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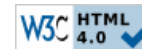
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thermoFAD

A thermoFluor-based Flavin Ad hoc Detection system

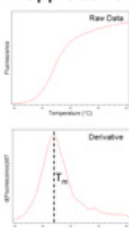
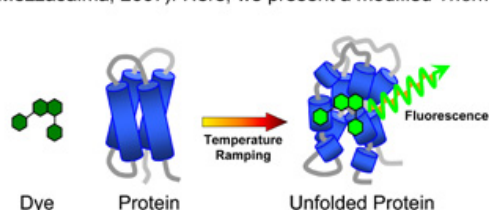
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Basic Principles of thermoFAD

Screening for optimal purification and storage conditions is one of 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.



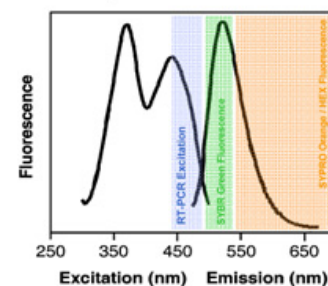
In a typical *ThermoFluor*® experiment, the unfolding temperature of a protein is monitored through evaluation of the fluorescence of a dye such as SYPRO Orange in a Real-Time PCR instrument. The derivative of the sigmoidal thermogram obtained by *ThermoFluor*® experiment allows direct evaluation of 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 strength, additives, ligands, ...) that could improve protein purification and stability.

References:

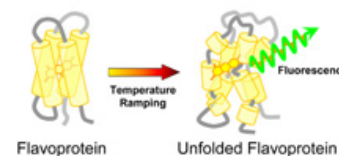
Pantoliano, M.W., Petrella, E.C., Kwasnoski, J.D., Lobanov, V.S., Myslik, J., Graf, E., Carver, T., Asef, E., Springer, B.A., Lane, P., and Salem, F.R. (2001) *J Biomol Screen* 6, 429-440.
 Ericsson, U.B., Hallberg, B.M., Dettla, G.T., Dekker, N., and Nordlund, P. (2006) *Anal Biochem* 357, 289-298.
 Mezzasalma, T.M., Kranz, J.K., Chan, W., Struble, G.T., Schalk-Hil, 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.

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.

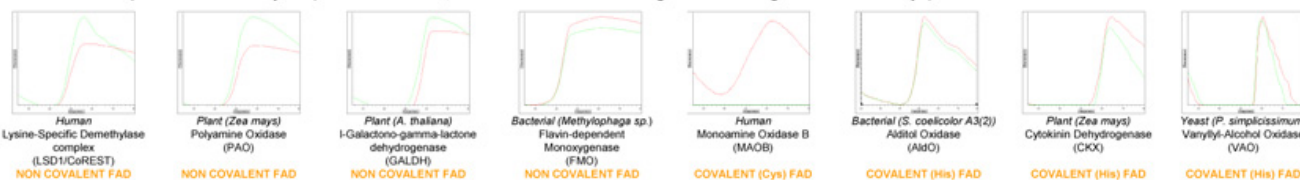


This is thermoFAD



Targets

In order to validate our *thermoFAD* 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: *thermoFAD*, GREEN: *ThermoFluor*® using SYPRO Orange as fluorescent dye).



Applications

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

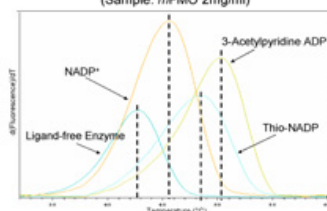
- Buffers (pH, Ionic Strength)
- Detergents
- Ligands (substrates, inhibitors)
- Other Additives

Testing various protein concentrations

pH / Buffer Screening

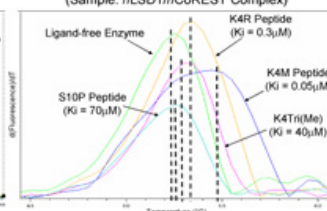
Testing Ligand Affinity

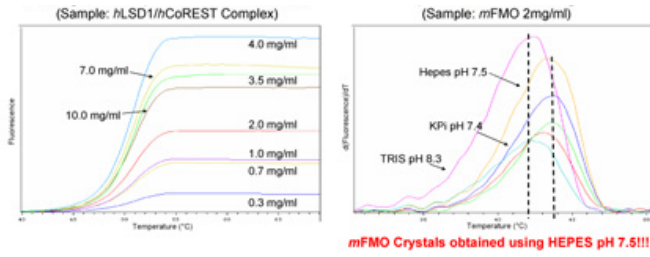
(Sample: mFMO 2mg/ml)



Probing Inhibitor Binding

(Sample: hLSD1/hCoREST Complex)





The above examples show how it is possible to obtain preliminary results about ligand affinity to flavoproteins by using the *thermoFAD* 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: 20 μ l

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