

Determination of Pseudokinase-ligand Interaction by a Fluorescence-based Thermal Shift Assay

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[Abstract] This protocol describes a robust technique for the measurement of pseudokinase-ligand interaction by a fluorescence-based Thermal Shift Assay (TSA). Pseudokinases are kinase-like proteins that have recently emerged as crucial regulators of signal transduction and may therefore represent a novel class of drug targets. Unlike kinases, the catalytic efficiency of pseudokinases is rather poor or non-existent, making it difficult to dissect the function of their nucleotide binding sites. Thermal denaturation-based methods have proven to be a powerful method for determining ligand binding capacity to purified pseudokinases and can inform on the potential drugability of the nucleotide binding site. This assay takes advantage of a change in fluorescence arising when a fluorescence dye, in this instance SYPRO[®] Orange, binds to hydrophobic patches that become exposed when a protein undergoes thermal denaturation. Ligand binding to a protein is known to increase its thermal stability which is reflected by a shift observed in the thermal denaturation curve between the unliganded protein and the liganded protein. This generalized protocol can also be tailored to other protein families. In addition, thermal denaturation-based methods can be used to identify optimal buffer conditions that may increase protein stability.

Materials and Reagents

1. Purified protein (stock solution preferably at a concentration above 20 μM) (Murphy *et al.*, 2013)
2. 1 M Dithiothreitol (DTT) stock (Astral Scientific, catalog number: C-1029)
3. DMSO (high grade) (Sigma-Aldrich, catalog number: D-1435)
4. MilliQ water
5. Nucleotide solutions (10 mM stock prepared in 20 mM Tris) (pH 8)
 - a. ATP (Sigma-Aldrich, catalog number: A2383)
 - b. ADP (Sigma-Aldrich, catalog number: A2754)
 - c. AMP-PNP (Sigma-Aldrich, catalog number: A2647)
 - d. GTP (Sigma-Aldrich, catalog number: G8877)
6. Divalent cations salt solutions (50 mM stock in MilliQ water)
 - a. MnCl_2 (Sigma-Aldrich, catalog number: 203734)
 - b. MgCl_2 (Sigma-Aldrich, catalog number: M8266)
7. Kinase inhibitor solutions (2 mM stock in 100% DMSO) (such as the pan-kinase inhibitors, Staurosporine, Sigma-Aldrich, catalog number: S5921)
8. Thermal Shift Assay buffer (see Recipes)
9. SYPRO[®] Orange (Sigma-Aldrich, catalog number: S5692) (see Recipes)

Equipment

1. RT-PCR tubes GST-RG01 (Gene Targets Solutions)
2. 1.5 ml microfuge tube
3. Qiagen/Corbett Rotor-Gene[®] 3000 RT-PCR machine (QIAGEN) (Murphy *et al.*, 2013)

Software

1. Microsoft Excel or Prism

Procedure

1. Thermal Shift Assay (TSA) test run for the determination of optimal amount of protein
 - a. Prepare a dilution series of your protein sample in buffer ranging from 1 to 10 μM final concentration in a total volume of 25 μl . Add 1 μl of 1x SYPRO[®] Orange to each tube.

- b. Perform a thermal cycler run using the parameters as described in step 3. Analyze the melt-curve (see step 4) and determine the optimal amount of protein that gives at least 30 fluorescence unit. Reduce amount of protein if the fluorescence signal is saturated.

Note: Typical final protein concentration is around 5 μ M. The Rotor-Gene® 3000 is equipped with a gain optimization function that can be adjusted for optimal signal (refer to manufacturer's manual).

2. Assay

- a. Start by defining the number of conditions that you need to test, including appropriate controls such as protein sample in buffer only if testing nucleotide binding, or in presence of DMSO if testing compounds. This will determine the total volume of master mix of protein needed to conduct the entire experiment. Each reaction is conducted in a total volume of 25 μ l.
- b. Prepare on ice just prior use a master mix containing your protein in the Thermal Shift Assay buffer using the concentration of protein that gave the peak fluorescence (without signal saturation) in step 1.
- c. Dispense 23 μ l of protein/buffer, add 1 μ l of ligand (10 to 200 μ M) or 1 μ l of DMSO/buffer for the control experiment and 1 μ l of diluted SYPRO® Orange (1:100). Prepare tubes in duplicate. If the protein to test is highly unstable at room temperature, it is recommended to prepare the tubes on ice. Nucleotide solutions are typically used at a final concentration of 200 μ M final, divalent cations at 1 mM final and kinase inhibitors at 40 μ M final.

3. Thermal cycler program

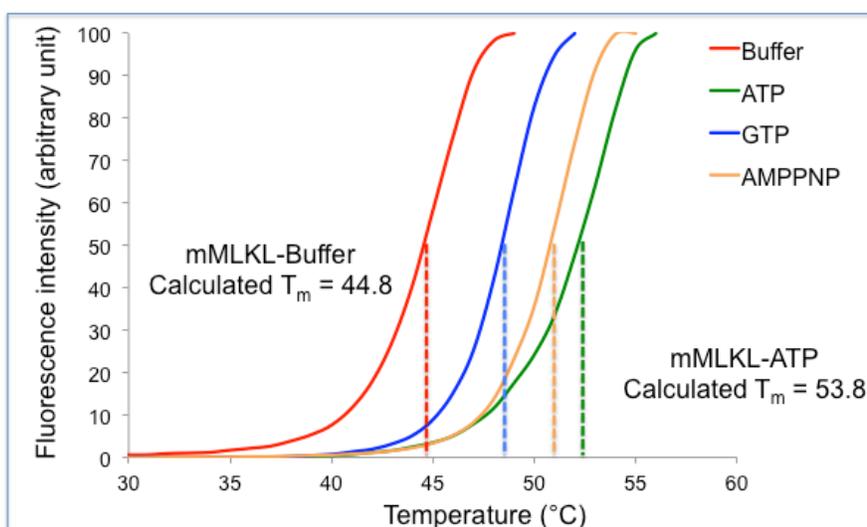
- a. Fluorescence-based Thermal Shift Assay can be performed using instruments that combine both sample temperature control and dye fluorescence detection. In this instance, we used the Qiagen/Corbett Rotor-Gene® 3000 RT-PCR machine.
- b. Start at 25 °C, hold at 25 °C for 2 min, increase temperature of 1 °C/min for 65 cycles (25 °C to 90 °C) reading fluorescence intensity every °C. Return to 25 °C. Excitation is at 470 nm (green channel) and emission is at 555 nm (yellow channel).

4. Data analysis

- a. Save data as fluorescence intensity vs temperature. Export file in a format suitable for import into Microsoft Excel or Prism for analyses.
- b. The highest fluorescence density is used as a cutoff for the data, therefore remove all data after the high fluorescence density peak. Fit fluorescence intensity curve to a Boltzmann sigmoidal curve using Prism.

- c. Obtain the melting temperature (T_m) of the protein in buffer/DMSO, which correspond to the midpoint for the protein unfolding curve. Similarly, obtain the T_m of the protein when ligand is added.
- d. Calculate $\Delta T_m = T_m \text{ ligand} - T_m \text{ buffer/DMSO}$. A positive ΔT_m indicates that the ligand stabilizes the protein from denaturation, hence binds to the protein. A value \geq to 2-3 $^{\circ}\text{C}$ is an indicator of ligand binding.
- e. Below is an example of a typical TSA experiment.

Note: If PRISM software is not available, non-linear regression analysis of experimental data can be conducted using a Microsoft Excel spreadsheet (Brown, 2001).



	Buffer	ATP	AMPPNP	GTP
T_m	44.8	53.8	50.1	48.6
ΔT_m	-	9	6.2	3.8

Figure 1. Practical example of a TSA experiment. (Top) Thermal Shift Assay data were obtained for mouse MLKL in the presence and absence of nucleotides. The midpoint for the unfolding transition in the absence of ligand was observed to be 44.8 $^{\circ}\text{C}$ while the T_m shifted to 53.8 $^{\circ}\text{C}$ in presence of ATP, inducing a shift of 9 $^{\circ}\text{C}$. (Bottom) Table summarizing the T_m and T_m values obtained for mMLKL in presence of ATP, GTP and AMPPNP.

Recipes

1. Thermal Shift Assay buffer

The composition of the buffer used to dilute the protein can be optimised according to each protein. The following recipe was used to study pseudokinases.

20 mM Tris (pH 8)

150 mM NaCl

1 mM DTT

2. SYPRO® Orange

1 to 100 dilution in 100% high grade DMSO

Notes

1. While the thermal denaturation of a single domain proteins mostly results in a single transition phase, multidomain proteins may exhibit distinct transition states. Nevertheless if a ligand binds tightly to one domain, it could result in a change in the thermal denaturation profile, providing a tractable method for assaying ligand binding. There are also cases in which a ligand may bind tightly but does not induce a change in the T_m .
2. The protocol described has been adapted to the Qiagen/Corbett Rotor-Gene® 3000 RT-PCR machine. The amount of protein required per experiment and the SYPRO® Orange dilution may need to be adapted depending on the RT-PCR machine used.

Acknowledgments

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