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Differential amino acid reactivity with chemical probes can provide valuable information on the functionality and ligandability of proteins in native biological systems. Here, we present a quantitative, multiplexed chemical proteomic protocol for in-depth reactivity and ligandability profiling of cysteines in proteins in quiescent and stimulated T cells. This protocol illuminates dynamic immune state-dependent alterations in cysteine reactivity, revealing chemoselective and stereoselective small-molecule interactions with cysteines in structurally and functionally diverse proteins that lack chemical probes.

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Highlights
Cysteine reactivity profiling to compare biochemical changes across T cell states
Cysteine ligandability profiling to discover small-molecule-protein interactions
Stereoisomeric covalent probes facilitate mechanism-of-action studies
Protocol

Multiplexed proteomic profiling of cysteine reactivity and ligandability in human T cells

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SUMMARY

Differential amino acid reactivity with chemical probes can provide valuable information on the functionality and ligandability of proteins in native biological systems. Here, we present a quantitative, multiplexed chemical proteomic protocol for in-depth reactivity and ligandability profiling of cysteines in proteins in quiescent and stimulated T cells. This protocol illuminates dynamic immune state-dependent alterations in cysteine reactivity, revealing chemoselective and stereoselective small-molecule interactions with cysteines in structurally and functionally diverse proteins that lack chemical probes.

For complete details on the use and execution of this protocol, please refer to Vinogradova et al. (2020).

BEFORE YOU BEGIN

Naïve T cell isolation

© Timing: 2 h

1. Isolate peripheral blood mononuclear cells (PBMCs) over standard Lymphoprep (STEMCELL Technologies) gradient using slightly modified manufacturer’s instructions.
   a. Layer 25 mL of freshly isolated blood on top of 12.5 mL of Lymphoprep in a 50 mL Falcon tube avoiding mixing of blood with Lymphoprep.
   b. Centrifuge the tubes at room temperature (931 g, 20 min, 23°C) with brake off.
   c. Transfer the plasma and Lymphoprep layers containing PBMCs to new 50 mL Falcon tubes with a 2:1 dilution with PBS.
   d. Pellet the cells (524 g, 8 min, 4°C) and combine the cells into 2 Falcon tubes by washing the rest of the tubes with PBS (10 tubes combined into 1 using 40 mL of PBS).
   e. Combine pellets from 2 Falcon tubes into 1 Falcon tube with PBS (20 mL and 20 mL wash), count the cell number, and spin down the cells (524 g, 8 min, 4°C).

Optional: In case red blood cell contamination is significant (the pellet is completely red), use RBC lysis buffer (Thermo Fisher) before proceeding to the next step.

2. Isolate T cells from fresh PBMCs using EasySep Human T Cell Isolation Kit (STEMCELL Technologies, negative selection) according to manufacturer’s instructions.
T cell activation for mass-spectrometry analysis (activated T cells)

© Timing: 3–4 days

3. Pre-coat non-tissue culture treated 6-well plates using a cocktail of αCD3 (5 µg/mL, BioXCell) and αCD28 antibodies (2 µg/mL, BioXCell) in PBS (2 mL/well) and keep at 4°C overnight (or 37°C for 2 h on the day of the experiment).

4. The next day, transfer the plates to a 37°C incubator for 30 min - 1 h followed by a wash with PBS (2 x 5 mL/well).

5. Re-suspend freshly isolated T cells obtained in step 2 in RPMI media supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 1 x 10⁶ cells/mL, plate into the pre-coated 6-well plates (3–7 mL/well) and keep at 37°C in a 5% CO₂ incubator for 3 days.

6. Following this incubation period, combine the cells in 50 mL Falcon tubes, pellet (524 g, 5 min, 4°C), and wash with PBS (10 mL). Pellet the cells after the wash (524 g, 5 min, 4°C).

7. Transfer the cells into an Eppendorf tube in 1 mL of PBS, pellet and either re-suspend in fresh RPMI media for in situ treatments or flash-freeze and keep at −80°C until further analysis (in vitro treatments). The cell count typically increases 1.5–2 fold compared to the originally plated cells.

T cell expansion for mass-spectrometry analysis (control T cells)

© Timing: 13–15 days

8. Pre-coat a non-tissue culture treated 6-well plate with αCD3 (1.5 µg/mL) antibody in PBS (3 mL/well) and keep at 4°C overnight.

9. The following day transfer the plates to a 37°C incubator for 30 min - 1 h followed by a wash with PBS (2 x 5 mL/well).

10. Re-suspend freshly isolated T cells isolated in step 2 in RPMI media (10% FBS, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL)), containing αCD28 antibody (1 µg/mL) at 1 x 10⁶ cells/mL, plate into the pre-coated 6-well plate (3–7 mL/well) and keep at 37°C in a 5% CO₂ incubator for 3 days. Typically, 30–60 x 10⁶ of freshly isolated T cells were selected for an expansion from each donor to afford 800–1600 x 10⁶ expanded T cells on average.

11. Combine the cells in 50 mL Falcon tubes, pellet (524 g, 5 min, 4°C), and wash with PBS (10 mL).

12. Re-suspend the cells in RPMI media containing recombinant IL-2 (10 U/mL) at 2.5 x 10⁵ cells/mL and keep at 37°C in a 5% CO₂ incubator for 10–12 days, splitting the cells every 3–4 days with IL-2 containing media to keep cell density below 2 x 10⁶ cells/mL.

13. After 13–15 days of cell expansion (3 day pre-activation on the plate + 10–12 days of expansion), pellet the cells (524 g, 5 min, 4°C), wash with PBS (10 mL). Pellet the cells after the wash (524 g, 5 min, 4°C), wash with PBS (10 mL), and transfer into Eppendorf tubes (1 mL PBS), spin down (524 g, 5 min, 4°C), flash-freeze and keep at −80°C until further analysis.

14. Combine pellets from 2 Falcon tubes into 1 Falcon tube with PBS (20 mL and 20 mL wash), count the cell number, and spin down the cells (524 g, 5 min, 4°C).

15. Re-suspend the cells in fresh RPMI media for in situ treatments or flash-freeze and keep at −80°C until further analysis (in vitro treatments).

Optional: For in situ treatments, re-suspend the cells in fresh RPMI media (3 x 10⁶ cells/mL) and treat with stereoisomeric probes (5 µM or 20 µM, Figure 1) at 37°C in a 5% CO₂ incubator for 3 h. Following the treatment, pellet the cells (524 g, 5 min, 4°C), wash with PBS (10 mL), transfer into Eppendorf tubes (1 mL PBS), spin down (524 g, 5 min, 4°C), flash-freeze and keep at −80°C until further analysis.

Note: T cell activation can be evaluated and monitored using flow cytometry-based analysis of activation markers CD25 and CD69 or ELISA analysis of secreted proinflammatory cytokines (e.g., IL-2, IFN-γ). Viability of cells can be measured using Trypan blue or flow cytometry-based approach using near-IR LIVE-DEAD stain (Invitrogen).
**Note:** T cell viability at the end of expansion should be >80% as measured by Trypan blue.

**KEY RESOURCES TABLE**

Media should be stored at 4°C and warmed to 37°C prior to use. IL-2 should be added to the media right before use. Lymphoprep should be stored at 4°C and used cold for better separation during the blood layering step.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Antibodies           |        |            |
| PB anti-human CD4    | BD Pharmeden | Cat#: 558116 |
| PerCP/Cyanine5.5 anti-human CD8a | BioLegend | Cat#: 301032 |
| FITC anti-human CD69  | BioLegend | Cat#: 310904 |
| PE anti-human CD25   | BioLegend | Cat#: 302606 |
| Monoclonal mouse anti-human CD3 (clone OKT3) | Bio X Cell | Cat#: BE0001-2 |
| Monoclonal mouse anti-human CD28 (clone 9.3) | Bio X Cell | Cat#: BE0248 |
| Biological samples   |        |            |
| Human blood          | Scripps Research Normal Blood Donor Services | https://nbds.scripps.edu |

(Continued on next page)
### Materials and Equipment

**IA-DTB stock solution (store in the dark at -80 °C)**

| Reagent | Final concentration | Amount |
|---------|----------------------|--------|
| IA-DTB  | 10 mM                | 5 mg   |
| DMSO    | n/a                  | 921.8 µL |
△ CRITICAL: EPPS pH needs to be adjusted after re-suspension.

△ CRITICAL: Ammonium bicarbonate causes eye, skin, and respiratory tract irritation. Avoid inhalation and contact with eyes, skin, and clothing.
CRITICAL: Formic acid is highly irritating and corrosive. It can cause severe burns to eyes, skin, and the respiratory system. Wear appropriate protective gear (lab coat, gloves, eye goggles) and handle large volumes in a well-ventilated chemistry hood.

| Solvent A (store at 20°C–23°C) | Reagent       | Final concentration | Amount |
|-------------------------------|---------------|----------------------|--------|
| LC-MS grade H₂O              | 95% (v/v)     | 950 mL               |
| LC-MS grade CH₃CN            | 5% (v/v)      | 50 mL                |
| Formic Acid                  | 0.1% (v/v)    | 1 mL                 |

| Solvent B (store at 20°C–23°C) | Reagent       | Final concentration | Amount |
|-------------------------------|---------------|----------------------|--------|
| LC-MS grade H₂O              | 20% (v/v)     | 200 mL               |
| LC-MS grade CH₃CN            | 80% (v/v)     | 800 mL               |
| Formic Acid                  | 0.1% (v/v)    | 1 mL                 |

| Formic acid (store at 4°C) | Reagent       | Final concentration | Amount |
|---------------------------|---------------|----------------------|--------|
| LC-MS grade H₂O           | 80% (v/v)     | 30 mL                |
| Formic acid               | 20% (v/v)     | 7.5 mL               |

CRITICAL: Formic acid is highly irritating and corrosive. It can cause severe burns to eyes, skin, and the respiratory system. Wear appropriate protective gear (lab coat, gloves, eye goggles) and handle large volumes in a well-ventilated chemistry hood.

| TMT tag stock solution (store in the dark at –80°C) | Reagent | Final concentration | Amount |
|-----------------------------------------------------|---------|----------------------|--------|
| TMT tag                                              | 20 µg/µL|                      | 5 mg   |
| Dry acetonitrile                                     | n/a     |                      | 250 µL |

CRITICAL: CH₃CN is a flammable liquid that is harmful upon inhalation or in contact with skin and eyes. Wear appropriate protective gear (lab coat, gloves, eye goggles) when working with it.

STEP-BY-STEP METHOD DETAILS

The timing for each step is calculated for the preparation of one 10-plex mass-spectrometry sample (10 separate treatment channels, Figure 2).

Sample preparation and treatment

Timing: 4 h

This step involves preparation of the proteome, treatment with the broad cysteine-reactive desthiobiotin-containing chemical probe (Figure 3), reduction and alkylation of free unreacted thiols.

1. Thaw the cell pellets on ice (calculate the amount of cells that will yield >1 mg of proteome), re-suspend in cold PBS (550–600 µL) and lyse by sonication (probe sonicator, 2 × 8 pulses, 40% duty cycle, output setting = 4).
2. Normalize protein concentration to 1.5–2.0 mg/mL using a standard DC protein assay (Bio-Rad).
Optional: Separate soluble and particulate proteomic fractions by ultracentrifugation (100,000 g, 45 min).

Optional: Treat total proteome, soluble or particulate fractions with scout fragments KB02 or KB05 (Backus et al., 2016) (500 μM final concentration) for 1 h at ambient temperature for broad ligandability analysis.

3. Treat the resulting proteomes (500 μL, 1.5–2.0 mg/mL) with iodoacetamide polyethyleneoxide desthiobiotin (IA-DTB (Santa Cruz), 5 μL of 10 mM stock in DMSO, final concentration: 100 μM) for 1 h at ambient temperature.

△ CRITICAL: IA-DTB is light sensitive, but can be stored for several months as DMSO aliquots at −80°C in the absence of light in amber 1.5 mL Eppendorf tubes.

4. Add ice-cold MeOH (600 μL), CHCl\textsubscript{3} (100 μL), and H\textsubscript{2}O (100 μL), vortex the mixture and centrifuge (10,000 g, 10 min, 4°C) to afford a protein disc at the interface of CHCl\textsubscript{3} and aqueous layers (Figure 2B).

5. Aspirate both layers without perturbing the disk.

△ CRITICAL: if the disk does not form properly, aspirate just the top layer.

6. Add cold methanol (1 mL) to the disk and vortex. Pellet the proteins (10,000 g, 10 min, 4°C), and re-suspend the resulting pellets in 90 μL of buffer containing 9M urea, 10 mM DTT and 50 mM triethylammonium bicarbonate (1/20 dilution of 1.0 M stock solution, pH 8.5) by thorough pipetting up and down or sonication.
CRITICAL: Avoid having excessive formation of bubbles, a cloudy solution is acceptable at this stage.

7. Heat the resulting mixture at 65°C for 20 min, then cool the sample to room temperature.
8. Add iodoacetamide (10 μL, 500 mM solution in H2O), and incubate the samples in the dark at 37°C for 30 min with shaking.

△ CRITICAL: DTT and IA should be stored at 4°C and the stock solutions in water should be made fresh on the day of the experiment.

Trypsin digestion and streptavidin enrichment

 Timing: 5 h

This step describes trypsin digestion of the proteins, streptavidin enrichment of desthiobiotin-containing peptides, and elution of the enriched peptides from streptavidin resin.

9. Following the labeling with iodoacetamide, dilute the samples with 305 μL of Buffer A (TEAB buffer, 50 mM, pH 8.5) to afford a 2.0 M final urea concentration, add trypsin (4 μL of 0.25 μg/μL trypsin in trypsin buffer, containing 25 mM CaCl2), and digest the proteins at 37°C for 2 h.

△ CRITICAL: Dilution of urea to 2.0 M concentration is critical to ensure proper enzymatic activity of trypsin.

 Pause point: The tryptic digest step can be performed overnight in a 37°C incubator with agitation.

10. The following day, dilute the samples with 2X wash buffer, 400 μL, 50 mM TEAB, pH 8.5, 150 mM NaCl, 0.2% NP-40), add streptavidin-agarose beads (50% slurry in 2X wash buffer) to each sample (50 μL/sample) and rotate the bead mixture for 2 h at rt.
11. For a 10-plex sample, wash streptavidin-agarose bead slurry in 2X wash buffer (550 μL, 50% slurry; 2 X 1 mL, 50 mM TEAB, pH 8.5, 150 mM NaCl, 0.1% NP-40) and bring up to the initial volume in the 2X wash buffer prior to the addition to the sample.
12. After incubation, pellet the beads by centrifugation (2,000 g, 1 min), transfer the beads to BioSpin columns and wash (3 X 1 mL wash buffer, 3 X 1 mL PBS, 3 X 1 mL H2O).
13. Elute the peptides by addition of 300 μL of 50% aqueous CH3CN containing 0.1% FA.
14. Evaporate the eluate to dryness using SpeedVac vacuum concentrator.

△ CRITICAL: Excessive washes of the beads after the enrichment step are critical for removal of non-specific binding peptides, which detrimentally affect the coverage of labeled peptides.

 Pause point: The samples can be stored at −20°C or −80°C for several months.
**TMT tag labeling**

- **Timing:** 2 h

15. Re-suspend the peptides in 100 μL Buffer B (200 mM EPPS, pH 8.0) with 30% CH₃CN, vortex and spin down (2,000 g, 1 min).

16. Add TMT tags (3 μL/channel in dry CH₃CN, 20 μg/μL; Figure 2B) to the corresponding tubes and allow the reaction to proceed for 60 min.

17. Quench the reaction by the addition of 5% hydroxylamine (3 μL per sample), vortex, spin down, and leave at room temperature for 15 min.

18. Add FA (5 μL, Final concentration: 5% v/v) to each tube, vortex the tubes, spin down and combine all of the labeled channels in a single low binding 1.5 mL Eppendorf tube.

19. Dry the final combined sample in a SpeedVac vacuum concentrator and keep at −80°C until the desalting and high pH fractionation step.

**Note:** The dry sample will look like viscous oil due to the presence of salts that will be removed in the following step.

**Δ CRITICAL:** TMT tags can decompose over time. Re-suspend the solid tags in dry acetonitrile and avoid storing them at room temperature for prolonged time, as well as extensive number of freeze/thaw cycles. Avoid using Tris buffer, as TMT tags are unstable in the presence of primary amines.

**Desalting and high pH fractionation**

- **Timing:** 3 h

In this step the sample is desalted and fractionated to afford better LC/MS separation at the stage of the mass-spectrometry analysis.

20. Re-suspend the samples in solvent A (1 mL, 95% H₂O, 5% CH₃CN, 0.1% FA), acidify with 20 μL of 20% FA in H₂O, and desalt by passing through Sep-Pak C18 cartridges (55–105 μm, Waters) to avoid loading excessive salts on the HPLC column (300Extend-C18, 3.5 μm, 4.6 × 250 mm).
   a. Condition the cartridge with CH₃CN (3 × 1 mL);
   b. Equilibrate the cartridge with solvent A (3 × 1 mL);
   c. Load the sample by dropwise addition and wash the cartridge with the flow-through from the sample (1 × 0.5 mL);
   d. Desalt by passing solvent A (3 × 1 mL);
   e. Elute the peptides by adding 1 mL of solvent B (80% CH₃CN, 20% H₂O, 0.1% FA);

21. Evaporate the eluent to dryness using SpeedVac vacuum concentrator.

**Note:** At this stage there should be no visible salts in the Eppendorf tube.

**□ Pause point:** The samples can be stored at −20°C or −80°C for several months.

22. Re-suspend the sample in solvent A (500 μL) with sonication and fractionate into a 96 deep-well plate using HPLC (Agilent).
   a. Add 20 μL of 20% FA to each well in a 96-well collection plate to acidify the eluting peptides.
   b. Elute the peptides onto a capillary column (ZORBAX 300Extend-C18, 3.5 μm) and separate at a flow rate of 0.5 mL/min using the following gradient: 100% Ammonium bicarbonate (Buffer C; 10 mM) from 0–2 min, 0%–13% acetonitrile from 2–3 min, 13%–42% acetonitrile from 3–60 min, 42%–100% acetonitrile from 60–61 min, 100% acetonitrile from 61–65 min, 100%–0% acetonitrile from 65–66 min, 100% buffer C from 66–75 min, 0%–13% acetonitrile...
c. Evaporate the eluent to dryness in the plate using SpeedVac vacuum concentrator.

23. Re-suspend the peptides in solvent B (80% CH$_3$CN, 20% H$_2$O, 0.1% FA; 75 µL/well) and combine all the wells from each column in the 96-well plate (Columns 1–12 become fractions f1–f12) into a new clean Eppendorf tube (1.5 mL, 12 fractions total, Figure 2B), following an additional wash with the same solvent (200 µL/column, washing wells in each row).

24. Remove the solvent using SpeedVac vacuum concentrator.

Pause point: The samples are ready for mass-spectrometry analysis and can be stored at –80°C for several days to months.

**TMT-ABPP liquid chromatography mass-spectrometry (LC-MS/MS/MS) analysis**

This step describes LC-MS/MS/MS analysis conditions for each proteomic fraction and the Orbitrap Fusion mass-spectrometer settings for the analysis.

25. Re-suspend the resulting 12 combined fractions (Figure 2B) in solvent A (10 or 14 µL, depending on the injection loop size) and analyze on the Orbitrap Fusion mass-spectrometer (5 µL or 10 µL injection volume).

26. Analyze the samples by liquid chromatography tandem mass-spectrometry using an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000 Series Rapid Separation LC system and autosampler (Thermo Scientific Dionex).

a. Elute the peptides onto a capillary column (75 µm inner diameter fused silica, packed with C18 (Waters, Acquity BEH C18, 1.7 µm, 25 cm) and separate at a flow rate of 0.25 µL/min using the following gradient: 5% CH$_3$CN (0.1% FA) in H$_2$O (0.1% FA) from 0–15 min, 5%–35% CH$_3$CN (0.1% FA) from 15–155 min, 35%–95% CH$_3$CN (0.1% FA) from 155–160 min, 95% CH$_3$CN (0.1% FA) from 160–169 min, 95%–5% CH$_3$CN (0.1% FA) from 169–170 min, and 5% CH$_3$CN (0.1% FA) from 170–200 min.

b. The voltage applied to the nano-LC electrospray ionization source was 1.9 kV.

c. Data was acquired using an MS3-based TMT method adapted from Wang, Y. et al. (Wang et al., 2019)

i. Begin the scan sequence with an MS1 master scan (Orbitrap analysis, resolution 120,000, 400–1700 m/z, RF lens 60%, automatic gain control [AGC] target 2E5, maximum injection time 50 ms, centroid mode) with dynamic exclusion enabled (repeat count 1, duration 15 s).

ii. Select the top ten precursors for MS2/MS3 analysis.

iii. MS2 analysis consisted of: quadrupole isolation (isolation window 0.7) of precursor ion followed by collision-induced dissociation (CID) in the ion trap (AGC 1.8E4, normalized collision energy 35%, maximum injection time 120 ms).

iv. Following the acquisition of each MS2 spectrum, synchronous precursor selection (SPS) enabled the selection of up to 10 MS2 fragment ions for MS3 analysis.

v. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (collision energy 55%, AGC 1.5E5, maximum injection time 120 ms, resolution was 50,000).

vi. For MS3 analysis, use charge state–dependent isolation windows. For charge state z = 2, the MS isolation window was set at 1.2; for z = 3–6, the MS isolation window was set at 0.7.

27. Extract the MS2 and MS3 files from the raw files using RAW Converter (version 1.1.0.22; available at [http://fields.scripps.edu/rawconv/](http://fields.scripps.edu/rawconv/)), upload to Integrated Proteomics Pipeline (IP2), and search using the ProLuCID algorithm (Figure 2B, publicly available at [http://fields.scripps.edu/downloads.php](http://fields.scripps.edu/downloads.php)) using a reverse concatenated, non-redundant variant of the...
Human UniProt database (release-2012_11; SwissProt and Trembl entries were combined and filtered to choose a single protein isoform for each Ensembl gene identifier such that there’s one protein per gene. Reviewed entries (SwissProt) were chosen if available, otherwise the longest Unreviewed (Trembl) entry was used.

**Note:** For future applications, newer versions of the database should be generated and used.

a. Search cysteine residues with a static modification for carboxamidomethylation (+57.02146 Da) and up to one differential modification for the desthiobiotin (DTB) tag (+398.2529 Da) (Figure 2B).

b. Search the N-termini and lysine residues with a static modification corresponding to the TMT tag (+229.1629 Da).

c. Trypsin was selected as the enzyme of choice. Peptides were required to be at least 6 amino acids long, to have at least one tryptic terminus, and to contain the DTB modification.

d. Filter ProLuCID data through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%.

e. Perform the MS3-based peptide quantification with reporter ion mass tolerance set to 20 ppm with Integrated Proteomics Pipeline (IP2).

**EXPECTED OUTCOMES**

It is expected that this protocol will, on average, lead to enrichment and identification of >10000 cysteine-containing peptides (from >5000 proteins) per each 10-plex TMT-based experiment. This level of proteomic coverage allows for broad characterization of cysteine reactivity and/or small molecule-interactions across different proteomes. Using lower amounts of proteome is possible, but might lead to decreases in proteomic coverage. When combined with an unenriched quantitative proteomics workflow, this protocol allows for the proteome-wide discovery of biochemical changes in proteins that reflect dynamic alterations in cysteine reactivity.

The use of a 10-plex multiplexing strategy, in principle, allows for profiling up to 9 different electrophilic compounds in a single TMT-ABPP experiment (along with a DMSO control sample); however, in practice, we recommend performing each compound and DMSO control treatment in duplicate to facilitate interpretation of data, resulting in 4 compounds assayed per TMT-ABPP experiment. This approach informs on the extent of cysteine engagement by electrophilic compounds and proteome-wide selectivity of these compounds, as well as structure-activity relationships for each quantified cysteine in a single experiment. This information facilitates mechanism-of-action studies related to phenotypic screening, where identifying structurally similar inactive compounds and their target landscapes is a powerful strategy for streamlined target identification. In particular, the deployment of compounds that can be stereochemically differentiated in their phenotypic effects and proteomic reactivity provides excellent active and inactive controls for mapping biologically relevant targets, identifying actionable sites of ligandability on diverse proteins in cells, and for performing follow-up functional studies on these proteins. A representative cysteine reactivity landscape for four stereoisomeric probes EV-96, EV-97, EV-98, and EV-99 is shown in Figure 4.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**TMT-ABPP R value calculation for cysteine state-dependent reactivity dataset**

This section of the protocol describes the filters applied at the stage of data compilation and processing.

1. At the individual TMT experiment level, the following filters should be applied to remove low-quality peptides:
   a. Remove non-unique peptides;
   b. Remove half-tryptic peptides;
   c. Remove peptides with more than one internal missed cleavage site;
d. Remove peptides with low (<10,000) sum of reporter ion intensities in both expanded or activated channels;

e. Remove peptides with high variation (coefficient of variance >0.5) between the replicate expanded or activated channels if their sum of reporter ion intensities is greater than 5,000.

f. Calculate R values (activated vs. expanded) for each peptide using the average reporter ion intensities of activated and expanded TMT channels.

g. Once the R values are calculated, two types of grouping should be performed to aggregate peptide quantification data:

i. Group together overlapping peptides with the same modified cysteine (e.g., different charge states, high pH fractionation fractions, or tryptic termini), then average their R values, and report the shortest unique tryptic peptide;

ii. Group together multiple modified cysteines on a tryptic peptide, then report the averaged R values for further data processing.

2. Report the median value derived from at least two biological replicates as the final R value for each peptide with a maximum ratio cap of 20.

Figure 4. Target landscape of stereoisomeric acrylamide electrophiles

Heat map showing cysteines engaged >50% by stereoisomeric acrylamide compounds (5 μM, 3 h). For inclusion in the map, cysteines were also required to show equivalent or greater engagement by the relevant stereoisomeric electrophile at 20 μM. Stereoselective target engagement for EV-96 and EV-97 is highlighted in red and green, respectively. (Adapted from Vinogradova et al. (Vinogradova et al., 2020))
Data processing and analysis for IA-DTB reactivity dataset

3. Proteins must have at least three unique quantified peptides in either particulate or soluble fraction in the TMT-ABPP experiments within the state-dependent dataset to be analyzed.

**Note:** Proteins with lower amount of quantified peptides in the reactivity profiling experiment can also be analyzed for reactivity changes, if 1) no subcellular fractionation into soluble and particulate fractions is performed in reactivity profiling and 2) a fully matched (donor, treatment conditions) unenriched proteomics data is available.

**Optional:** If the sample was fractionated into soluble and particulate fractions, the fraction with the most quantified unique peptides was selected for analysis for each protein. If a protein had an equal number of unique quantified peptides in both fractions, the peptide R ratios (activated vs. expanded) from both fractions were averaged.

4. To account for potential donor variations in protein expression level, proteins are required to have at least one peptide R ratio within 1.5-fold of the protein expression level measured in unenriched proteomics (TMT-exp) experiments (if available) and excluded from the analysis if all peptide R ratios are greater than 2.0 or less than 0.5.

5. For proteins with 5 or more quantified peptides, a cysteine can be considered for potential change in reactivity if its peptide R value differs more than two-fold from both the median R value of all quantified cysteines on the same protein and from the protein expression level measured in TMT-exp experiments (if available). For proteins with three or four quantified peptides, a cysteine was considered for potential change in reactivity if its peptide R value differed more than two-fold from the protein expression level measured by TMT-exp data, with an additional requirement that the maximum peptide R ratio differed more than 2-fold from the minimum peptide R ratio. All the cysteines that passed the initial filters described above were manually curated to remove low quality profiles.

LIMITATIONS
Like many untargeted mass spectrometry-based proteomic platforms, the cysteine reactivity profiling method described herein requires a substantial amount of protein (~0.5–1.0 mg of protein per cell state) for optimal sensitivity. This quantity of protein may be challenging to acquire for rare cell states, leading to lower sensitivity and cysteine coverage. Some dynamic cysteine reactivity changes may be lost upon cell lysis and adapted protocols where the IA-DTB probe is added immediately upon cell lysis might address this limitation. Low stoichiometry liganding events that produce < 50% engagement of a cysteine (i.e., < 50% reduction in IA-DTB reactivity for that cysteine) may be challenging to quantify with accuracy. Averaging the cysteine reactivity changes across several replicate experiments can increase confidence in lower stoichiometry liganding events, and this confidence can be further strengthened if such events show additional features indicative of specific binding (e.g., stereoselectivity).

TROUBLESHOOTING
**Problem 1**
Low intensity of the mass-spectrometry signal. (steps 25 and 26)

**Potential solutions**
- Run a BSA digest standard to test the performance of the mass spectrometer;
- Increase the amount of proteome used;
- Troubleshoot the enrichment step by performing quality control of all essential reagents (IA-DTB, streptavidin beads);
Sonicate the Eppendorf tube with the sample and solvent A before transferring the sample into the mass-spectrometry vial;

**Problem 2**
High levels of unlabeled peptides. (steps 12, 25, and 26)

**Potential solutions**
- Add additional wash steps after the enrichment step;
- Perform quality control of essential reagents (IA-DTB, TMT tags);

**Problem 3**
High levels of channel variability between TMT channels. (steps 15–19)

**Potential solutions**
- Make sure to fully close the Eppendorf tubes and carefully inspect them at each step to ensure absence of leaks;
- Perform quality control of TMT tags;

**Problem 4**
High pressure and/or clogging of the mass-spectrometry column. (steps 25 and 26)

**Potential solutions**
- Make sure you are not overloading the column;
- When possible, use a trap column to avoid clogging of the main column;
- If you see visible gooey residue after high pH fractionation step and removal of solvent using SpeedVac – pass each sample through a C18 tip to remove any salts that could clog the instrument. Re-make your buffers used for high pH fractionation of future samples;
- If you see visible precipitate at the final step of the protocol, spin down the sample (10,000 g; 5 min) and avoid getting the precipitate into the sample loading vial;

**Problem 5**
Low peptide numbers from the IP2 search results. (step 27)

**Potential solutions**
- Check if you are using the right FASTA database for the search (e.g., the correct organism);
- Double-check the search parameters, including the expected masses of static and differential modifications.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Benjamin F. Cravatt (cravatt@scripps.edu).

**Materials availability**
All chemical probes generated in this study are available from the lead contact with a completed Materials Transfer Agreement.
Data and code availability
Raw proteomic data is available from the lead contact upon request. Processed proteomic data is
provided in Data S1 of Vinogradova et al. (Vinogradova et al., 2020)

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AUTHOR CONTRIBUTIONS
E.V.V. and B.F.C. conceived the project and drafted the manuscript.

DECLARATION OF INTERESTS
B.F.C. is a founder and scientific advisor to Vividion Therapeutics. B.F.C. and E.V.V. are co-inventors
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