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CETSA®: Explore your Chemistry and Navigate the Biology to Enhance your Drug Discovery Project

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Chapter 1: The need to discover new medicines

One of society's most pressing challenges today is to find new drugs that treat life-threatening and incurable diseases. However, despite increasing investment in research and development, the majority of candidates still fail in clinical trials. It's thought that over half of these failures are due to a lack of efficacy in proof-of-concept trials – a subject of much debate in the industry.

These efficacy failures come at a considerablely high cost. Not only are substantial investments made for no gain, but a significant amount of lost time may also mean that pursuing that target is no longer a practical proposition. This is particularly detrimental for smaller companies that have invested all their resources in only one asset, and may mean they have to close down as a result. Another possible impact is on pharmaceutical companies that need to release new drugs to maintain their existing market share. In such situations, the company must either buy in a late-stage candidate drug (CD) from elsewhere, at great cost, or give up on a market in which they may have spent years developing.

Ultimately, the high rate of efficacy failures means that the resources put into failed compounds could have been invested in others that had a better chance of becoming new, life-changing therapies. This makes finding ways to minimize late-stage failures more vital than ever before. We therefore need to be able to better predict which compounds will fail much earlier in the drug discovery pipeline so that only the very best ones are prioritized from the outset.

For a drug to be effective and safe, the compound must selectively bind to the target protein at the intended site of action, a process known as target engagement (TE). As such, measurements are already routinely used for selecting CDs with sufficient TE, but the high rate of efficacy failures during clinical trials shows that these conventional approaches are not working.

One reason for this is that many traditional assays do not provide sufficiently relevant TE assessments so cannot reliably predict efficacy failures. Additionally, rigorous TE measurements must be performed in the early stages of drug discovery to ensure the correct prioritization decisions are made as early as possible in the pipeline.

Meeting these requirements can seem daunting; however, there is already one promising solution. The Cellular Thermal Shift Assay (CETSA®) enables direct, physiologically relevant TE quantification within intact cells and tissues, based on the fundamental principle of thermal shift assays (TSAs; see Box 1).

We need to be able to better predict which compounds will fail much earlier in the drug discovery pipeline so that only the very best ones are prioritized from the outset.

Box 1: What are thermal shift assays?

Thermal shift assays (TSAs) are a well-established technique for measuring ligand binding to purified proteins. They involve subjecting the protein to varying temperatures ('heat shocks') and quantifying its thermal denaturation by producing melt curves. The key principle is that when a ligand is bound to a protein, the melting is altered, which is known as a 'thermal shift'and can be used to quantify the affinity of the ligand-protein interaction. CETSA® is based on this fundamental principle but leverages the novel insight that the melt curves can be measured in intact cells and tissues (Martinez Molina et al., 2013).

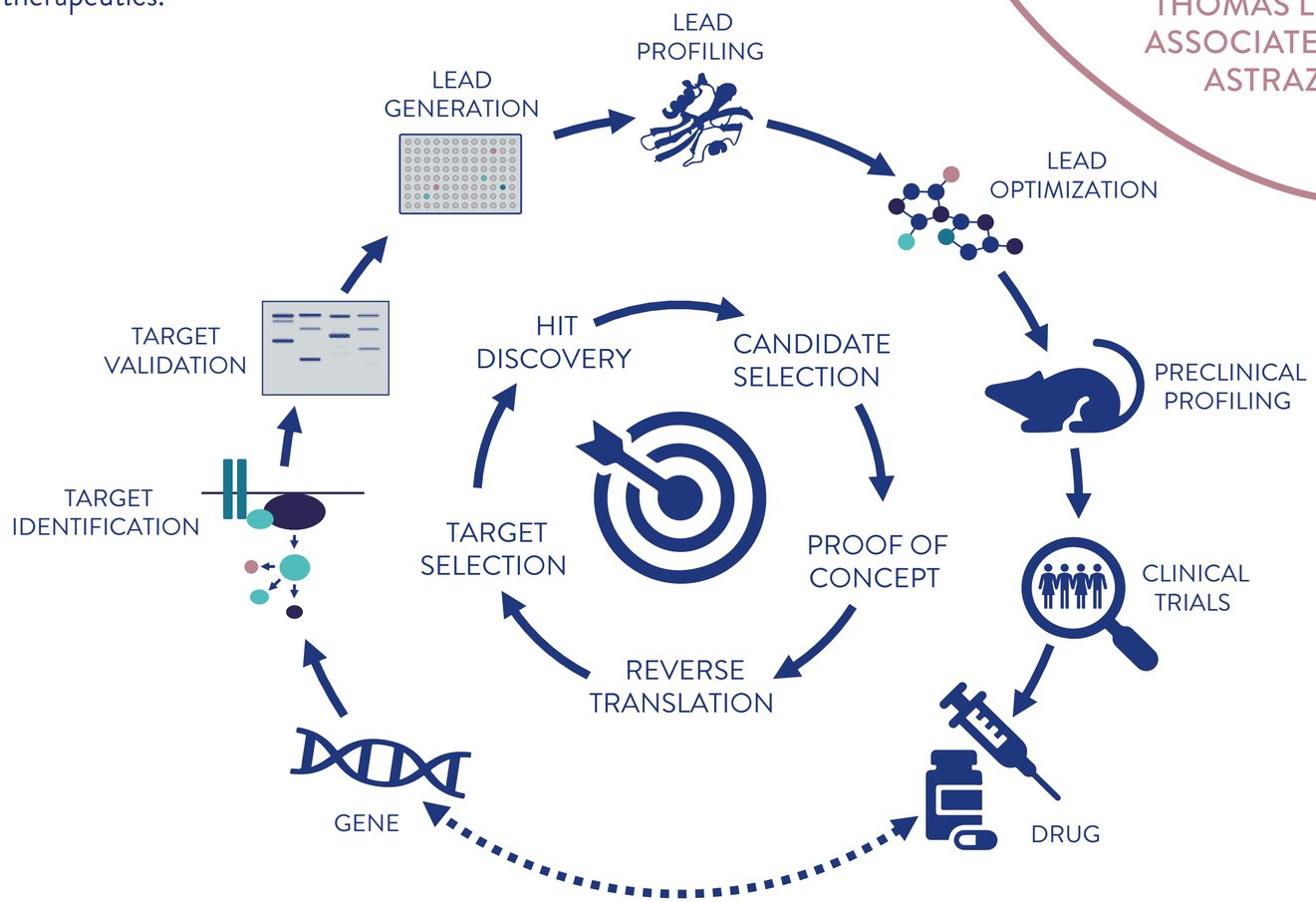
CETSA® is an extremely flexible assay capable of being applied throughout the drug discovery value chain, as highlighted by Thomas Lundback, Associate Director AstraZeneca, “At AstraZeneca we are applying CETSA® HT in high-throughput screening and lead optimization to identify and characterize hit and lead compounds for a number of novel targets.” CETSA® brings real value at these stages. Used at the lead generation phase, it can uncover novel compounds that would have been missed by other assays. At lead optimization, it can help to guide the process and keep the chemistry on track, as well as confirm that your final compounds have engagement in *in vivo* PK/PD and efficacy models.

Several leading pharmaceutical and biotechnology companies prosper from the many benefits CETSA® brings: **It provides added value including quicker project timelines, more robust data, and higher-quality leads, providing greater returns on investment than any traditional assay could deliver.**

Employing CETSA® in your discovery efforts offers you an opportunity to achieve your own goals as well as benefit your company, the pharmaceutical industry, and society as a whole.

In this eBook, we outline how you can implement CETSA® during lead generation and lead optimization to drive the development of the right compounds and get the most value out of your drug discovery projects. This includes expert insights from our in-house drug discovery specialist, Stina Lundgren, who has over 12 years’ experience working as a drug discovery researcher. We also discuss the unique benefits you can gain from adopting CETSA®, and how its widespread implementation has advanced the field of drug discovery to meet society’s need for new therapeutics.

> “At AstraZeneca we are applying CETSA® HT in high-throughput screening and lead optimization to identify and characterize hit and lead compounds for a number of novel targets.
>
> THOMAS LUNDBACK, ASSOCIATE DIRECTOR ASTRAZENECA



Chapter 2: Why CETSA® is the best way to measure target engagement

CETSA® is based on the principle of thermal shift assays (TSAs; see Box 1) but leverages the novel insight that the melt curves of proteins can be measured in intact cells (Martinez Molina et al., 2013). The premise of CETSA® involves heating proteins in cells, which then unfold and aggregate at a specific temperature, known as T_{agg} (or T_m). When a compound binds to the target protein in the cell, the protein becomes more or less resistant to heat, causing a shift in the thermal denaturation profile T_{agg} /T_m (also known as thermal shift).

In order to measure target engagement of a compound using CETSA®, cells are heated in the presence and absence of the compound, after which the unfolded and aggregated proteins are typically removed. The remaining soluble protein indicates the amount of protein that has stayed folded (i.e., it is a readout of the thermostability of the protein). A protein detection method, such as Western blot, is then used to quantify this remaining soluble protein, which is plotted against the temperature to give the CETSA® melt curve, a concentration-response experiment at a single temperature then determines the TE potency at half-maximal effect concentration (CETSA® EC_{50}).

CETSA® has revolutionized the measurement of TE, which has previously relied on traditional biophysical assays. Traditional cell-based approaches involve either tags, labels or artificial substrates, and recombinant purified protein-based assays (e.g., surface plasmon resonance (SPR)) only measure drug binding on immobilized protein in a cell-free environment.

In contrast, CETSA® provides a direct measurement of compound binding in a physiologically relevant manner (i.e. within the complex natural environment of lysates, intact cells, and tissues) without modifying the compounds or target proteins. In other words, CETSA® is a label-free method. As it measures drug-target binding directly, it can be easily translated between different cell and tissue types, allowing rapid translation of TE between sample matrices.

The physiologically relevant and label-free measure of affinity means that CETSA® TE potencies are the true reflection of the direct binding of the compound to the target in the natural environment of cells and tissues. Moreover, it is much quicker to set up than traditional assays, which involve more time-consuming assay development. Ultimately, this means that CETSA® can help reduce timelines while providing you with the data you need to make better-informed decisions to secure drug discovery success.

CETSA® target engagement potencies are the true reflection of the direct binding of the compound to the target in the natural environment of cells and tissues.

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CETSA® is available in two formats with different applications in drug discovery (also see Figure 1):

- **CETSA® Navigate** – a targeted method focusing on a single selected target. Detection is based on either Western blot (CETSA® Classics) or is plate-based using dual antibody proximity assay (CETSA® HT). The format is useful in targeted drug discovery from lead generation through to late lead optimization for confirming and quantifying target engagement, and can be applied for primary screening, hit confirmation and translational studies.

- **CETSA® Explore** - the protome-wide mass spectrometry-based method, also known as thermal proteome profiling (TPP) or thermal stability profiling. It has been demonstrated useful in Mechanism-of-Action (MoA) analysis, as well as in phenotypic target deconvolution and safety assessments (which is the focus of the CETSA® MS eBook).

In the next few chapters, we will discuss CETSA® Navigate, which includes CETSA® HT and CETSA® Classics and their respective applications in the lead generation and optimization stages of drug discovery.

Navigate your Chemistry and Explore the Biology

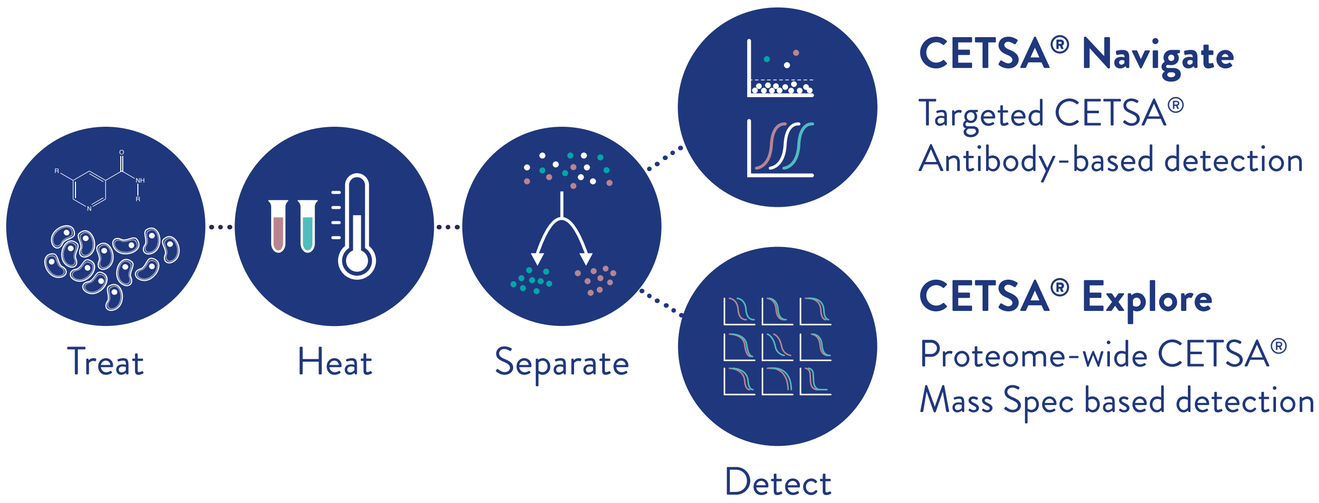


Figure 1: The two different CETSA® formats: CETSA® Navigate and CETSA® Explore.

Chapter 3: How CETSA® overcomes challenges in early drug discovery

CETSA® has many applications during both the early lead generation and later lead optimization stages of drug discovery (Figure 2). Adopting the assay can help you overcome the challenges often encountered during these phases (see Box 2). Although some of these benefits are unique to each stage (see Chapters 4 and 5), implementing CETSA® also have general advantages that can enhance the drug discovery process.

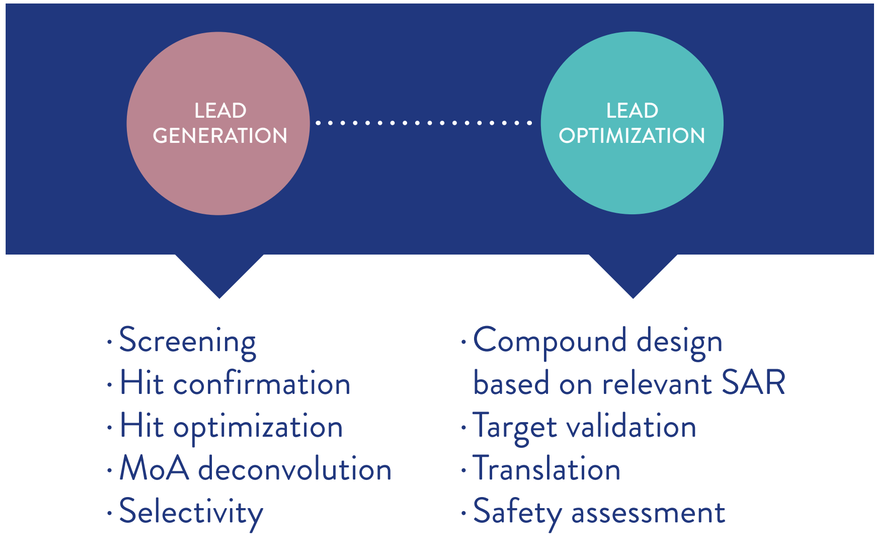


Figure 2: CETSA® applications during target identification, lead generation and lead optimization stages of drug discovery.

Box 2: The goals and challenges of lead generation and lead optimization

Lead generation is one of the earliest stages of the drug discovery process and includes hit identification and confirmation. In general terms, hit identification is the process of identifying compounds ('hits') that have the desired affinity against the intended protein target. High-throughput screening (HTS) is typically used to assay libraries comprising hundreds of thousands of compounds directly against the target to quickly discard inactive compounds and quickly compile hit compound series. One key challenge here is that it is very easy to generate false positives. These can be extremely time-consuming to identify and subsequentely eliminate, resulting in substantial costs and timeline delays. Worse still, if the false positives are not identified early then this gives misleading results that can cause costly efficacy failures at later stages. As such, it is essential to implement tools that allow you to identify and discard false positives quickly.

After lead compounds have been identified, **lead optimization** begins. The aim at this stage is to optimize the compound properties to ensure the development of safe and effective candidate drugs (CDs) that can perform their intended function. Structure-activity relationships (SARs) analysis is used for guidance of the design of potent and selective compounds. Additional tests, such as safety optimization and drug metabolism and pharmacokinetics (DMPK) analysis, are also used.

In late-lead optimization, before CD selection, a smaller set of compounds (pre-CDs, i.e. those that have successfully passed through lead optimization) are further investigated. The aim here is to ensure the selected CDs are safe for clinical testing as well as suitable for formulations and drug development. This requires additional tests, such as rigorous solubility and stability analysis and safety assessments. In vivo efficacy models are used to examine the pharmacokinetic and pharmacodynamic (PK/PD) properties of the compounds for dose predictions as well as to strengthen the project hypothesis. Lead optimization can be extremely time-consuming and costly, so tools such as CETSA® that helps you to speed up timelines and minimize delays are very valuable during this stage of drug discovery.

The general benefits of using CETSA® in the drug discovery process

CETSA® is an effective assay at all stages of drug discovery as it enables you to confirm hits and prioritize compounds in primary cells that are relevant for the intended therapeutic function of the drug. Once established, CETSA® can be used to correlate TE in the cell with functional readouts to strengthen target validation and establish the structure-activity relationship (SAR). As CETSA® can performed in live cells or tissue, the CETSA® EC_{50} values give you a consistent reference point that can help with translational studies as your compounds move from lead generation into lead optimization. This will ensure that your project stays on track and that you are prioritizing only the very best compounds at each stage.

What's more, you only have to set up CETSA® once as you can use the same assay repeatedly without investing any more time in assay development. Adopting this quick and easy method is valuable not just for meeting your project's deadlines and ensuring you stay under budget, but it also ensures that you make your prioritization decisions based on robust, relevant data, dramatically minimizing the risk of late-stage failure.

In this way, CETSA® enables you to priotitize, helping you to save not only a significant amount of time and financial resources but also avoid the opportunity costs of investing in the wrong compound series. You can make informed and confident decisions about which compounds are likely to succeed, which allows you to invest your time and budget in only the very best compounds, helping to expedite the development and approval of new therapies.

The robust, relevant data generated by CETSA® can also help biotechs complete outlicensing deals with pharmaceutical companies by providing strong evidence that CDs will be successful. By facilitating out licensing deals, biotechs can then go on to investigate further targets and compounds, ultimately helping to fuel the drug discovery and development pipeline and get needed therapeutics to patients.

CETSA® can be used to correlate target engagement in the cell with functional readouts to strengthen target validation and establish the structure-activity relationship (SAR).

Box 3: Why adopting CETSA® earlier is better

Adopting CETSA® in the earlier stages of drug discovery is crucial to minimize costs and ensure only the very best compounds progress through to the later stages. During lead generation, you can quickly screen tens of thousands of compounds using CETSA® HT with the confidence that you will generate highly reliable data on which to base your earliest prioritization decisions. As you pass the compounds on from lead generation to lead optimization, CETSA® HT and CETSA® Classics can help you to quickly and reliably identify which compounds have insufficient TE and drop them from the investigation accordingly, long before you have made costly investments into their development.

Chapter 4: How CETSA® HT enables more effective lead generation

CETSA® HT uses homogeneous, dual-antibody detection for high-throughput screening in a microtiter plate format. In the proof of concept study, Almqvist et al. (2016) used CETSA® HT along with AlphaScreen® technology to perform a primary compound library screen, which enabled measurements of an intracellular drug binding to a well-known cancer target (human thymidylate synthase (TS)).

Almvqist and colleagues used this CETSA®-based assay to screen a library consisting of almost 11,000 compounds in live, non-engineered cells expressing physiological levels of TS. The screen identified all known drugs that act on TS within the annotated validation library. Even more interestingly, it also identified novel compounds that can bind to and inhibit TS. Overall, CETSA® HT offers a reliable high-throughput screening method in natural cellular environments that can successfully be applied in compound screening for hit identification and confirmation during lead generation.

There are several unique benefits of using CETSA® during the lead generation phase. For example, in cases of unknown target function, the assay can be used to obtain a chemical starting point for your compound at the primary screening stage. CETSA® is also much quicker and easier to set up compared to traditional assays, which typically require time-consuming development (e.g., probe synthesis) that can take 6 months or more. As such, CETSA® can save you a significant amount of time and speed up your project timeline by up to a year. Perhaps most importantly, you can use CETSA® to directly discard false positives to ensure you do not pass ineffective compounds on to the lead optimization phase.

> “Perhaps the most important unique benefit of using CETSA® during lead generation is that it allows you to directly discard false positives, allowing you to fail faster and prioritize only the very best compounds.
>
> STINA LUNDGREN,
> DRUG DISCOVERY EXPERT

CETSA® HT in action

Recent studies have demonstrated how CETSA® HT can enhance primary screening during lead generation. In one of these studies, Shaw et al (2018a) investigated Androgen Receptor (AR) as a target for the treatment of prostate cancer. Novel AR antagonists have historically been hard to identify due to the difficulties of distinguishing between direct AR-binders and inhibitors that act on AR co-regulators. The researchers applied CETSA® HT to a compound library containing AR-binders, as well as to another library consisting of compounds known to target AR co-regulators. They found that CETSA® HT accurately identified direct AR-binders and, unlike functional cellular assays, could differentiate them from co-regulator inhibitors.

In another study, Shaw *et al* (2018b) developed two CETSA® HT assays directed against two additional oncology targets: B-Raf and poly(ADP-ribose) polymerase 1 (PARP1). By using these assays to screen large and diverse compound libraries, the team showed that CETSA® HT could efficiently identify intracellular binding inhibitors for both PARP1 and B-Raf, as well as rank the compounds according to cellular TE to drive SAR. Additionally, the study showed a good correlation between the CETSA® HT assay for PARP1 with other screening approaches.

Additionally, the use of CETSA® HT for primary screening on a set of 0.5M compounds was recently reported by Kirsten Tschapalda et al at SLAS 2020 San Diego. In this study, the group conducted a Semi-Automated CETSA®-based screen on CRAF, resulting in a hit rate of 0.2%. The identified hits were then validated by CETSA® EC_{50} determinations, which confirmed a total of 374 active compound hits. Using CETSA® for primary screening enabled the team to unlock new chemical space by exploring ways for the compound to interact with the target, such as binding to a different site.

These studies show how you can apply CETSA® HT in lead generation to reliably identify hits that are more likely to have the intended functionality and efficacy during later-stage trials. Such research exemplifies how implementing CETSA® could lead to the development of novel therapeutics for life-threatening diseases, including cancer.

> “Adopting CETSA® can reduce your project timeline by 6-12 months and expedite the progress of compounds from lead generation into the lead optimization stage.
>
> STINA LUNDGREN,
> DRUG DISCOVERY EXPERT

Chapter 5: How CETSA® Classics enables more effective lead optimization

Originally introduced as part of the original proof of concept experiment by Martinez Molina et al., (2013), CETSA® Classics utilizes Western blot-mediated detection for the interrogation of individual target proteins. Martinez Molina and the team used human cell lines and mouse models to show that drug binding-induced thermal shifts could be measured for 10 different drug targets from several different protein families. Since then, CETSA® Classics has been reported for a range of low-throughput applications (Martinez Molina et al. 2016), mainly as a way to interrogate a small number of compounds in lead optimization.

Applying CETSA® Classics to your lead optimization studies offers you a variety of unique benefits. Western blot-based CETSA® experiments are relatively simple to set up because they only require one antibody directed at the protein target (and there is usually a wide range of detection antibodies available). Furthermore, the only equipment required is readily available in most biochemistry labs (for a detailed protocol, see Jafari et al. 2014).

The CETSA® Classics format is particularly useful in translational studies because of the ease of transfer between different cell and tissue samples and the fact that these experiments are typically performed on only a few candidate compounds.

CETSA® Classics in action

CETSA® Classics can also be applied successfully in lead optimization (Ishii et al., 2017). Here, Ishii investigated a lead compound on the target—receptor interacting protein 1 kinase (RIPK1)—which is implicated in a variety of diseases such as multiple sclerosis and chronic inflammatory diseases. The researchers demonstrated a robust and feasible CETSA® assay for measuring TE of the lead compound (reversible RIPK1 inhibitors) in both mouse peripheral blood and animal tissues.

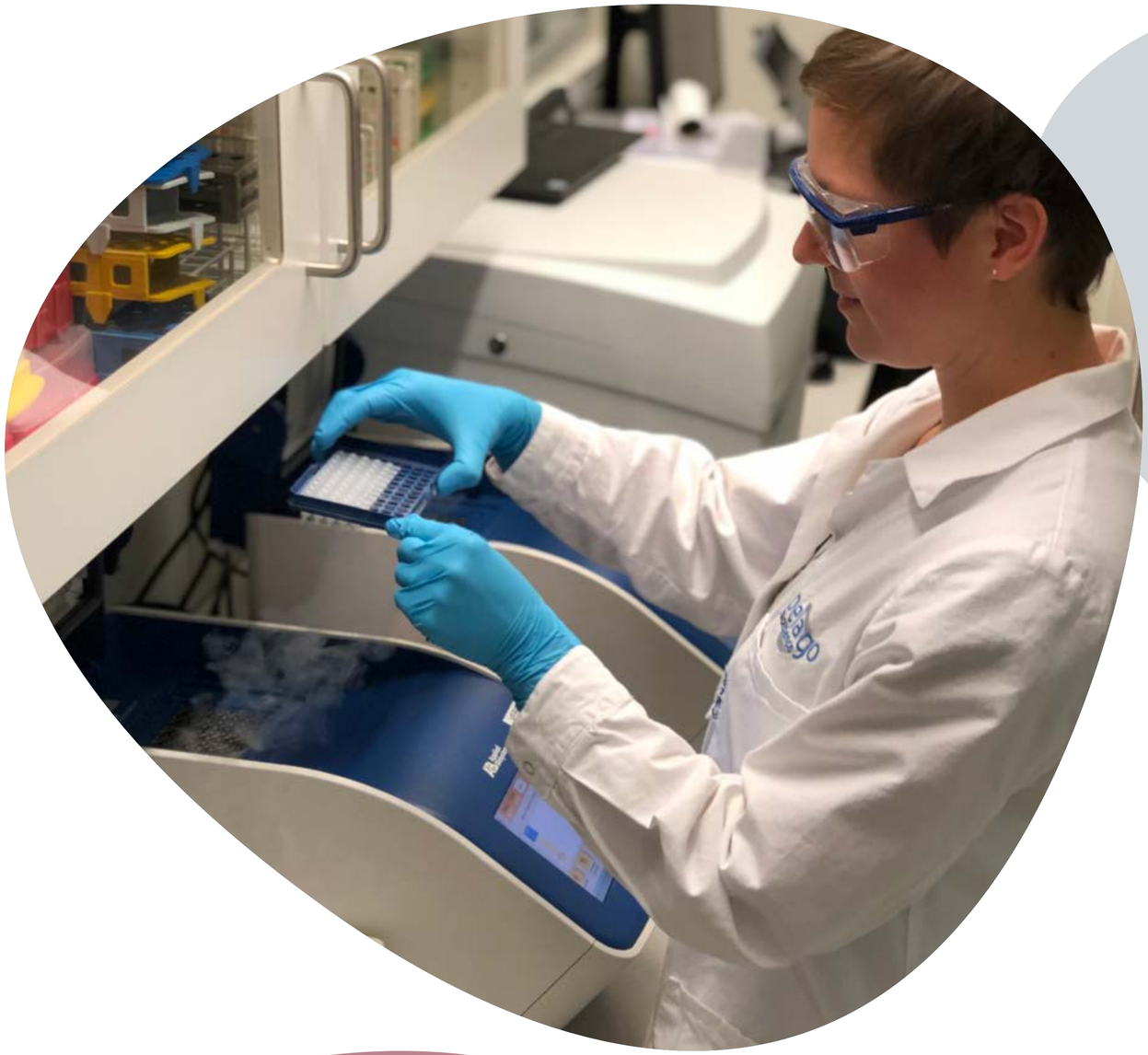
Box 4: The future evolution of CETSA® across a range of new applications

CETSA® has the potential to inspire innovation and change in the drug discovery industry and society as a whole, so that we can all work together to start reducing attrition rates and more efficiently develop new therapies to help patients. By using CETSA® for CD confirmation, you could increase your chances of developing high-quality candidate drugs for clinical development.

There are other possible future applications for CETSA® currently under assessment, beyond those already discussed in this eBook. For example, there is the potential for applying CETSA® to protein degradation-focused drug discovery (i.e. molecular glues and proteasome-targeting chimeras (PROTACs)).

By using CETSA® alongside a semi-automated system the researchers could evaluate the SAR using native RIPK1 in culture cell lines and enabled them to estimate the drug occupancy ratio in mouse peripheral blood mononuclear cells. In addition, they were able to monitor the *in vivo* drug TE in spleen and brain tissue. This study demonstrates how CETSA® Classics can be used to directly assess drug TE in *in vivo* animal experiments for lead optimization and highlights how you too could use this tool to enable more effective drug discovery.

CETSA® has primarily been applied to soluble proteins which, upon heating, denaturate to form insoluble aggregates by exposing hydrophobic surfaces. Case studies on how to overcome the challenges associated with deploying CETSA® on insoluble protein targets such as membrane-bound proteins were published by Aarti et al in 2019. The studies describe thorough experimental work undertaken to develop assay protocols for challenging multi-pass transmembrane proteins. This is hugely valuable to drug discovery researchers—by demonstrating how CETSA® can be used for membrane-bound proteins such as ion channels and transporters, scientists now have an alternative to biochemical or biophysical methods (Arti et al 2019).



Chapter 6: Driving drug discovery forward

The adoption of CETSA® is key to improve success rates and bring new therapeutics forward. This novel TE assay enables direct, physiologically relevant measurements of drug-target engagement that can be used to make confident decisions about which project and compounds to prioritize. What's more, by expediting timelines, CETSA® allows the companies using the assay gain a competitive edge over those that don't.

CETSA® can be applied as early as primary screening during lead generation, allowing you to 'fail faster' and progress only the very best compounds to the next stage of drug discovery. Implementing this approach and developing other potential applications will save the industry significant amounts of time and financial investment, as well as reduce attrition rates to help solve drug discovery's major problems.

The future success of drug discovery relies on us all taking responsibility and working together to find ways of reducing attrition rates. We already have one valuable tool in CETSA® and, as this becomes even more widely adopted in the industry, it is likely that we will see overall late-stage efficacy success rates improve, resulting in new drug approvals that directly benefit patients.

Book a complimentary consultation with us to learn how CETSA® can help boost the success of your drug discovery project.

BOOK YOUR CONSULTATION

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About Pelago Bioscience: Science with Engagement

The Home of CETSA®, Pelago Bioscience AB, was founded to provide and develop the patented Cellular Thermal Shift Assay (CETSA®) in 2013. CETSA® was invented at Karolinska Institute, a prestigious medical university in Stockholm, Sweden, and developed by Pelago to deliver *in situ* target engagement studies to expedite clinical drug discovery and diagnostics development. In keeping with the company's core message, "Science with Engagement," Pelago has vast experience and expertise in drug discovery, including the areas of screening and assay technology development as well as lead generation and lead optimization research. By tapping into this wealth of knowledge, you can ensure you effectively prioritize your projects to make an impact and get the most value out of your drug discovery projects.

Michael Dabrowski, CEO

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