

Protein Thermal Shift Assay is an Excellent High Throughput Screening Strategy To Identify Optimal Buffer Conditions And Ligands Required For Maximizing YraM Protein Crystallization Success



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ABSTRACT

The protein thermal shift (PTS) assay is an attractive and efficient screening tool for measuring protein thermal stability, identifying suitable buffer conditions and measuring protein-ligand interactions. Protein-Ligand interactions study can be of high value for protein crystallography. PTS provides a high-throughput fluorescence-based approach for the crystallographers assessing a range of parameters impacting the thermal stability of their protein of interest. In this study, we demonstrate the advantage of this new screening tool utility for X-ray crystallographers looking for ligands to co-crystallize their protein of interest. The YraM Lipoprotein, encoded by the gene (HI1655), was identified as crucial for the growth and viability of Haemophilus influenzae (NTHi) but function is still unknown. There are no effective vaccines available for this strain and thus is a major target for drug design. Therefore, we have selected this candidate protein (YraM), buffer and ligand panel to evaluate the protein thermal shift assay as a fast and efficient technique for identifying the optimal conditions that would lead to successful crystallization and provide better understanding of the NTHi pathogen and better design of drug therapies to combat this pathogen.

MATERIALS AND METHODS

Protein and Reagents

Protein (YraM) and the ligand (Maleamic acid) were kindly provided by Dr. Mark Saper at University of Michigan.

Protein Thermal Shift Assay

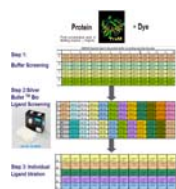
To monitor the PTS assay, PTS Dye was used. The dye does not fluoresce in aqueous solution but is highly fluorescent in non polar environments. When a protein starts to unfold or melt, the dye binds to exposed hydrophobic parts of the protein, resulting in a significant increase in fluorescence emission. The thermal shift assay was conducted in the StepOnePlus™ Real Time PCR System. The protein melt reaction mix was added to the wells of the 96-well PCR plate. The plate was heated from 25 to 99°C with a heating rate of 1°C/min. The fluorescence intensity was measured with Ex/Em: 490/530 nm.

Analysis of Thermal Shift data

We have developed a MATLAB based software, TmTool™ that can analyze fluorescent readings from the protein melt experiments performed using all Applied Biosystems Real-Time PCR Instruments. The software allows the user to calculate a melting temperature (T_m) from their melt curve data using the Boltzmann equation.

EXPERIMENTAL WORKFLOW

Figure 1. PTS Experimental workflow for identifying optimal conditions for YraM Protein Thermal Stability



RESULTS

Figure 2. Buffer Screen with YraM

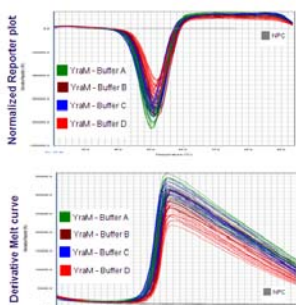


Figure 2. shows the Buffer screen PTS data for YraM from StepOnePlus™ showing the Normalized reporter and derivative melt profile of YraM protein in buffer A, B, C, and D. Data was collected at 1°C intervals from 25°C through 99°C.

Figure 3. Effect of Salt Concentration on YraM Thermal Stability

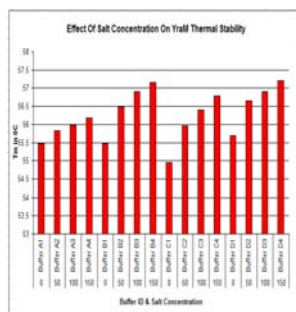


Figure 3. YraM protein showed increased thermal stability with increase in salt concentration. Different amount of salt concentration was titrated for each of the buffer condition tested against YraM. Among the buffers and the salt concentrations titrated the D4 buffer provided the highest T_m for the YraM protein.

Figure 4. Variation of T_m of YraM as a function of salt concentration

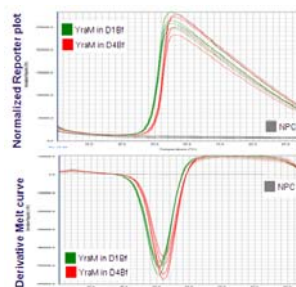


Figure 4. Shows Example of PTS data from StepOnePlus™ showing the Normalized reporter and derivative melt profile of YraM protein in buffer D1 and D4.

Figure 5. Screening against a Panel of Ligand Mixes



Figure 5. Shows Example of ANOVA Analysis of Positive Ligand Hit identification for YraM using commercially available Silver Bullet BIO™ Screening plates

Table1. List of Positive Ligand Hits for YraM

Number	Additive	Final concentration (mM)	ΔT_m in °C	P-Value
1	MgCl ₂	10	1	<.0001
2	CdCl ₂	1	1	<.0001
3	CoCl ₂	1	0.5	<.0001
4	Maleamic acid	10	1	<.0001

Figure 6. Individual ligand Titration (MgCl₂)

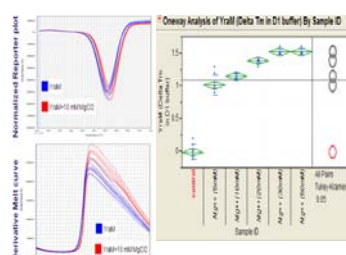
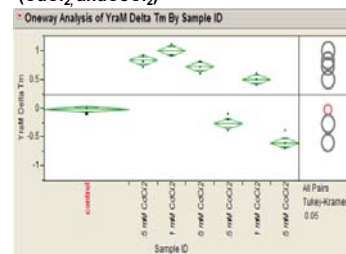


Figure 6a. shows the melt curve plots for YraM with and without ligand from StepOnePlus™ Real-Time PCR system. Blue curve represents YraM without ligand and Red curve represents YraM with 10 mM MgCl₂. YraM with 10mM MgCl₂ had shown a degree shift in T_m in comparison to the protein alone. Figure 6b. shows the ANOVA analysis plots for YraM ΔT_m analysis of control with respect to different MgCl₂ concentration titrated. The results indicated that with increase in MgCl₂ concentration improves the thermal stability of the YraM protein.

Figure 7. Individual ligand Titration (CdCl₂ and CoCl₂)



Figures 7. shows the ANOVA analysis plots for YraM ΔT_m analysis of control with respect to different concentration of CdCl₂ and CoCl₂ titrated. The results indicated that with increase in salt concentration improves the thermal stability of the YraM protein.

Figure 8. Individual ligand Titration (Maleamic acid)

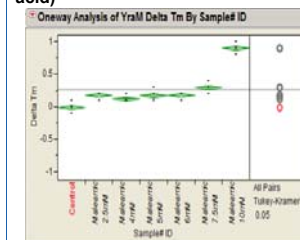


Figure 8. shows The ANOVA analysis of the concentration – dependent stabilization of YraM protein by Maleamic acid. The ΔT_m value was plotted against the compound concentration. The ΔT_m of the protein in buffer D4, was measured at Maleamic acid concentration between 2.5 and 10 mM. There are 12 replicates per condition tested. Compressed diamonds indicated high level of replicates reproducibility.

CONCLUSIONS

The PTS results from the buffer screen and salt titrations test against YraM suggest that identifying the most stabilizing buffer/ salt condition for the YraM protein can lower the tendency for aggregation and improve the success rate of crystallization. The PTS results from Ligand mix/ individual ligand screen is promising in identifying potential ligand candidates for YraM. This can increase the likelihood of YraM protein crystallization. This work demonstrates that the protein thermal shift assay is a widely applicable approach for the systematic screening and identification of buffer conditions and ligands which maximize protein thermal stability. All Applied Biosystems' Real-Time PCR instruments are compatible with the protein thermal shift testing workflow.

REFERENCES

- Saper et al. - Published 15 April 2008 in Wiley InterScience
- Ericsson et. al. - Published 15 October 2006, in Analytical Biochemistry
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