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Acquisition of data at multiple gains within a single thermal melt experiment using the Rotor-Gene Q instrument

Sai Hari A Gandham[1], David E Volk[1,2] and David G Gorenstein[1,2]*

Abstract

Background: Rotor-Gene Q instrument was used to perform high-resolution protein thermal melt studies to characterize protein-small-molecule interaction. Fluorescent dye (1-anilino-8-naphthalenesulfonate (1,8-ANS)) is used as a reporter of protein unfolding to measure the protein melting temperature (T_m). Variations in the fluorescence yield upon titration of small molecules with the protein resulted in poor melting curves at low gain while a high gain setting caused signal saturation leading to data loss.

Findings: Acquisition of data at multiple gains within a single experiment provided high-quality data for samples with both low and high fluorescence yields. The melting temperatures were measured for all the samples in one run, while avoiding loss of data due to signal saturation. This method was successfully used to measure the binding constant by titration of a small-molecule ligand with the target protein.

Conclusion: Protein thermal melt experiments using the Rotor-Gene Q instrument have been made feasible for samples that show variations in fluorescence yield. Furthermore, since protein melting is irreversible, using multiple gains in the same experiment prevented loss of sample and saved gain optimization time.

Keywords: Thermal stability; Protein melting temperature; Rotor-Gene Q; Fluorescence yield; Gain optimization; Multiple-gain protocol; Small-molecule binding

Introduction

Protein thermal melt (PTM) experiments are gaining importance to study thermodynamic stability of a protein by measuring their protein melting temperature (T_m). A change in T_m upon binding of a small-molecule ligand to target protein enables this technique to be extended for high-throughput screening and characterization of binding (Pantoliano et al. 2001; Matulis et al. 2005). This label-free screening method requires no modification to protein or ligand. Further, PTM experiment uses small sample volume of at least 10 μl containing low amount of protein (10 μM) and a fluorescent reporter dye like SYPRO-Orange (SO) or 1-anilino-8-naphthalenesulfonate (1,8-ANS). These dyes have low fluorescence

intensities when there is no interaction with protein. As protein unfolds with increasing temperature, the dye molecules interact with the protein's hydrophobic interior and show an increase in their fluorescence intensity thus acting as a reporter of protein unfolding. This helps to measure the stability of protein (T_m) in the presence and absence of a small-molecule compound to determine the differential shift in protein stability (ΔT_m) upon compound binding. This technique and its theoretical principles are well documented (Lo et al. 2004; Cimmperman et al. 2008; Zhang and Monsma 2010). We utilized a conventional real-time PCR instrument called Rotor-Gene Q (QIAGEN, Inc., Hilden, Germany) which is ideal for performing high-resolution

* Correspondence: David.G.Gorenstein@uth.tmc.edu
[1]Center for Proteomics and Systems Biology, Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, Houston, TX 77030, USA
[2]Department of Nanomedicine and Biomedical Engineering, The University of Texas Health Science Center, Houston, TX 77030, USA

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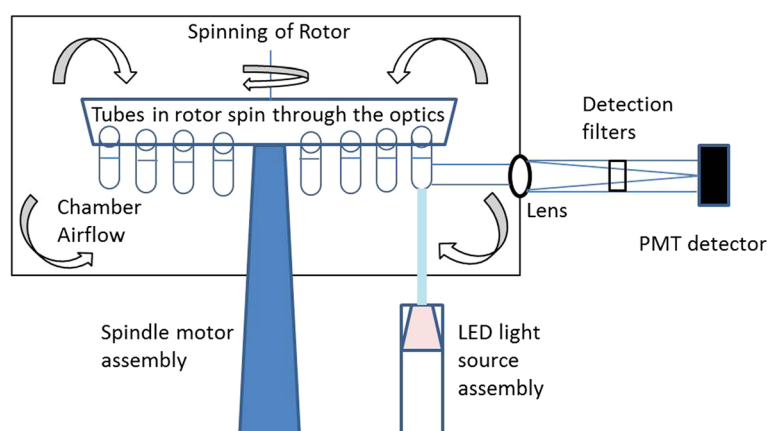
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Figure 1 Schematic illustration for the working of Rotor-Gene Q instrument is shown. The samples in the optically clear PCR tubes spin past the optics while the LED light excites the sample and the PMT detector detects the fluorescence emission from each sample. The airflow in the chamber maintains uniform temperature across all samples.

melting of nucleic acids and protein because of its unique rotating sample holder that provides uniform temperature across all samples. A schematic of the Rotor-Gene Q instrument cross-sectional view is provided to show the excitation and emission light paths and the position of the samples to enable rapid high-throughput and precise detection of fluorescence. The excitation and emission wavelength pairs are set up before the start of experiment to choose the right LED light source and the detection filter. The samples in the rotary holder (interchangeable for 36 or 100 samples) spin at 400 rpm in an enclosed chamber that allows precise temperature control (Figure 1). Also, the flexibility of Rotor-Gene Q for using several excitation and emission filter pair combinations in a single run allows for monitoring a wide array of samples using different dyes in the same experiment (Köppel et al. 2009). The major drawback that we found was with choosing the

gain for the detector that has to be set at a fixed value before starting the experiment. Several gain optimization runs were needed to choose the right signal intensity for a given concentration of protein and buffer system used. In our case of small-molecule compound screening, a high gain setting caused saturation of fluorescence signal resulting in loss of data for high fluorescence yield samples while a low level of gain does not provide sufficient signal to observe the melting transition for low fluorescence yield samples (Figure 2) to accurately measure the protein stability.

Findings

By choosing the same excitation and emission filter pair (excitation wavelength of 365 nm and emission wavelength of 460 nm for ANS dye) in several channels and assigning different gains for each channel,
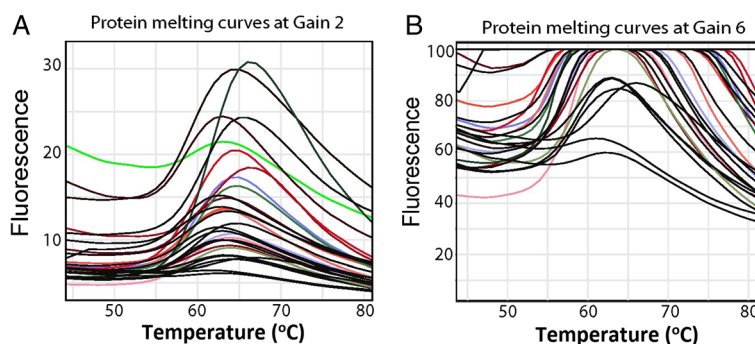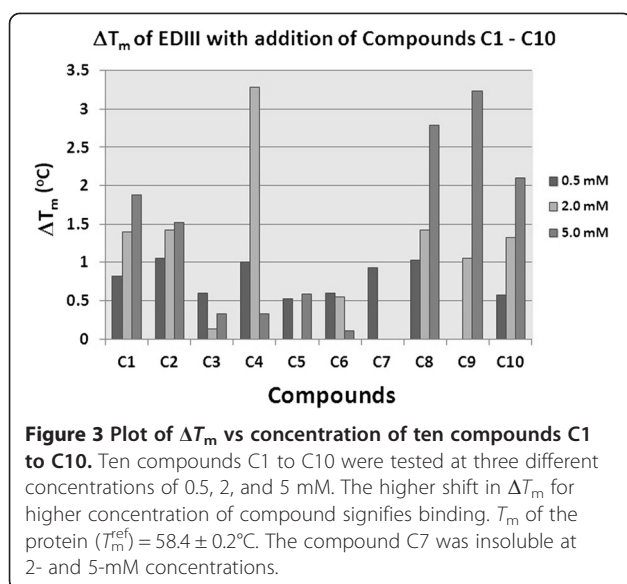

Figure 2 Fluorescence melting curves at gains 2 and 6. (A) The fluorescence melting curves for the ten compounds C1 to C10 at gain 2 are shown. It was optimal for most samples that had relatively higher fluorescence yield. **(B)** Melting curves at gain 6 show optimal melting transition for samples with low fluorescence yield while samples with high fluorescence yield showed saturation of fluorescence intensity, and hence, their ΔT_m could not be calculated at this gain setting.

Table 1 ΔT_m of protein for ten compounds (C1 to C10)

Concentration of compounds C1 to C10 (mM)		The values of ΔT_m at different gains used in the same experimental run (°C)					Average ΔT_m (°C)
		Gain −2	Gain 0	Gain 2	Gain 4	Gain 6	
0.5		0.83	0.93	0.93	0.63	-	0.82 ± 0.14
2	C1	1.13	1.63	1.43	-	-	1.39 ± 0.25
5		1.63	2.13		-	-	1.87 ± 0.35
0.5		0.83	1.33	1.13	0.93	-	1.05 ± 0.22
2	C2	1.13	1.63	1.63	1.33	-	1.42 ± 0.24
5		1.13	1.63	1.83		-	1.52 ± 0.36
0.5		0.33	0.83	0.63	0.63	-	0.60 ± 0.21
2	C3	−0.08	0.33	0.13	0.13	-	0.12 ± 0.16
5		0.43	0.33	0.43	0.13	-	0.32 ± 0.14
0.5		0.63	1.13	1.33	0.93	-	1.00 ± 0.30
2	C4	2.93	3.63	3.43	3.13	-	3.27 ± 0.31
5		−0.08	0.43	0.63		-	0.32 ± 0.36
0.5		0.43	0.63	0.63	0.43	-	0.52 ± 0.12
2	C5	−0.18	0.13	0.13	−0.18	-	−0.03 ± 0.17
5		0.33	0.63	0.83	-	-	0.59 ± 0.25
0.5		0.63	0.83	0.63	0.33	-	0.60 ± 0.21
2	C6	0.63	0.83	0.63	0.33	0.33	0.55 ± 0.22
5		0.33	0.33	0.13	−0.08	−0.18	0.11 ± 0.23
0.5		0.43	1.43	0.93	-	-	0.93 ± 0.05
2	C7	−0.18	0.13	-	-	-	−0.03 ± 0.21
5		-	-	-	-	-	-
0.5		1.13	1.43	1.13	0.83	0.63	1.03 ± 0.31
2	C8	1.13	1.63	1.63	1.33	-	1.43 ± 0.24
5		2.43	2.83	3.13		-	2.79 ± 0.35
0.5		-	0.33	0.13	−0.38	−0.38	−0.08 ± 0.36
2	C9	-	1.33	1.13	0.93	0.83	1.05 ± 0.22
5		2.63	3.63	3.63	3.13	3.13	3.23 ± 0.42
0.5		0.43	0.83	0.63	0.43	-	0.58 ± 0.19
2	C10	1.13	1.63	1.43	1.13	-	1.33 ± 0.24
5		1.83	2.33	2.33	1.93	-	2.10 ± 0.26

Hyphen indicates samples that did not show fluorescence melting signal above threshold noise level or resulted in signal saturation (data loss) due to high fluorescence intensity. The ΔT_m of protein was measured for the ten compounds C1 to C10 at three concentrations (0.5, 2, and 5 mM) at five levels of gain set within a single experimental run. T_m of the protein ($T_\mathrm{m}^{\mathrm{ref}}$) = 58.4 ± 0.2°C.

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Figure 3 Plot of ΔT_m vs concentration of ten compounds C1 to C10. Ten compounds C1 to C10 were tested at three different concentrations of 0.5, 2, and 5 mM. The higher shift in ΔT_m for higher concentration of compound signifies binding. T_m of the protein (T_m^{ref}) = 58.4 ± 0.2°C. The compound C7 was insoluble at 2- and 5-mM concentrations.

protein unfolding was monitored at multiple gains in a single experiment. This enabled the monitoring of samples that had different yields of fluorescence intensity in the same experiment without significant increase in the run time. This method is novel and to the best of our knowledge, it has not been used previously. The use of the ANS dye with the Rotor-Gene Q instrument has been demonstrated to work with our model protein to study binding of small-molecule compounds by using different levels of gain in a single PTM experiment.

Model system used

Recombinantly expressed and purified envelope protein domain III (EDIII) from dengue virus (serotype-2 WT

strain 11608) was used at a concentration of 10 μM in a total sample volume of 10 μl. The structure of EDIII has seven beta strands that form a beta barrel. The loops at the top contain the critical neutralizing epitopes, and thus, this protein is important for the development of vaccines and therapeutics targeting the dengue virus. Optimization for maximum fluorescence signal and melting transition was performed using various buffer systems at different concentrations of protein, salt, and ANS dye. PBS buffer (×0.5 containing 5.95 mM phosphates, 68.5 mM NaCl, 1.35 mM KCl, at pH 7.4) and 50 μM ANS provided the optimal fluorescence melting transition for 10 μM protein, and the protein melting temperature of the protein (T_m^{ref}) was determined as 58.4 ± 0.2°C. Computational methods (data not shown) were used to identify several small-molecule compounds that showed possible interactions with our protein. Ten compounds (C1 to C10) from this set were tested at three different concentrations for each (0.5, 2, and 5 mM) to obtain the protein melting curves and the first derivative of melting curves (dF/dT) for different levels of gain.

Results

Melting temperatures were obtained for the protein without the compounds (T_m^{ref}) and with the compounds (T_m^{com}) using their corresponding optimal melting curves. The protein melting temperature values were measured by the Rotor-Gene Q-Pure Detection software version 2.0.2 (Build 4). The differences in melting temperatures ($\Delta T_m = T_m^{com} - T_m^{ref}$) were calculated (Table 1) where an increase in ΔT_m with increasing concentration of compound signified binding (Figure 3). Based on this initial testing, compound 8 (C8) was found to bind the protein in a dose-dependent manner, among several others. A titration

Table 2 The ΔT_m of protein at various concentrations of C8

[C8] mM	ΔT_m at different levels of gain (°C)												Average ΔT_m (°C)
	−2	−1	0	1	1.3	1.7	2	2.3	3	3.5	4	5	
0.25[a]	-	1.7	1.2	2.2	1.9	1.2	0.9	1	0.7	1.5	1	0.9	1.29 ± 0.45
0.75	1.7	2.7	2.9	2.7	2.7	2.7	1.7	2.2	1.7	1.5	1.7	2	2.22 ± 0.50
1	2.9	3	2.9	3	2.9	2.9	2.4	2.2	2.2	2.2	2.2	2.2	2.55 ± 0.36
1.5	2.7	3	3.5	3.2	2.7	2.9	2.7	2.7	2.7	2.7	2.5	2.5	2.82 ± 0.28
2	3.2	3.4	3.7	3.7	3.4	3.2	3.2	3.2	3	3	3.2	3	3.27 ± 0.23
2.5	3	3.4	3.7	3.5	3.4	3.2	3.2	3	2.9	3	2.9	2.9	3.19 ± 0.26
3	3.2	3.5	3.7	3.7	3.4	3.4	3.2	3.2	3.2	3.2	3	3	3.31 ± 0.23
3.5	3	3.7	3.7	3.7	3.5	3.4	3.4	3.2	3.2	3.2	3.2	3.2	3.40 ± 0.23

[a]T_m could not be calculated for −2 gain due to low fluorescence signal, and the melting curves were below threshold. ΔT_m of protein for ten compounds (C1 to C10). The ΔT_m of protein was measured at different gain levels set within the same experimental run that were used to determine the binding affinity; T_m of the protein (T_m^{ref}) = 58.4 ± 0.2°C. The ΔT_m were calculated by the Rotor-Gene Q-Pure Detection software version 2.0.2 (Build 4).

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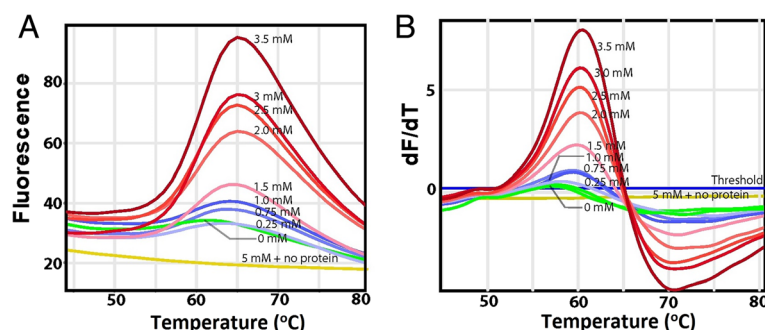
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Figure 4 Titration of compound 8 with the target protein. (A) Fluorescence melting curves showing change in fluorescence intensity with increasing temperature for different concentrations of C8 shown in the figure. **(B)** First derivative of melting curves (dF/dT) is plotted and the temperature at maximum dF/dT is the protein melting temperature (T_m). The data for gain level of 5 are shown.

experiment was performed for increasing concentrations of C8 from 0.25 up to 3.5 mM to obtain melting curves and the first derivative curves at different gains to calculate the protein melting temperature and their corresponding ΔT_m (Table 2). The fluorescence intensity data for a gain level of 5 is shown in Figure 4. The plot of ΔT_m vs C8 concentration was fit using nonlinear regression equation for one-site-specific binding $[\Delta T_m = \Delta T_m(max)*[C8]/(K_d + [C8])]$ using GraphPadPrism (v 5.07), and the binding affinity (K_d) was determined to be 516.2 µM (Figure 5). The values of protein melting temperature that could be measured at all the different gains were used in the fit, and the variations in ΔT_m could be observed by the error bars. The results clearly indicate that our method provides the best use of the Rotor-Gene Q instrument for optimization of gain and for performing protein melts for several samples by collecting data simultaneously at multiple gains in a single run thereby increasing the throughput.
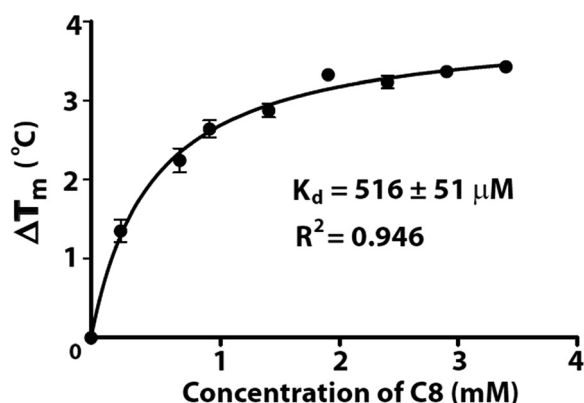
Conclusion

Optimization of gain can be a challenging task, and it is impossible to use fixed gain for samples that have varying fluorescence yields in a single experiment. Here, we have demonstrated a novel method for using the Rotor-Gene Q instrument for PTM using multiple levels of gain in the same experiment. Since PTMs are mostly irreversible, our method should help to save precious sample and time.

Availability and requirements

Project name: Rotor-Gene Q-Pure Detection v2.0.2 (Build 4)
Operating system(s): PC
Programming language: Unknown
Other requirements: None
License: Commercially available from Qiagen
Any restrictions to use by non-academics: license needed

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Figure 5 The ΔT_m vs [C8] data was fit to a one-site-specific binding non-linear regression equation. The error bars represent the variation in ΔT_m at different levels of gain. The data used for the fit are provided in Table 2.

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