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DRUG DISCOVERY

Direct Binding Assays for Pharma Screening

Assay Tutorial:
ThermoFluor Miniaturized Direct-Binding Assay for HTS & Secondary Screening

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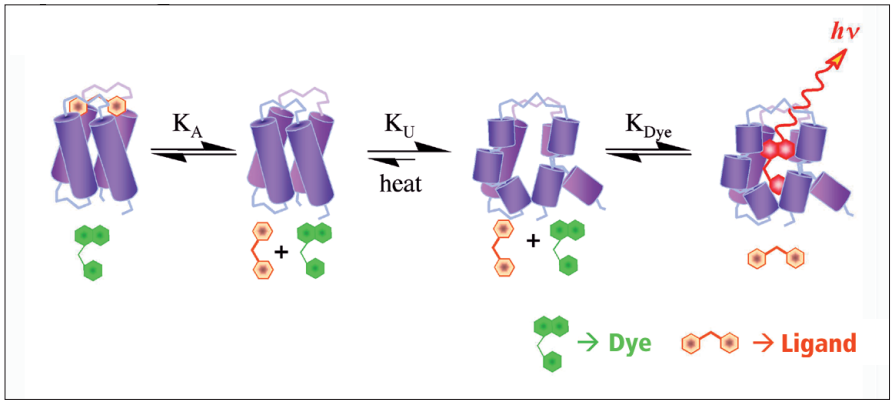


Figure 1. Equilibrium-binding ligand (K_A, orange) increases protein thermal stability (K_U). Fluorescent dyes (K_{Dye}, green to red) allow detection of protein integrity but occasionally decrease thermal stability. The protein free energy of unfolding ($\Delta G_{U(T)}$) follows a well-described thermodynamic function.[1]

Pharmaceutical companies have developed a variety of technologies for high-throughput screening (HTS) to test compounds for biological activity, including assays in whole organisms/tissues, cell assays, enzymological assays, and direct-binding assays. With the increasing size of screening decks, even screens with low hit rates produce large numbers of "screening positives."

Unfortunately, depending on the assay, these can represent numerous types of activities, including desirable mechanisms (reversible equilibrium-binding to the expected target) and less desirable mechanisms that may require unique secondary assays to deconvolute the mechanism *(Table)*. To address this, **3-Dimensional Pharmaceuticals** (Exton, PA), which was acquired by **Johnson & Johnson** (New Brunswick, NJ) last month, has developed ThermoFluor®, a miniaturized biophysical binding assay for use both in HTS and as a secondary screening technique.

ThermoFluor exploits a well-known biophysical phenomenon: small changes in the intrinsic melting temperature of proteins in the presence of ligands are related to the equilibrium-binding constant (K_A; *Figure 1*).[1,2] The temperature dependence of protein stability ($\Delta G_{U(T)}$) is a well-described, thermodynamic property. Compounds that interact preferentially with the native form of the protein will shift the equilibrium to the left, increasing the T_m, the temperature where half the protein is unfolded.

Mechanism of Screening Positives and Effect on Protein Thermal Stability

Molecular Functional Assay	Protein Thermal Stability
NONSPECIFIC EFFECTORS	
Highly colored compounds	Decrease $\Delta I_{U\text{-}F}$
Fluorescent compounds	Increase I_F, may decrease $\Delta I_{U\text{-}F}$
Adsorption of protein:	
To plastics	Decrease $\Delta I_{U\text{-}F}$, may lower T_m
To precipitated compounds	Decrease $\Delta I_{U\text{-}F}$, may lower T_m
Interaction with substrate/co-factor	Substrate/co-factors not typically present; Independently test effects
INHIBITION MECHANISM	
Reversible, noncovalent	Increase thermal stability
Covalent (±reversible)	Increase, or may decrease T_m Concentration-independent effect upon saturation·
Denature protein	Increase I_F, decrease $\Delta I_{U\text{-}F}$, lower T_m
ALLOSTERIC/ACCESSORY SITES	
May or may not give functional effect	All binders detected
	Test substrate/co-factor competition independently
	Discern binding sites through competition with known ligands

Compounds that have deleterious effects on protein stability will shift the equilibrium to the right, decreasing the T_m.

Protein stability as a function of temperature can be measured in numerous ways. Optical methods include absorbance, fluorescence, circular dichroism, and light scattering. ThermoFluor monitors changes in the fluorescent intensity of an environmentally sensitive dye that is highly quenched in aqueous environments but increases in fluorescence on binding to the hydrophobic core of nonnative proteins, thus giving a fluorescent readout of protein stability as the temperature is increased.

ThermoFluor utilizes a 384-well format and determines T_m with high statistical precision (±0.2°C, 4-µL reactions at ~0.05 mg/mL, ~200 ng/well).

Data Processing

Dye fluorescence as a function of temperature is fit to a 6-parameter function to obtain the T_m *(Figure 2A)*. In the presence of an equilibrium-binding ligand, T_m increases. The magnitude of the change in T_m (ΔT_m) is proportional to the free-ligand concentration, the affinity of the ligand, and the enthalpy of the protein unfolding. Since the enthalpy of a given protein is constant (and can be determined calorimetrically), a concentration-response curve for every ligand will have a similar shape, with a tighter-binding ligand giving a greater increase in T_m at a given concentration.

Concentration-response curves for three carbonic anhydrase inhibitors (CAII) all show the same change in T_m with change in inhibitor concentration *(Figure 2B)*. Excellent agreement is obtained between the ThermoFluor-determined K_A and values obtained by other direct-binding methods.

When K_A values are compared to enzyme assays, good agreement is obtained, with discrepancies usually explained by different mechanisms of action or assay conditions (e.g., ThermoFluor is typically run in the absence of substrates). Interestingly, there is no upper limit on the affinity that can be measured by ThermoFluor: tighter-binding ligands simply give a greater ΔT_m .

This powerful biophysical method is applicable not only for HTS, but also serves to triage screening hits and to rank affinities during lead-optimization steps.

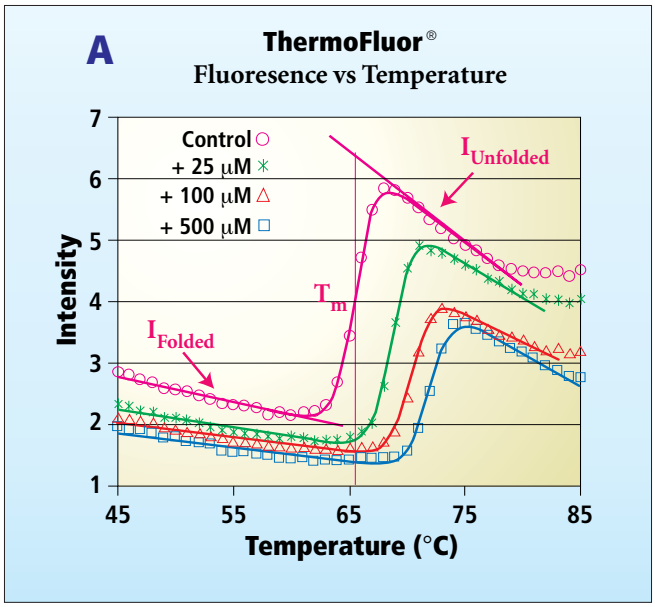
ThermoFluor Signature of "Screening Positives"

Biological screening of large compound decks using functional assays identifies compounds with numerous types of undesirable interferences. Common interferences, and their effect on thermal stability, are listed *(Table)*.

Compounds that absorb light at either the excitation or emission wavelength of the dyes will result in a quenching of the fluorescent signal. The T_m of a protein, however, is not dependent on the size ($\Delta I_{U\text{-}F}$) of the unfolding transition, thus these compounds rarely interfere. Fluorescent compounds often increase the initial fluorescence (I_F) or may cause a smaller change in fluorescence ($\Delta I_{U\text{-}F}$), but rarely have an effect on the protein's T_m.

Like other biophysical measurements, ThermoFluor uses higher protein concentration than do functional assays (0.1–2 µM protein), thus the loss of protein to plastics widely used in HTS operations is less apparent. If protein loss does occur, reliable screening data is still obtained with only a decrease in $\Delta I_{U\text{-}F}$ and occasionally a decrease in T_m (for multimeric proteins, the T_m is dependent on the protein concentration). Fortunately, commonly used biological excipients (e.g., reductants, glycerol, PEG, surfactants) rarely interfere with the assay.

It has recently been demonstrated



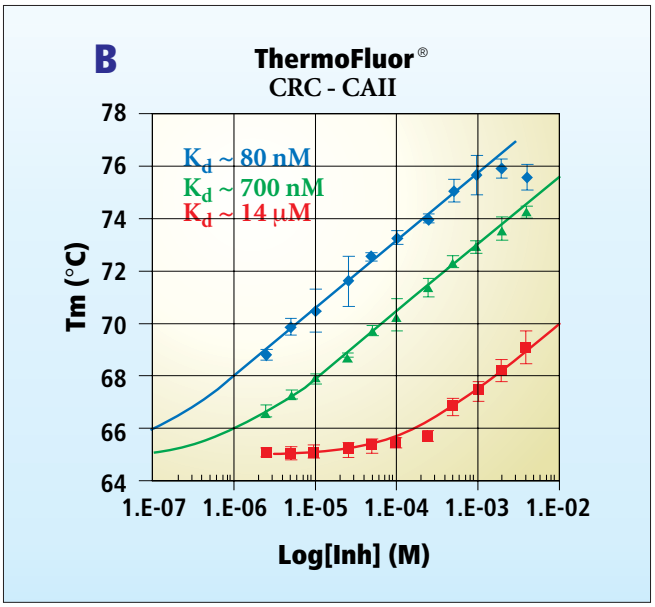


Figure 2. Binding constants from changes in protein stability. A) CAII (4 µL at 0.05 mg/mL) in ANS buffer + varying concentrations of methazolamide heated at 1°C/min. Fluorescence intensity monitored by CCD imaging using ThermoFluor; note quenching of ANS fluorescence at high methazolamide concentrations. B) Concentration response curves for CAII stabilization by dichlorophenamide (blue circles), methazolamide (green triangles), and sulfanilamide (red squares) gives the indicated dissociation constants.

that many promiscuous inhibitors found in pharmaceutical screening decks form small aggregates that may destabilize or inactivate proteins.[3] These promiscuous activities were minimal at high protein concentrations, where the molar amount of aggregate is substantially lower than the molar concentration of protein, i.e., exactly the conditions employed in ThermoFluor.

When explicitly tested, such compounds either decrease $\Delta I_{U\text{-}F}$, lower the T_m, or have no effect and are not identified by ThermoFluor as "screening positives." In addition, other compounds that interact with substrates or co-factors that could result in false-positives are never observed in ThermoFluor.

ThermoFluor and Inhibition Mechanisms

Concentration-response curves obtained from ThermoFluor not only allow ranking of inhibitors based on their affinity, but also allow an initial classification of inhibitory mechanisms and, thus, rapid triage of screening hits. Most drugs act by reversible, noncovalent equilibrium-binding mechanisms and give an increase in protein thermal stability.

Some (for example, aspirin) act by covalent binding mechanisms and are irreversible. The effect of these compounds on thermal stability varies, depending on the relative free energy of the covalently modified protein. When the covalent modification increases thermal stability, the concentration-response curve shows saturation on adding one-molar equivalent of ligand.

Other compounds may appear as screening positives in functional assays due to detrimental effects on protein stability (e.g., urea would be a "screening positive" in many assays). Compounds that act as protein denaturants usually decrease thermal stability in ThermoFluor.

An example of how ThermoFluor can be used to distinguish inhibitory mechanisms of two Protein Tyrosine Phosphatase 1B (PTP1B) inhibitors is demonstrated in *Figure 3*. An oxamic acid containing inhibitor[4] increases the T_m, as expected for an equilibrium binding ligand, whereas a second compound[5] decreases thermal stability. The effect of the latter compound is consistent with inhibition due to protein destabilization.

Stoichiometry

A final observation that we are just beginning to appreciate is the stoichiometric relationship between ligands and thermal stability. The equation detailed in *Figure 2* predicts an increase in T_m with increasing concentration of free ligand,

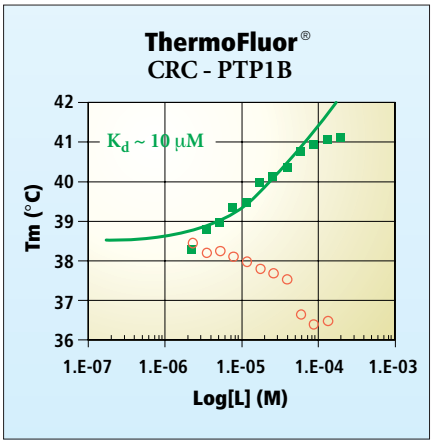


Figure 3. Concentration-response curves for two PTP1B inhibitors. A compound with an oxamic acid (green squares) inhibits as an equilibrium-binding ligand. An arylbenzo naptholfuran5 (red circles) decreases protein stability.

yet most assays are designed based on knowledge of only the total ligand concentration. When examining tight-binding ligands, the free concentration may be appreciably lower than the total concentration, introducing sigmoidicity into the concentration-response curve.

By simultaneously varying the protein and ligand concentration in the assay, the binding stoichiometry can be determined. The technique has recently been used to identify an active minor component from a well, and to quantitate compound loss due to interaction with plastics used in HTS.

Conclusion

The best screening hits and lead compounds show activity regardless of the assay employed; thus compounds with overlapping activity in binding, functional, and cell-based assays are given the highest priority.

For companies specializing in structure-based drug design, however, a simple, direct-binding assay has an additional advantage when probing the subtleties of compound binding (e.g., pH and detergent effects, cooperativity between binding sites) to elucidate mechanistic issues. ThermoFluor technology has thus become a central tool for drug discovery efforts at 3-Dimensional Pharmaceuticals. GEN

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References

1. J.A. Shellman.The Thermodynamic Stability of Proteins. *Ann. Rev. Biophys. Chem.* 16, 115-137 (1987).

2. M. W. Pantoliano, E. C. Petrella, J. D. Kwasnoski, V. S. Lobanov, J. Myslik, E. Graf, T. Carver, E. Asel, B. A. Springer, P. Lane, and F. R. Salemme. "High-density miniaturized thermalshift assays as a general strategy for drug discovery." *J Biomol. Screen* 6:429-40 (2001).

3. S. L. McGovern, E. Caselli, N. Grigorieff, & B.K. Shoichet. "A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening." *J. Med. Chem.* 11:1712-22 (2002).

4. H. S. Andersen, L. F. Iversen, C. B. Jeppensen, S. Branner, K. Norris, H. B. Rasmussen, K. B. Møller, & N. P. H. Møller. "2-(oxalylamino)-benzoic acid is a general, competitive inhibitor of protein-tyrosine phosphatases." *J. Biol. Chem.* 275:7101-7108.

5. J. Wrobel, J. Sredy, C. Moxham, A. Dietrich, Z. Li, D. R. Sawicki, L. Seestaller, L. Wu, A. Katz, D. Sullivan, C. Tio, & Z-Y Zhang. PTP1B Inhibition and antihyperglycemic activity in the ob/ob mouse model of novel 11-Arylbenzo[b] naptho[2,3-d]furans and 11-Arylbenzo[b]naptho[2,3-d]thiophenes. *J. Med. Chem.* 42:3199-3202 (1999).