.4		56.9		58.1		57.7	

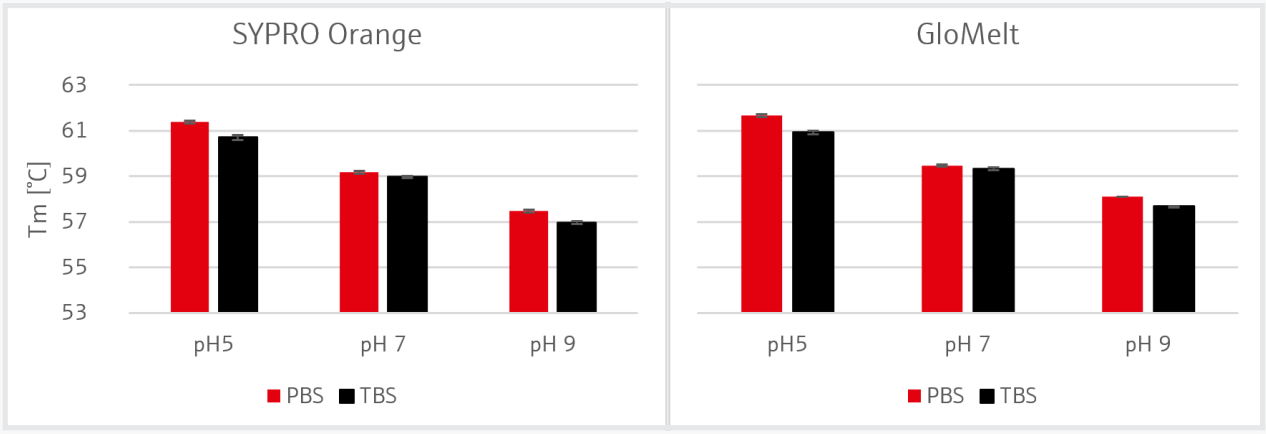

Fig. 3: pH-dependent shift in melting temperature of α-chymotrypsinogen in PBS and TBS buffers detected via temperature-dependent fluorescence shifts of SYPRO® Orange or GloMelt™, respectively. Fluorescence data was measured and analyzed using qTOWERiris in combination with qPCRsoft.

The results of the influence of the salt concentration on the thermal stability of α-chymotrypsinogen are shown in Table 3 and Figure 4. Alpha-chymotrypsinogen is most stable under low salt conditions (20 mM NaCl). At this concentration, protein stability is higher in PBS than TBS buffer. At high salt concentrations (4000 mM NaCl), the protein is more stable in TBS than PBS buffer.

Both thermal shift assays yielded comparable results. Contrary to the pH-dependent results, melting temperatures determined with GloMelt™ were lower than those using SYPRO® Orange. This effect was more pronounced when using TBS (3.0 °C on average) than PBS (1.6 °C on average). This is due to interactions of the buffer system with the fluorescent dye which is altered in the presence of NaCl salt. However, the overall trend observed in protein stability is unaffected by this shift.

Table 3: Impact of the salt (NaCl) concentration on melting temperature of α-chymotrypsinogen in PBS and TBS buffers detected via temperature-dependent fluorescence shifts of SYPRO® Orange or GloMelt™, respectively. Fluorescence data was measured and analyzed using qTOWERiris in combination with qPCRsoft.

	SYPRO® Orange				GloMelt™			
	PBS		TBS		PBS		TBS	
mM	Tm	Mean Tm	Tm	Mean Tm	Tm	Mean Tm	Tm	Mean Tm
20	61.1	61.1	60.5	60.5	60.7	60.7	58.8	58.7
	61.1		60.5		60.7		58.6	
	61.2		60.6		60.7		58.7	
500	60.3	60.3	59.6	59.6	58.3	58.3	57.1	57.0
	60.2		59.6		58.3		57.0	
	60.3		59.7		58.3		57.0	
2000	59.1	59.0	60.0	59.9	56.4	56.4	56.0	56.0
	59.0		59.9		56.4		56.1	
	59.0		59.7		56.3		56.0	
4000	53.1	52.9	55.3	55.4	51.5	51.5	51.9	51.8
	52.9		55.4		51.6		51.8	
	52.8		55.4		51.4		51.8	

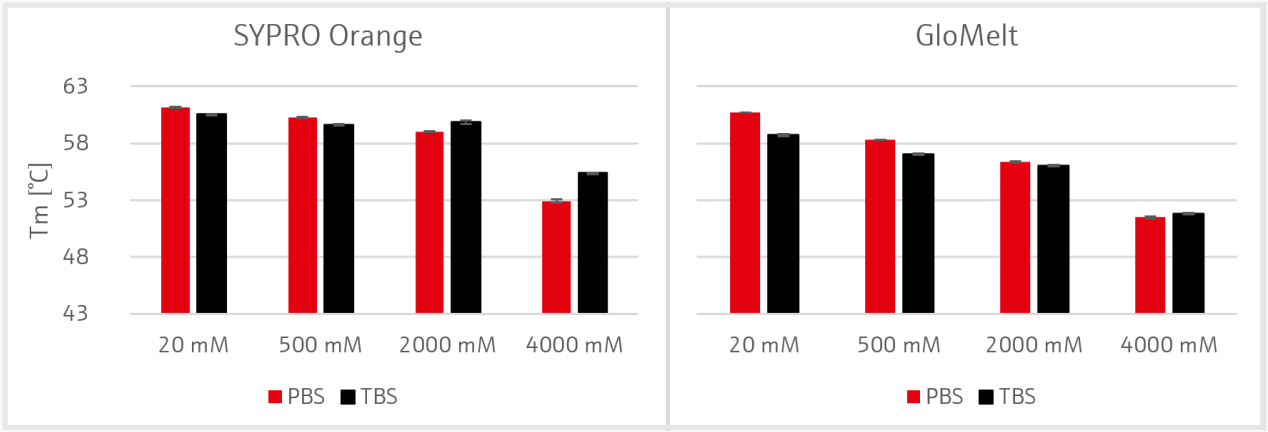

Fig. 4: Impact of the salt (NaCl) concentration on melting temperature of α-chymotrypsinogen in PBS and TBS buffers detected via temperature-dependent fluorescence shifts of SYPRO® Orange or GloMelt™, respectively. Fluorescence data was measured and analyzed using qTOWERiris in combination with qPCRsoft.

Thermal shift assays in 384 well format

In the 384 well format the pH-dependency of the thermal stability of α-chymotrypsinogen was shown as a proof-of-principle. The setup was the same as in the 96 well format. The fluorescence intensity of the two dyes were inversely affected by the pH of the buffer. While SYPRO® Orange showed the highest fluorescence intensity at low pH (5)(Figure 5), GloMelt™ yielded higher fluorescence at high pH (9) (Figure 6). SYPRO® Orange also showed higher background signals and lower overall fluorescence intensity increase compared to GloMelt™. The same trends were observed in the 96 well experiments (data not shown). The fluorescence intensity differences did not affect the measurement of the melting temperatures (Table 4, Figure 7). As in the 96 well format assay, GloMelt™ melting temperatures were slightly higher than those determined using SYPRO® Orange. However, overall results were the same – showing highest protein stability at low pH (5), consistent with the fact that the α-chymotrypsin is physiologically present in low pH environments.

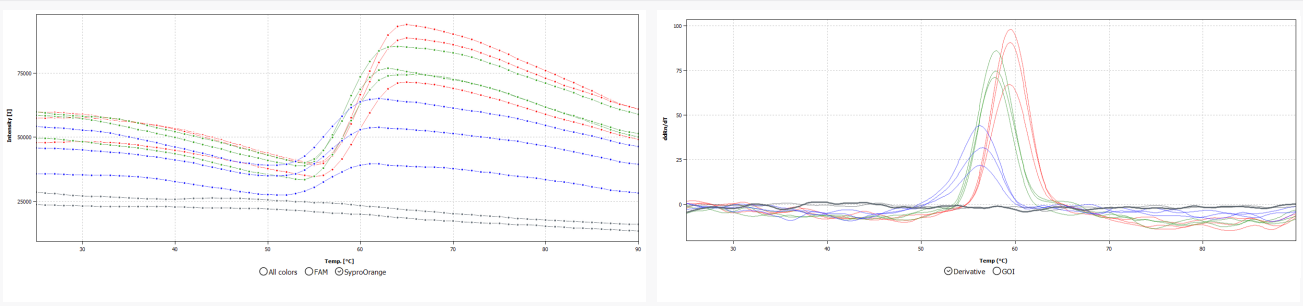

Fig. 5: **(Left)** - Melting curves of α-chymotrypsinogen in PBS buffers of different pH (red – pH 5, green – pH 7, blue – pH 9). Fluorescence data of SYPRO® Orange acquired using qTOWERiris 384 with color module protein 1 with gain 5. **(Right)** - First derivative of melting curve to generate peaks at the temperature with the highest change in fluorescence. Melting temperatures calculated using qPCRsoft are listed in Table 2.

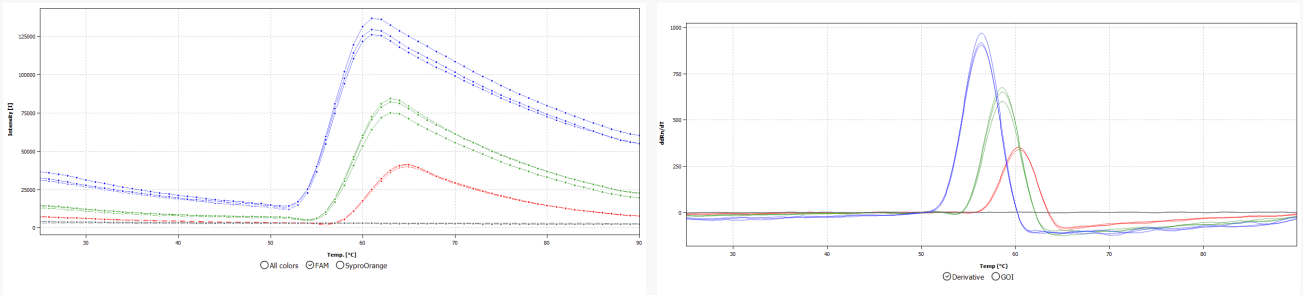

Fig. 6: **(Left)** - Melting curves of α-chymotrypsinogen A in PBS buffers of different pH (red – pH 5, green – pH 7, blue – pH 9). Fluorescence data of GloMelt™ acquired using qTOWERiris 384 with color module 1 with gain 5. **(Right)** - First derivative of melting curve to generate peaks at the temperature with the highest change in fluorescence. Melting temperatures calculated using qPCRsoft are listed in Table 2.

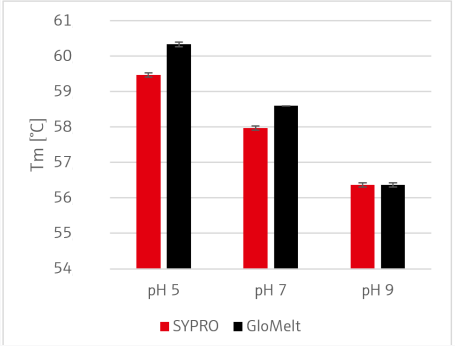

Fig. 7: pH-dependent shift in melting temperature of α -chymotrypsinogen in PBS buffers detected via temperature-dependent fluorescence shifts of SYPRO® Orange or GloMelt™, respectively. Fluorescence data was measured and analyzed using qTOWERiris 384 in combination with qPCRsoft.

Table 4: Melting temperatures of α-chymotrypsinogen in PBS buffers of different pH. Determined on qTOWERiris 384 using SYPRO® Orange (detected with color module protein 1) and GloMelt™ (detected with color module 1), each with gain 5. Melting temperatures were calculated using the Melting curve analysis implemented within qPCRsoft.

	SYPRO® Orange		GloMelt™	
pH	**Tm**	**Mean Tm**	**Tm**	**Mean Tm**
5	59.5	59.5	60.4	60.3
	59.5		60.3	
	59.4		60.3	
7	58.0*	58.0	58.6	58.6
	58.0		58.6	
	57.9		58.6	
9	56.6	56.4	56.4	56.4
	56.3		56.4	
	56.2		56.3	

Summary

Thermal cyclers of the qTOWERiris Series can be used to assess thermal protein stability rapidly and precisely in the 96 as well as 384 well format. This enables users to efficiently screen conditions in which to store proteins for maximal stability as well as assess impacts of small compounds or ligands and cofactors on the stability of a given protein. A choice of various color modules allows users to choose between different fluorophores such as SYPRO® Orange and GloMelt™. Thus, providing maximum flexibility in the optimization of the testing procedure. The high-performance optic system precisely detects minute alterations in fluorescence. The accurate and homogenous temperature control of the thermal block enables the user to run high-resolution melting curves (0.1 °C/s) to reliably detect exact melting temperatures. Furthermore, the melting curve analysis implemented within the qPCRsoft facilitates the analysis of the acquired data, computing the melting temperatures directly from the derivative of the fluorescence data.

Figure 10: qTOWERiris Series

Recommended device configuration

Table 5: Overview of required equipment and recommended accessories.

Article	Article number	Description
qTOWERiris incl. Color Module 1	844-00853-x*	Real-time PCR system designed in the 96-well format, operable via PC, customizable with up to 6 color modules. Available in 100 V, 115 V, and 230 V version, incl. color module 1 for excitation and detection of fluorescent dyes – FAM™, SYBR®Green, ATTO425 and Cyan500.
qTOWERiris 384 incl. Color Module 1	844-00858-x*	Real-time PCR system designed in the 384-well format, operable via PC, customizable with up to 6 color modules. Available in 100 V, 115 V, and 230 V version, incl. color module 1 for excitation and detection of fluorescent dyes – FAM™, SYBR®Green, ATTO425 and Cyan500.
Color Module Protein 1	844-00530-0	Color Module for the excitation and emission of fluorescence dyes like SYPRO®Orange.

*x=2 for 230 V, x=4 for 115 V and x=5 for 100 V

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