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thermoFAD

A thermofluor-based Flavin *Ad hoc* Detection system

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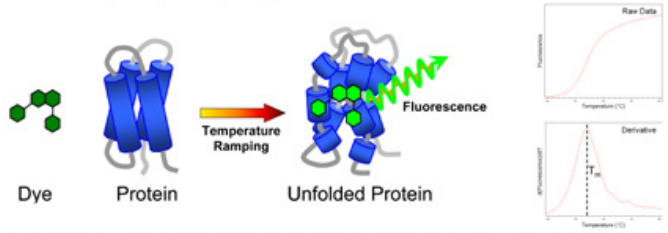
Basic Principles of thermoFAD

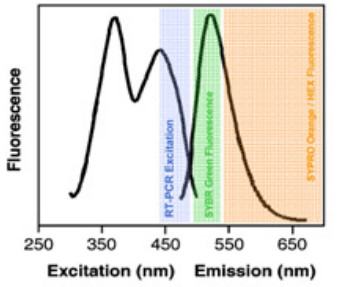
Screening for optimal purification and storage conditions is one the most important preliminary investigations in the biochemical characterization of a protein. Usually, this characterization requires time, and a large amount of lab work. *Thermofluor*© techniques (Pantoliano, 2001) can be of great help at this stage gathering a large amount of information by using small amounts of protein in a mid/high throughput approach (Ericsson, 2006; Mezzasalma, 2007). Here, we present a modified *Thermofluor*© approach that simplifies the screening in the case of flavoproteins.



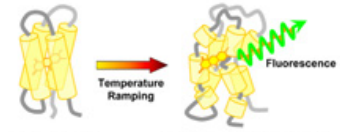
In a typical *Thermofluor*© experiment, the unfolding temperature of a protein is monitored through evaluation of the fluorescence of a dye such as SYPRO Orange in a Real-Time PCR instrument. The derivative of the sigmoidal thermogram obtained by *Thermofluor*© experiment allows direct evaluation of the melting temperature [T_m] of the protein under analysis. Using a medium/high throughput screening approach it is possible to evaluate the differences in T_m and thus to determine the conditions (pH, ionic strenght, additives, ligands, ...) that could improve protein purification and stability.

The excitation and emission ranges of FAD and FMN (Ghisla, 1974) are compatible with the common excitation and detection ranges used in RT-PCR.

As the fluorescence of flavin cofactors in flavoproteins is usually quenched by the protein environment (Munro, 1999), it is possible to measure the unfolding temperature of a flavoprotein using *Thermofluor*© by monitoring the increase in cofactor fluorescence.



This is thermoFAD



References:

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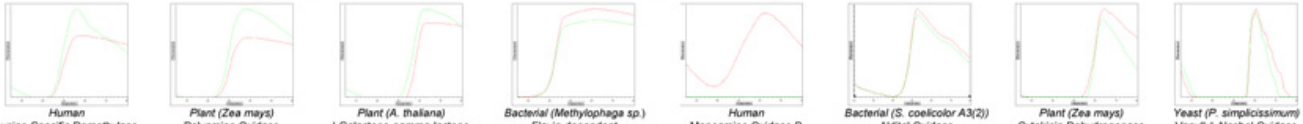
Ericsson, U.B., Hallberg, B.M., Detitta, G.T., Dekker, N., and Nordlund, P. (2006) *Anal Biochem* **357**, 289-298.
Mezzasalma, T.M., Kranz, J.K., Chan, W., Struble, G.T., Schalk-Hihi, C., Deckman, I.C., Springer, B.A., and Todd, M.J. (2007) *J Biomol Screen* **12**, 418-428.
Ghisla, S., Massey, V., Lhoste, J.M., and Mayhew, S.G. (1974) *Biochemistry* **13**, 589-597.
Munro, A.W., Noble, M.A. (1999) in: Methods in Molecular Biology (Chapman, S.K. and Reid, G.A., Eds.), Flavoprotein Protocols,vol. 131, pp 25-48, Humana Press Inc., Totowa, NJ, USA.

Targets

In order to validate our *thermo*FAD technique, we have chosen several flavoproteins under study in our lab. In particular, we have probed flavoproteins bearing non-covalent (mFMO, LSD1,...) and covalent flavin binding (AldO, MAO,...). The last ones are known for their characteristic fluorescence-quenching due to covalent flavinylation that, theoretically, can cause detection problems during thermal protein-unfolding. As a benchmark of the efficiency and sensitivity of our approach, we compared the results obtained with thermoFAD with conventional thermofluor measurements done using SYPRO Orange as fluorescent probe for denaturation. The results are in perfect agreement for the whole set of flavoproteins under analysis. (RED: *thermo*FAD, GREEN: *Thermofluor*© using SYPRO Orange as fluorescent dye).



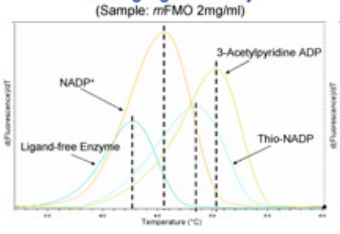
Human Lysine-Specific Demethylase complex (LSD1/CoREST)	*Plant (Zea mays)* Polyamine Oxidase (PAO)	*Plant (A. thaliana)* l-Galactono-gamma-lactone dehydrogenase (GALDH)	*Bacterial (Methylophaga sp.)* Flavin-dependent Monoxygenase (FMO)	*Human* Monoamine Oxidase B (MAOB)	*Bacterial (S. coelicolor A3(2))* Alditol Oxidase (AldO)	*Plant (Zea mays)* Cytokinin Dehydrogenase (CKX)	*Yeast (P. simplicissimum)* Vanyllyl-Alcohol Oxidase (VAO)
NON COVALENT FAD	NON COVALENT FAD	NON COVALENT FAD	NON COVALENT FAD	COVALENT (Cys) FAD	COVALENT (His) FAD	COVALENT (His) FAD	COVALENT (His) FAD

Applications

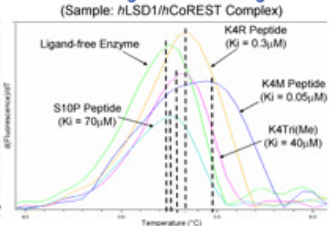
thermoFAD allows the fast and realiable evaluation of many purification and storage conditions for flavoproteins:

- **Buffers (pH, Ionic Strength)**
- **Detergents**
- **Ligands (substrates, inhibitors)**
- **Other Additives**

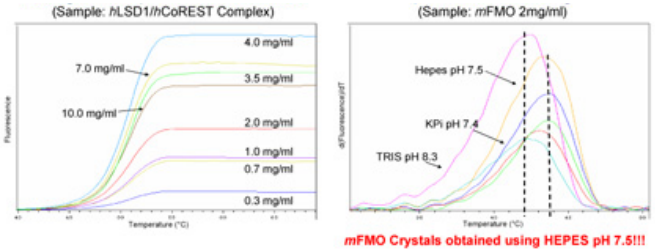
Testing Ligand Affinity
(Sample: *m*FMO 2mg/ml)



Probing Inhibitor Binding
(Sample: *h*LSD1/*h*CoREST Complex)



Testing various protein concentrations **pH / Buffer Screening**



*m*FMO Crystals obtained using HEPES pH 7.5!!!

The above examples show how it is possible to obtain preliminary results about ligand affinity to flavoproteins by using the *thermo*FAD approach. The measured T_m shifts, compared to the ligand-free enzymes, are in **good agreement with biochemical data measured with conventional affinity/inhibition assays**.

Experimental requirements:

Basic Real-time PCR instrument

Protein concentration: 0.5 ~ 2.0 mg/ml

Sample volume: 20µl

Sample purity: because of the specificity of the detection, *thermo*FAD works also on partially-purified proteins.