Breaking Sensitivity Barriers in DSF: Application of a Novel FRET-Based Probe from QRET technologies for Nanomolar Protein Stability and Ligand Interaction studies.

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Abstract

Differential Scanning Fluorimetry (DSF) is a pivotal technique for assessing protein stability and ligand interactions. However, it faces sensitivity and protein requirement limitations that restrict throughput for affinity-based drug discovery screening. Recently, a Förster Resonance Energy Transfer (FRET)-based probe was reported which enabled highly sensitive detection of protein denaturation and ligand binding at nanomolar concentrations.[2,3] In this study, we evaluated this probe against a popular DSF dye, *e.g* SYPRO Orange, demonstrating improved sensitivity and lower sample requirements for protein stability across various AstraZeneca targets as well as illustrating its utility in protein-ligand interaction analysis.

Introduction

Protein stability is fundamental to cellular processes and underpins the development of biologics and reliable *in vitro* assays.[1] Understanding the influence of solvent composition and temperature on protein stability is essential for optimising conditions for expression, purification and storage.[1,2] Thermal Shift Assays (TSA), especially DSF using SYPRO Orange dye, provide rapid, label-free detection of protein thermal stability and protein-ligand interactions *via* fluorescence readout during protein unfolding. However, standard DSF is limited by sensitivity and protein requirements, posing challenges for high-throughput screening (HTS). Recent advances[2] have introduced a FRET-based probe capable of detecting protein unfolding and ligand interactions at nanomolar concentrations, providing a potential solution to these limitations. Herein, we assess this probe from QRET technologies against SYPRO Orange across a range of protein targets from our portfolio as part of an early proof-of-concept (PoC), with the aim of establishing a more cost-effective and sensitive screening platform to accelerate affinity-driven hit identification projects.

Thermal Shift Assay (TSA) Description

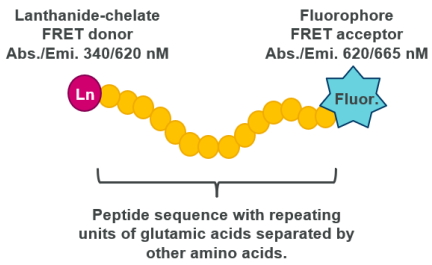


Figure 1. FRET-probe design for TSA from *QRET technologies* (FRET-Probe-400 kit).[3]

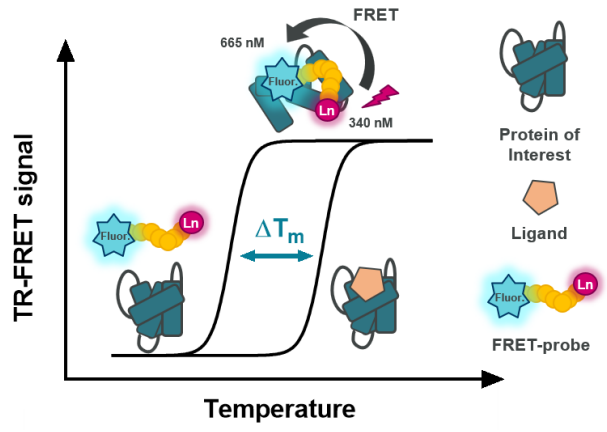


Figure 2. TSA principle with FRET-probe.[2,3]

Analysis & experimental set up

The FRET-probe assay employs a dual-labelled peptide, featuring a lanthanide donor and an acceptor fluorophore at opposite ends (Figure 1). During thermal shift analysis, protein unfolding exposes hydrophobic regions that facilitate probe binding, bringing the labels closer together and increasing the FRET signal.[2,3] Protein-ligand interactions are reflected by shifts in melting temperature (ΔT_m), altering the FRET profile.

After plate heating, FRET signals are measured using a Pherastar FSX reader, and melting curves are fitted (Boltzmann model, fixed slope = 1) in GraphPad Prism:

$$Y = Bottom + \frac{Top - Bottom}{1 + \exp(\frac{V_{50} - X}{Slope})}$$

Results

A. Comparative Analysis of the FRET-Probe Sensitivity Against the SYPRO Orange Dye

Figure 3. Early PoC studies for T_m determination with Transcription factor a) A vs b) B using proprietary buffers (pH 5 or 7) from *QRET technologies* FRET-Probe-400 kit. *For SYPRO Orange, in-house assay buffer was used at pH 7.5.

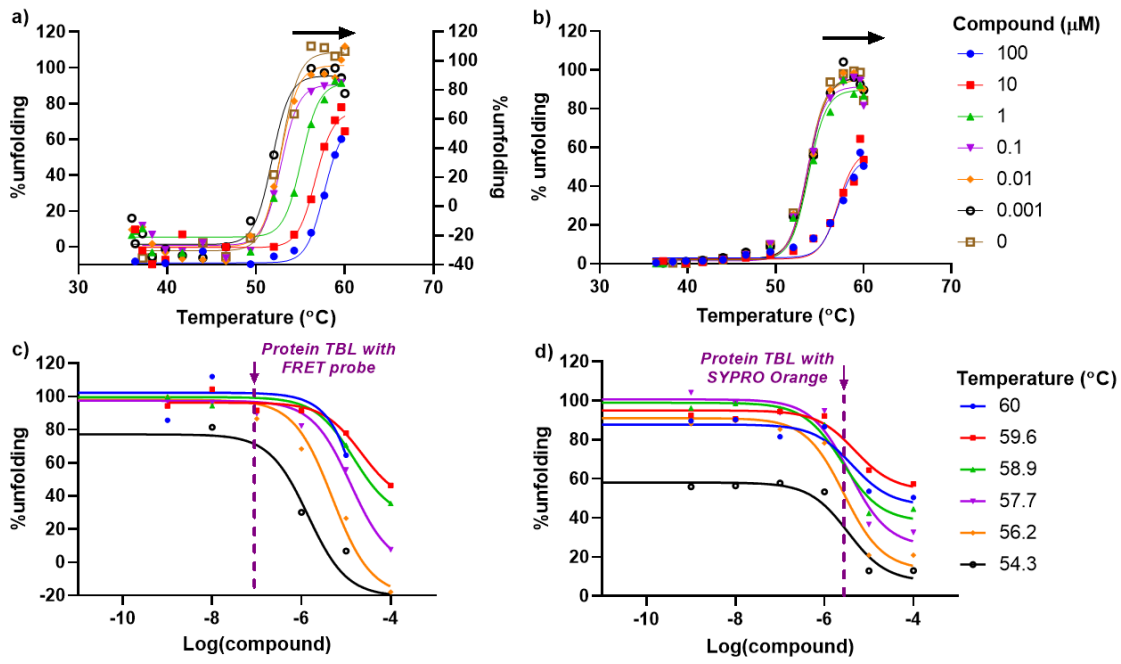
FRET-probe protein-specificity and pH-dependence from our early PoC are demonstrated in Figure 3. Next, we show that across multiple targets, the FRET-probe reliably measures protein stability using 20–40 times less protein than SYPRO Orange, with signals remaining stable for 3h (Table 1).

Table 1. Protein scope and assay parameters using the FRET-probe from *QRET technologies*.

	Transcription factor A		Scaffolding protein		ATPase		Transcription factor B		GTPase	
Probe	SYPRO	FRET	SYPRO	FRET	SYPRO	FRET	SYPRO	FRET	SYPRO	FRET
[Protein]	2.5 uM	125 nM	5 uM	125 nM	5 uM	250 nM	2.5 uM	Inactive	5 uM	Inactive
S:B	5	2-3	11	2-3	5	2	4	-	3	-
Tm (ºC)	63		54-55		43-44		39-40	-	47	-

B. Scaffolding Protein Stabilisation with Ligand & Concentration Effects

Figure 4. Scaffolding protein stabilisation across ligand titration between a) FRET-probe & b) SYPRO Orange and respective ligand Dose-Response Curves (DRC) with protein concentration of c) 125 nM vs d) 5.0 μM.



Using less protein with the FRET-probe lowers the Tight Binding Limit (TBL), allowing broader ligand concentration ranges for observing ΔTm (Figure 4a vs b) and more defined IC_{50} in the T_m range (Figure 4c vs d). This could enable ΔTm assessment with reduced ligand quantities, minimising compound insolubility risk and benefiting single-point screening.

Conclusions

- The FRET-probe enabled Thermal Shift Assays for most of the tested targets, reducing protein use up to 40-fold versus the SYPRO Orange dye.
- This enhanced sensitivity support lower-concentration, single-point screening formats and hence would reduce insolubility risks.
- Future work aims to expand the target scope and further validate this approach to enable a more accessible and cost-effective protein stability screening platform.

References

1. Gooran N et al. *Int. J. Mol. Sci*. **2024**;25(3):1764.
2. Mahran R et al. *Sci.Rep.* **2023**;13(1):20066.
3. https://qrettech.com/

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