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Thermal Proteome Profiling to Identify Protein-ligand Interactions in the Apicomplexan Parasite *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is a single-celled eukaryotic parasite that chronically infects a quarter of the global population. In recent years, phenotypic screens have identified compounds that block parasite replication. Unraveling the pathways and molecular mechanisms perturbed by such compounds requires target deconvolution. In parasites, such deconvolution has been achieved via chemogenomic approaches—for example, directed evolution followed by whole-genome sequencing or genome-wide knockout screens. As a proteomic alternative that directly probes the physical interaction between compound and protein, thermal proteome profiling (TPP), also known as the cellular thermal shift assay (CETSA), recently emerged as a method to identify small molecule–target interactions in living cells and cell extracts in a variety of organisms, including unicellular eukaryotic pathogens. Ligand binding induces a thermal stability shift—stabilizing or destabilizing proteins that change conformationally in response to the ligand—that can be measured by mass spectrometry (MS). Cells are incubated with different concentrations of ligand and heated, causing thermal denaturation of proteins. The soluble protein is extracted and quantified with multiplexed, quantitative MS, resulting in thousands of thermal denaturation profiles. Proteins engaging the ligand can be identified by their compound-dependent thermal shift. The protocol provided here can be used to identify ligand-target interactions and assess the impact of environmental or genetic perturbations on the thermal stability of the proteome in *T. gondii* and other eukaryotic pathogens.

Keywords: Thermal proteome profiling, CETSA, Toxoplasma, Parasite, Proteomics

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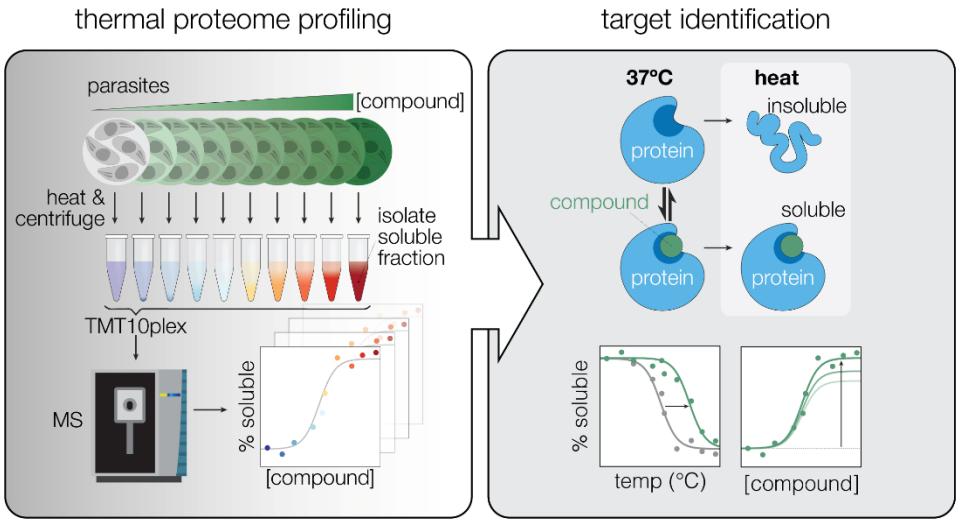
Graphic abstract:



Thermal proteome profiling for target identification in the apicomplexan parasite *T. gondii*.

Background

Target deconvolution is a major challenge for the wealth of compounds identified through phenotypic screening. Chemogenomic approaches, such as directed evolution or drug screens, have been the favored tools for target identification in eukaryotic parasites (Paquet *et al.*, 2017; Cowell *et al.*, 2018; Luth *et al.*, 2018; Rosenberg *et al.*, 2019; Harding *et al.*, 2020). Such approaches require culturing parasites and host cells under compound treatment for extended periods and often identify pathways indirectly affected by a small molecule rather than the target itself. In contrast, several proteomic methods developed in the past decade directly identify interactions between compounds and protein targets (McClure and Williams, 2018; Conway *et al.*, 2021). For example, enrichment of interacting proteins can be performed with derivatized compounds for affinity-purification followed by mass spectrometry (MS). However, these approaches require specialized chemistry and introduce a linker and other chemical groups to the compound of interest, which may affect its behavior.

Thermal proteome profiling (TPP), also known as the cellular thermal shift assay (CETSA), offers a label-free approach that can be performed in a variety of formats that preserve cellular physiology, including *in situ* (Dai *et al.*, 2019; Mateus *et al.*, 2020b). Interactions with a target are identified by a compound-dependent shift in the protein's thermal profile. Cells or cell extracts are treated with the compound and heated to induce thermal denaturation. Aggregated proteins are removed, and soluble proteins are quantified by MS to generate melting curves for each protein. TPP has recently identified the targets of antiparasitic compounds in the apicomplexan parasites *Plasmodium falciparum* (Dziekan *et al.*, 2019; Lu *et al.*, 2020) and *Toxoplasma gondii* (Herneisen *et al.*, 2020), as well as in the trypanosome *Leishmania donovani* (Corpas-Lopez *et al.*, 2019).

The application of TPP extends beyond target deconvolution (Becher *et al.*, 2018; Dai *et al.*, 2018 and 2019; Sridharan *et al.*, 2019; Mateus *et al.*, 2020b). Alterations to protein state and stability may arise from conformational changes, post-translational modifications, altered localization, and interactions with other proteins and biomolecules such as metabolites and nucleic acids. For example, we performed TPP on parasites lacking mitochondrial DegP2 to identify proteins with altered stability based on the loss of this protease (Harding *et al.*, 2020). Genetic perturbations in conjunction with functional proteome profiling are in the early stages (Mateus *et al.*, 2020a) and may be especially well-suited to map the unannotated parts of parasite proteomes.

While TPP has been performed predominantly in mammalian systems, it is expanding to other organisms (Corpas-Lopez *et al.*, 2019; Dziekan *et al.*, 2019; Volkening *et al.*, 2019; Lu *et al.*, 2020; Herneisen *et al.*, 2020; Harding *et al.*, 2020; Jarzab *et al.*, 2020). We believe this approach pairs particularly well with the study of eukaryotic parasites,

whose evolutionary divergence complicates identifying molecular pathways by genomic annotation or bioinformatic analysis. For that reason, we provide a detailed protocol describing our thermal profiling pipeline developed for the organism *T. gondii*. Below, we identify key considerations for selecting a TPP workflow appropriate for the researcher's biological question. Step-by-step guidelines follow.

Types of experiment

In this protocol, we stratify steps by choice of material and treatment. TPP can be performed on live parasites or parasite lysates and by melting samples over a range of 10 temperatures ("temperature range") or over a range of 10 compound concentrations melted at a single temperature ("concentration range"). These variations give rise to four permutations described in the *Procedure* as *(B) Lysate Temperature Range Experiment*, *(C) Parasite Temperature Range Experiment*, *(D) Lysate Concentration Range Experiment*, and *(E) Parasite Concentration Range Experiment*. Each experiment has advantages and disadvantages (Franken *et al.*, 2015; Dai *et al.*, 2019; Mateus *et al.*, 2020b). For example, experiments using live cells are more physiological but combine direct and indirect effects. Lysate experiments may more directly identify ligand-protein interactions, but loss of cellular compartmentalization can also lead to non-physiological interactions. Concentration range experiments yield more information-rich thermal profiles, but real interactions may be missed if the thermal challenge temperatures are suboptimal (too low or too high) and the overall coverage of the proteome is reduced due to global denaturation.

Treatment conditions

We have performed thermal profiling experiments on extracellular parasites to avoid the added complexity of the host proteome and confounding effects from compound permeability and host metabolism. Compound treatments are often performed on intracellular parasites; however, the appropriate concentration of compound for the thermal profiling experiment should be determined by assays using extracellular parasites. The thermal profiling experiment should mimic assay conditions as closely as possible. Considerations include the amount of time needed for the compound to arrive at diffusion and binding equilibria, the buffer in which the equilibration takes place, and equilibration temperature (*e.g.*, room temperature vs. 37°C). Mammalian studies have often performed the incubation in PBS (Reinhard *et al.*, 2015; Savitski *et al.*, 2014; Franken *et al.*, 2015). We have used a buffer composition resembling the ionic makeup of the host cytosol (Herneisen *et al.*, 2020; Harding *et al.*, 2020). Buffers should lack serum, which would overwhelm parasite signals that can be quantified by MS.

We aim to process 25 µg of protein per reference sample, which in our experience corresponds to the material from 1×10^7 extracellular parasites of the type I RH strain. We subject concentration-range samples to at least two different thermal challenge temperatures; we have found 54°C and 58°C to work well while still providing sufficient coverage of the proteome. The thermal challenge temperatures may need to be optimized for each experiment; further commentary is provided in the *Data analysis* section.

Lysis conditions

The final lysis buffer composition should contain 0.5-1% IGEPAL CA-630 (also known as NP-40), which provides a balance between solubilizing membrane proteins without re-solubilizing aggregated proteins (Reinhard *et al.*, 2015). For most experiments, the lysis buffer should contain protease inhibitors (and optionally phosphatase inhibitors, depending on the focus of the experiment) and benzonase for digestion of nucleic acids prior to the SP3 cleanup. If the compound of interest is thought to affect proteases, phosphatases, or nucleic acid binding activity, then these supplements should be omitted until after the *Separation of Soluble and Aggregated Protein*. Our lysis buffers have had an ionic composition similar to PBS (Herneisen *et al.*, 2020) and an intracellular-like buffer (Harding *et al.*, 2020), depending on the application. The ionic composition of the buffer (*e.g.*, presence of ATP and metabolites) can substantially influence the melting behavior of proteins (Lim *et al.*, 2018; Sridharan *et al.*, 2019). The concentration of parasite lysate also influences melting behavior; therefore, it is crucial to count the number of parasites prior to lysis and use a consistent lysis buffer volume for the number of parasites. Following harvest, parasites should be resuspended at least once in a wash buffer that is similar in composition to the lysis buffer (but lacking detergents) to dilute cell culture contaminants, such as serum proteins.

Materials and Reagents

1. T12.5 flask (*e.g.*, Corning Falcon Tissue Culture Flasks, catalog number: 29185-298)
2. T175 flask (*e.g.*, CELLSTAR® Filter Cap Cell Culture Flasks, catalog number: 82050-872)
3. 15-cm dish (*e.g.*, Corning Falcon® Tissue Culture Dishes, catalog number: 25383-103)
4. Corning® 150 ml Bottle Top Vacuum Filter, 0.22 µm Pore 13.6 cm^2 CA Membrane (Corning, catalog number: 430624)
5. 50 ml conical tube (Corning, catalog number: 430829)
6. Human foreskin fibroblast (HFF) cells (ATCC, catalog number: SCRC-1041)
7. *T. gondii* cell lines (RH, *e.g.*, ATCC 50838 or PRA-319)
8. *T. gondii* filter (Whatman Pop-Top and Swin-Lok Plastic Filter Holders for 47 mm membrane filter size, *e.g.*, VWR catalog number: 28163-089, with GE Healthcare Whatman Nuclepore Hydrophilic Membrane 3 or 5 µm circles, catalog number: 111112 or 111113)
9. Cell scraper (Corning® Small Cell Scraper, catalog number: 3010)
10. Protein low-bind tube (*e.g.*, Eppendorf™ LoBind Microcentrifuge Tubes, 1.5 ml Thermo Fisher Scientific, catalog number: 13698794)
11. 8-strip PCR tubes (*e.g.*, Genesee Scientific, catalog number: 27.125 U)
12. Thickwall polycarbonate open-top ultracentrifuge tubes (0.2 ml, 7 × 20 mm; Beckman Coulter, catalog number: 343775)
13. Protein low-bind 96-well plate (Eppendorf, catalog number: 951032905)
14. Syringes 20 ml (BD Biosciences, catalog number: 302830)
15. Hydrophobic Sera-Mag Speed Beads (GE Healthcare, catalog number: 65152105050250, ~50 mg/ml, keep at 4°C until use)
16. Hydrophilic Sera-Mag Speed Beads (GE Healthcare, catalog number: 45152105050250, ~50 mg/ml, keep at 4°C until use)
17. DMEM (Thermo Fisher Scientific, catalog number: 11965118, keep at 4°C until use)
18. Newborn Calf Serum USA origin, heat Inactivated, sterile-filtered, suitable for cell culture (Sigma-Aldrich, catalog number: N4762-500ML, keep at -80°C until use)
19. 10 mg/ml gentamicin (Life Technologies, catalog number: 15710072, room temperature)
20. 200 mM L-glutamine (Life Technologies, catalog number: 25030081, keep at -20°C until use)
21. 250 U/µl benzonase (Sigma-Aldrich, catalog number: E1014-25KU, store at -20°C)
22. 100× Halt Protease Inhibitor Cocktail (Life Technologies, catalog number: 87786)
23. IGEPAL® CA-630 viscous liquid (Sigma-Aldrich, catalog number: I3021-50ML)
24. 10× PBS suitable for tissue culture (*e.g.*, VWR, catalog number: 45001-130)
25. DC Protein Assay (Bio-Rad, catalog number: 5000116)
26. Tris(2-carboxyethyl)phosphine (TCEP; Pierce, catalog number: 20490; keep at -20°C until use)
27. Methyl methanethiosulfonate (MMTS; Thermo Fisher Scientific, catalog number: 23011, keep at 4°C)
28. Ethyl alcohol, Pure 200 proof, HPLC/spectrophotometric grade (Sigma-Aldrich, catalog number: 459828-1L)
29. Sequencing-grade trypsin (*e.g.*, Promega, catalog number: V5113, keep at -80°C until use)
30. Triethylammonium bicarbonate buffer 1.0 M, pH 8.5 (Sigma-Aldrich, catalog number: T7408-100ML, keep at 4°C)
31. Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, catalog number: 23290, keep at 4°C until use)
32. TMT10plex Isobaric Label Reagent Set (Thermo Fisher Scientific, catalog number: 90110, keep at -20°C until use)
33. 50% hydroxylamine (Thermo Fisher Scientific, catalog number: 90115)
34. Pierce high pH fractionation kit (Thermo Fisher Scientific, catalog number: 84868, keep at 4°C until use)
35. Ultra-high-performance liquid chromatography (UPLC)-MS acetonitrile (Thermo Fisher Scientific, catalog number: A9561)
36. UHPLC-MS water (Thermo Fisher Scientific, catalog number: W81)
37. Pierce Formic Acid, LC-MS Grade (Thermo Fisher Scientific, catalog number: 28905)
38. DMEM + 3% CFS (see Recipes)

39. PBS (see Recipes)
40. 10% IGEPAL CA-630 (also known as NP-40) (see Recipes)
41. 10× CETSA buffer (see Recipes)
42. CETSA wash buffer (see Recipes)
43. CETSA lysis buffer (see Recipes)
44. 1 M TCEP stock solution (see Recipes)
45. 200 mM MMTS stock solution (see Recipes)
46. Buffer A (see Recipes)
47. Buffer B (see Recipes)

Equipment

1. CO_2 incubator (Thermo Fisher Scientific Forma Steri-Cycle 370, catalog number: 370)
2. Clinical benchtop centrifuge (Eppendorf, model: Centrifuge 5810R, catalog number: 022625101)
3. Microcentrifuge (Eppendorf, model: Centrifuge 5424R [discontinued], alternatives include Centrifuge 5425/5425 R)
4. Minicentrifuge (VWR Galaxy Mini Centrifuge, catalog number: 37000-700)
5. Hemocytometer (VWR Counting Chamber, catalog number: 1517O-173)
6. Thermal cyclers (Bio-Rad C1000 Touch™ Thermal Cycler with Dual 48/48 Fast Reaction Module, catalog number: 1851148 and Bio-Rad C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module, catalog number: 1851197)
7. Benchtop ultracentrifuge (Beckman Ultra MAX [discontinued], alternatives include the Optima MAX-XP and Optima MAX-TL)
8. Thermo mixer (Eppendorf, model: ThermoMixer C, catalog number: 5382000023 with 1.5 ml SmartBlock, catalog number: 5360000038)
9. Magnetic stand (Invitrogen Dynamag 2, catalog number: 12321D)
10. Vacuum centrifuge (Savant™ Universal SpeedVac™ Vacuum System, catalog number: SPD111V and, catalog number: UV5450)
11. Lyophilizer (Labconco FreeZone Triad Freeze Dryer, catalog number: 794001030)
12. Orbitrap mass spectrometer (Thermo Fisher Scientific Q Exactive HFX [discontinued] or Exploris 480, catalog number: BRE725533) with optional FAIMS Pro Interface (Thermo Fisher Scientific, catalog number: FMS02-10001)
13. MS-coupled LC system (Thermo Fisher Scientific EASY-nLC 1200, catalog number: LC140) with Acclaim PepMap 100 75 µm × 2 µm nanoViper trapping column (Thermo Fisher Scientific, catalog number: 164946) and PepMap RSLC C18 3 µm, 100A, 75 µm × 15 cm analytical column (Thermo Fisher Scientific, catalog number: ES900)
14. Pierce formic acid, LC-MS grade (Life Technologies, catalog number: 28905)
15. UPLC-MS acetonitrile (Thermo Fisher Scientific, catalog number: A9561)
16. UPLC-MS water (Thermo Fisher Scientific, catalog number: W81)

Software

1. Proteome Discoverer, version 2.4 (Thermo Fisher Scientific)
2. R, version 4.0 or later: https://cran.r-project.org/
3. Tidyverse package, version 1.3: https://cran.r-project.org/web/packages/tidyverse/index.html
4. TPP package, release 3.12: https://bioconductor.org/packages/TPP/

Procedure

This protocol assumes readers are familiar with *T. gondii* parasite and host cell propagation. For standard reviews, see Roos *et al.* (1994) and Jacot *et al.* (2020).

A. Parasite harvest

1. Infect T175 flasks or 15-cm dishes with confluent HFFs with 2×10^7-5×10^7 RH tachyzoites each, which is equivalent to parasites from one fully lysed T12.5 flask, 40-48 h before the assay. Enough T175's should be infected to harvest 4×10^8 parasites for the assay. The yield may vary depending on host cell age, parasite strain, and treatment; in our experience, 3-4 15-cm dishes are usually sufficient to achieve this number of parasites.
2. When the parasites have fully lysed from the monolayer, scrape the flask and collect the media containing extracellular parasites. A fully lysed monolayer contains an abundance of extracellular parasites and few remaining attached host cells. Remove host cell debris by passing the media through a 3 µm filter into one 50 ml conical vial per flask or dish.
3. Concentrate the parasite solution by centrifuging the conicals at 1,000 × *g* for 10 min at room temperature in a centrifuge with swinging bucket rotors. Discard the supernatant. Resuspend the parasite pellet in 1 ml of wash buffer (lysis buffer without detergents, inhibitors, or enzymes) and transfer the parasite suspension to a 1.5 ml protein low-bind tube.
4. Create a 1:500 dilution of the parasite suspension and count using a hemocytometer.
5. Centrifuge the parasites at 1,000 × *g* for 10 min at room temperature. Discard the supernatant.
6. Depending on the desired treatment, proceed to section *(B) Lysate Temperature Range Experiment, (C) Parasite Temperature Range Experiment, (D) Lysate Concentration Range Experiment, or (E) Parasite Concentration Range Experiment*.

B. Lysate temperature range experiment

1. Parasite lysis
   a. Resuspend the parasite suspension in 100 µl lysis buffer per 2×10^7 parasites (see lysis considerations in the *Background*). Sufficient parasites (4×10^8) should be harvested for at least 1.1 ml of lysate, with a small amount of excess to account for pipetting error in the steps below.
   b. Allow lysis to proceed on ice for 15 min with occasional mixing by pipetting.
2. Compound treatment
   a. Prepare a compound dilution in the lysis buffer at 2× the desired final concentration and a vehicle solution with an equivalent amount of DMSO (or appropriate vehicle). Aliquot 550 µl of each solution into a 1.5 ml protein low-bind tube.
   b. Combine 550 µl of parasite lysate with 550 µl of the 2× compound or vehicle solution and gently pipette to mix. The compound is now at the desired final concentration.
   c. Aliquot 100 µl of the parasite suspension with vehicle or compound into ten labeled PCR tubes corresponding to the anticipated melting temperatures (see below).
   d. Allow the solution to equilibrate at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
3. Thermal challenge
   a. Briefly collect the liquid in the bottom of the tubes using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. Place the PCR tubes in the appropriate orientation on the thermal cycler, such that the tubes with lysate match the desired temperature.
   Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added; the precise sequence depends on the temperature gradient that can be

achieved by the user's thermal cycler. We have used melting temperatures of 37°C, 41°C, 43°C, 47°C, 50°C, 53°C, 56°C, 59°C, 63°C, and 67°C split across two PCR strip tubes in 48-well thermal cyclers.

c. Allow denaturation to occur for 3 min.
d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
e. Briefly collect evaporated liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
f. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

C. Parasite temperature range experiment

1. Compound treatment
   a. Prepare a compound solution at 2× the desired final concentration and a vehicle solution with an equivalent amount of DMSO (or appropriate vehicle). Aliquot 550 µl of each solution into a 1.5 ml protein low-bind tube.
   b. Combine 550 µl of parasite suspension with 550 µl of the 2× compound or vehicle solution and gently but thoroughly pipette to mix. The compound is now at the desired final concentration.
   c. Aliquot 100 µl of the parasite suspension with vehicle or compound into ten labeled PCR tubes corresponding to the anticipated melting temperatures (see below).
   d. Allow the compound to equilibrate with the parasites at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
2. Thermal challenge
   a. Briefly collect evaporated liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. Place the PCR tubes in the appropriate orientation on the thermal cycler, such that the tubes with lysate match the desired temperature.
      Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added; the precise sequence depends on the temperature gradient that can be achieved by the thermal cycler. We have used melting temperatures of 37°C, 41°C, 43°C, 47°C, 50°C, 53°C, 56°C, 59°C, 63°C, and 67°C split across two PCR strip tubes and 48-well thermal cyclers.
   c. Allow denaturation to occur for 3 min.
   d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
3. Parasite lysis
   a. Briefly collect evaporated liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. Add 20 µl of 6× lysis buffer to each tube and gently pipette to mix (see treatment considerations in the *Background*). Allow the parasites to lyse on ice for at least 15 min.
   c. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

D. Lysate concentration range experiment

1. Parasite lysis
   a. Resuspend the parasites suspension in 100 µl lysis buffer per 2×10^7 parasites (see lysis considerations in the *Background*). Sufficient parasites (4×10^8) should be harvested for at least 1.1 ml of lysate, with a small amount of excess to account for pipetting error in the steps below.
   b. Allow lysis to proceed on ice for 15 min with occasional mixing by pipetting.
2. Compound treatment
   a. Prepare a dilution series of ten concentrations of the compound, including vehicle alone, at 2× the desired final concentration in lysis buffer. Aliquot 110 µl of the 2× compound solution into a PCR tube.
      Note: We advise ensuring that the same concentration of vehicle is maintained across all samples by preparing the dilution series into a lysis buffer containing a vehicle concentration equal to that of the highest compound concentration.

b. Aliquot 110 µl of the parasite lysate into the PCR tubes containing 2× compound solution. The compound is now at the final desired concentration, and the volume in each tube is 220 µl.
c. Use a multichannel pipette to gently mix the lysate and transfer half the volume (110 µl) to another set of PCR tubes. There are now two sets of 10 tubes with 110 µl per tube.
d. Allow the solution to equilibrate at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).

3. Thermal challenge
   a. Briefly collect the liquid in the bottom of the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. To induce thermal denaturation, place one set of tubes on a thermal cycler pre-warmed to 54°C and the other set of tubes in a deep-well thermal cycler pre-warmed to 58°C (see considerations in the *Background*).
      Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added.
   c. Allow denaturation to occur for 3 min.
   d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
   e. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

E. Parasite concentration range experiment

1. Compound treatment
   a. Prepare a concentration range of ten compound solutions, including vehicle, at 2× the desired final concentration in lysis buffer. Aliquot 110 µl of the 2× compound solution into a PCR tube.
      Note: We advise ensuring that the same concentration of vehicle is maintained across all samples by preparing the dilution series into a lysis buffer containing a vehicle concentration equal to that of the highest compound concentration.
   b. Aliquot 110 µl of the parasite suspension into the PCR tubes containing 2× compound solution. The compound is now at the final desired concentration, and the volume in each tube is 220 µl.
   c. Use a multichannel pipette to gently mix the parasite suspension and transfer half the volume (110 µl) to another set of PCR tubes. There are now two sets of 10 tubes with 110 µl of parasites in compound solution.
   d. Allow the compound to equilibrate with the parasites at room temperature or at 37°C for at least 5 min (see treatment considerations in the *Background*).
2. Thermal challenge
   a. Briefly collect the liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. To induce thermal denaturation, place one set of tubes on a thermal cycler pre-warmed to 54°C and the other set of tubes in a deep-well thermal cycler pre-warmed to 58°C (see considerations in the *Background*).
      Note: The thermal cycler program should be started in advance so that the wells are at temperature when the tubes are added.
   c. Allow denaturation to occur for 3 min.
   d. Quickly remove the tubes from the thermal cycler and place on ice for 5 min.
3. Parasite lysis
   a. Briefly collect evaporated liquid in the tubes by using a mini-centrifuge with PCR tube adapter. Centrifuge for approximately 3 s.
   b. Add 20 µl of 6× lysis buffer to each tube and gently pipette to mix (see treatment considerations in the *Background*). Allow the parasites to lyse on ice for at least 15 min.
   c. Proceed to the step described below, *F. Separation of Soluble and Aggregated Protein*.

F. Separation of soluble and aggregated proteins

Below, we describe the two separation methods we have used for isolation of soluble proteins. For more information, see Note 1.

1. Ultracentrifugation method
   a. Transfer the heat-challenged lysates (a volume of approximately 100 µl) to ultracentrifuge tubes pre-chilled on a bed of ice.
      Note: The minimum volume of these tubes is 100 µl. Using lower volumes risks unbalancing the centrifuge rotor.
   b. Load the tubes into a pre-chilled TLA-100 rotor in a benchtop ultracentrifuge (*e.g.*, Beckman Ultra MAX) chilled to 4°C. The TLA-100 rotor can fit up to 20 tubes, which is enough for the two treatment conditions of a temperature-range experiment or two challenge temperatures of a concentration-range experiment. The tubes must be appropriately balanced to avoid damage to the rotor and ultracentrifuge.
   c. Centrifuge the samples at 100,000 × *g* for 20 min at 4°C using an ultracentrifuge. To calculate the appropriate rpm, use the rotor radius specifications and an online calculator such as https://www.beckman.com/centrifuges/rotors/calculator.
   d. Gently remove the rotor, taking care not to disturb the tubes, and immediately transfer the tubes to ice. If available, work in a cold room.
   e. Remove the top ~80% by volume of the supernatant and transfer to a pre-chilled protein low-bind tube. It is critical not to disrupt the pellet, which contains aggregated proteins and the membranous fraction.
   f. Proceed to the next section, *F. Protein Cleanup and digestion with the SP3 protocol.*
2. Filter plate method
   a. Pre-wet the filter plate with 100 µl of 1× lysis buffer (with compound/treatment, if applicable). Place the filter plate on top of a 96-well plate. Centrifuge at 500 × *g* in a swinging-bucket centrifuge for 5 min, until the solution passes through the filter and into the 96-well plate. Discard the solution.
   b. Place the filter plate over a clean protein low-bind or polypropylene 96-well plate. Transfer the heat challenged lysates (~100 µl) to the equilibrated filter plate and centrifuge at 500 × *g* for 5 min at 4°C to separate the soluble protein from aggregates. Soluble proteins pass through the filter into the 96-well plate.
   c. Transfer the soluble fraction from the 96-well plate to protein low-bind tubes. The volume of the soluble protein solution is reduced relative to the input volume and should be measured prior to the next step, *G. Protein Cleanup and Digestion*.

G. Protein cleanup and digestion with the SP3 protocol

1. Quantify protein abundance
   a. Determine the protein concentration in the reference sample (37°C for temperature-range experiments and the lowest compound concentration for concentration-range experiments) using a protein quantification assay, *e.g.*, the DC Protein Assay (Bio-Rad), according to the manufacturer's instructions. Diluent solutions should contain the compound of interest or vehicle, if applicable, as it may substantially alter absorbance readings. The amount of protein determined in this step will be used to calculate the amount of SP3 beads to use for sample cleanup and trypsin to add for digestion. We typically quantify 20-60 µg of soluble protein in the reference sample. The following steps assume a yield of 50 µg in the reference sample; adjust volumes accordingly for lower amounts of protein.
   b. Transfer a volume corresponding to 50 µg of protein in the reference sample to a new protein low-bind tube. Transfer the same volume of the remaining samples to protein low-bind tubes as well. Raise the volume to 100 µl with lysis buffer.
2. Reduce cysteines
   a. Add 0.5 µl of a 1 M TCEP solution to each sample. The concentration of TCEP is now 5 mM.
   b. Incubate the samples at 55°C for 10 min, *e.g.*, on a heat block or thermomixer.
3. Alkylate cysteines
   Remove the tubes from 55°C and allow them to cool to room temperature. Add 7.54 µl of a 200 mM MMTS stock solution to bring the concentration to 15 mM. Allow the reaction to occur for 10 min at room

temperature. Note: alternative protocols alkylate with iodoacetamide (IAA) in the dark. We prefer MMTS for in-solution digests due to its rapid reaction rate, stability, and lower non-specific alkylation (Müller and Winter, 2017), which can increase the number of peptide identifications following MS analysis. The choice of alkylating agent will determine search modification on cysteine, *i.e.*, methylthio (+45.988 Da) for MMTS or carbamidomethyl (+57.021 Da) for IAA.

4. Clean up samples using the SP3 protocol (Hughes *et al*., 2019). For more information, see Note 2.
   a. Prepare enough hydrophobic and hydrophilic Sera-Mag beads at 50 µg/µl for a 1:10 bead/protein (wt/wt) ratio relative to the reference sample. For example, to process 10 samples with a 50 µg reference sample, prepare 5 mg of beads.
      i. In a 1.5 ml tube, combine 50 µl of the 50 mg/ml hydrophobic beads with 50 µl of the 50 mg/ml hydrophilic beads.
      ii. Place the beads on a magnetic rack and allow them to separate. Use a P200 pipette to remove and discard the supernatant.
      iii. Wash the beads in 100 µl MS-grade water. Place the beads on a magnetic rack and again discard the supernatant.
      iv. Resuspend the beads in 100 µl of MS-grade water for a final concentration of 50 µg/µl.
   b. Add 10 µl of 50 µg/µl beads to each sample. The bead/protein (wt/wt) ratio is now at least 10:1.
   c. Bind the proteins to the beads by adding a 4× volume of 100% HPLC-grade ethanol. For example, to the combined volume of 100 µl sample with 0.5 µl TCEP, 7.54 µl MMTS, and 10 µl Sera-Mag beads, add 472 µl 100% ethanol. Note: the protein solution is now 80% ethanol by volume. We found this proportion to be optimal for binding of *T. gondii* proteins to the Sera-Mag beads.
   d. Allow the proteins to aggregate with the beads by placing the tubes in a thermomixer and shaking at 1,000 rpm at 24°C for at least 10 min. The beads should “clump” upon binding protein.
   e. Place the tubes on a magnetic rack and allow the beads to separate, which takes approximately 30 s. Discard the supernatant into a waste stream that is appropriate for 80% ethanol.
   f. Wash the beads three times with 180 µl 80% ethanol, which can be prepared by diluting HPLC-grade ethanol with HPLC-grade water. Each time, allow the beads to magnetically separate from the solution for 30 s and dispose of the supernatant into an appropriate waste stream.
      Note: In the final rinse, remove as much of the ethanol wash solution as possible to minimize carryover during the enzymatic digestion step. We remove nearly all of the liquid by centrifuging the beads at 16,000 × g for 30 s and double-stacking a P200 and P10 tip to remove the supernatant.
5. Digest proteins into peptides
   a. Prepare a trypsin digest solution in 50 mM TEAB at a 1:50 (wt/wt) protein:trypsin ratio. Prepare enough stock solution for the number of samples to be processed, *e.g.*, 20 samples for a temperature range experiment with a control and treatment condition and with 10 melting temperatures each.
   b. Add 35 µl of digest solution to each tube. Gently move the beads into the liquid with the tip of a pipette, but avoid pipetting the beads, as they are sticky.
   c. Place the tubes in a thermo mixer warmed to 37°C and shake at 1,000 rpm overnight (16-18 h).
   d. Centrifuge the tubes at 16,000 × *g* for 1 min at room temperature to pellet the beads and collect evaporated liquid.
   e. Place the tubes on a magnetic rack and allow the beads to separate for 30 s. Transfer the aqueous supernatant, which contains the digested peptides, to a new protein low-bind tube.

After the peptides have been eluted, samples can be snap-frozen in liquid nitrogen and dried in a lyophilizer with a condenser temperature of -80°C and chamber pressure of approximately 0 mbar. The peptides are typically lyophilized to a powder in four hours or fewer. The lyophilized peptides can be stored at -80°C for several months.

H. Tandem mass tag labeling

Sample multiplexing is performed with isobaric mass tags, which are commercially available in 10-plex and 16-plex format (Werner *et al*., 2014; Li *et al*., 2020). We keep working stocks of TMT 10-plex reagents at

concentrations of 6.66 µg/µl (100 µg per 15 µl) in acetonitrile at -80°C for 3-6 months. We perform labeling at 2:1 (wt/wt) TMT:peptide (Zecha *et al*., 2019).

A TMT labeling scheme should be selected in advance of labeling. Each temperature or concentration is labeled with one TMT channel. Two full 10-plex labeling reactions are performed per experiment: the 10 melting temperatures with vehicle and compound for temperature-range experiments or the 10 compound concentrations melted at two temperatures for concentration-range experiments. We have observed reporter ion interference when labeling sequentially (Brenes *et al*., 2019). Therefore, the labeling schemes shown in **Figure 1** are recommended.

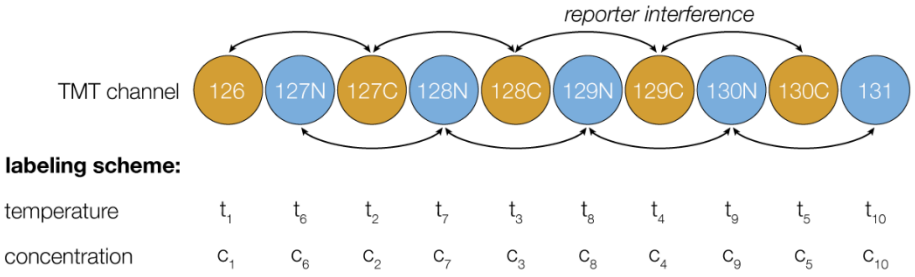


Figure 1. Recommended TMT labeling strategy for temperature- and concentration-range experiments. t_1/c_1 refers to the lowest temperature or concentration in the experiment.

1. Quantify peptide abundance with the Pierce fluorometric peptide assay
   Before starting, quantify the number of peptides in the reference sample (37°C for temperature range experiments and the lowest compound concentration for concentration range experiments) using the Pierce Fluorometric Peptide Assay according to manufacturer's instructions. If peptides have been lyophilized, resuspend in 35 µl 50 mM TEAB pH 8.5. A 1:20 dilution is often sufficient to place the sample within the range of the standard curve, *e.g.*, 0.5 µl of sample + 9.5 µl water. For a standard whole-proteome TMT reaction, use 25-50 µg of peptides in 35 µl of 50 mM TEAB, diluting the sample as necessary. Use equivalent volumes and dilutions of the non-reference samples. The steps below are written for samples containing 50 µg of peptides in 35 µl.
2. TMT labeling reaction
   a. Equilibrate the TMT reagents at room temperature for 3 min.
      Note: Record the reagent lot number and isotopic corrections for the batch. This information may be used to create a custom quantification method in Proteome Discoverer that corrects for isotopic impurities arising from natural carbon isotopes.
   b. Centrifuge the TMT reagents at 13,000 × *g* for 1 min and resuspend each vial in 120 µl of 100% MS-grade acetonitrile. Create 15 µl aliquots and store at -80°C for up to 6 months.
      Note: If resuspended TMT reagents will not be used for extended periods of time, lyophilize the reagents and store as a powder at -20°C.
   c. Add 15 µl of TMT reagent (100 µg) to the reference sample (50 µg protein in 35 µl 50 mM TEAB). If working with more or less peptide input, maintain the final vol/vol ratio of acetonitrile (TMT reagents) to aqueous buffer (TEAB buffer). For example, if labeling only 25 µg of peptides in 35 µl of TEAB, add 50 µg of TMT reagent in 7.5 µl and 7.5 µl of 100% acetonitrile to bring the final composition to 30% vol/vol acetonitrile.
   d. Centrifuge the tubes at 13,000 × *g* for 30 s to collect the liquid.
   e. Place the tubes in the thermomixer and shake at 400 rpm for 60 min at room temperature.
   f. Quench unreacted TMT reagent by adding 3.2 µl of 5% hydroxylamine per 50 µl reaction. Place the tubes in the ThermoMixer and shake at 400 rpm for 15 min at room temperature.

g. Combine the samples in a 1.5 ml protein low-bind tube. Use the same pipette tip for all transfers to avoid losing peptides due to contact with new surfaces. The volume should now be approximately 530 µl.
h. Flash-freeze the pooled sample in liquid nitrogen and lyophilize until dry. Note: sample volume may alternatively be reduced via vacuum centrifugation.
i. Dry samples may be stored at -80°C for several months.

3. Desalting and fractionation
TMT-labeled samples should be fractionated prior to MS data acquisition to reduce isolation interference during MS analysis. We perform high pH reversed-phase peptide fractionation using HPLC (*e.g.*, with Shimadzu LC-20AD; see Herneisen *et al.*, 2020) or the Pierce High pH Reversed-Phase Peptide Fractionation Kit according to manufacturer's instructions, which we have found provide equivalent coverage of the *T. gondii* proteome and also function as a desalting step. We pool samples into eight fractions for LC-MS. The fractions can be lyophilized and stored at -80°C indefinitely.

I. MS data acquisition

Data acquisition methods are highly dependent on facilities. At a minimum, TMT-labeled samples should be acquired using sufficient resolution to resolve the reporter ions and with a long gradient to separate the complex peptide mixtures and reduce co-isolation interference. Here, we describe the data acquisition protocol for our Exploris 480 orbitrap with FAIMS Pro interface coupled to an Easy-nLC 1200 system.

1. Sample resuspension and injection
a. Resuspend each lyophilized fraction in Buffer A to an estimated concentration of 0.5-1 µg peptides/µl. We typically resuspend each sample in 25 µl. Ensure that the lyophilizate is completely solubilized; it may help to thoroughly wash the sides of the tube and collect the liquid by centrifuging at 16,000 × *g* for 1 min.
b. Transfer each resuspended fraction to an autosampler tube. Once the samples are resuspended, they should be kept at 4°C.
c. Inject 0.5-1 µg of peptides for MS analysis (typically 1-2 µl). Samples belonging to the same TMT labeling experiment can be injected sequentially (*i.e.*, the set of fractions). We perform a blank injection between different TMT labeling experiments to reduce carryover.

2. LC gradient
Our samples are separated over a 90-min gradient described in **Table 1**. The gradient includes an optional 12-minute seesaw for column maintenance. Our LC system includes a commercial trapping column (Acclaim PepMap 100 75 µm × 2 µm nanoViper) connected to a 15 cm commercial analytical column (PepMap RSLC C18 3 µm, 100A, 75 µm × 15 cm).

Table 1. LC gradient used for TMT10-labeled *T. gondii* proteome

Time	Duration	%B
00:00	00:00	1
01:00	01:00	6
42:30	41:30	21
63:15	20:45	36
73:30	10:15	50
74:00	00:30	100
88:00	14:00	100

91:00	03:00	2
94:00	03:00	2
97:00	03:00	98
100:00	03:00	98

3. MS acquisition settings
Method parameters for the orbitrap Exploris 480 with FAIMS Pro interface are summarized in **Table 2**. In our experience, alternating between compensation voltages of -50 and -65 yielded best coverage of the *T. gondii* proteome. The ddMS^2 resolution of 30,000 has been optimized for the TurboTMT scan option (Bekker-Jensen *et al.*, 2020); users who elect not to use this setting should opt for a higher resolution.

Table 2. MS acquisition settings for Orbitrap Exploris 480 with FAIMS Pro interface

Parameter	Setting
Global	
Ion source	
Ion Source Type	NSI
Spray Voltage	Static
Positive Ion (V)	1800
Gas Mode	Static
Ion Transfer Tube Temp (°C)	270
FAIMS Mode	Standard Resolution
FAIMS Gas	Time Dependent
FAIMS Gas Table	
0 min	3 L/min gas
1 min	0 L/min gas
MS Global Settings	
Infusion Mode	Liquid Chromatography
Expected LC Peak Width (s)	30
Advanced Peak Determination	False
Default Charge State	2
Internal Mass Calibration	Off
EXP 1: TMT MS2 FAIMS – 50 CV	

Full Scan	
Orbitrap Resolution	120000
Scan Range (m/z)	350-1200
FAIMS Voltages	On
FAIMS CV (V)	-50
RF Lens (%)	40
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	Disabled
Intensity	
Filter Type	Intensity Threshold
Intensity Threshold	5.0e3
Charge State	
Include charge state(s)	2-5
Include undetermined charge states:	False
Dynamic Exclusion	
Dynamic Exclusion Mode	Custom
Exclude after n times	1
Exclusion duration (s)	30
Mass tolerance	10 ppm
Exclude isotopes	True
Perform dependent scan on single charge state per precursor only	True
Precursor Fit	
Fit threshold (%)	70
Fit window (m/z)	0.7

Data Dependent	
Data Dependent Mode	Cycle Time
Time between Master Scans (sec)	2
ddMS^2	
Multiplex Ions	False
Isolation Window (m/z)	0.7
Isolation Offset	Off
Collision Energy Mode	Fixed
Collision Energy Type	Normalized
HCD Collision Energy (%)	36
Orbitrap Resolution	30000
TurboTMT	TMT Reagents
Scan Range Mode	Define First Mass
First Mass (m/z)	110
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Centroid
EXP 2: TMT MS2 FAIMS – 65 CV	
Full Scan	
Orbitrap Resolution	120000
Scan Range (m/z)	350-1200
FAIMS Voltages	On
FAIMS CV (V)	-65
RF Lens (%)	40
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Profile

Polarity	Positive
Source Fragmentation	Disabled
Intensity	
Filter Type	Intensity Threshold
Intensity Threshold	5.0e3
Charge State	
Include charge state(s)	2-5
Include undetermined charge states:	False
Dynamic Exclusion	
Dynamic Exclusion Mode	Custom
Exclude after n times	1
Exclusion duration (s)	30
Mass tolerance	10 ppm
Exclude isotopes	True
Perform dependent scan on single charge state per precursor only	True
Precursor Fit	
Fit threshold (%)	70
Fit window (m/z)	0.7
Data Dependent	
Data Dependent Mode	Cycle Time
Time between Master Scans (sec)	2
$ddMS^2$	
Multiplex Ions	False
Isolation Window (m/z)	0.7
Isolation Offset	Off
Collision Energy Mode	Fixed
Collision Energy Type	Normalized
HCD Collision Energy (%)	36

Orbitrap Resolution	30000
TurboTMT	TMT Reagents
Scan Range Mode	Define First Mass
First Mass (m/z)	110
AGC Target	Standard
Maximum Injection Time Mode	Auto
Microscans	1
Data Type	Centroid

Data analysis

A. Protein quantification with Proteome Discoverer

Following MS data acquisition, RAW files are processed using any of several analysis pipelines to obtain protein quantification from the MS/MS scans and reporter ion abundances. This protocol describes data processing using the Proteome Discoverer 2.4 software. Alternatives are documented elsewhere (Perez-Riverol *et al*., 2014; Franken *et al*., 2015).

1. Load the data into Proteome Discoverer 2.4 by creating a new study and analysis.
   a. Select processing and consensus workflows that are appropriate for the instrument used for data acquisition and the reporter ion-based quantification method. We use the common templates provided by Thermo Fisher for the Q Exactive for our orbitrap platforms.
   b. Select TMT 10-plex as a quantification method. We create custom quantification methods with lot-specific corrections, but the default quantification method will suffice.
   c. Add the RAW spectrum files as fractions (if following the protocol here, add 8 RAW files per experiment). We analyze each set of fractions separately. For example, the vehicle treatment of a temperature range experiment would be analyzed separately from the compound treatment.
2. Adjust settings in the Processing Workflow to conform to the experiment. We use default settings for the Minora Feature Detector, Spectrum Selector, and Percolator nodes (strict targeted FDR of 0.01 based on q-value with a relaxed FDR of 0.05). Major adjustments to the Sequest search engine node include
   a. Inputting the correct protein database (for *T. gondii* RH strains, the most recent release of the GT1 annotated proteins *.fasta, which can be found at https://toxodb.org/toxo/app/downloads/Current_Release/TgondiiGT1/fasta/data/).
   b. Selecting the desired dynamic modifications. We have used Oxidation (+15.995 Da) on M, Phosphorylation (+79.966 Da) on S/T/Y, and Acetylation (+42.011 Da) on the N terminus of the protein. Including additional dynamic modifications will increase the search space but may be common practice based on the conditions used in the protein workup steps.
   c. Selecting the appropriate static modifications: TMT 6-plex (+229.163 Da) on the peptide N terminus and K, and methylthio (+45.988 Da) on C. Note that use of other alkylating agents (*e.g.*, IAA) will require an alternative modification on cysteine. For hyperperplexing with SILAC, see Note 3.
3. Adjust the settings in the Consensus Workflow to enable downstream processing of melting curves:
   a. Use only unique peptides for quantification.
   b. Turn off scaling.
   c. For temperature range experiments, set Normalization Mode to none; it is important not to normalize abundances by channel, as protein abundance is globally decreasing at higher melting temperatures.

d. For concentration range experiments, optionally set Normalization Mode to none. Data can be normalized in the TPP R package (see next section). We have also opted to normalize in Proteome Discoverer and forgo normalization in the TPP package.
e. Optionally adjust the co-isolation threshold or Average Reporter S/N threshold. Lowering these thresholds may increase quantification but lower data quality.

4. Upon completion of the analysis, export the protein-level quantification as a *.txt file.

B. Curve Fitting

Curve fitting is performed using the TPP R package, which has been extensively documented (Franken *et al*., 2015; Childs *et al*., 2019; Kurzawa *et al*., 2020). Recently, alternative thermal proteome profiling data analysis packages have been proposed (Dziekan *et al*., 2020), and users may develop their own custom normalization and curve fitting approaches. The output file from Proteome Discoverer must be modified to match the input format of the TPP package. **Tables S1-S4** represent example output from Proteome Discoverer. **Tables S5-S9** show the streamlined tables used as input to the TPP package, and **Table S10** is representative output.

Table S1. Temperature range, cells, replicate 1 output from the Proteome Discoverer 2.4 software.
Table S2. Temperature range, cells, replicate 2 output from the Proteome Discoverer 2.4 software.
Table S3. Temperature range, lysate, replicate 1 output from the Proteome Discoverer 2.4 software.
Table S4. Temperature range, lysate, replicate 2 output from the Proteome Discoverer 2.4 software.
Table S5. Temperature range, cells, replicate 1 trimmed input to the TPP R package.
Table S6. Temperature range, cells, replicate 2 trimmed input to the TPP R package.
Table S7. Temperature range, lysate, replicate 1 trimmed input to the TPP R package.
Table S8. Temperature range, lysate, replicate 2 trimmed input to the TPP R package.
Table S9. An example configuration table specifying the experiments, conditions, and replicates used for curve fitting in the TPP R package.
Table S10. Example output from the TPP R package.

C. Anticipated Results

In a typical temperature-range experiment, we detect over 3,000 proteins, of which ~80% have quantification values sufficient for curve fitting. Our other proteomics experiments identify 4,600-4,800 proteins, indicating that the thermal challenge inherent to the thermal profiling approach reduces proteome coverage. We perform experiments in biological duplicate. **Figure 2A** reveals replicate variability in calculated protein melting temperatures. To generate a reference dataset, we performed thermal profiling on live parasites or lysates belonging to the *T. gondii* RH/TIR1 strain and hyperplexed the samples with SILAC (Harding *et al*., 2020; Herneisen *et al*., 2020); see Note 4. Aggregates were separated using the filter plate method described in section F of the *Protocol*. As observed for other organisms (Jarzab *et al*., 2020), proteome-wide thermal stability is greater in lysates than in cells (**Figure 2B**). To include thermostable proteins in our analysis, we calculated the numerical area under the curve (AUC) using the trapezoidal rule (**Figure 2C**). In contrast to the melting temperature, which requires at least 50% thermal denaturation, the AUC metric can be calculated for all proteins with complete thermal profiles. **Figure 2D** shows the relationship between melting temperature and AUC.

Such a reference dataset can be used to select temperature ranges and thermal challenge temperatures for experiments involving compound treatment. **Table 3** summarizes the distribution of melting temperatures from parasites and lysates from two different sets of experiments. The first experiment melted parasites or lysates over a temperature range of 37-67°C and separated soluble proteins from aggregates by ultracentrifugation (Herneisen *et al.*, 2020). The second experiment, presented here, melted parasites or lysates over a temperature range of 41-73°C and separated aggregates with a filter plate. Researchers may reference the distribution most similar to their intended workflow. To detect compound-dependent thermal stabilization, concentration range experiments should be performed slightly above the melting temperature of the protein target under vehicle-treated conditions (Franken *et al*., 2015). In cases in which the protein target is not known, we have opted to perform the thermal challenges at two temperatures corresponding to the median and third quartile temperatures

of the melting distribution. However, melting temperature often depends on the cellular environment. **Figure 2E** and **F** show melting temperatures and AUC values stratified by subcellular assignment by the MS-based LOPIT approach (Barylyuk *et al*., 2020). Some subcellular structures, such as the tubulin cytoskeleton and 20S subunit of the proteasome, prove particularly thermostable; detecting compound-dependent thermal shifts in proteins belonging to these substructures would require a high thermal challenge temperature. By contrast, proteins in the nucleus and nucleolus tend to be prone to precipitation, and using the median thermal challenge temperature would result in poor quantification and coverage of proteins in these substructures. Other organelles are particularly sensitive to cellular preparation; for example, components of the 60S ribosome co-melt in cells but exhibit disparate melting profiles in lysates. Therefore, researchers should leverage their observations and predictions about a compound's mechanism of action to select the most appropriate thermal profiling parameters.

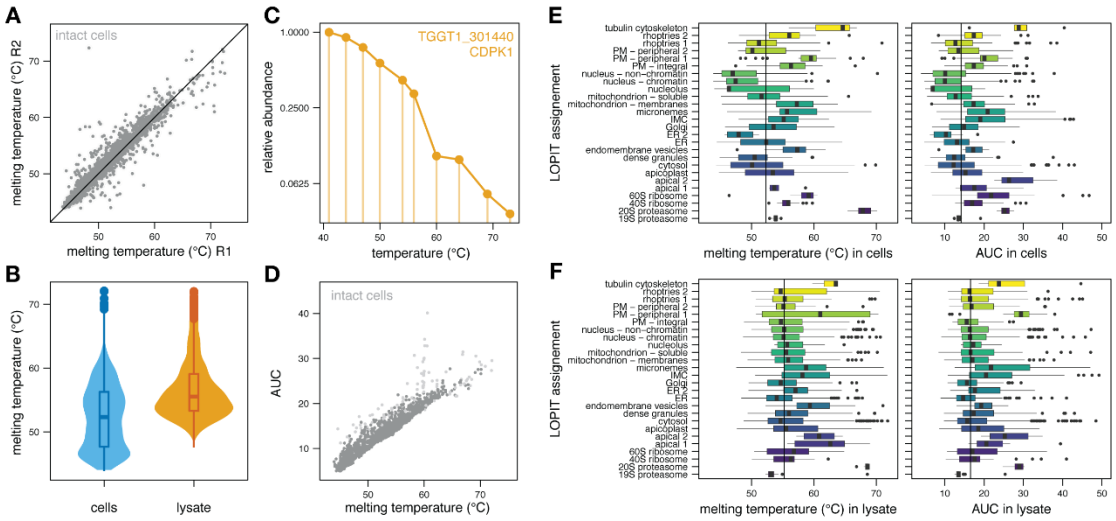


Figure 2. Melting behavior of the *T. gondii* proteome. (A) Reproducibility of melting temperatures of proteins quantified in both replicates of an intact cell melting experiment. **(B)** Distribution of average melting temperatures of proteins quantified in both replicates of an intact cell and lysate temperature range experiment. **(C)** Depiction of how area under the curve (AUC) is calculated by numerical integration using the trapezoidal rule for the protein CDPK1. **(D)** Relationship between average melting temperature and AUC of proteins for which both values are available. Points with a lighter shade of gray were poorly fit to a sigmoidal melting curve ($R^2 < 0.8$). **(E)** Distribution of average melting temperatures and AUC in cells or **(F)** lysates by LOPIT assignment from Barylyuk *et al.* (2020).

Table 3. Distributions of melting temperatures from *T. gondii* lysates and intact cells from two different sets of experiments

	Ultracentrifugation (Herneisen *et al*., 2020)		Filter plate (here)	
	Lysate	Cells	Lysate	Cells
Min	44.8	43.5	47.6	43.9
1st quartile	50.7	50.9	53.3	47.7
Mean	53.4	53.8	56.7	52.5
Median	52.7	53.4	55.5	52.3

3rd quartile	55.4	56.4	59.1	56.3
Max	65.6	66.9	72.0	72.1

Notes

1. Following thermal challenge and global protein denaturation, soluble protein is separated from unfolded protein aggregates. The original CETSA protocol described centrifugation in a minifuge at 20,000 × *g* (Jafari *et al.*, 2014), which was subsequently elevated to 100,000 × *g* in an ultracentrifuge to enhance the signal-to-noise ratio for MS analysis (Franken *et al.*, 2015). Filter plates can be used as an alternative with the benefit of higher throughput (Mateus *et al.*, 2018 and 2020a; Dziekan *et al.*, 2020). After the soluble protein has been separated from the aggregates, samples can be snap-frozen in liquid nitrogen and stored at -80°C for several months.
2. Solutions containing soluble proteins are cleaned up and processed using a modified SP3 protocol based on Hughes *et al.* (2019), which provides high capture and throughput that is well-suited for dilute and low-abundance TPP samples. Protein precipitation is not recommended as it can lead to uneven sample loss that degrades the quality of melting curves. The protocol has been optimized for *T. gondii* protein samples (Harding *et al.*, 2020; Herneisen *et al.*, 2020) and is compatible with TMT-labeling upon elution.
3. To reduce MS time and run-to-run variability, we have hyperplexed TPP experiments using SILAC, as described elsewhere (Herneisen *et al.*, 2020). This variation requires growing parasites in heavy and light SILAC media for 3 passages prior to the TPP experiment. Parasites grown in different media are treated as biological duplicates and are combined in equal weights prior to alkylation. Quantification values originating from the heavy samples are obtained by searching for peptides with heavy arginine (+10.008 Da) and the heavy Lysine-TMT6plex (+237.177 Da) modifications in Proteome Discoverer.
4. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD024912 and 10.6019/PXD024912.

Recipes

1. DMEM + 3% CFS (used for routine parasite passaging) per 500 ml

5 ml 200 mM glutamine
500 µl 10 mg/ml gentamicin
15 ml calf serum
Filter the supplemented DMEM through a bottle top filter into a clean glass bottle that has not been washed with detergent.

2. PBS

100 ml 10× tissue culture-grade PBS
900 ml deionized water
Filter-sterilize and store at room temperature

3. 10% IGEPAL CA-630 (also known as NP-40) (50 ml)

5 ml IGEPAL CA-630
45 ml deionized water
Store at 4°C for 6 months

4. 10× CETSA buffer (1 L)

(50 mM NaCl, 1.42 M KCl, 10 mM $MgCl_2$, 56 mM glucose, 250 mM HEPES pH 7.2)
2.922 g NaCl

105.86 g KCl
2 g $MgCl_2$
10.1 g glucose
59.575 g HEPES
Add deionized water to 1 L and adjust the pH to 7.2 with KOH
Sterile-filter the solution and store at 4°C

5. **CETSA wash buffer (1 ml)**
   (5 mM NaCl, 142 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES pH 7.2)
   100 µl 10× CETSA buffer
   900 µl deionized water

6. **CETSA lysis buffer (1 ml)**
   100 µl 10× CETSA buffer
   80 µl 10% IGEPAL CA-360
   10 µl Halt protease inhibitors
   1 µl benzonase
   809 µl deionized water

7. **1 M TCEP stock solution**
   1 g TCEP HCl
   3.489 ml deionized water
   Store as 500 µl aliquots at -80°C and as 20 µl working aliquots at -20°C

8. **200 mM MMTS stock solution**
   200 mg MMTS
   7.924 ml isopropanol
   Store as 500 µl aliquots at 4°C

9. **Buffer A (100 ml)**
   (0.1% formic acid in MS-grade water)
   100 ml MS-grade water
   100 µl >99% formic acid
   Sonicate for 10 min

10. **Buffer B (25 ml)**
    (80% acetonitrile and 0.1% formic acid)
    20 ml MS-grade acetonitrile
    25 µl >99% formic acid
    5 ml MS-grade water
    Sonicate for 10 min

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Competing interests

The authors declare no conflicts or competing interests.

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