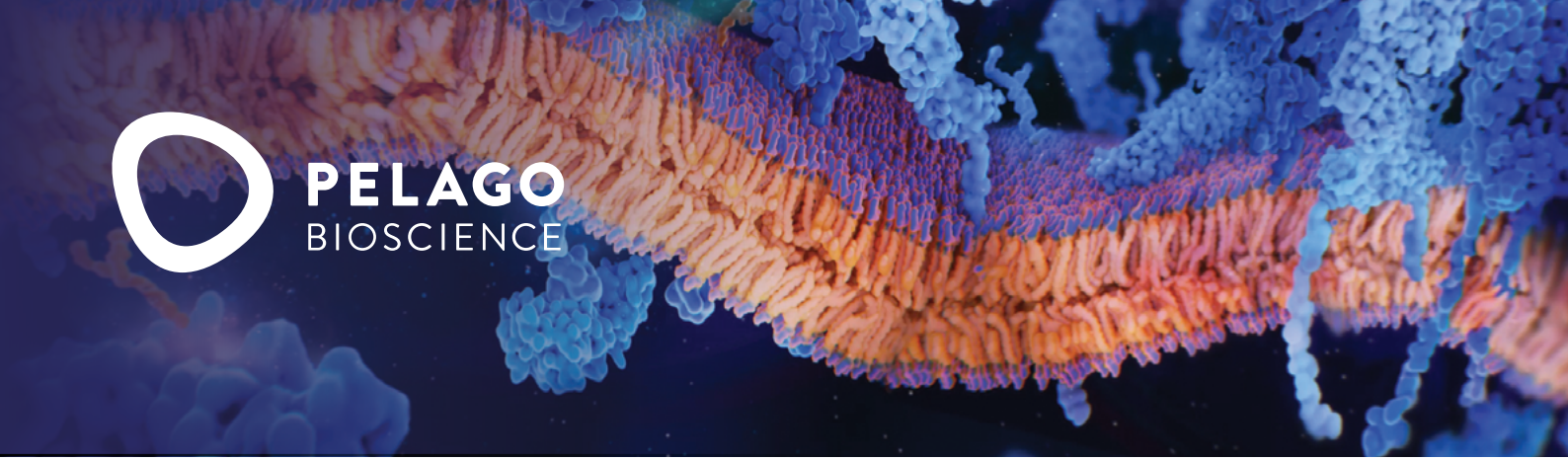


PELAGO BIOSCIENCE

Is your protein degrader effectively driving the biology you desire?

In the discovery and development of targeted protein degraders, as with any modality, assuring efficacy from the early stages of drug discovery is essential to prevent failure at later stages of clinical development. Correlating physiological relevant affinity (target engagement) with therapeutically relevant effect at each stage in the value chain increases the overall success rates in drug discovery and development.

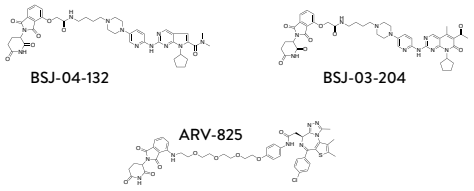
The Cellular Thermal Shift Assay (CETSA®), a patented technology by Pelago Bioscience based on ligand-induced changes in protein thermostability, allows the confirmation of target engagement and is a physiologically relevant measurement of protein-compound binding in live cells without modification of the proteins or the compound. Since target engagement is measured inside live cells and only occurs if the degrader reaches its target (either the POI or the E3 ligase), this also provides valuable insights on permeability and in-cell performance of the degrader.

CETSA® Navigate HT leverages on homogeneous bead-based approaches, such as AlphaLISA®, for protein detection and allows for primary screening in high throughput to identify binders to either the POI or the E3 ligase. With the AlphaLISA® protein detection kits, one can also, in the same cells, monitor the compound-mediated degradation of the POI and identify the optimal concentration to achieve effective degradation, thus assessing the relevant pharmacology.

By introducing an unbiased MS-based proteome-wide analysis with CETSA® Explore, one can achieve further insights of the mechanism of action of the degrader, not only simultaneously assessing the selectivity of target engagement with both the POI and the E3 ligase, but also observing downstream effects from the removal of the POI by degrader-mediated degradation and not only its inhibition, thus supporting the expression of the relevant phenotype.

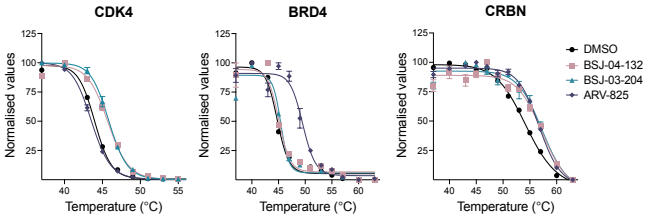
Confirming target engagement with either the POI or the E3 ligase using CETSA® Navigate HT

The PROTACs® BSJ-04-132, BSJ-03-204 and ARV-825 are Cereblon-dependent selective degraders for CDK4 (BSJ-04-132 and BSJ-03-204) or BRD4 (ARV-825).



Using CETSA® Navigate HT it was possible to confirm the selective engagement with their respective POI as well as with the E3 ubiquitin ligase Cereblon (CRBN), by incubating THP1 cells with 10 µM of each PROTAC® for 30 minutes.

Selective engagement of PROTACs® BSJ-04-132 and BSJ-03-204 with CDK4 can be confirmed by the presence of a thermal shift, whereas ARV-825 only showed a thermal shift with BRD4 and no effect on CDK4. All PROTACs® induced a thermal shift on CRBN, confirming engagement with this E3 ligase.

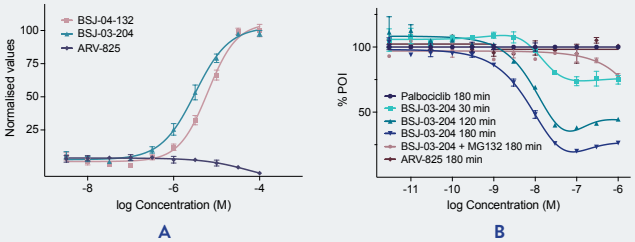


Is your protein degrader effectively degrading the POI?

The same AlphaLISA® SureFire® Ultra™ total CDK4 kit can be used to assess the target engagement (A) and, with minor modifications to the CETSA® protocol, the degradation profile of a protein degrader (B). In this case we detected the remaining levels of CDK4 after incubation of intact cells with a selective degrader based on the CDK4 inhibitor palbociclib. A time and concentration-dependent degradation of CDK4 was observed in the presence of BSJ-03-204, while incubation with palbociclib alone shows no effect on the levels of CDK4 even at the longest time point. Incubation with ARV-825, which targets BRD4 as POI, also showed no effect on the levels of CDK4 after 180 minutes. By co-incubating with the proteasome inhibitor MG-132, the BSJ-03-204-dependent degradation of CDK4 can effectively be blocked.

A maximum effect is also consistently observed at a concentration of 100 nM regardless of the incubation period, with higher concentrations resulting in a lower level of protein degradation. This points to this concentration being the optimal for the formation of ternary complexes and effective protein degradation.

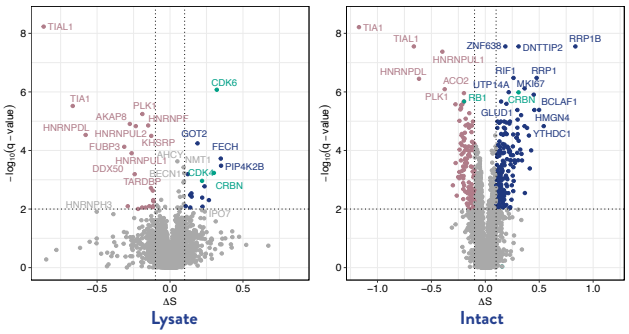
With two parallel assays that share the same detection method, the quantification of binding to the POI and degradation is achieved, correlating target engagement potency with degradation efficiency. This synergy will enable the quicker generation of optimized degraders.



PELAGO BIOSCIENCE

Selectivity profiling and MoA studies using CETSA® Explore

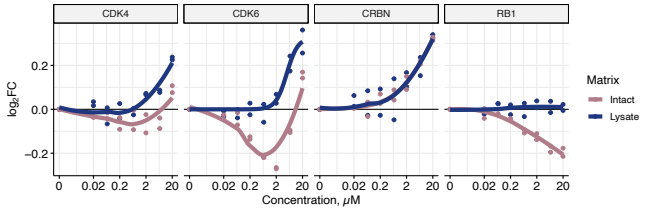
With CETSA® Explore it is possible to simultaneously identify direct binding events to both the POI and the E3 ligase and potential off-targets, or to observe downstream effects from direct binding interactions as well as from the removal of the POI by PROTAC®-mediated degradation [1]. The proteome effects of BSJ-03-204 in K562 cells were further investigated with CETSA® Explore in both intact and lysed K562 cells, by incubating these matrices with up to 30 µM of the PROTAC® for 60 and 15 minutes, respectively.



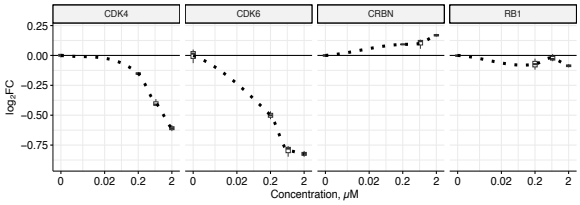
Volcano plots for lysate (Left), clearly show a stabilization of CRBN, CDK4 and CDK6, as well as of some known off-targets of palbociclib e.g., PIP4K2A, PIP4K2C, CSNK2A2 and PLK1. In intact cells (Right), CRBN is clearly shown as stabilized by BSJ-03-204. Protein hits in intact cells also may constitute pathway effects and RB1, the direct substrate of CDK4 and CDK6, is clearly destabilised. Several proteins involved in transcriptional regulation (RRP1B, RRP1, ZNF638, DNTTIP2, and RIF1) are also identified as protein hits in the intact cell experiment. However, CDK4 and CDK6 are not among the significant hits in the volcano plot. This happens as the volcano presents the relative abundance of each protein in the soluble fraction, which results from a weighed effect of thermal stability changes and compound-mediated degradation.

Individual concentration response (CR) curves for CDK4 and CDK6 show a clear concentration dependent stabilization of these targets in the presence of BSJ-03-204 in lysed cells as well as for the E3 ligase CRBN in both intact cells and lysate. However, the PROTAC®-mediated degradation of these POI in intact cells weighs more on the overall effect until the concentration goes beyond the hook effect for the ternary complex formation, preventing the detection of the stabilization observed in lysate. Moreover, the removal of CDK4/6 from the cell, leads to reduced phosphorylation of its substrate retinoblastoma protein (RB1) which is only observable in intact cells and is presented as a reduction of the solubility of this protein. The degradation of the POIs can be confirmed with quantitative proteomic analysis of unheated samples, where we can observe a clear reduction of the levels of CDK4/6, while CRBN and RB1 do not show any reduction.

CETSA® Explore CR Curves

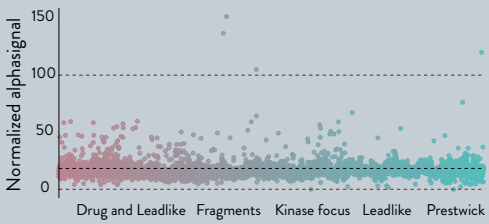


Quantitative Proteomics



CETSA® Navigate HT can be used to identify novel binders to either the POI or the E3 ubiquitin ligase

A library of 11,000 structurally diverse compounds was screened for binders to Cyclin-dependent kinase 4 (CDK4) using a CETSA® Navigate HT assay. The screen was performed in intact THP1 cells at a single concentration of 50 µM, in 384-well format. AlphaLISA® SureFire® Ultra™ total CDK4 protein kit was used for detection, and the screen resulted in a hit rate of 1.2% with a Z'-factor >0.8. Over 90% of the preliminary hits were confirmed in a hit confirmation screen, opening a path to the development of novel PROTAC® molecules targeting CDK4.



Pelago Bioscience is your partner to increase the likelihood for drug success

The prevention of failure at later stages of clinical development is of paramount importance in early drug discovery. Morgan *et al.* [2] have laid the ground for three pillars of survival for a successful program when reaching Phase II: (1) exposure at target site of action (cell penetration), (2) binding to the pharmacological target (target engagement and selectivity), and (3) expression of pharmacology. Later, Bunnage *et al.* [3] expanded the concept to other chemical probes like PROTACs® with the addition of a fourth pillar: expression of relevant phenotype. Pelago Bioscience is set to be your drug discovery research partner and offers a range of assays to support all these pillars, building trust and assuring the efficacy of your molecules from the early stages of development.

References

1. Chernobrovkin, Alexey L. *et al.* SLAS discovery : advancing life sciences R & D vol. 26,4 (2021): 534-546. doi:10.1177/2472555220984372
2. Morgan, Paul *et al.* Drug discovery today vol. 17,9-10 (2012): 419-24. doi:10.1016/j.drudis.2011.12.020
3. Bunnage, Mark E *et al.* Nature chemical biology vol. 9,4 (2013): 195-9. doi:10.1038/nchembio.1197