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Rapid, high-throughput assessment of protein stability on the Rotor-Gene Q cycler

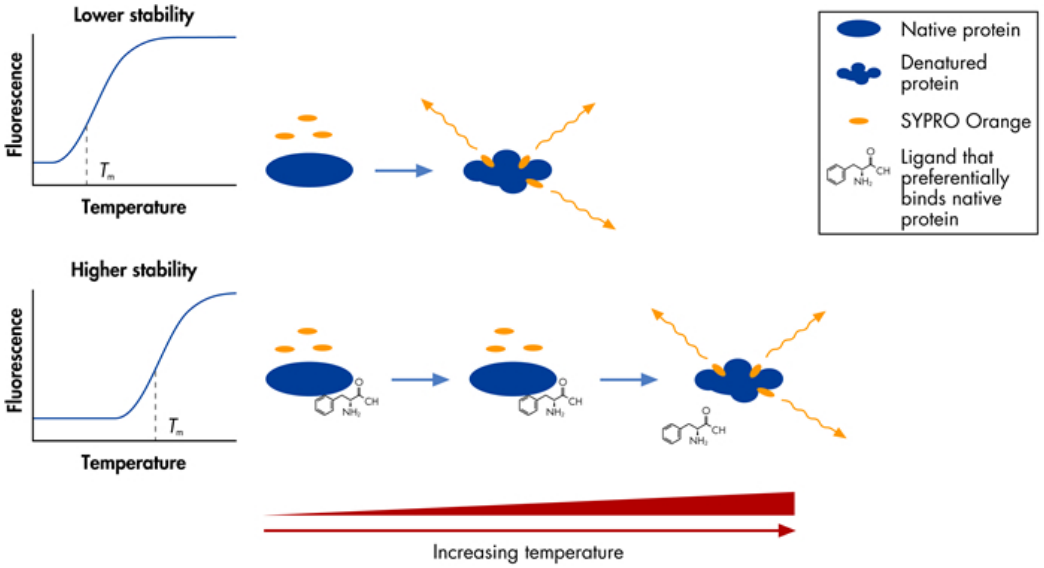
The assessment and optimization of protein stability is crucial for many diverse applications. Determination of the melting temperature of a protein provides a straightforward method of protein stability measurement. Such measurements can be easily performed using the Rotor-Gene Q cycler with up to 100 samples in parallel. The Rotor-Gene Q cycler provides unmatched temperature uniformity due to its unique centrifugal rotary design and single short optical pathway. Its detection precision is an important advantage for techniques such as real-time PCR or protein stability measurement. In this article, we describe how to set up and perform protein stability measurement using a Rotor-Gene Q cycler. We also present application data demonstrating the potential of this technique for drug discovery.

Introduction

Protein stability is a measure of the ability of a protein to remain in its native folded state with increasing temperature or denaturing conditions. The ability to measure protein stability is a useful tool with many practical applications. For example, it can be used to determine optimal buffer conditions for protein storage, or to identify and characterize protein-ligand binding. In addition, protein stability measurement can be used in drug discovery studies to screen mutations that may lead to changes in stability of critical proteins and to assess the suitability of ligands as drug candidates.

Here we describe a method for protein stability measurement called Differential Scanning Fluorimetry (DSF, also called Thermofluor analysis or protein melting). This method is based on the real-time measurement of fluorescence of a dye such as SYPRO Orange, which has affinity for hydrophobic parts of a protein (see figure "DSF principle"). SYPRO Orange has weak fluorescence in its unbound state in water and hydrophilic environments, but strongly fluoresces when bound to hydrophobic patches exposed by denatured proteins. In this method, the protein of interest is subjected to increases in temperature using the Rotor-Gene Q cycler, in the presence of SYPRO Orange. Fluorescence is measured and plotted against temperature, enabling determination of the melting temperature (T_m). The T_m is usually defined as the turning point of the sigmodial-like curve of fluorescence plotted against temperature. In the Rotor-Gene Q software, T_m is typically determined by assigning the minimum of the corresponding inverted first derivative curve (for details, consult the *Rotor-Gene Q User Manual*). A high melting temperature indicates high protein stability.

In this article, we describe DSF for soluble proteins. A complementary method has been described for hydrophobic (i.e., membrane) proteins, which require different assay conditions to soluble proteins (Alexandrov, A.I. et al. (2008) Microscale fluorescent thermal stability assay for membrane proteins. Structure **16**, 351).



DSF principle. *Fluorescence of SYPRO Orange is dramatically increased when it is bound to hydrophobic patches exposed upon protein denaturation. Protein stability (e.g., T_m) can be estimated by analyzing the temperature dependence of fluorescence intensity. Addition of ligands that preferentially bind to either the native or denatured protein will affect the T_m thereby allowing binding to be detected.*

Parameters for DSF on a Rotor-Gene Q cycler

Reaction conditions

The requirements for DSF are as follows:

- Purified protein of interest
- Rotor-Gene real-time cycler with HRM channel
- SYPRO Orange (cat. no. S5692 from Sigma Aldrich)

Various protein amounts, SYPRO Orange concentrations, detection settings, gain settings, and heating parameters were tested for DSF. The table below shows parameters that provided optimal results.

DSF reaction conditions

Parameter	Setting
Protein amount	1-10 µg
SYPRO Orange concentration	20X
Excitation	460 nm (HRM lamp)
Detection	510 nm (Green detection filter)
Gain	7
Heating increase	1°C each step
Heating wait	5 sec each step

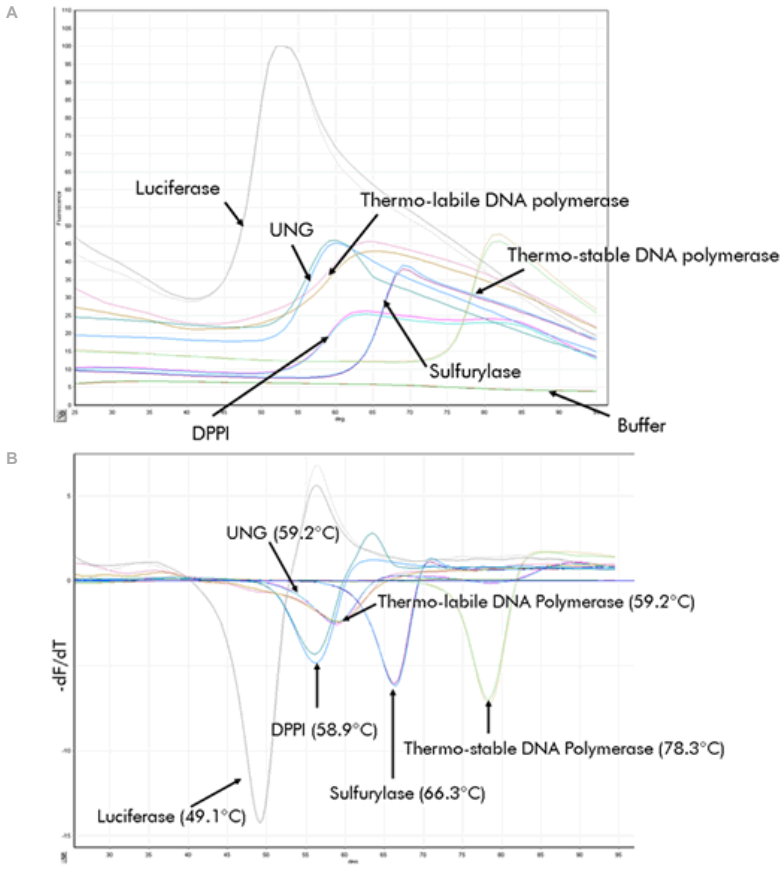
Rotor-Gene Q setup and data analysis

For experimental setup on the Rotor-Gene Q, follow the instructions provided in the "*Rotor-Gene Q Protein Stability Measurement Quick-Start Guide*".

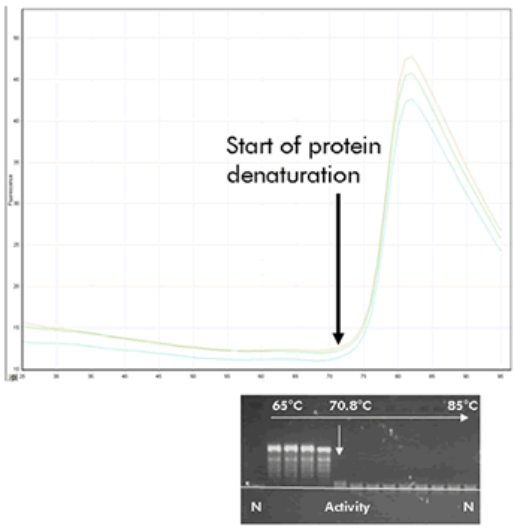
Results

Protein stability of various proteins

Using the reaction conditions and setup described above, protein stability was successfully measured for a range of different proteins, including thermolabile and thermostable DNA polymerase (see figure "Protein stability measurements of a range of proteins"). The data show a good correlation between the melting point of the thermostable polymerase determined by DSF and the loss of polymerase activity (see figure " Loss of DNA polymerase stability").



Protein stability measurements of a range of proteins *DSF was used for protein stability measurement of DPPI (DAPase) (10 µg) thermostable polymerase (4 µg), thermolabile polymerase (4 µg), luciferase (2 µg), UNG (4 µg), and sulfurylase (4 µg) using the Rotor-Gene Q, 72-well Rotor-Discs, and the reaction conditions described above. Experiments were performed in duplicate.* A *Raw data showing the increase of fluorescence of SYPRO orange during heating and denaturation of the proteins.* B *Negative first derivatives of raw data were used to estimate the melting points.*



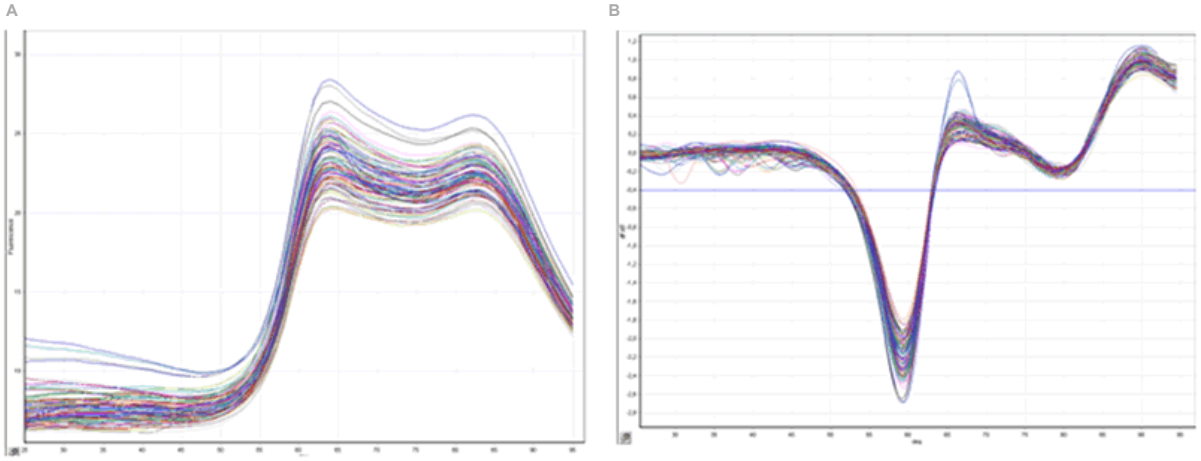
Loss of DNA polymerase stability. *DSF was used for protein stability measurement of thermostable polymerase (4 µg, triplicate samples). By measuring the polymerase elongation activity on M13 DNA, it was shown that the activity of the polymerase was lost at 70.8°C, coinciding with the point at which SYPRO orange fluorescence began to increase indicating protein unfolding.*

High reproducibility and superior performance to an alternative cycler

To test reproducibility of DSF on the Rotor-Gene Q, multiple measurements of protein stability were performed for DPPI (DAPase) using the QIAgility for reaction setup in 100-well Rotor-Discs and the Rotor-Gene Q. Reproducibility of results from multiple replicates was high with a standard deviation of only 0.08°C corresponding to less than 0.2% (see figure "Highly reproducible protein stability measurement").

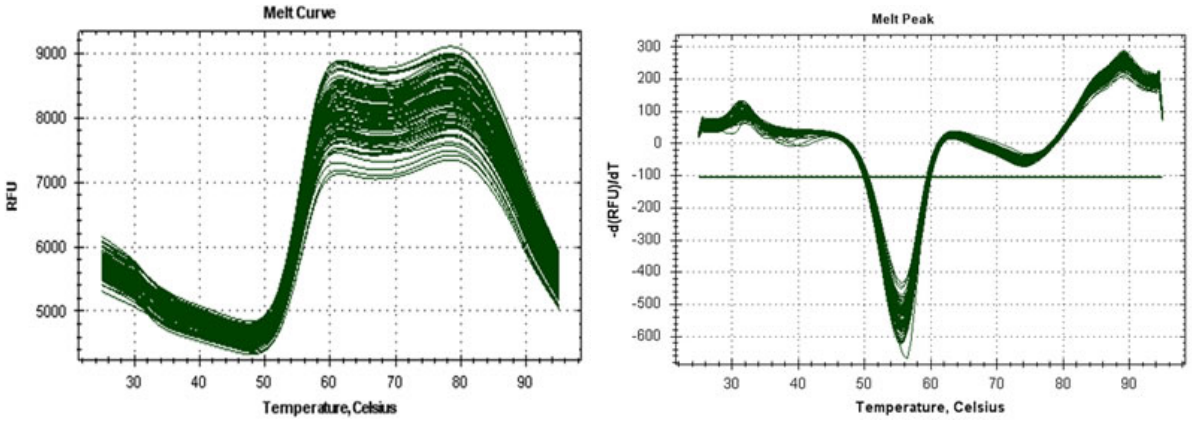
Intra-assay reproducibility on the Rotor-Gene Q was superior to results achieved using an alternative cyclers from Supplier B (see figure "Lower reproducibility using an alternative cycler"). The results of these experiments are summarized in the table "Intra-assay reproducibility of replicates".

In addition, tests of inter-assay reproducibility on the Rotor-Gene Q revealed highly reproducible melting point determination across multiple runs (see table "Inter-assay reproducibility across multiple runs").



Highly reproducible protein stability measurement. *DSF was used for protein stability measurement of DPPI (DAPase) (10 µg). Reactions were set up in Rotor-Disc 100 format using the QIAgility. DSF was performed on the Rotor-Gene Q using the reaction conditions described above. One hundred replicate reactions were performed in parallel.* A *Raw data.* B *Negative first derivative data. The mean* T_m *was calculated as 59.27 with a standard deviation of 0.08 indicating high reproducibility.*

A B



Lower reproducibility using an alternative cycler. *DSF was used for protein stability measurement of DPPI (DAPase) (10 µg). Reactions were set up in the appropriate format using a cycler from Supplier B (* A *and* B *). DSF was performed using the reaction conditions described above. Ninety-six replicate reactions were performed in parallel.* A *Raw data.* B *Negative first derivative data. The mean* T_m *was calculated as 55.53 with a standard deviation of 0.14.*

Intra-assay reproducibility of replicates

Format	Rotor-Gene Q	Supplier B
Mean T_m (°C)	59.27	55.53
Standard deviation	0.08	0.14
Temperature uniformity specified by manufacturer (°C)	0.01	0.4
Resolution	0.1°C	0.5°C

Comparison of intra assay reproducibility (precision) for the protein stability measurement of DPPI (10 µg) between the Rotor-Gene Q (Rotor-Disc 100 format) and a cycler from Supplier B . Reaction setup for all experiments was performed using the QIAgility.

Inter-assay reproducibility across multiple runs

Run	Run 1	Run 2	Run 3
Mean T_m	58.94	58.93	58.93
Number of replicates	34	3	3

Inter-assay reproducibility for protein stability measurement of DPPI (10 µg) with the Rotor-Gene Q. Reaction setup for all runs was performed manually. Reactions were performed in Rotor-Disc 72 format.

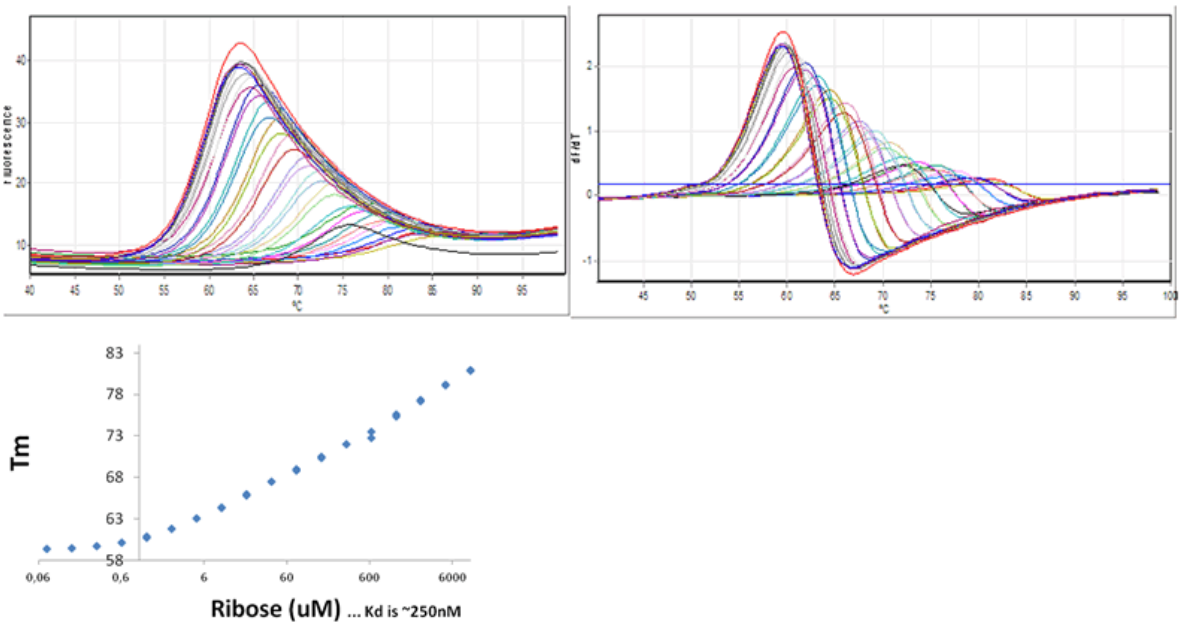
These results emphasize the importance of high temperature uniformity of the real-time cycler when performing protein stability measurement. To observe minute changes of stability of a folded protein by precisely measuring the melting point, any instrument temperature deviations must be minimized. Temperature uniformity (usually specified by the instrument manufacturer) is the critical parameter in this context. Due to its centrifugal rotary design and air-based temperature control, the Rotor-Gene Q provides at least one magnitude higher temperature uniformity than alternative cyclers that use peltier-element heating of metal blocks. The higher temperature uniformity results in higher resolution of the melting curves. The combination of high temperature uniformity and melting curve resolution provides protein stability results with high biological relevance.

It is well known that small differences in salt concentration or pH of the buffer system can influence stability measurements. To minimize any artificial influences from the buffer system in DSF experiments, an automated reaction set-up robot such as the QIAgility is recommended to eliminate manual pipetting errors and increase assay set-up precision.

These data enable comparison of results between different runs, reaction vessels, and cyclers. Data obtained with the Rotor-Gene Q and with the cycler from Supplier B differ by almost 4 °C under identical experimental conditions. This highlights the importance of performing regular temperature verification to ensure comparability of results over time. Verification of the accuracy of the temperature can easily be performed for Rotor-Gene Q cyclers within minutes by anyone who can start a run, using the Rotor-Disc QTV Kit. However, in block based instruments this task usually requires more time and labor and can only be performed by a service engineer.

Protein stability measurement in a ligand-binding study

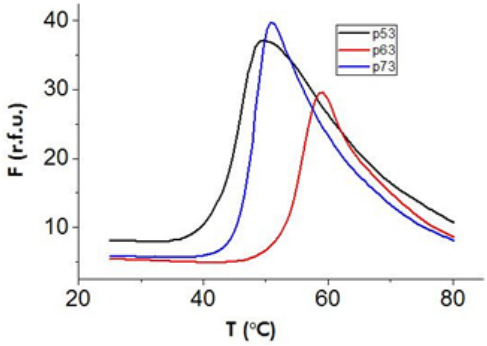
To test reproducibility and sensitivity of the DSF technique on the Rotor-Gene Q, ligand-binding studies were performed. *E. coli* ribose binding protein (1 µM) was mixed with various dilutions of ribose. Results indicated a dissociation constant (K_D) of approximately 250 nM and showed highly precise and reproducible measurements at all concentrations tested (see figure "Reproducible results over a concentration range").



Reproducible results over a concentration range. *E. coli ribose binding protein (1 µM) was mixed with various dilutions of ribose (2-fold dilutions from 10 mM to 40 nM, duplicate samples). DSF was used for protein stability measurement on the Rotor-Gene Q.* A *Raw data.* B *First derivative data.* C *Plot of* T_m*s against ribose concentration showing highly linear correlation. Data kindly provided by Philippe Marguet, Duke University, Durham, USA.*

Comparison of transcription factor stability in cancer research

DSF was used to measure the stability of 3 transcription factors: p53, p63, and p73. These transcription factors are involved in cell cycle control and development, and p53 is a tumor suppressor that is important for the prevention of cancer. Protein stability measurements were performed to determine whether relative stability influences the functionality of these proteins. Using DSF, the relative stability of the proteins was ranked as p53<p73<p63 (see figure "Relative protein stability").



Relative protein stability. *DSF melting curves for full-length p53, p63 and p73 proteins. Data kindly provided by Dmitry Veprintsev, MRC Laboratory of Molecular Biology, Cambridge, UK.*

Conclusions and applications

- The unique features of the Rotor-Gene Q cycler ensure precise simultaneous measurement of fluorescence and temperature, providing highly accurate and reproducible results in protein stability measurement by DSF.
- DSF on the Rotor Gene Q cycler allows high-throughput, straightforward measurement of protein-ligand complexes to compare different ligands and, if desired, optimization of their binding behavior.
- Long-term protein storage can be optimized by quick assessment of protein stability in different buffers to predict the most suitable formulation.
- Different protein family members or mutations can be easily compared by their differences in stability determined by their melting point.

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