Axxam Boot Camp Webinar

Q&A

Thermal Shift Assays meet HTS to discover and characterize novel protein binders

What is the minimal working concentration of the target protein you can proficiently use in TSA?

As a general rule, a good protein concentration is in the one-digit micromolar range. It is essential, however, to assess protein and dye (e.g., SYPRO® Orange) concentration for producing the optimal signal with the lowest protein consumption.
In our experience, the lowest protein concentration which proficiently generated a TSA signal was 0.5 µM.

Is the fluorescent signal generated by SYPRO® Orange dye dependent on experimental conditions such as pH, salt, etc.?

pH can modify the environment of the dye, changing its spectroscopic proprieties and thus altering its performance. This is especially true for a very acidic/basic pH. Salts can exacerbate their effect more on the protein behaviour, deviating its melting temperature. Detergents are in general not tolerated by SYPRO® Orange owing to their intrinsic hydrophobicity.
Protein-based blocking agents, such as BSA, are also not compatible with TSA, being proteins themselves with their own Tm.
Other additives can be used, although they must first be validated.

What are the normal variations you see in your Thermal Shift Assay (TSA) measurements? For example, if you have a compound that gives rise to a 1 degree increase in Tm, would you be able to see this?

The TSA technology and the QuantoStudio™12 instrument we use are quite precise. Usually, we consider +0.5 °C as a reliable shift in the melting temperature, which would anyway need to be confirmed with independent experiments.

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What is the temperature precision of the QuantStudio™ machine you are using? Could you use a qPCR machine instead?

In our experiments, the temperature ramp of our QuantStudio™ RT-qPCR instrument is usually set to + 0.5°C per cycle. Other qPCR instruments could be potentially used if they match the excitation/emission wavelengths of SYPRO® Orange, or those of other dyes.

Why do you add protein and SYPRO® Orange dye separately into each well? Why not start with a Protein+SYPRO® solution?

Although addition of the protein and SYPRO® solution together is possible, we prefer to first give the compound a chance to settle with the target protein, and then add the SYPRO® Orange dye, or other dye. Thus, we are sure that SYPRO® Orange, or any other dye used, does not interfere/compete with the binding site of compounds to the target protein.

Are the pre-plated plates for buffer/pH conditions optimization commercially available?

The pre-plated plates are not commercially available, and we have developed and prepared them in house. There are commercially available plates for crystallization purposes, but they contain components and reagents that do not match the same purpose of assay development and TSA. We have evolved the pre-plated plates internally after several years of trial and error, and thanks to the expertise and experience of our scientists. Furthermore, we also have recently assembled dedicated components for the optimization of TSA, which are only partially overlapping with reagents routinely applied for enzymatic or protein interaction assays for HTS. Indeed, we routinely add, for example, non-ionic and/or zwitterionic detergents, and other additives to the biochemical assays, which are not otherwise suitable for TSA. Thus, the optimization plates are tailored to the purpose, rather than being for general use.

Does the Axxam team routinely scan their buffer plate to assess the optimal SYPRO® Orange TSA profile for a given protein? Or is that a special service that is done only when the standard neutral pH SYPRO® Orange TSA protocol does not show any good Tm behaviour?

Assessing buffer pH for optimizing the SYPRO® Orange profile is not always mandatory; usually, we offer this as an optional phase. However, it was necessary, for example, for the protease that we mentioned earlier in the webinar, otherwise the project would have stalled. Instead, thanks to the pH screen, we were able to move on with the project.
In general, we make use of buffer optimization when the assay development results are sub-optimal under standard buffer conditions and need to be improved.

On the other side, it is preferable to use these platform routines when there is a specific request from our clients to characterize the target protein in detail. If a nice signal peak is observed immediately then we can proceed to testing the compounds. However, if a borderline trace signal appears with the protein and/or with the protein + a reference or a test compound, then it is best to screen the optimization plates with the aim of identification of the proper experimental condition to observe a measurable Tm and/or ΔTm shift induced by a compound.

Could TSA be extrapolated and useful for other proteins other than CDK?

In principle, TSA can be performed using all soluble proteins with a molecular weight higher than 15 kDa. A success rate of about 70% can be confidently predicted under standard conditions. For all the remaining "reluctant" target proteins, the scouting of alternative experimental conditions, including the optimization plates and our dye library, may enable a TSA analysis.

TSA is usually successful on a subset of about 70% of tested proteins. In your opinion, why are the remaining 30% resistant to be characterized by TSA?

Unfortunately, there is no clear explanation as to why 30% of the proteins are resistant to TSA, when SYPRO® Orange dye is employed. Proteins with a molecular weight < 15 kDa usually produce a signal that is too low to be detected by TSA. Furthermore, it is possible that SYPRO® Orange could be incompatible with some proteins for unknown reasons.

Can TSA be used for membrane proteins/protein complexes? If so, is SYPRO® Orange a problem and would one of the alternatives in your dye library be more suitable?

Unfortunately, TSA with SYPRO® Orange is not suitable for membrane proteins because these have naturally exposed hydrophobic regions/patches in general buried in the phospholipid bilayer, which allow the immediate binding of SYPRO® Orange, therefore giving rise to a very high fluorescence signal and preventing the occurrence of any sigmoidal fluorescence curve upon temperature increase. Moreover, purified membrane proteins are kept in solution with detergents, which also naturally have hydrophobic regions incompatible with SYPRO® Orange. It should also be noted that Alexandrov et al (2008) showed that it is possible to use TSA to characterize a membrane protein using CPM as the dye based on detection of cysteines, however, CPM excitation/emission wavelengths are not compatible with our QuantStudio™ RT-qPCR instrument.
A thorough characterization of the dye library is ongoing, and we cannot exclude the

possibility that a subset of dyes may show a more suitable physicochemical or spectroscopic profile to characterize membrane proteins.

Are there any restrictions on the size of the proteins that can be measured in TSA? Related to this question: can macromolecular complexes be measured using TSA?

Yes, in theory there is a restriction on the size of the proteins – a conventional limit of 15 kDa. Therefore, with any protein of a size lower than 15 kDa, it is hard to get a good TSA signal. This is because the SYPRO® Orange dye can bind to the internal hydrophobic regions of structured proteins. If a protein is too small then its number of buried hydrophobic regions is also small, and so the signal is also considerably low, if any. By contrast, if you have a large protein then it is necessary to tailor the quantity of protein as the SYPRO® Orange dye is able to bind multiple hydrophobic sites, thus potentially elevating the TSA signal.

Regarding the macromolecular complexes, yes, it is possible, in principle, to measure them using TSA. However, one must be careful with this because "macromolecular" means more than one protein, with each protein yielding an independent trace TSA signal. When there are numerous proteins in the macromolecular complex, the trace signals they generate are quite complex to be analyzed, and deconvolution would therefore pose potential problems. As we showed during the webinar, we were lucky with Transferrin as its two domains have two different melting temperatures. However, if you have two proteins with similar melting temperatures, then they show overlapping melting-temperature curves, thus making it quite difficult to analyze the results.

Have you managed to enable a TSA assay for Protein 3 using your new dye library?

We have not yet established a TSA assay for Protein 3. We are currently validating our dye library by using "standard" proteins yielding trackable TSA denaturation curves. The next step will be to assess the dye library using "TSA-reluctant" proteins, such as Protein 3 and others.

Is it possible to differentiate different members of an enzyme/protein family like the CDK or cyclophilin family with TSA?

We focused our studies only on one member of the CDK family. However, in theory, there are no foreseeable problems if you select other members of the CDK family. If you have a selective compound, then TSA is most likely to be the right technology to identify a difference between orthologous proteins, especially as it is expected that

different CDKs will have different melting temperatures.
Additionally, a compound that binds with different affinities to CDK members is expected to show varied ΔTm measurements.
Therefore, the technology certainly backs this hypothesis, but experimental data need to be generated to support the hypothesis.

What are the QC criteria that should be applied in protein production and formulation to have a protein target eligible for TSA analysis?

As a general rule, the target protein should:

- Have purity > 90%; Contaminating proteins could produce TSA traces that overlap the target one.
- Be dissolved in a buffer without detergents (or at least used below their CMC) or protein-based blocking agents such as BSA, which could impair the correct trace determination.
- Have a molecular weight > 15 kDa; otherwise, it could be tricky to obtain good TSA traces.

Is the binding mechanism of the developed dyes known? / Do all new dyes bind preferentially to unfolded protein?

The dye library has been developed based on the binding mechanism of SYPRO® Orange, thus, the new dyes are expected to bind and increase their fluorescence upon binding to hydrophobic patches of unfolded proteins. Thorough characterization of the new dyes is ongoing, and we cannot exclude the possibility that a subset of dyes may show different binding mechanisms.

Do your alternative dyes work in the same way as SYPRO® Orange, in the sense that they are quenched in aqueous environment and fluorescent when they bind to exposed hydrophobic patches of protein?

One criterion imposed by our medicinal chemistry colleagues for the selection of new dyes was their predictable ability to produce a low signal when in aqueous solution and, conversely, a higher signal when bound to hydrophobic patches. However, thorough characterization of the dye library is still ongoing, and we cannot exclude the possibility that a subset of dyes may show different binding mechanisms.

Would the TSA approach also work without adding a fluorescent dye by using intrinsic protein tryptophan fluorescence, which changes upon unfolding?

Although this is possible in principle, one can only conduct such experiment with a suitable instrument (the RT-qPCR in our laboratory is not suitable). With the correct instrumentation, it is not only possible but also preferable as you do not need a dye to

label your unfolded protein. Therefore, you would only use the protein plus a compound. However, sensitivity of the detection system based on tryptophan may be possibly lower, and limitations may apply when proteins do not contain tryptophan residues and/or tryptophan fluorescence upon melting is not modulated by the binding of compounds under investigations.

Why did you assemble a new dye library as SYPRO® Orange works so nicely and is reputed as the gold-standard for the technology?

Literature in the field reports that only 70% of soluble proteins work with the SYPRO® Orange dye. And we have gained corroborating evidence during our studies about this assumption. In order to increase the number of tractable proteins, we assembled a proprietary dye library. Thus, if the target protein is not TSA-detectable when using SYPRO® Orange, we have the possibility to screen our dye library with the aim of obtaining useful data using a different dye.

If your compound binds to hydrophobic patches, it might compete with the SYPRO® Orange dye. How do you handle that situation?

SYPRO® Orange binds non-specifically to hydrophobic patches, which are usually buried and inaccessible in soluble folded proteins. During a TSA experiment, the target protein unfolds and its hydrophobic patches are exposed, thus making them accessible to SYPRO® Orange for binding.

Therefore, it is unlikely that SYPRO® Orange and the compounds under investigation may compete for the same site, as compounds usually bind to pockets that are solvent-accessible.

In the rare instances of competition between SYPRO® Orange and the compounds, we might not exclude a false negative outcome.

Can you comment on the 2-order of magnitude differences observed between measured Kd values in TSA and Ki values with functional assay?

TSA-determined Kd values may not reflect actual affinities between compounds and target protein when the affinity is much lower than the working concentration of the target enzyme. This evidence implies that the Kd values and compound affinity may be affected by experimental conditions in some cases.

Additionally, it is important to underline that the TSA experiment was performed using only the CDK enzyme in this case, while the kinase assay was performed using the CDK–cyclin complex; thus, the latter represents the more accurate physiological

behaviour of the catalytically-competent holoenzyme. Moreover, structure of CDK2 is modified by binding to cyclin, thus resulting in modified affinities of test compounds between CDK-only and CDK-cyclin complex.

Is the intensity of the absolute fluorescent value relevant for the analysis by TSA and for Kd determination?

The higher the intensity of the fluorescence signal of the TSA trace, the easier it is to calculate the melting temperature (with software). In other words, the signal is strong enough for the correct Tm determination above a certain threshold by using both the mathematical methods – the midpoint and the derivative.

How much is the Kd dependent on the concentration of enzymes or buffers used? Is it comparable to physiological conditions?

In our presentation, we showed that TSA-determined Kd values may not reflect actual affinities between the compounds and the target protein, when the affinity is much lower than the working concentration of the target enzyme. This evidence implies that Kd values may be affected by experimental conditions in some cases.
We have no evidence of shifted Kd values due to variable pH or buffer conditions. However, it is quite reasonable to think that compound affinity may be influenced by experimental conditions. At the same time, detailed knowledge of the physiological conditions of the target protein is, in most cases, inadequate, and therefore, assessing different buffer conditions is crucial to enable the analysis of a target protein by TSA.

Does ΔTm at a single concentration also correlate with Kd, and by extension Ki from the HTRF assay?

It is very hard to extrapolate reliable affinity and inhibition parameters by using a single compound concentration. Based on our experience, testing different compounds at one fixed concentration against the same target protein may help us confidently rank compounds according to their relative affinity and potency, and in the case of enzymes, based on the corresponding Delta Tm shift.

How do you determine specific heat capacity (Cp) of your protein when using Boltzmann fit?

The Genedata software algorithm automatically estimates the initial values of key thermodynamic variables, including ΔHu and ΔCp, and sets them close to the optimal ones. Thermodynamic values can also be fixed if they are known through different technologies, such as Isothermal Titration Calorimetry, (ITC).

Can you explain negative shifts? And any correlation between consistent negative shifts and binding?

Negative shift may account for compounds being able to misfold the target proteins, making them more susceptible to thermal denaturation. Potential issues related to negative-shifting compounds are not related to their questionable binding with the target protein, instead, it is essential to understand whether the unfolding mechanism is target-specific. In the latter case, it is important to validate whether the compounds promoting a negative shift are non-specific aggregators or protein denaturants.

Would you consider compounds inducing negative shifts if they have shown inhibition of the enzymatic activity?

Compounds inhibiting enzymatic activity and inducing negative shifts in TSA can certainly be considered. However, it would be quite difficult to understand why they induce a decreasing melting temperature, and evidence would be needed to support it. Moreover, we must presume that the binding of such compounds trigger destabilization of the protein. Therefore, this also may occur allosterically, and not really on the orthosteric site or the active site, which is an interesting mechanism that should be further investigated. Additionally, there needs to be evidence to support this, because, for example, it would be of concern to further develop a "denaturing" agent as a pharmaceutically relevant drug. It should be demonstrated that the compound is not a generic destabilization agent of unrelated proteins, but that its mechanism is target-specific.

Did you find CDK binders that stabilize but do not inhibit functional activity? Or did you screen using only the kinase domain?

Regarding the first part of the question: We only assessed a limited number of compounds and all of these except one (which we showed during the webinar) were found to be binders as well as inhibitors.

Regarding the second part of the question: we used the full-length protein and not just the kinase domain.

What is the value in creating a correlation plot between midpoint values and derivative values to analyze a HTS campaign with TSA?

Thanks to the correlation plot, we can immediately identify outlier compounds because they deviate from the bisector. Such an outlier compound is that whose melting temperature, calculated using the midpoint method, is not equal or close to its melting temperature calculated using the first derivative method.

This compound can be flagged and analyzed separately.
Moreover, the upper-right corner of the correlation plot shows putative binders of the target protein because they increase the Tm of the latter as compared with the neutral reference.

Hits identified from the HTS campaign on peptidase displayed marked a different Hill slope when tested on activity assay. Can you comment on that?

This is true especially when we analyze the destabilizer compound. We cannot exclude the possibility that such a compound is a promiscuous inhibitor or an aggregator.
These are recent data, and more experiments are needed to understand the mechanism of action underlying such destabilizer compounds.

For the aspartic protease TSA screen, it appears that the destabilizer compound may be a so-called promiscuous inhibitor.
Did the Axxam team try to add detergent to see if there was any effect on eliminating The steep Hill slope inhibition by the destabilizer?

This is a very good question! We have not yet tried using detergent because the priority of the project was to study the stabilizers rather than the destabilizers.
However, addition of detergent is planned in the future.
We also intend to characterize more deeply these destabilizers. We should highlight that the addition of a detergent should be done with great care as TSA technology is sensitive to hydrophobicity, by definition, because of the SYPRO® Orange dye, which implies that different detergents need to be scouted. In particular, we will definitely need to stay well below the critical micelle concentration (CMC) otherwise the background signal will increase excessively.
On the contrary, addition of detergents in the activity test is feasible.
However, alteration of the buffer condition between TSA and enzymatic assay may possibly affect the correlation of Kd value and Ki value, respectively. In general, there is sufficient evidence in literature to support that in some cases it is possible to add detergents and to further characterize a compound that may show such behaviour.

When you screen for stabilizers, is it better to screen in the conditions where protein is the most stable or you will pick stabilizers more efficiently in (slightly) sub-optimal buffer conditions?

In general, it is advisable to screen the target protein in the most stable conditions for TSA, even if in some cases these conditions may be different from the ones used in an

activity assay. Indeed, TSA under the most stable conditions for the target is expected to elicit the highest ΔTm shifts. However, exceptions may apply, and the slightly Sub-optimal buffer condition may be the preferred choice, for example, where you are dealing with “undruggable” proteins and structural proteins known to have scarce anchoring sites for binders.

Do you have experience in running TSA on RNA?

We know from literature that TSA on RNA is feasible with the right dye(s). We have recently gained evidence that characterization of binding of compounds to RNA Which induce an increase in ΔTm of RNA itself is feasible for a number of structured RNA molecules and compounds.