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Drug Target Identification Methods After a Phenotypic Screen

Pelago Bioscience supports preclinical drug discovery and development projects with their expertise in biologically relevant assay systems. Their unique core technology, the Cellular Thermal Shift Assay (CETSA®) has multiple assay formats that makes it a keystone of decision-making throughout the drug discovery pipeline.

Here's a common scenario – your team has just completed a phenotypic screening campaign, cleaned out the false positives through hit triage, and now you have modestly optimized validated leads with cell-based activity. The problem is, you have no idea how your compounds work, and whether they act on a target or set of targets that are worth pursuing. What do you do?

In this minireview, we summarize available methods for identifying a drug's target when it is not known at the outset, with a focus on:

- Target deconvolution methods used in industry
- Strengths and limitations of commonly used methods
- The utility of label-free methods like CETSA® for probeless target deconvolution

Read this article online to see all of the resources and download the PDF reference poster.

We assume that your team has already established the presence of a worthwhile target, such as by cleaning out all your technical false positives including many AICS and PAINS, evaluating how known MoAs behave in your screening cascade, and identifying an active/inactive pair of compounds in your series (such as an active/inactive enantiomer pair) to suggest specific target-based pharmacology, rather than a non-specific mode of action like redox cycling.

Why Target Identification and Target Deconvolution Matter

Target identification is the process of figuring out what biological molecule(s) your drug acts on to elicit its effects (also called "target deconvolution" when referring to the process after a screen). Whether you are trying to optimize a new molecule's properties or trying to mitigate an existing molecules' off-target toxicities, it's helpful to know how exactly your drug is causing the effects that it is. While there are rare cases of molecules advancing in the clinic without a full understanding of the biological mechanism of action (such as for RNA splicing modulator branaplam), knowing what target you're modulating makes life easier in many ways.

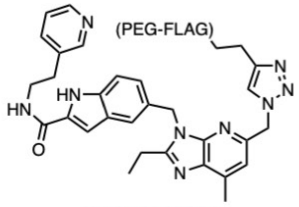
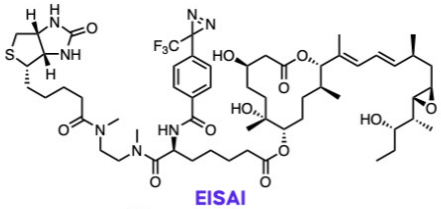
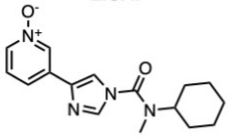
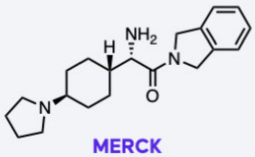
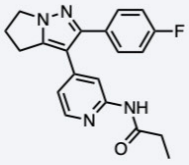
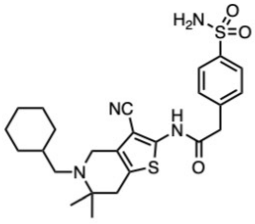
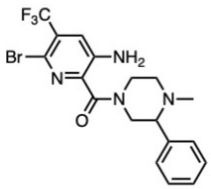
With knowledge of the target, you can start to build high-throughput biochemical or biophysical assays for measuring activity and target engagement, develop more focused assessments of selectivity, enable structure-based design, have a better idea of what biomarkers to look for, and better understand the biological outcomes of experiments in the context of what's been done before.

Target Deconvolution Methods Currently Used in Industry

While proof-of-concept has been demonstrated for numerous target identification methods in academic labs (see some excellent reviews below in "Further Reading"), only a handful of approaches are regularly used in industry. These can generally be categorized in three categories:

- Chemical probe-based methods (e.g. affinity pulldown, photoaffinity labeling)
- Label-free methods (e.g. thermal stability profiling, chemical/proteolytic stability profiling)
- Indirect methods (e.g. resistance screening, chemogenomic profiling)

Target Identification Methods in Phenotypic Drug Discovery

	METHOD	INDUSTRY APPLICATION	TARGET (LIGAND)	EXAMPLE REFERENCE
Chemical Probe-Based	**Affinity-Based Pulldown**	 KYOWA KIRIN	NAMPT (K542)	Yamaguchi, D. et al. *Sci. Rep.* **2019**, *9*, 7742.
	Photoaffinity Labeling	 EISAI	SF3b (pladienolide)	Kotake, Y. et al. *Nat. Chem. Biol.* **2007**, *3*, 570.
	Activity-Based Protein Profiling	 PFIZER+	ALDH2 (BIA 10-2474)	Huang, Z. et al. *ACS Chem. Biol.* **2019**, *14*, 192.
Label-Free Methods	**Thermal Stability Profiling** (CETSA®)	 MERCK	DPP9 (ICeD-2)	Moore, K. P. et al. *ACS Chem. Biol.* **2022**, *17*, 2595.
	Chemical/Proteolytic Stability Profiling (e.g. SPROX, DARTS, LiP, PP..)	 BAYER+	Fungal CK1 (BAYE-004)	Piazza, I. et al. *Nat. Commun.* **2020**, *11*, 4200.
Indirect Methods	**Resistance Screening**	 NOVARTIS	Dengue NS4B (NITD-688)	Moquin, S. A. et al. *Sci. Transl. Med.* **2021**, *13*, issue 579.
	Chemogenomic Profiling	 NOVARTIS	Fungal Sec14p ('compound 1')	Pries, V. et al. *Cell Chem. Biol.* **2018**, *25*, 279.

Chemical Probe-Based Methods for Target Deconvolution

The most widely employed methods of target identification are chemical proteomics methods that modify the ligand of interest into a chemical probe, which is used to "fish out" a protein target of interest. In general, these methods require that the ligand be modified with a identification/reporter tag, such as an azide or alkyne, that allows the ligand to be put on solid support, or otherwise be recovered after incubation with potential targets, such as through chemical conjugation to a motif that is easily purified from the experimental mixture (e.g. a biotin tag).

The three most common chemical probe-based methods are:

- Affinity-based chemoproteomics (a.k.a. affinity chromatography or "pulldown")
- Photoaffinity labeling
- Activity-based proteome profiling (ABPP)

Affinity-based chemoproteomics typically involves immobilizing a pulldown probe on a solid phase and exposing the solid-supported probe to protein extracts such as cell lysate. Washing of the solids (typically beads) removes non-specific binders, leaving behind potential targets bound to the solid-supported probe. Detection of the protein targets is then possible by a number of methods, such as gel electrophoresis, tryptic digestion, and peptide identification by MS/MS. A wide range of tools (e.g. kinobeads), protocols (e.g. competitive format), and detection strategies (e.g. SILAC, iTRAQ) have been developed (see Further Reading), but the overall principle depends on chemical probes that can be recovered protein extracts.

- ***Strengths:*** tried-and-true approach with extensive usage, probes can be reversible, relatively high probability of success
- ***Limitations:*** requires probe development and SAR, potent probes are needed (KD = nM), and cannot be done in live cells
- ***Recent industry example:*** Kyowa Kirin scientists used a FLAG-TAG-based probe to identify NAMPT as a target of their lead compound K542, which was identified from phenotypic screening

Photoaffinity labeling involves modification of the ligand with a photoaffinity group, such as a diazirene, which can be activated by light to release a reactive intermediate that covalently conjugates the ligand to the nearest molecule in a mixture, which is hopefully the target protein. The ligand must also be modified to have an identification/reporter tag such as an alkyne, which allows the covalent protein-ligand conjugate to be isolated from the mixture through onjugation to an isolation tag like biotin via a bioorthogonal reaction such as azide-alkyne click chemistry. Identification of the protein(s) that have been photochemically conjugated to the ligand of interest is then possible through a number of methods, such as through gel electrophoresis, tryptic digestion, and peptide identification by MS/MS.

- ***Strengths:*** irreversible binding to target provides strong evidence of target engagement, can be used to identify a binding site on the target, and can be done in living cells and with insoluble proteins
- ***Limitations:*** requires two modifications to the ligand of interest, including a photoreactive group and a purification tag, though combined linkers have been developed. Requires more experimentation as photoreactive group may not necessarily react with target of interest
- ***Recent industry example:*** Eisai scientists used a photoaffinity probe based on the natural product pladienolide to identify its target as SF3b.

Activity-based proteome profiling (ABPP) leverages probe molecules that covalently and promiscuously react with a class of enzyme's active sites, such as beta-lactones for serine hydrolases. Like the above methods, the probe molecules must possess reporter tags to facilitate target characterization, such as fluorophores, biotin, or latent modifiable handles such as alkynes or azides. If the ligand of interest has a suspected covalent warhead, such as a beta-lactone, activity-based proteome profiling can be applied to identify its target. If the ligand of interest does not have an applicable covalent warhead, competitive activity-based proteome profiling can be used instead, in which competition between the ligand of interest and a separately-developed activity-based probe is used to identify a target within a suspected target class.

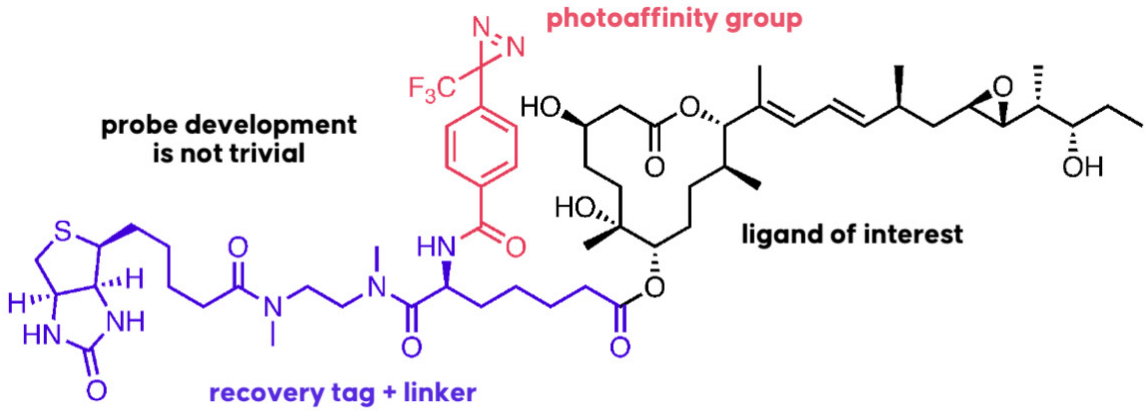
- ***Strengths:*** irreversible binding to target provides strong evidence of target engagement, can be used to identify a binding site on the target, and can be done in living cells and with insoluble proteins
- ***Limitations:*** the reactive probes used depend on reactivity of the target (e.g. specific enzymes), making the approach limited to identifying active sites of specific classes of enzymes
- ***Recent industry example:*** Pfizer scientists and the Cravatt lab used ABPP to identify aldehyde dehydrogenases (ALDH2 in particular) as undesired targets of Bial's FAAH inhibitor BIA 10-2474, a compound that notoriously caused a patient's death in the clinic.

Label-Free Methods for Target Deconvolution

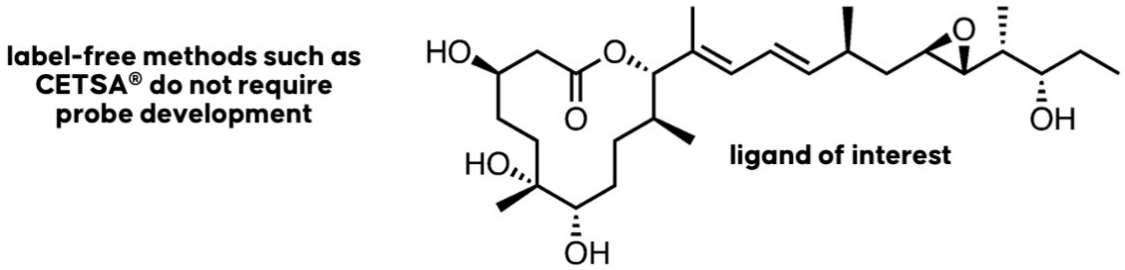
Probe-based methods offer clear evidence of ligand-target interactions and can provide direct evidence of binding sites, but they come with limitations, including the need for resource-intensive chemical probe development and the fact that they often only work with cell lysates or specific cell fractions, limiting their applicability in live or primary cells. Moreover, chemical probes can exhibit promiscuity and non-specific binding, resulting in false positives and negatives. Conversely, label-free methods for target deconvolution are gaining popularity, as they do not require ligand labeling and can potentially overcome some of these limitations.

Probe-Based vs. Label-Free Approaches

Chemical Probe - Target ID



Label-Free Target ID



Probe-based vs. label-free approaches to target ID. Probe development is not trivial, and label-free methods such as CETSA® offer more direct alternative approaches to target ID.

The most commonly approach to label-free target deconvolution is stability proteomics, which in general look at the ability of compounds to bind to proteins, protecting them from denaturation against a variety of stimuli such as oxidation or proteolysis. The Stability of Proteins from Rates of Oxidation, or "SPROX" method, for example, looks at the ability of compounds to stabilize proteins from methionine oxidation, while the Drug Affinity Responsive Target Stability, or "DARTS" method looks at the ability of compounds to stabilize proteins from protease degradation. While chemical and proteolysis approaches are now well-known in academia and in industry, beyond the target deconvolution of Bayer's fungicide BAYE-004, there are limited published examples of use by industry groups of such methods for the target identification of compounds with previously unknown targets.

CETSA®-Based Methods for Target Deconvolution

Methods based on the **Cellular Thermal Shift Assay**, first published in Science in 2013, are increasingly used approaches to target deconvolution by industry groups. Instead of depending on the ability compounds to stabilize their target proteins to chemical degradation, Cellular Thermal Shift Assay (CETSA®) methods look at the ability of compounds to influence the thermal stability of proteins. Combining CETSA with MS-based protein detection enables unbiased assessment of the proteome (CETSA® MS, a.k.a. CETSA® Explore, Compressed CETSA® MS, Thermal Protein Profiling/TPP, Proteome Integral Solubility Alteration/PISA).

- ***Strengths:*** no modification of compound of interest is needed, reversible molecules can be used, can be done in living cells, and has high proteome coverage (6-8k proteins) among label-free methods. The approach does not depend on target enzymatic activity or reactivity, making it amenable to detecting allosteric binding interactions. It also allows multiple targets to be detected at the same time, allowing visualization of pathway effects to support a proposed MoA.
- ***Limitations:*** the approach does not provide binding site information, requires a soluble target, and may be less sensitive than probe-based methods.
- ***Recent industry example:*** Merck scientists recently applied a CETSA®-based method (CETSA® MS, or Proteome Integral Solubility Alteration [PISA]), to identify the target of a compound capable of killing HIV-1 infected cells discovered from a phenotypic screen.

Indirect Methods for Target Deconvolution

Indirect methods, including genomic approaches or phenotypic profiling approaches, attempt to identify a target without directly measuring a direct target-compound interaction. Until the development of label-free direct methods, indirect methods were the only ways to identify targets without chemical probe development. While indirect methods can be high throughput and provide pathway-level information, narrowing down the specific target, especially for first-in-class mechanisms, often requires additional experimentation.

In infectious diseases or oncology, targets are often detected through resistance screening. Mutations in the pathogen/cancer cell line that generate resistance to the drug of interest often provide strong clues to the identity of the target of interest (e.g. recently in Novartis's discovery of a NS4B inhibitor for dengue, the discovery of the diarylquinoline antimycobacterials, or in the identification of DHODH as a target for AML), which can be followed up with experiments to confirm the suspected target.

Chemogenomics methods such as haploinsufficiency profiling and homozygous profiling (HIP-HOP) have also been used successfully in industry, such as by Novartis to identify Sec14p as an antifungal target. A CRISPR-Cas-based genetic screening method has been recently used to identify NAMPT as a target of Karyopharm's oncology asset KPT-9274, as another example.

In general, such methods have been most successful in infectious diseases and oncology research, where cell viability is an effective readout, and are often paired with direct- and label-free methods to confirm the ultimate targets.

Other Approaches to Target Deconvolution

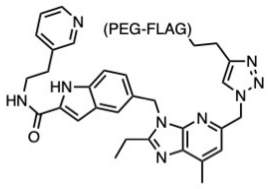
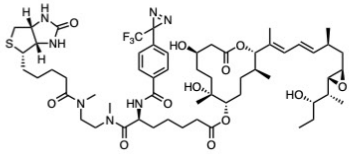
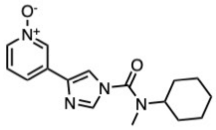
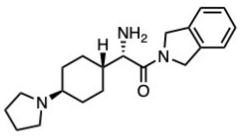
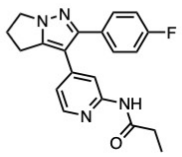
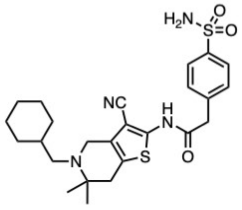
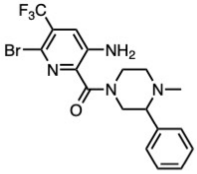
While there are many other direct methods for target deconvolution, including molecular biology approaches such as yeast display, phage display, mRNA display, ribosome display, and chemical biology approaches such as protein microarrays, such approaches have found limited use to date in industry for the identification of previously unknown targets, likely in part due to the biological system manipulation involved, which both makes them more challenging to execute and makes results potentially less relevant.

Emerging indirect methods include attempting to build large datasets that will allow the "fingerprinting" of compounds, to enable target identification based on comparison of compound phenotypic or morphological profiles (e.g. via cell painting) to the effects observed when specific targets are modulated (e.g. via RNAi, CRISPR, or other small molecules). The use of a reference collection of gene-expression profiles from human cells cultured with small molecules ("Connectivity Map"), for example, was used by the Broad Institute to identify the target of the natural product gedunin as Hsp90. More details on what to expect in the future can be found in reviews in the Further Reading section.

When Label-Free Methods like CETSA® MS Stand Out for Target Deconvolution

Although there are various methods for target deconvolution used in the industry, there is growing interest in label-free methods such as the Cellular Thermal Shift Assay (CETSA®). This non-invasive and convenient approach has been leveraged to solve many drug target mysteries, including the long-awaited identification of the target of the antimalarial quinine, which impressed many industry observers, including Derek Lowe. New applications of CETSA® continue to be published on a regular basis, including this recent study by Georg Winter's group on the target identification of orpinolide.

Target Identification Methods in Phenotypic Drug Discovery

	METHOD	STRENGTHS	LIMITATIONS	INDUSTRY APPLICATION	EXAMPLE REFERENCE
Chemical Probe-Based	**Affinity-Based Pulldown** (e.g., kinobeads, SILAC, click, iTRAQ)	• Probes can be reversible • Relatively high probability of success	• Requires probe linker modification • Potent reversible probe needed (K_D = nM) • Requires cell lysis	 KYOWA KIRIN, NAMPT	Yamaguchi, D. et al. *Sci. Rep.* **2019**, *9*, 7742.
	Photoaffinity Labeling (e.g., diazirines, competitive labeling)	• Irreversible binding, can provide binding site information • Can be done in living cells and for insoluble proteins	• Requires photoreactive group and purification tag group • Relatively lower probability of success	 EISAI, SF3b	Kotake, Y. et al. *Nat. Chem. Biol.* **2007**, *3*, 570.
	Activity-Based Protein Profiling (e.g., sulfonyl fluorides, beta-lactones)	• Irreversible binding, can provide binding site information • Can be done in living cells and for insoluble proteins	• Requires reactive probe for target class • Limited to specific classes of enzymes	 PFIZER, ALDH2+	Huang, Z. et al. *ACS Chem. Biol.* **2019**, *14*, 192.
Label-Free Methods	**Thermal Stability Profiling** (CETSA® Explore / TPP / PISA)	• No modification of compound needed • Reversible molecules can be used • Can be done in living cells • High protein coverage among label-free methods	• Does not provide binding site information • May have lower sensitivity than probe-based methods • Requires soluble target	 MERCK, DPP9	Moore, K. P. et al. *ACS Chem. Biol.* **2022**, *17*, 2595.
	Proteolytic or Chemical Stability Profiling (e.g., PP, DARTS, LiP-MS, SPROX)	• No modification of compound needed • Reversible molecules can be used • Provides alternative methods of detection	• Can theoretically localize binding site to peptide regions • Cannot be conducted in live cells • May be limited in protein coverage (e.g., SPROX requires methionines)	 BAYER, FUNGAL CK1	Piazza, I. et al. *Nat. Commun.* **2020**, *11*, 4200.
Indirect Methods	**Resistance Screening** (e.g. anti-infectives, cytotoxics)	• No modification of compound needed • Reversible molecules can be used • Provides relatively strong evidence for target	• Only applicable in certain indications (e.g., anti-infectives, cancer) • Can be time and biology resource-intensive	 NOVARTIS, DENV NS4B	Moquin, S. A. et al. *Sci. Transl. Med.* **2021**, *13*, issue 579.
	Chemogenomic Profiling (e.g. HIP-HOP, CRISPR mutagenesis scanning)	• No modification of compound needed • Reversible molecules can be used • Unbiased for target abundance	• Only applicable in certain settings (e.g., antifungals, cancer) • Can be time and biology resource-intensive	 NOVARTIS, SEC14P	Pries, V. et al. *Cell Chem. Biol.* **2018**, *25*, 279.

CETSA® offers several advantages, including the ability to work with living cells, applicability to reversible molecules, and high protein coverage. Although it may not provide information about the binding site and has certain limitations, CETSA® is a valuable method that should be considered by researchers looking to identify drug targets after a phenotypic screen both on its own and in combination with other methods.

If your team needs to identify the target of a new molecule, it may be worth consulting the team at Pelago Bioscience. Pelago was founded by the scientists that invented the CETSA® approach, and employs many former industry scientists (including from AstraZeneca) who understand the nuances needed to make data relevant to industry. With experience across hundreds of programs, and with 10 years' experience in CETSA®, Pelago's team can help not only execute experiments, but quickly analyze and interpret the data based on everything they've seen before.

LEARN MORE ABOUT CETSA®

As the field continues to evolve, it is crucial to stay informed about new and emerging techniques for target deconvolution, which will ultimately contribute to the development of more effective and safer drugs. We hope that this collection of additional references will serve as a helpful guide for your target deconvolution efforts in the future.

Further Reading

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