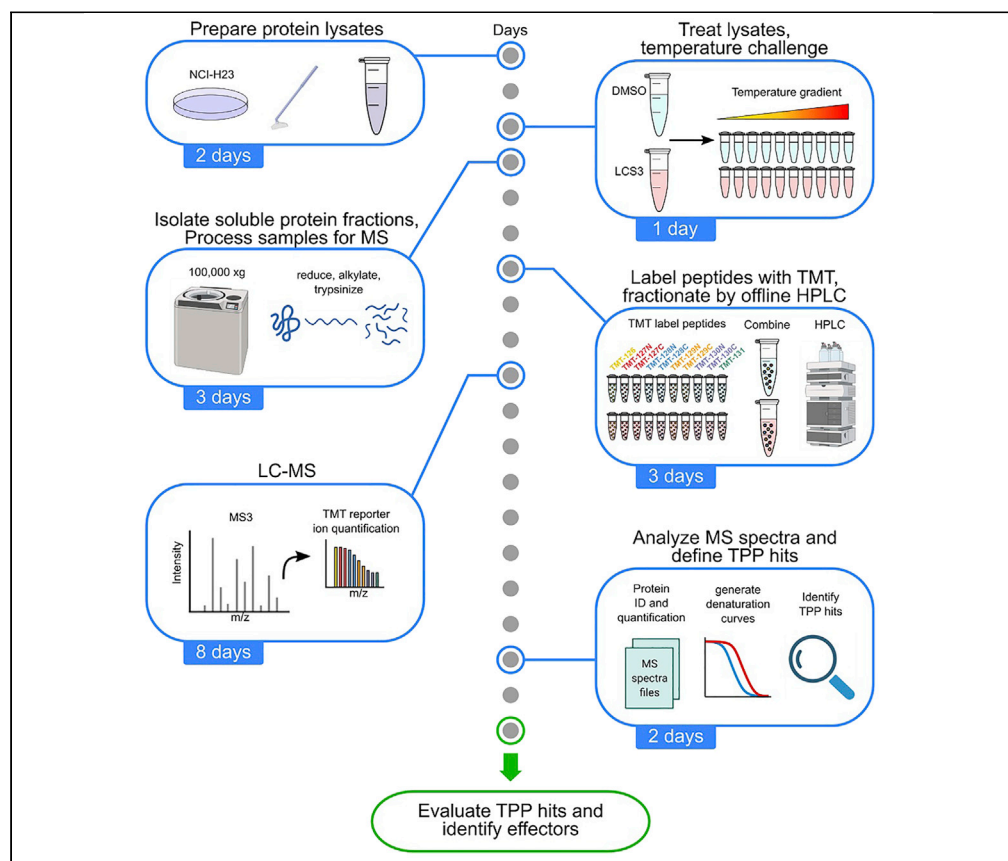# STAR Protocols

Protocol

Tandem mass tag-based thermal proteome profiling for the discovery of drug-protein interactions in cancer cells

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Highlights

Protocol for profiling small-molecule-protein interactions through thermal dynamics

Streamlined sample preparation, labeling, and proteome assessment techniques

Nonparametric analysis of response curves for hit prioritization of candidates

Strategy for follow-up validation and identification of drug binders and effectors

Identification of effector targets is imperative to the characterization of the mechanisms of action of novel small molecules. Here, we describe steps to identify effector drug-protein interactions in lysates derived from cancer cell lines using a thermal proteome profiling (TPP) protocol. Building on existing TTP approaches, we detail the use of an in-solution trypsin digestion technique to streamline sample preparation, a nonparametric analysis to rank proteins for prioritization, and a follow-up strategy for identifying effector interactors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Tandem mass tag-based thermal proteome profiling for the discovery of drug-protein interactions in cancer cells

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SUMMARY

Identification of effector targets is imperative to the characterization of the mechanisms of action of novel small molecules. Here, we describe steps to identify effector drug-protein interactions in lysates derived from cancer cell lines using a thermal proteome profiling (TPP) protocol. Building on existing TTP approaches, we detail the use of an in-solution trypsin digestion technique to streamline sample preparation, a nonparametric analysis to rank proteins for prioritization, and a follow-up strategy for identifying effector interactors. For complete details on the use and execution of this protocol, please refer to Johnson et al. (2022).[1]

BEFORE YOU BEGIN

The TPP approach builds on the principle of the cellular thermal shift assay and the knowledge that, when heated, proteins will denature and become insoluble in a way that is dependent on their biophysical properties in solution.[2,3] TPP relies on the phenomenon in which the temperature required to denature proteins can change in the presence of protein-ligand interactions, such as with a small molecule or metabolite. In the protocol, protein lysates are treated with a small molecule of interest, challenged with a temperature gradient, and then soluble non-denatured proteins are isolated by ultracentrifugation. Proteins are digested and multiplexed using isobaric tandem mass tag (TMT) labels, and the relative abundances of isolated proteins are determined by quantitative mass spectrometry (MS).[4] Since its conception, TPP has been used to study a range of small molecules in a diverse set of biological contexts, including yeast and zebrafish embryos.[5,6]

The first-published TPP protocol described both varying the compound concentration and temperature ranges to identify ligand-protein interactions.[3] The TPP protocol described here focuses on the temperature range method, and we highly recommend reviewing other published works to understand the application of the concentration range method.[2,3,7] A TPP protocol that applies the temperature range method in the unicellular parasite *Leishmania donovani* is also worth reviewing due to its comprehensive annotation of sample preparation and data analysis steps.[8] The protocol below describes the specific steps for performing TPP on H23 lung adenocarcinoma cells treated

with a small molecule compound (2 μM LCS3), that we previously used successfully to identify two disulfide reductase effector targets of this drug.[1] We describe how to perform TPP and the adaptations made for the specific purpose of discovering LCS3 effector targets, as a representative example for identifying ligand-protein interactions using TPP in human cancer cell lines. We present a detailed description of the preparation of cell lysates, treatment, and temperature challenge. We use an in-solution trypsin digest method, which eliminates the gel electrophoresis and extraction steps and quickens the protocol by approximately 2 days. We apply the nonparametric analysis of response curves (NPARC) analytical method[9] and describe suggested follow-up experiments in the expected outcomes section that can be used to prioritize TPP hits for the purpose of discovering small molecule effector protein targets.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate buffered saline	Gibco	Cat# 14190250
0.25% Trypsin-EDTA	Gibco	Cat# 25200114
RPMI media	Gibco	Cat# 11875119
Fetal bovine serum	Gibco	Cat# 1283020
Bovine serum albumin	Sigma	Cat# A3912-100g
DMSO	Thermo Fisher	Cat# BP231-100
LCS3	Chembridge	Cat# 5181525
NaOH	Sigma	Cat# S8045
EtOH	Sigma	Cat# V001229
HEPES	Sigma	Cat# H3375
Trypsin + rLysC mix	Promega	Cat# V5073
Trifluoroacetic acid	Sigma	Cat# 91707
Iodoaceteamide	Bio-Rad	Cat#1632109
DTT	Sigma	Cat# D9779-5g
Ammonium bicarbonate	Sigma	Cat# A11213
Glycine	Sigma	Cat# W328712
Critical commercial assays		
BCA assay kit	Thermo Fisher	Cat# P123227
TMT 10plex isobaric label reagent set plus TMT11-131C label reagent	Thermo Fisher	Cat# A34808
Deposited data		
TPP dataset	This paper	PRIDE: PXD030294
Software and algorithms		
R Environment	The R Project for Statistical Computing	https://www.r-project.org/
R Studio	Rstudio Team	https://www.rstudio.com/
TPP analysis script for this study	This paper	https://zenodo.org/record/6612536#.YpqH_cXMKUk
BioRender 2021	BioRender	https://biorender.com/
DAVID Bioinformatics Database v6.8	Huang et al.[10]	https://david.ncifcrf.gov/
Enrichr	Chen et al. (2013)[11]	https://maayanlab.cloud/Enrichr/
Proteome Discoverer	Thermo Fisher	https://thermo.flexnetoperations.com
Other		
Thickwall polycarbonate centrifuge tubes	Beckman Coulter	Cat# 343775
Orbitrap Fusion™ mass spectrometer	Thermo Fisher	Cat# IQLAAEGAA PFADBMBCX
LTQ Orbitrap Velos™ mass spectrometer	Thermo Fisher	Discontinued
TLA-100 fixed-angle rotor	Beckman Coulter	Cat# 343840
TL-100 ultracentrifuge	Beckman Coulter	Discontinued
1100 series HPLC	Agilent	Discontinued
SimpliAmp Thermal Cycler	Thermo Fisher	Cat# A24811
Eppendorf® ThermoMixer® C	Sigma	Cat# EP5382000023

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HPLC Column Poroshell HPH-C18 (150 × 2.1 mm)	Agilent	Cat# 693775-702T
MicroAmp™ Optical 8-Tube Strip with Attached Optical Caps 0.2 mL	Thermo	Cat# A30588
1.5 mL Safe-Lock tubes	Eppendorf	Cat# 022363204
Corning Cell Scrapers	Sigma	Cat# CLS3011
Sep-Pak tC18 Vac 50 mg Cartridge	Waters	Cat# WAT054960
CentriVap Benchtop Centrifugal Vacuum Concentrator	Labconco	Cat# 7810011
H23 cell line (KRAS^{G12C}, lung adenocarcinoma, derived from 51-year-old male)	ATCC	Cat# CRL-5800

MATERIALS AND EQUIPMENT

Ultracentrifuge

The ultracentrifugation step for isolation of soluble, non-denatured proteins is an essential step in this protocol. For this purpose, we used a TL-100 tabletop ultracentrifuge with a TLA-100 fixed-angle rotor. This rotor has 20 tube cavities so that the entire array of 2 samples loaded into thickwall poly-carbonate tubes can be processed simultaneously (10 tubes each), which reduces variability.

TPP analysis

The original TPP protocol ranked protein significance by analyzing the difference in melting points between two treatment conditions.[3] A more recent nonparametric analysis, called NPARC, was published by the same laboratory.[9] NPARC is a functional data analysis that assesses thermal stability changes by comparing competing nonlinear regression models by their goodness-of-fit. NPARC was reported to outperform melting point-focused analysis in the identification of protein targets.[9] We applied both analyses and only NPARC identified GSR and TXNRD1 as hits, which we successfully validated using *in vitro* enzymatic reactions. Thus, our protocol recommends using the NPARC analysis published by Childs et al.[9]

STEP-BY-STEP METHOD DETAILS

Prepare protein lysates

⏱ Timing: 48 h

1. Seed cells and harvest cell suspension.
   a. Seed 5 million H23 cells in RPMI media supplemented with 10% FBS into two 15 cm plates.
   b. Harvest 24 h later.
      i. Wash twice with ice cold PBS.
      ii. Gently scrape cells off each plate using a cell scraper in 1.5 mL ice cold PBS.
      iii. Transfer each cell suspension into a 15 mL conical tube and keep on ice.
2. Lyse cells in a dry ice-ethanol bath.
   a. Prepare a dry ice-ethanol bath by submerging dry ice in ethanol.
   b. Submerge the PBS-cell suspension in conical tubes under the surface of the dry ice-ethanol bath. Maintain the tube submerged for 1 min to ensure complete freezing.
   c. Remove the conical tubes from the dry ice-ethanol bath and allow them to thaw at ambient temperature or by gentle warming with your hands.
   d. Once lysates have thawed, re-submerge each tube in the dry ice-ethanol bath, and repeat this freeze-thaw process for three cycles in total.
3. Extract protein from cell lysate and determine protein concentration.
   a. Centrifuge the conical tubes at 4,000 × *g* for 10 min at 4°C.
   b. Pool the supernatants by transferring into a single 15 mL conical tube.
   c. Determine protein concentration by BCA assay.

Table 1. Samples for temperature challenge

	37°C	41°C	44°C	47°C	50°C	53°C	56°C	59°C	63°C	67°C	No heat
DMSO-1	1	2	3	4	5	6	7	8	9	10	11
DMSO-2	12	13	14	15	16	17	18	19	20	21	22
LCS3-1	23	24	25	26	27	28	29	30	31	32	33
LCS3-2	34	35	36	37	38	39	40	41	42	43	44

    d. Aliquot supernatants into 1.5 mL microcentrifuge tubes and store at −80°C.

    ⚠ CRITICAL: Wear gloves and be sure to not contact dry ice or ethanol with skin as both can cause serious freezing injury.

    Alternatives: Cells can be mechanically lysed by liquid nitrogen freeze-thaw, or with shearing cells through a 23 g needle. We advise against the use of detergents or sonication in order to ensure maintenance of the native configuration of soluble proteins. If using liquid nitrogen, be sure to use safety goggles to protect from eye injury.

Treat protein lysates and temperature challenge

🕐 Timing: 3 h

This step involves the incubation of protein lysates with the compound and subsequent temperature challenge. Proteins which are in contact with a small molecule will typically exhibit ligand-induced thermal stabilization, a well-established biophysical concept.[12]

4. Thaw samples on ice and aliquot 1.2 mg of protein lysate into four 1.5 mL microcentrifuge tubes and dilute to 1.2 mL with PBS to achieve a protein concentration of 1 mg/mL.
5. Treat lysates with approximately the small molecule EC_{80} or vehicle control, in duplicate. We solubilize LCS3 in DMSO and use 0.1% DMSO as a vehicle control.
6. Gently pulse vortex the samples and incubate for 20 min at room temperature. After the 20 min incubation, put treated lysates on ice.
7. Gently pulse vortex samples again and aliquot each lysate sample into 11 × 0.2 mL PCR tube aliquots of 100 μL as shown in Table 1.

    ⚠ CRITICAL: The next section describes the temperature challenge, which is a crucial series of steps. If using a thermal cycler with a trusted gradient setting, all samples can undergo temperature challenge simultaneously using the gradient heat setting. However, the accuracy of thermal cycler gradient is dependent on the heating and cooling elements of the thermal block, and measured thermal profiles have been reported to not reflect gradient settings.[13] Due to this uncertainty, we instead ran each temperature condition on the thermal cycler alone on a static heating temperature setting, without gradient. Thus, for our heating method it is rational to aliquot the samples into PCR tubes grouped according to temperature. Grouping samples in this manner also minimizes the risk that samples of a same temperature are handled unequally.

    Note: If using a dry ice-ethanol bath, the ethanol can remove permanent marker ink used for labeling. Consider using an ethanol-resistant permanent marker for labeling the PCR tubes. The temperature challenge step is best accomplished by two persons working together in an 'assembly line' as shown in Figure 1.

8. Perform temperature challenge. The organization we used for this setup is shown in Figure 1.
    a. Set thermal cycler to 37°C and place samples 1, 12, 23, 34 on the thermal block and set timer for 3 min.

Figure 1. Organization for temperature challenge incubations

b. Remove samples from thermal cycler and place samples in a room temperature water bath for 3 min.
c. Snap freeze samples in a dry ice-ethanol bath and then incubate on dry ice.
d. Increase thermal cycler to next in sequence temperature and repeat sample heating, water bath incubation, and snap freezing for 41, 44, 47, 50, 53, 56, 59, 63, 67°C groups. Snap freeze the 'No heat' samples (11, 22, 33, 44) without heating.

⏸️ Pause point: The protocol can be paused here by storing the samples at −80°C indefinitely.

Isolate soluble protein fractions

🕓 Timing: 2 h

9. Thaw samples on ice and transfer to 200 μL thickwall polycarbonate centrifuge tubes that fit the TLA-100 rotor.
10. Ultracentrifuge samples.
    a. Place centrifuge tubes (samples 1–10, 12–21) into rotor cavity and insert rotor into ultracentrifuge.
    b. Centrifuge at 100,000 × g for 20 min at 4°C.
    c. Remove the rotor and transfer centrifuge tubes onto the tube stand.
    d. Carefully pipette 90 μL of lysate carefully from the surface as to not capture denatured proteins pelleted by ultracentrifugation. The lysate should be transferred into 1.5 mL microfuge tubes.
    e. Repeat ultracentrifugation steps with samples 23–32, 34–43.

⚠ CRITICAL: Be sure to properly balance the ultracentrifuge to reduce the risk of instrument damage or bodily harm during the 100,000 × g ultracentrifugation.

⏸️ Pause point: The protocol can be paused here by storing the samples at −80°C indefinitely.

Reduce, alkylate, and digest proteins

🕓 Timing: 24 h

11. Prepare reagents for protein reduction and alkylation.[14]
    a. Prepare 200 mM HEPES and adjust to pH 8.0 using 2 N NaOH in water.
    b. Prepare 200 mM dithiothreitol (DTT) in 200 mM HEPES.
    c. Prepare 400 mM of fresh iodoacetamide (IAA) in 200 mM HEPES.

⚠ CRITICAL: DTT and IAA are oxygen-sensitive and light-sensitive, respectively, and should be prepared immediately before use.

12. Reduce and alkylate proteins.
    a. Add 5 μL of 200 mM DTT to each sample and incubate for 30 min at 55°C in a thermomixer.
    b. Add 10 μL of 400 mM IAA to each sample and incubate for 30 min at room temperature while wrapped in aluminum foil or in a dark area.
    c. Add 5 μL 200 mM DTT to each sample to quench the alkylation reaction.
13. Digest proteins.
    a. Resuspend lyophilized Trypsin/Lys-C immediately before use by adding 200 μL of 200 mM HEPES pH 8.0 into each vial of 20 μg Trypsin/Lys-C (concentration: 0.1 μg/μL).
    b. Prepare a digestion solution by adding resuspended Trypsin/Lys-C to 200 mM HEPES pH 8.0 to a concentration of 5 μg/mL with enough volume to add 400 μL to each sample.

    Note: For an experiment with 44 samples at this step, 17.6 mL volume is be needed and we recommend preparing a 19 mL digestion solution containing 95 μg Trypsin/Lys-C (950 μL of resuspended Typsin/Lys-C mix).

    c. Add 400 μL of digestion solution to each sample to achieve approximately a 1:50 enzyme:-protein ratio (2 μg of trypsin to approximately 100 μg of protein in each sample based on the 1.2 mg per 1.2 mL starting amount) and a dilution of approximately 1:5.
    d. Digest proteins for 16 h at 37°C in on a thermomixer.

    ⏸ Pause point: The protocol can be paused here by storing the samples at −80°C indefinitely.

Quality check peptides by mass spectrometry

⏱ Timing: 48 h

14. As a quality check (QC) for the protein digest, transfer approximately 10% (v/v) of each sample (50 μL) into new 1.5 mL microfuge tubes and add 5 μL of 10% trifluoroacetic acid (TFA) to each tube.
15. Desalt peptide QC samples using Stop And Go Extraction (STAGE) tip protocol[15] or a comparable desalting method.
16. Speed vacuum peptides to dryness and reconstitute in 15 μL of water.
17. Determine the approximate peptide concentration by a UV-Vis spectrophotometer.
18. Acidify the reconstituted peptides to 1% (v/v) formic acid and inject approximately 200 ng for MS analysis on a LTQ Orbitrap Velos™ or similar mass spectrometer to assess sample quality.

    Note: Users should observe a typical peptide chromatographic elution profile with a multitude of resolved peaks, and an abundance of multiply charged precursor masses (z = 2–4+) in acquired MS1 spectra.

    Note: Our mass spectrometer for QC is equipped with a 75 μm internal diameter column and therefore a 200 ng injection amount is appropriate. If the dimensions of the user's system setup are different, the injection amount should be scaled up or down to achieve an appropriate on-column loading quantity.

TMT label peptides

⏱ Timing: 3 h

Described below are the steps for TMT labeling peptides. We use an 11-plex TMT set, such that the 11th channel (TMT11-131C) contains an untreated H23 lysate mix that is not subjected to temperature challenge or ultracentrifugation, to enhance the discovery of underrepresented peptides. The 11 samples of each treatment condition should be multiplexed in a single 11-plex TMT set.

19. Prepare reagents for TMT labeling.
    a. Reconstitute lyophilized TMT reagent by adding 500 μL of acetonitrile to each 5 mg TMT reagent tube (10 μg/μL).
    b. Prepare 10 mL of 1 M glycine. Store at 4°C after use.
20. TMT-label the samples.
    a. Add sufficient TMT reagent to achieve a minimum ratio of 2:1 for TMT:peptide (μg:μg) to each sample based on the total concentration of peptide in your sample as determined as part of the QC procedure in step 17. Pulse vortex samples and incubate at room temperature for 30 min.
    b. Repeat the labeling step once more to ensure complete labeling.
    c. Add 15 μL 1 M glycine to each sample to quench the TMT reagent and then pulse vortex. Incubate at room temperature for 15 min to ensure complete quenching.
    d. Speed vacuum samples to dryness to ensure removal of acetonitrile.
    e. Reconstitute the first TMT-labeled sample in 1 mL water by pipetting. Transfer this 1 mL of solution to the next TMT-labeled sample (do not discard the tube) and reconstitute by pipetting. Repeat for all remaining samples to pool all 11 TMT-labeled samples into a single 1.5 mL microcentrifuge tube.

Note: During peptide reconstitution by pipette, some volume can be lost to the sides of the tube. To minimize sample loss, centrifuge all the reconstituted sample tubes for 1 min at 10,000 × *g* and recover the liquid to the final 1 mL of reconstituted solution.

Note: After TMT labeling, TMT reagent tube lids should be enclosed in parafilm and stored at −80°C, while taking care to ensure proper sealing of the tube lids. We have observed stability in the TMT reagent across multiple freeze-thaw cycles and months in storage.

⏸ **Pause point:** The protocol can be paused here by storing the samples at −80°C indefinitely.

Fractionate peptides by offline high-performance liquid chromatography (HPLC)

⏱ Timing: 48 h

To reduce the complexity of the TMT labeled mixture to increase overall proteome coverage, we prepare peptides with a fractionation-concatenation scheme prior to mass spectrometry (MS) analysis. Below are the steps for offline fractionation of peptides with an Agilent 1100 series HPLC. If using an alternative HPLC, users should consult manufacturer's instructions for that instrument. We perform peptide fractionation using a gradient of acetonitrile and 10 mM ammonium bicarbonate, pH 8, with an Agilent Poroshell HPH-C18 column (150 × 2.1 mm) designed for stability in high pH conditions. Each set of TMT-labeled samples is separated into 48 fractions and concatenated into a final set of 12.[16]

21. Acidify all four TMT labeled and pooled samples to pH < 3 by diluting a 10% (v/v) solution of TFA to 1% (v/v) in each sample.
22. Desalt TMT-labeled peptides using a 50 mg C18 SepPak as follows:
    a. Desalt the TMT-labeled peptides using a tC18 50 mg SepPak or equivalent (e.g., TopTip, Strata-X column) on a vacuum manifold.
    b. Precondition the column using two sequential loadings each of 1 mL of acetonitrile + 0.1% TFA and 1 mL of water + 0.1% TFA.

    c. Load the sample and rinse three times with 1 mL of water + 0.1% formic acid.

    d. Elute twice into the same collection tube using 600 μL of 60% acetonitrile so that the final elution volume is 1.2 mL.

Note: The vacuum should be set to provide a flow rate of approximately 1 drop per second.

23. Speed vacuum the desalted TMT-labeled peptide solution to dryness.
24. Reconstitute dried TMT-labeled peptides in 100 μL water and measure the concentration using a UV-Vis spectrophotometer or alternative assay. Dilute your sample to a concentration of 20 μg/μL and add bring to approximately pH 8 by adding 10% (v/v) of a 100 mM ammonium bicarbonate solution.

Note: Reconstitute lyophilized peptides in a volume appropriate for injection to your HPLC system. A good rule of thumb is to reconstitute your sample in a maximum total volume corresponding to the capacity of your HPLC injection loop.

Note: On system start-up and prior to injection, system performance should be verified using an appropriate standard. On our system, we use an on-column injection of 10 μg of a tryptic digest of bovine serum albumin, assessing peak elution reproducibility across the history of the column.

25. Vortex, centrifuge samples at 20,000 × *g* for 1 min to pellet any particulate and transfer the supernatant to a sample vial appropriate for injection on the HPLC system taking care to not transfer any pelleted material.
26. Fractionate samples by HPLC.

    a. Configure your system with a mobile phase A of 10 mM ammonium bicarbonate in water (pH 8), and mobile phase B of 10 mM ammonium bicarbonate in 80% acetonitrile.

    b. Program the HPLC to inject at least 100 μg of peptide sample onto the column and fractionate using a gradient running from 5% – 45% acetonitrile with collection of 48 fractions into a 96-well plate across the appropriate elution window.

    c. Collect fractions from HPLC using a 96-well plate.

    d. Concatenate the 48 fractions into 12 by pooling equal-interval HPLC fractions (i.e., fraction 1 = A1 + B1 + C1 + D1; fraction 2 = A2 + B2 + C2 + D2, etc.).

Note: The appropriate elution window will depend on the chosen column and HPLC system dead volume. Determining the appropriate elution window will require optimization for each HPLC system.

⏸ **Pause point:** The protocol can be paused here by storing the samples at −80°C indefinitely.

LC-MS

⏱ **Timing: 8 d**

Below are the steps for running fractionated, TMT-labeled peptides on an Orbitrap Fusion™ or similar mass spectrometer using a standard tandem MS/MS acquisition method. An example method containing the complete mass spectrometer parameters is provided in Table S1. This method is provided as a guideline, and users should always optimize parameters to provide the optimal results with their own MS system. For sample injection, our nanoflow ultra-high performance HPLC (nano-UHPLC) system uses a direct on-column injection setup (i.e., no trap column) with an analytical column 25 cm in length, prepared in-house and packed with 3 μm C18 beads. The injection and acquisition routine is controlled using Thermo XCalibur (v3.0 or greater) software.

Table 2. Liquid chromatography gradient conditions

Time (mins: seconds)	Duration (mins: seconds)	Flow rate (nl/min)	% buffer A: % buffer B
0:00	0:00	425	97:3
2:00	2:00	425	93:7
96:00	94:00	425	72:28
113:00	17:00	425	40:60
114:00	1:00	425	20:80
120:00	6:00	425	20:80

Note: The mass spectrometer should be optimized and calibrated according to the manufacturer's specifications. Performance of the nano-UHPLC chromatography columns and MS performance should be verified using an appropriate standard. On our system, we use a tryptic digest of BSA to monitor peak elution performance over the lifetime of the chromatography column, and a tryptic digest of a whole cell lysate to ensure optimal operation of the mass spectrometer (e.g., peptide signal, MS/MS identification rate).

27. Speed vacuum the peptide fractions from HPLC to dryness.
28. Reconstitute dried peptides in a volume of 1% formic acid, 1% DMSO appropriate for your nano-UHPLC system configuration and column loading capacity based on the amount of starting material used during HPLC fractionation. A good rule of thumb for a 75 μm internal diameter column is to aim for a final concentration of 200 ng/μL.
29. Load the peptide sample into a 96-well plate and cover with a silicone Axymat.
    a. Program the nano-UHPLC to inject 1 μL of the reconstituted peptides on-column, and gradient elute the peptides with a 2-h ramp of water + 0.1% formic acid (buffer A) and 80% acetonitrile + 0.1% formic acid (buffer B) (Table 2).
    b. Configure the MS to perform tandem MS/MS acquisition of eluted peptides, ensuring the use of parameters appropriate for TMT reporter ion observation (i.e., MS/MS acquisition in a high-resolution analyzer, such as the Orbitrap).

Note: An example method describing data-dependent acquisition with MS1 and MS/MS scanning with the Orbitrap mass analyzer is provided in Table S1.

Mobile phase A: Water, 0.1% formic acid.

Mobile phase B: 80% acetonitrile, 0.1% formic acid.

Note: After drawing sample into the sample loop, sufficient volume should be flowed to move the peptides to the column front. The required volume will depend on the nano-UHPLC system configuration in your lab. On our system, we maintain sample injection volumes at <4 μL, and load by flowing a total volume of 10 μL through the sample loop at a maximum pressure of 400 bar. The nano-UHPLC flow rates and elution conditions will depend on the dimensions of the analytical column used and the capabilities of each system. On our column step, we use a flow rate of 425 nL/min, which provides a consistent operation at a pressure of ∼300 bar.

Analyze mass spectrometry data

⊙ Timing: 2 d

The steps below describe how to analyze the TPP mass spectrometry data. Our code is available, which integrates the original TPP code,[3] the NPARC analysis code,[9] and contains alternate graphics settings for generating denaturation curves. Our code can be downloaded at https://doi.org/10.5281/zenodo.7294843.

Note: We recommend downloading the open source NPARC R package because it contains a comprehensive walkthrough of the NPARC workflow, data preprocessing, function definition and NPARC model fitting.[9] Our justification to use NPARC analysis in lieu of the original TPP analysis is outlined in the materials and equipment section.

30. Use Proteome Discoverer or a comparable proteomics software package such as MaxQuant or FragPipe to identify and quantify proteins in the raw MS spectra files using standard data processing protocols.

Note: In Proteome Discoverer, example processing and consensus workflows that are appropriate for high-resolution MS/MS data are provided with the software and can be used with only minor modifications to the default settings.

Note: Based on the above-described MS acquisition method in which we utilize the Orbitrap analyzer to acquire both the MS1 survey and MS2 fragment ion scans, we recommend the use of 20 parts-per-million (ppm) mass accuracy settings for both precursor and fragment ion tolerance settings. To ensure peptide identification, an appropriate protein sequence database should be acquired from a standard repository, such as UniProt, enzyme specificity classified as trypsin with one allowed missed cleavage, fixed modification masses of +57.02146@C, and variable modification masses of +15.9949@M, +229.16293@K, and +229.16293@peptide N-terminus. Filter data based on the scoring scheme used by the software package, such as Percolator in Proteome Discoverer, to provide a 1% false discovery rate at the protein level and export the resulting data for downstream analysis.

31. Analyze protein abundance data using the open source NPARC R package.[9]

   a. Install all R packages required.

```
> install.packages(''limma'')

> install.packages(''plyr'')

> install.packages(''dplyr'')

> install.packages(''reshape2'')

> install.packages(''ggplot2'')

> install.packages(''xlsx'')

> install.packages(''BiocManager'')

> BiocManager::install(''TPP'')
```

   b. Parse raw data off of Proteome Discover in the sample format given. Read the configuration file formatted according to the samples run.

```
>df_dmso <- read.table(''isobarquant_dmso.txt'', quote=NULL, header=TRUE, fill=TRUE, sep='\t')

> df_lcs3 <- read.table(''isobarquant_lcs3.txt'', quote=NULL, header=TRUE, fill=TRUE, sep='\t')

> config <- read.xlsx(''dup_LCS3_TPP-TR_config.xlsx'', sheetName=''Sheet 1'', check.names=FALSE)
```

   c. Removing proteins with values of 0 or duplicates in the raw data.

```
>datacleanup <- function(datframe){

  datcolnames <- colnames(datframe)

  datframe  <- ddply(datframe,'gene_name',colwise(median,datcolnames[2:length(colnames
(datframe))]))
```

```
  datframe_noNA <- subset(datframe, datframe$rel_fc_126>0.6)

  return(datframe_noNA)}

>df_dmso_noNA <- datacleanup(df_dmso)
```

d. Create a list containing the TMT channel values of all the samples.

```
>dfs <- list(df_dmso_noNA,df_dmso_noNA_2,df_lcs3_noNA,df_lcs3_noNA_2)

>names(dfs) <- c(``Vehicle_1'',``Vehicle_2'',``LCS3_1'',``LCS3_2'')
```

e. Run the analyzeTPPTR() function and save the results to a text file.

```
>TRresultsSp <- analyzeTPPTR(configTable = config,

            methods = ``splinefit'',

            data = dfs,

            resultPath = resultPath3,

            plotCurves =FALSE)
>write.table(TRresults,''TRresults.txt'',quote=FALSE,sep='\t',col.names=TRUE,row.names=FALSE)
```

Note: This generates a NPARC analysis, "splinefit" file to rank proteins by false discovery rate (FDR) adjusted p-value. The default method is "meltcurvefit", which looks for significant differences in melting points between treatment conditions. Our rationale for using the NPARC analysis is detailed in the materials and equipment section.

f. Visualize the median curves for each sample after normalization.

```
> TR_med <- as.data.frame(apply(TRresultsSp [,2:41],2, median, na.rm = TRUE))

> TR_mean <- as.data.frame(apply(TRresultsSp [,2:41],2,mean,na.rm=TRUE))

> temperatures <- rep(c(``37'',``41'',``44'',``47'',``50'',``53'',`56',`59',`63',`67'),
times=4)

> treatment <- rep(c(``DMSO_1'',``DMSO_2'',``LCS3_1'',``LCS3_2''),each=10)

> mediancurve <- cbind(TR_med, TR_mean, temperatures, treatment)

> qplot(data=mediancurve, x=temperatures, y=TR_mean, color=treatment, group=treatment)
```

g. Obtain a list of hits. Proteins with Benjamini-Hochberg adjusted p-value < 0.01 can be considered hits.

```
> splinepass <- filter(TRresultsSp, p_adj_NPARC< 0.01)

> splinepasscomp <- splinepass[complete.cases(splinepass),]

> merge(TRresultsSp,data.frame(splinepasscomp$Protein_ID), by=``Protein_ID'')

> targetspline <- (splinepasscomp$Protein_ID)

> targetspline <- grep(``'',targetspline,value=TRUE)

> cat(targetspline, sep=``\n'')
```

h. Generate denaturation curves for all hits.

```
> trData <- tpptrImport(configTable = config, data = dfs)

> normResults <- tpptrNormalize(data=trData)

> Biobase::featureNames(normResults$normData$Vehicle_1)

> trData_targetspline <- lapply(normResults$normData, function(d)

+ d[Biobase::featureNames(d) %in% targetspline,])

> resultPathsp2 = file.path(getwd(), 'target_splinep0.01')

>  trData_targetspline  <-  tpptrCurveFit(data=trData_targetspline, resultPath=result-
Pathsp2, nCores=4)
```

Note: There is no singular rule for assessing denaturation curve robustness, but we recommend visually assessing all significantly ranked denaturation curves and prioritizing hits that have both high NPARC significance and denaturation curves with robust characteristics. The NPARC FDR-adjusted p-value is a method to prioritize proteins for follow-up studies, but visual examination of denaturation curves can yield additional information. Figure 2 shows denaturation curves from two validated hits (TXNRD1 and GSR), and three hits that were not validated (DNAJC9, NCAPD3, SAP130). As an example, Figure 2 shows that both GSR and DNAJC9 have comparatively similar –log adjusted p-values (4.9 and 4.5, respectively). However, the goodness-of-fit of each GSR sample nonlinear regression line is much greater than that of DNAJC9. Furthermore, the GSR replicates are much closer in agreement than DNAJC9. Lastly, the DNAJC9 vehicle replicate lines do not reflect a discernible pattern. Thus, while subjective, it is important to visually inspect all significant denaturation curves for robust characteristics such as goodness-of-fit, agreement between replicates, and pattern.

EXPECTED OUTCOMES

Using the above protocol, we consistently generate robust denaturation curves for many proteins. In our setup we quantified melting curves for 5593 proteins in the lung adenocarcinoma cancer cell line H23 and identified 77 candidate effector proteins. Ultimately, two proteins (GSR and TXNRD1) were found to mediate crucial biological properties of LCS3.[1] The depth of proteome coverage will be dependent on the size of the proteome of the organism under investigation, the offline fractionation scheme, if used, the LC-MS conditions, and the performance of the mass spectrometer used.

Figure 3 shows an example of a typical chromatogram showing many resolved peaks across the entire elution window. Users should expect the HPLC fractionation or mass spectrometer QC to show similar chromatograms.

NPARC analysis provides a prioritized list of thermally-affected proteins. A TPP experiment is expected to identify many proteins that bind the ligand; only some, or one, may impart the biological phenotype. The affected melting curves of some TPP hits may result from indirect actions of the ligand—for example, by affecting the protein-protein interaction of a protein-ligand complex. Such information may provide important biological information on the function of the effector protein-ligand complex. Thus, it is critical to evaluate the initial TPP hits using various evaluation strategies as outlined in the section below. The majority of approved human drugs target G-protein-coupled receptors, enzymes, ion channels, and transporter proteins.[17] Thus, TPP hits that have defined protein activities such as these may be prioritized in subsequent target validation experiments.

Strategies for evaluation of TPP hits and identification of effectors

The steps outlined above will result in a list of proteins that have been thermally profiled, ranked by NPARC analysis, and manually inspected for denaturation curve robustness. These steps will potentially
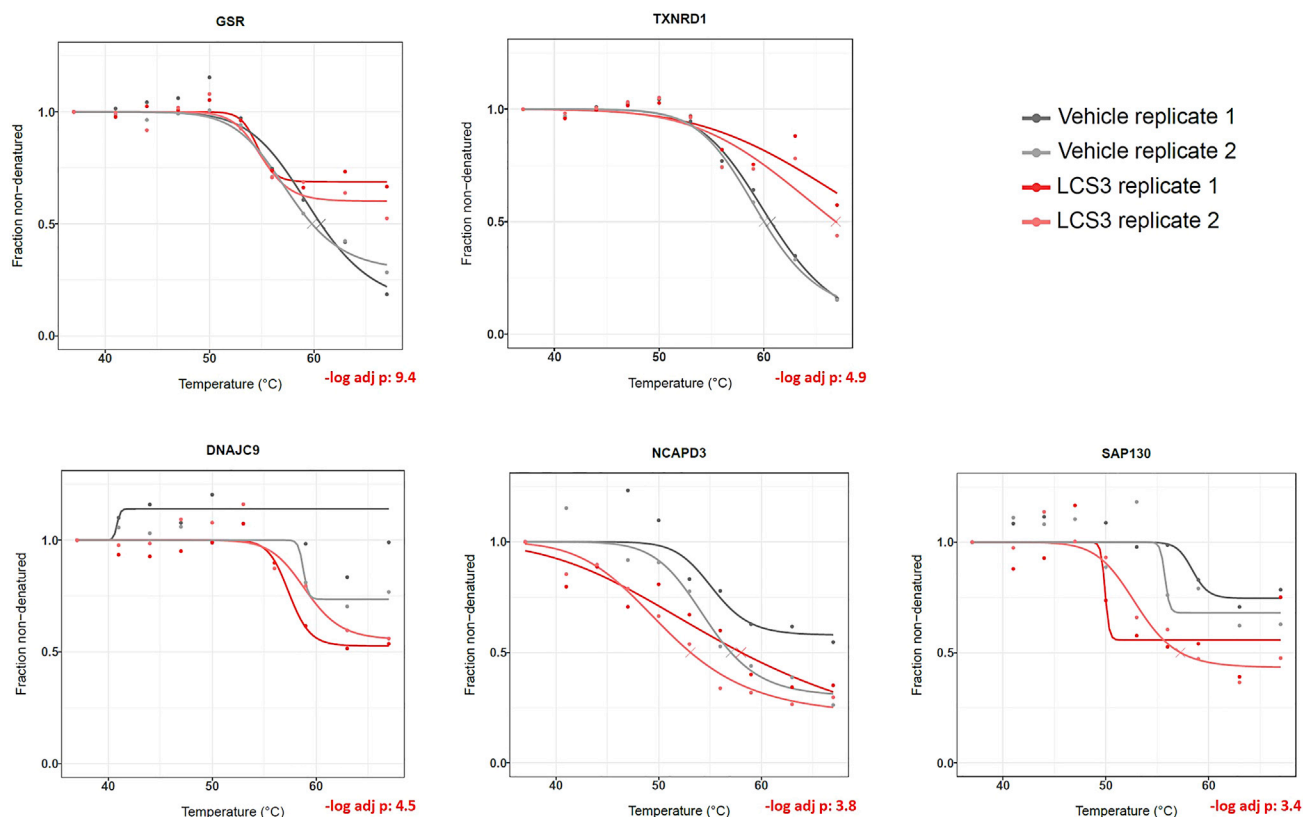
Figure 2. Select TPP denaturation curves
The –log adjusted p-values are specified in red. TXNRD1 and GSR curves were published previously.[1]

produce a list of dozens of TPP hits that will require further prioritization to uncover effector targets—this task is challenging as it depends heavily on the compound and specific characteristics of the phenotype it induces in cells. Further investigation into the identification of effector targets may require protein-specific, tailor-made strategies. It is not uncommon for a small molecule, even optimized 'selective' drugs, to bind multiple proteins; however, there is usually only one or few that impart the biological phenotype associated with the compound. In this section, we will describe suggested strategies that can aid in identifying effector targets in a rational manner, but the ultimate approach will depend on myriad considerations specific to the compound and the phenotypes it engenders.

Following the completion of a TPP experiment, the typical objective is to use TPP data to identify biologically meaningful small molecule-protein binders. It also is unlikely that all TPP hits translate to real protein-small molecule interactions and not all small molecule binding proteins will be biologically meaningful. Thus, two objectives should be defined: (1) to identify proteins that bind or are affected by the compound of interest and (2) to identify protein binders that are biologically meaningful effector targets.

We propose using numerous orthogonal approaches to identify true effector targets. No singular approach is likely to identify the effector targets of any small molecule. There are very few published examples of TPP being used to identify primary effector targets.[1,18–21] A recently published review comprehensively describes recent developments in TPP applications and details all protein targets, including off-targets, that have been identified by TPP to-date.[22]

In our investigation into the protein targets of LCS3, we used a multidimensional, integrative approach to identify effector protein targets. We analyzed expression at the transcriptome and
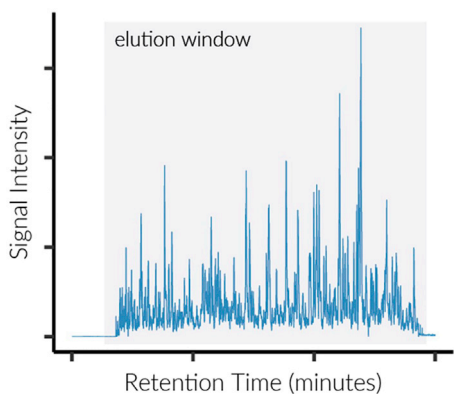
Figure 3. Example chromatogram

proteome levels to understand the phenotype induced by LCS3. Both datasets suggested that lung cancer cells treated with LCS3 activate genes involved in the response to oxidative stress. This led us to focus on TPP hits which fall into the functional category of redox homeostasis. We noted that both GSR and TXNRD1 are homologous, both structurally and by amino acid sequence, which indicates they may have a common interaction with a small molecule. We therefore validated these proposed targets as bona fide TPP hits by performing disulfide reductase activity assays using the respective purified proteins. We also performed functional studies to assess the biological significance of these enzymes in the context of lung adenocarcinoma. Lastly, we conducted a CRISPR screen to identify genes that are key to regulating cellular sensitivity to LCS3.

As mentioned, each TPP experiment will require follow up in a unique manner. In the sections ahead, we outline the rationale for taking the above target validation steps and describe how such strategies can provide a template for investigation in other TPP experiments. We emphasize that this list of strategies is not exhaustive and that each TPP investigation is expected to generate unique results and research directions.

Transcriptome and proteome expression profiling. Understanding how cells regulate gene expression in response to a compound will be key in identifying its mechanism of action and effector targets. RNA sequencing and microarray profiling are examples of methods used to quantify relative mRNA transcript abundance. Label-free, stable isotope labeling by amino acids in cell culture (SILAC), TMT, or other proteomics techniques may be used to quantify relative protein abundances in a sample.[23] Genes or biological processes that are significantly upregulated or downregulated should be noted. Gene Ontology (GO) analysis of biological processes can offer insights into affected biological pathways. The Gene Ontology Resource (www.geneontology.org) provides external tools that integrate GO analysis. Our preference was to use DAVID Bioinformatics (https://david.ncifcrf.gov/) because of its utility and ease of use.[10] A main objective of expression profiling is to identify a defined cellular response or gene signature that is induced by the compound of interest. The open resource, computational systems biology research tool Enrichr (https://maayanlab.cloud/Enrichr/) predicts proteins that mediate a transcriptional response. Identifying a well-characterized cellular response to the compound of interest can help define upstream effectors and mechanism of action.

Functional annotation of TPP hits. After generating a prioritized list of TPP hits, it is rational to determine if there are common themes among the hits. In our LCS3 TPP experiment, the TPP hits in the functional category of redox homeostasis were overrepresented compared to the total proteome. This category aligned with the oxidative stress response that was observed in our gene expression profiling data and thus we were interested to further explore proteins in this functional category. The DAVID Functional Annotation Tool allows the simultaneous querying of GO pathways, InterPro (to find enriched protein domains), and other analyses. As an example, the InterPro search identified 'FAD/NAD-linked

reductase, dimerisation' as the top-ranked protein domain that was enriched in the LCS3 TPP hits, of which GSR and TXNRD1 were included. Small molecules are likely to interact with proteins that have homologous structural domains and therefore any approach to find overrepresented structural domains within a list of TPP hits may yield important information regarding effectors.

Unbiased genetic screening. Unbiased genetic screens provide a starting point for identifying genes with non-redundant cellular functions.[24] Genetic screens involving libraries of siRNA, shRNA, or sgRNA can complement TPP and provide insights into which biological processes treated cells become reliant on. Accordingly, TPP hits that mediate such processes may be prioritized for further functional studies.

Comparison with established agents that have known protein targets. When considering TPP hits as plausible effector targets, it is rational to investigate established inhibitors that target the protein target of interest. For example, if EZH2 (a histone-lysine N-methyltransferase enzyme) is a top TPP hit, then it is rational to contrast the bioactivities of the small molecule of interest with those of other established EZH2 inhibitors. If the biological response induced by the small molecule of interest mimics that of the established inhibitor, there is increased likelihood that they exert bioactivity via a common target or mechanism.

Evaluating cell line sensitivities. When investigating TPP hits as potential effector targets, it is useful to characterize the response to the compound in diverse cell lines or cell types. Each cell line provides a unique genetic context and understanding which cell types are sensitive or resistant to the small molecule of interest is useful in determining predictors of response and in identifying the effector target. In the field of cancer, the Cancer Dependency Map (https://depmap.org/portal/) and Genomics of Drug Sensitivity in Cancer (www.cancerrxgene.org) databases are open resources that describe gene expression, genomic, and drug sensitivity data for over one thousand cancer cell lines and hundreds of established compounds. Such databases allow for the predictive modeling of drug sensitivity[25] and can be useful when investigating TPP hits.

Validation of small molecule-protein interaction. A major milestone in validating a TPP experiment is discovering that the small molecule of interest affects the function of a TPP hit. Even if a validated TPP hit is not the main effector target, identification of any off-target interaction with a small molecule of interest is valuable data. If commercially available purified protein exists for the TPP hit, it will avoid the need for protein overexpression and purification. If the TPP hit is an enzyme, one will need to establish its enzymatic activity and test for agonism or antagonism by the compound. Validating any novel protein activity assay adds an additional layer of complexity into this target validation step.

If the protein of interest does not have an established activity readout, it may be useful to validate protein-small molecule binding by immunoprecipitation, cellular thermal shift assay (CETSA), or alternative physical interaction determination experiments such as surface plasmon resonance (SPR), biolayer interferometry (BLI) or scintillation proximity assays (SPAs). Additionally, *in silico* molecular docking can elucidate probable spatial protein regions of small molecule binding. In the absence of agonism or antagonism data, such methods can produce evidence to support claims of protein-small molecule interactions.

Functional studies to demonstrate importance of effector target. If protein agonism, antagonism, or direct binding by the small molecule of interest has been established, one still needs to assess the biological significance of the protein-small molecule interaction, as not all protein binders are effectors. One should establish if downstream molecular processes are affected in a way that is consistent with the hypothesized target interaction. For example, if a histone acetyltransferase is suggested to be the target, observing changes in histone acetylation may be supportive of such a claim. If cellular and biochemical experiments of downstream molecular processes reveal evidence of protein inhibition, then it builds a case that the proposed target is a bona fide effector.

In cancer target studies, the generation of resistant cell lines from originally sensitive cell lines by step-wise dose escalation with continuous compound exposure can allow for the identification of biological processes and genes that promote treatment resistance. Such studies may be important in uncovering the effector target and compensatory mechanisms that govern cell survival upon treatment.

Creating variant target proteins harboring amino acid changes to a predicted binding site and then evaluating the essentiality of this amino acid using *in vitro* assays and *ex vivo* phenotype assays can also assist in establishing that a target protein is the biologically meaningful effector.

Lastly, functional studies to modify the expression of the proposed target, either through vector-mediated overexpression, or RNAi-mediated gene silencing, or CRISPR-Cas9-mediated knockout, may shed light on the functional significance of the proposed protein target. As an example, if over-expression of the proposed protein target decreases sensitivity to the small molecule of interest, then it may be plausible that the protein target is an effector.

Using orthogonal strategies such as those described above can aid in establishing which TPP hits are main effectors of the compound of interest.

QUANTIFICATION AND STATISTICAL ANALYSIS

The NPARC analysis used for this protocol[9] uses the Benjamini-Hochberg method to control false discovery and corrects for multiple hypothesis testing. All p-values are Benjamini-Hochberg corrected and the significance cutoff is $p < 0.01$.

LIMITATIONS

Target identification by TPP

Due to the broad dynamic range of protein abundances in all cells and technical limitations, mass spectrometry is not able to detect all proteins in any given proteome. Due to the lack of detergents used during cell lysis, this protocol favors the detection of more abundant, soluble, mainly cytosolic, proteins and thus is biased against the detection of membrane-bound proteins. Thus, no denaturation curves will be produced for many proteins. For users that may be interested in enriching for membrane-bound proteins, consider reviewing two methods protocols that specifically interrogate membrane proteins[26] and the cell surface proteome.[27]

There is also no assurance that any small molecule will be bioactive through a mechanism that involves direct binding of proteins that is discoverable by TPP. While most therapeutics in clinical use and development seek to target proteins, other drugs such as cisplatin and doxorubicin, exert bioactivity mainly through the targeting of nucleic acids in proliferating cells.[28]

Analysis

The significance determined by NPARC analysis does not always translate into strength of a drug-protein interaction, which can obfuscate interpretation of TPP data. It is unlikely that any user can systematically interrogate all TPP hits. It will often be difficult to evaluate TPP hits that lack established activity assay protocols.

TROUBLESHOOTING

Problem 1

MS quality check in step 18 identifies poor quality sample.

Potential solution

Run another label-free sample of known quality with the same MS settings. If the other sample is shown to be of good quality, then retry the TPP quality check samples. If TPP quality check samples

are still of poor quality, start the experiment from step 4 if sufficient lysate is available. If sufficient lysate is unavailable, restart the experiment from step 1.

Problem 2
Denaturation curves produced in step 31 are flat instead of sigmoidal.

Potential solution
During protein discovery and quantification, TMT channel abundances may have been scaled or normalized incorrectly. Higher temperature incubations of lysate samples consistently result in decreased median protein abundances, so repeat the protein quantification using non-scaled abundance settings.

Problem 3
Median denaturation curve in step 31 is not smooth and has points that deviate significantly from the line of best fit.

Potential solution
This is likely due to human error. Be sure to handle all temperature conditions uniformly during sample processing in step 8. Use timers and have two persons to perform the temperature challenge. Be careful to not disturb the pellet with a pipette tip when extracting supernatant after ultracentrifugation.

Problem 4
Insufficient protein abundance in step 4.

Potential solution
We have observed that freeze-thaw lysis of two 15 cm plates of 5 million cells yields an excess of the 4.8 mg of protein that is required for this protocol. If harvest quantity is insufficient or uncertain in advance, the quantity of cells seeded and harvested in step 1 can be increased.

Problem 5
Too many or too few TPP hits in step 31.

Potential solution
In our experiments, a p-value threshold of 0.01 was suitable to create a list of 50–100 significant TPP hits. If desired, this p-value threshold could be more or less stringent (i.e., 0.05 or 0.001).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gregg Morin (gmorin@bcgsc.ca).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The Orbitrap Fusion Method Summary is uploaded as Table S1. Processed TPP data (NPARC analysis) are uploaded as Table S2 and raw data have been uploaded as PRIDE accession PXD030294.

Code used for TPP analysis is available at (https://doi.org/10.5281/zenodo.7294843).

All additional information needed to reanalyze data reported in this publication is available from the lead contact, Gregg Morin (gmorin@bcgsc.ca).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.102012.

AUTHOR CONTRIBUTIONS

Methodology, F.D.J., A.L., C.S.H., W.W.L., G.B.M.; Formal Analysis, F.D.J., A.L., W.W.L., G.B.M.; Visualization, A.L., C.S.H., W.W.L.; Writing—Original Draft, F.D.J., C.S.H., W.W.L., G.B.M.; Writing—Review and editing, F.D.J., C.S.H., W.W.L., G.B.M.; Resources, W.W.L.; Supervision, W.W.L., G.B.M.

DECLARATION OF INTERESTS

W.W.L. is a consultant of HyperBio Therapeutics.

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