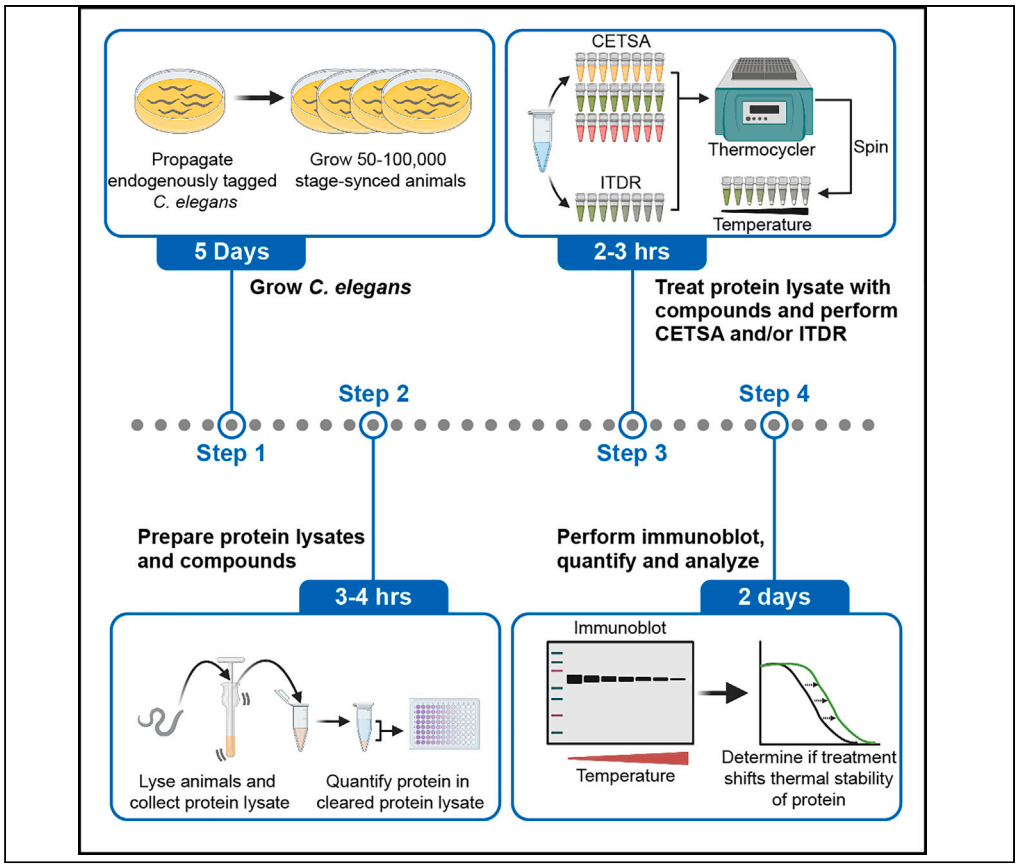
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to assess receptor-ligand binding in *C. elegans* using adapted thermal shift assays



The *Caenorhabditis elegans* genome encodes a greatly expanded number of nuclear hormone receptors, many of which remain orphaned. Here, we present a protocol to assess ligand-receptor binding in *C. elegans* using an adapted cellular thermal shift assay and isothermal dose response. We describe steps for growing *C. elegans* and preparing lysates and compounds. We also detail how to perform and quantify these assays. This protocol can be used to study any soluble receptor.

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

Samantha Y. Tse, Read Pukkila-Worley

read.pukkila-worley@umassmed.edu

Highlights

Protocol to assess *C. elegans* ligand-receptor binding using thermal shift assays

Accessible protocol to de-orphan nuclear hormone receptors

Adaptable to any soluble receptor in *C. elegans*

Tse & Pukkila-Worley, STAR Protocols 4, 102477 September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102477

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to assess receptor-ligand binding in *C. elegans* using adapted thermal shift assays

Samantha Y. Tse[1,4] and Read Pukkila-Worley[1,2,3,*]

[1]Program in Innate Immunity, Division of Infectious Diseases and Immunology, Department of Medicine, UMass Chan Medical School, Worcester, MA 01655, USA

[2]Twitter: @RPWLab

[4]Technical contact: samantha.tse@umassmed.edu

[3]Lead contact

*Correspondence: read.pukkila-worley@umassmed.edu
https://doi.org/10.1016/j.xpro.2023.102477

SUMMARY

The *Caenorhabditis elegans* genome encodes a greatly expanded number of nuclear hormone receptors, many of which remain orphaned. Here, we present a protocol to assess ligand-receptor binding in *C. elegans* using an adapted cellular thermal shift assay and isothermal dose response. We describe steps for growing *C. elegans* and preparing lysates and compounds. We also detail how to perform and quantify these assays. This protocol can be used to study any soluble receptor.

For complete details on the use and execution of this protocol, please refer to Peterson et al. (2023).[1]

BEFORE YOU BEGIN

Culturing worms

Timing: 5 days

Here, we present protocols to determine binding between a nuclear hormone receptor and its ligands in *C. elegans* – the cellular thermal shift assay (CETSA) and the isothermal dose response (ITDR).[2,3] In the example described below, we used a *C. elegans* strain that carries a 3×FLAG-tagged NHR-86 protein, which we showed binds the pathogen-derived metabolite phenazine-1-carboxamide (PCN) as well as a synthetic immunostimulatory xenobiotic (R24), but not a negative control phenazine, phenazine-1-caboxylic acid (PCA).[1]

The protocols described here require transgenic *C. elegans* that express a tagged protein under study. We recommend that CRISPR-Cas9 be used to knock-in a tag at its endogenous locus and have had success using a 3×FLAG sequence in such experiments. Protocols for this purpose have been described in detail elsewhere and will not be discussed here.[4,5] In theory, the tagged protein could also be expressed on an extrachromosomal array, and a different tag (e.g., HA, GFP, His) could be used. It is important to identify the experimental conditions, such as antibody concentration and immunoblotting conditions, that optimize protein detection. In addition, users should be aware that the addition of a tag could itself change the thermal stability of the protein.[6] Lastly, phenotypic assays should be used to ensure that the tagged protein is functional. Of note, a useful repository for these strains is the modEncode project, which created many strains expressing GFP- and FLAG-tagged nuclear hormone receptors.[7]

These protocols can theoretically be applied to any stage of *C. elegans*. Here we use larval stage L4 *C. elegans* 3xFLAG::NHR-86 animals. If other stages are required, scale up or down the number of worms to obtain the optimal protein concentration.

1. Chunk *C. elegans* 3xFLAG::NHR-86 onto 10-cm NGM plates containing a 10×-concentrated 16 h overnight culture of *E. coli* OP50 and allow to grow to gravid adults (~3 days) at 20°C.

   Note: Chunking wild-type animals onto 8–10 10-cm NGM plates will typically yield ~200,000 synchronized L1s. Although no difference in brood size was noted with the transgenic 3xFLAG::NHR-86 strain, tagging other nuclear hormone receptors may affect the brood size, and the number of plates required would need to be adjusted accordingly.

2. Synchronize *C. elegans* to the L1 stage by hypochlorite treatment of gravid adult animals followed by incubation in M9W on a roller overnight ("egg prep") for 18–20 h.
3. Drop 5000–6000 animals onto 10-cm NGM plates containing 2-mL 10× *E. coli* OP50 that has been spread into a large lawn. Prepare enough plates to drop ~100,000 animals in total (~20 10-cm plates).
   a. If there are not enough animals, the worms can be dropped to as many plates as possible and egg prepped again 3 days later.
4. Allow animals to reach the L4 stage at 20°C (~2 days).

Preparing ligands

Timing: 30 min

5. Prepare compounds fresh when possible.
   a. CETSA: Prepare 500 µL of each compound to be tested at 2× concentration in PBS.
      i. 4% DMSO (solvent control): 20 µL DMSO (filter-sterilized) + 480 µL PBS
      ii. 900 µM PCN: 20 µL PCN at 22.4 mM (5 mg/mL) + 480 µL PBS.
      iii. 900 µM PCA: 20 µL PCA at 22.4 mM (5 mg/mL) + 480 µL PBS.
      iv. 140 µM R24: 2 µL R24 at 35 mM + 18 µL DMSO + 480 µL PBS.
   b. ITDR: Prepare 8 concentrations of R24 in PBS at 2× concentration.
      i. 4% DMSO (solvent control): 20 µL DMSO (filter-sterilized) + 480 µL PBS.
      ii. 700 µM R24: 10 µL R24 at 35 mM + 10 µL DMSO + 480 µL PBS.
      iii. 400 µM R24: 5.7 µL R24 at 35 mM + 14.3 µL DMSO + 480 µL PBS.
      iv. 200 µM R24: 2.8 µL R24 at 35 mM + 17.2 µL DMSO + 480 µL PBS.
      v. 20 µM R24: 2.8 µL R24 at 3.5 mM + 17.2 µL DMSO + 480 µL PBS.
      vi. 2 µM R24: 2.8 µL R24 at 0.35 mM + 17.2 µL DMSO + 480 µL PBS.
      vii. 0.2 µM R24: 2.8 µL R24 at 0.035 mM + 17.2 µL DMSO + 480 µL PBS.
      viii. 0.02 µM R24: 2.8 µL R24 at 0.0035 mM + 17.2 µL DMSO + 480 µL PBS.
      ix. 0.002 µM R24: 2.8 µL R24 + 0.00035 mM + 17.2 µL DMSO + 480 µL PBS.
   c. Keep compounds at room temperature (22°C).

   Note: Final concentrations of DMSO can often be limited by the solubility of the compound. For the compounds tested here, 2% DMSO was the lowest concentration of DMSO possible to keep compounds in solution. Of note, DMSO concentrations of 0.5%–3% have been used successfully and did not lead to variable results.

   Note: Many solvents can be used in this assay. If a compound can only be used with a specific solvent that is not described here (*i.e.*, DMSO), perform a CETSA to determine if the solvent alone has major effects on the thermal stability of your protein of interest. It is also important to keep the amount of solvent the same in each condition.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-FLAG M2	Sigma-Aldrich	Cat# F1804; RRID: AB_262044
Goat Anti-Mouse IgG – H&L, Polyclonal, HRP-conjugated	Abcam	Cat# ab6789; RRID:AB_955439
Bacterial and virus strains		
Escherichia coli OP50		WB Cat# WBStrain00041969; RRID:WB-STRAIN:WBStrain00041969
Chemicals, peptides, and recombinant proteins		
Streptomycin sulfate	Sigma	Cat# S9137-25G
Gibco™ Bacto™ Tryptone	Fisher	Cat# DF0123173
Fisher BioReagents™ Yeast Extract (Granulated)	Fisher	Cat# BP9727500
Sodium chloride (NaCl)	Fisher	Cat# S25542A
Sodium hydroxide (NaOH)	Sigma	Cat# S5881-500G
Bacto™ Peptone	VWR	Cat# 90000-264
Granulated agar, Fisher BioReagents	Fisher	Cat# BP97442
Magnesium sulfate ($MgSO_4$)	Fisher	Cat# M65500
Calcium chloride ($CaCl_2$)	Sigma	Cat# C3881
Potassium phosphate monobasic (KH_2PO_4)	Fisher	Cat# BP362
Potassium phosphate dibasic (K_2HPO_4)	Fisher	Cat# P288
Cholesterol	Sigma	Cat# C3045-25G
Nystatin	Fisher	Cat# BP29495
Halt™ Protease and Phosphatase Inhibitor Cocktail (100×)	Thermo Fisher Scientific	Cat# 78440
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D8418
RPW-24 (R24)	MedChemExpress	Cat# HY-W035409
Phenazine-1-carboxamide	Princeton BioMolecular Research	Cat# PBMR030086
Phenazine-1-carboxylic acid	Ark Pharm	Cat# AK-98673
Gibco™ Phosphate-Buffered Saline (PBS), pH 7.4	Thermo Fisher Scientific	Cat# 10010023
β-Mercaptoethanol (β-ME)		
NuPAGE™ LDS Sample Buffer, 4×	Thermo Fisher Scientific	Cat# NP0007
Ponceau S Staining Solution	Thermo Fisher Scientific	Cat# A40000279
1× phosphate-buffered saline (PBS), pH 7.4	Thermo Fisher Scientific	Cat# 10010023
NuPAGE™ 12%, Bis-Tris, 1.0 mm, Mini Protein Gels	Thermo Fisher Scientific	Cat# NP0349BOX
NuPAGE™ MES SDS Running Buffer (20×)	Thermo Fisher Scientific	Cat# NP0002
1× Tris-Buffered Saline (TBS)	Thermo Fisher Scientific	Cat# J60764.K7
Tween-20	Sigma	Cat# P1379
Critical commercial assays		
DC Protein Assay	Bio-Rad	Cat# 5000111
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific	Cat# 34095
Software		
ImageJ (Version 2.1.0/1.53c)	Fiji	RRID: SCR_002285
GraphPad Prism (Version 9.5.0)	GraphPad	RRID: SCR_000306
Experimental models: Organisms/strains		
C. elegans: Strain: RPW401 *nhr-86 (ums14[3xFLAG::NHR-86])*; *agIs44 [irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry]*	This study	N/A
Other		
C1000 Thermal Cycler	Bio-Rad	Cat# 1851148
Kontes 0.5 mL Potter-Elvehjem Tissue Grinder with PTFE Pestle and Unground Glass Tube, Size: 18 (see below)	VWR	Cat# KT886000-0018

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Centrifuge fitted with FA-24 × 2 rotor	Eppendorf	Cat# 5425R
Trans-Blot Turbo Transfer Packs, 0.2 µm nitrocellulose, Midi	Bio-Rad	Cat# 1704159
ChemiDoc MP Imaging System	Bio-Rad	Cat# 12003154

MATERIALS AND EQUIPMENT

M9W buffer		
Reagent	Final concentration	Amount
KH_2PO_4	22 mM	3 g
Na_2HPO_4	42 mM	6 g
NaCl	86 mM	5 g
1M $MgSO_4$ (after autoclave)	1 mM	1 mL
dH_2O		Up to 1 L

- Autoclave.
- Store at room temperature (22°C) up to 6 months.

LB broth		
Reagent	Final concentration	Amount
Bacto Tryptone	1% (w/v)	10 g
Bacto Yeast Extract	0.5% (w/v)	5 g
NaCl	0.5% (w/v)	5 g
2 N NaOH		Adjust to pH 7.0
dH_2O		Up to 1 L

- Autoclave.
- Store at room temperature (22°C) up to 6 months.

NGM (nematode growth medium) plates		
Reagent	Final concentration	Amount
NaCl	1% (w/v)	10 g
Bacto Peptone	0.5% (w/v)	5 g
Agar	0.5% (w/v)	5 g
dH_2O		965 mL
Autoclave and cool to 55°C before adding supplements below		
1 M $MgSO_4$	1 mM	1 mL
1 M $CaCl_2$	1 mM	1 mL
5 mg/mL cholesterol	5 µg/mL	1 mL
1 M KPO4 Buffer	25 mM	25 mL
2.5% streptomycin sulfate	0.02%	7.5 mL
10 mg/mL Nystatin	10 µg/mL	1 mL

- For 10-cm plates, pour 25 mL per plate.
- Store plates at room temperature (22°C) for 1 week or 4°C for up to 1 month.

1 M KPO_4		
Reagent	Final concentration	Amount
KH_2PO_4	796 mM	108.3 g
K_2HPO_4	204 mM	35.6 g
pH to 6.0		
dH_2O		Up to 1 L

2 N NaOH

- 4 g NaOH in 50 mL dH_2O.

1 M $MgSO_4$

- Dissolve 12.04 g in 100 mL dH_2O.
- Autoclave.

1 M $CaCl_2$

- Dissolve 14.7 g in 100 mL dH_2O.
- Autoclave.

5 mg/mL cholesterol

- Dissolve 0.25 g cholesterol in 50 mL 99.5% ethanol.
- Filter-sterilize through 0.2 μm filter.

10 mg/mL Nystatin

- Suspend 500 mg in 30 mL 70% ethanol, bring up to 50 mL final volume with ethanol.
- Store at −20°C for 6 months.

TBST (TBS + 0.2% Tween-20)

- Add 2 mL Tween-20 to 998 mL 1× TBS.

10× *E. coli* OP50

- Prepare 2.5% streptomycin sulfate in water and filter-sterilize: 2.5 g streptomycin sulfate in 100 mL dH_2O.
- Inoculate a single colony of *E. coli* OP50 in 1 L LB broth supplemented with 7.5 mL 2.5% streptomycin sulfate.
- Shake at 250 rpm in 37°C for 16–18 h.
- Spin at 2000 × *g* for 15 min at 4°C.
- Resuspend pelleted bacteria in 100 mL (10×) of M9W.
- Store at 4°C up to 1 week.

PTFE tissue grinder

- The tissue grinder we use is a modified homogenizer where we attach the polytetrafluoroethylene (PTFE) pestle to a speed-adjustable rotor.

Thermocyclers

The success of performing CETSA or ITDR is dependent on how consistently each sample is exposed to each temperature on the thermocycler. We use 2 independent C1000 Touch™ Thermal Cyclers (Bio-Rad) containing dual 48/48-well fast modules (4 independent blocks total), which allows for samples to be exposed to 4 separate temperatures at a time. Note that we do not use the temperature gradient function for two reasons: 1) we cannot choose specific temperatures in between the highest and lowest temperatures, and 2) there is bleed-over in temperature between rows that prevents accurate exposure to specific temperatures. Other thermocyclers can also be used so long as independent modules are available. We do not recommend performing the CETSA with a thermocycler containing only one module.

Alternatives: The Veriti™ Thermal Cyclers (Applied Biosystems) can be outfitted with VeriFlex™ Blocks that can run up to 6 independent temperatures, which may simplify treating the protein lysates at different temperatures.

STEP-BY-STEP METHOD DETAILS

Lysing animals

Timing: 3 h

Disrupting the cuticle of *C. elegans* to lyse them effectively is essential to the success of this protocol. We have found that a PTFE homogenizer attached to a rotor gives us optimal lysing of *C. elegans*.

1. Once animals have reached the L4 stage, collect all animals in a 15 mL conical tube by washing plates with M9W and centrifuging for 30 s at 1500 × *g* to pellet worms.
   a. Wash worms twice with 10 mL M9W and repeat centrifugation.
   b. For the last wash, resuspend animals in 1× PBS and repeat centrifugation.

   Note: The pellet size should be between 0.5–1 mL in size.

   c. Allow the pellet to form tightly on ice and remove supernatant by aspirating to the pellet.
2. Prepare a solution of 1× PBS and 2× Halt Protease and Phosphatase Inhibitor Cocktail (dilute 100× Halt Protease and Phosphatase Inhibitor Cocktail to 2×). Keep solution on ice.
   a. Estimate the pellet size of the animals.
   b. Add 1 volume of 1× PBS + 2× Halt Protease and Phosphatase Inhibitor Cocktail. Flick the bottom of the conical gently to mix. Keep on ice.

   Pause point: *C. elegans* animals at this point can be flash frozen (liquid nitrogen or dry ice/ethanol) and stored at −80°C until ready for lysis.

   ⚠ CRITICAL: Detergents that are commonly used to lyse cells may disrupt downstream ligand-protein interactions.

3. Lyse animals with a modified PTFE homogenizer (see materials and equipment) by transferring animals with a glass Pasteur pipet to the mortar.
   a. Insert pestle into the mortar containing the animals and turn on rotor. Move mortar up and down at least 10 times. Keep lysed animals on ice.
   b. Continue to homogenize until there are no visible animals and the lysate becomes 'milky white.'
   c. Repeat until entire volume of animals has been lysed.
4. Transfer the lysate into an ice-chilled 1.5 mL microcentrifuge tube and centrifuge at 20,000 × *g* for 20 min at 4°C.

a. There will be some volume loss during the homogenization process.

5. Transfer supernatant, without disturbing the pellet, into a new ice-chilled 1.5 mL centrifuge tube and quantify protein concentration.
6. Estimate the protein concentration using the *DC* protein assay (BioRad) using the manufacturer's instructions.

Note: Any protein estimation assay can be used to quantify protein. Follow manufacturer's instructions.

Alternatives: If a modified PTFE homogenizer is not available, sonication can also be used to break open *C. elegans* animals to release and extract protein.

7. Proceed directly to "performing the CETSA."

Performing the CETSA

Timing: 2.5 h

The following protocol is adapted for *C. elegans* samples from the original description of CETSA.[2,3] For CETSA, whole cell lysates from *C. elegans* will be treated with different ligands of choice and then be subjected to increasing temperatures on a thermocycler. If the exogenous ligand binds to the *C. elegans* protein, the protein will be stabilized at higher temperatures against denaturation and aggregation compared to control samples. Although the following protocol is specific for the addition of exogenous ligands, this protocol can also be used to assess the thermal stability of *C. elegans* proteins in the presence or absence of endogenous ligands (i.e., in *C. elegans* mutants that do not express the ligand in question compared to wild-type animals).

8. Dilute the protein in the lysate from **Step 5** to 5–10 mg/mL using ice-cold PBS supplemented with 1× Halt Protease and Phosphatase Inhibitor.
   a. 100,000 L4 animals will yield about 1 mL at 10 mg/mL of protein. The protein will then be diluted 1:1 with the compound of choice (final concentration: 5 mg/mL protein) in a following step.
   b. Equilibrate diluted protein lysate to room temperature (22°C) for 10 min.
9. Prepare PCR tubes. Cut 8-tube PCR strips to the number of treatment conditions. For our example, we will treat the lysate from 3xFLAG::NHR-86 *C elegans* animals with either DMSO (solvent control), PCN, R24 (treatments) or PCA (negative control). Hence, we will prepare 8 sets of 4-tube PCR strips for 8 different temperatures.
   a. The first tube in each strip will be control and the second, third, and fourth tubes in each strip will be PCN, PCA, and R24, respectively.
10. Transfer 25 µL of diluted and equilibrated protein lysate to each PCR tube.
11. Using a multichannel, transfer 25 µL of 4% DMSO (final 2% DMSO) or PCN, PCA, or R24 to the first, second, third, and fourth sets of tubes, respectively, and mix well.
    a. To ensure that the samples are exposed to the different temperatures on the thermocyclers at the same time, adding the solvent control or treatment can be staggered 5 min apart to allow the temperatures to ramp up for 'Set 2' (see **Step 13**).
12. Incubate lysate with compounds at room temperature (22°C) for 15 min.
    a. The time for incubation can be adjusted and optimized. Incubation times as little as 5 min and as long as 1 h can be used depending on the protein and compound of interest. These parameters can be determined empirically.
13. During incubation, set the temperatures for the thermocycler. Set 4 thermocycler blocks at one time (see below).
    a. After incubation with the first 4 temperatures (Set 1), the thermocyclers can be set to the next 4 temperatures (Set 2).

b. Set 1:

Block #	Temperature (°C)
1	45
2	48
3	51
4	54

c. Set 2:

Block #	Temperature (°C)
1	57
2	60
3	63
4	66

14. Expose the treated protein lysates to each temperature in 'Set 1' (4-tube strip per temperature) for 3 min by placing tubes directly onto heated blocks.
    a. Close the heated lids while incubating.
    b. Remove tubes from each block and allow samples to cool to room temperature (22°C) for 3 min.
    c. Transfer samples on ice until protein lysates have been exposed to all temperatures. Repeat for temperature 'Set 2.'

⚠ CRITICAL: It is imperative that all samples be exposed to each temperature for the same amount of time for consistent results.

15. Transfer each sample into ice-chilled 1.5 mL microcentrifuge tubes and spin at 20,000 × *g* for 20 min at 4°C.
16. Prepare labeled 8-tube PCR strips for gel electrophoresis.
    a. Label 1 PCR tube for each sample (total 16 PCR tubes).
    b. In each PCR tube, transfer 10 μL 4× LDS sample buffer and 2 μL β-mercaptoethanol (β-ME) (5% of total volume).
17. Carefully transfer 28 μL of the supernatant (without disturbing the pellet) from **Step 14** into the labeled PCR tubes of **Step 15** and mix gently. Pellets may or may not be visible depending on the temperature.

⚠ CRITICAL: To prevent carryover of protein from the sides of the microcentrifuge tubes, do not touch the pipette tips to the sides and take care not to touch pellet at the bottom of the tubes.

Pause point: Samples can be frozen at −20°C until gel electrophoresis and immunoblotting. Use samples within 1–2 days.

Immunoblot analysis

Timing: 2 days

The primary readout for CETSA is immunoblot analysis to assess the solubility of protein at increasing temperatures when exposed to different treatment conditions. The protocol here

describes one method by which you can process and develop your samples. Use any optimized immunoblotting protocol for your protein of interest at this step.

18. Incubate samples at 70°C for 10 min.
19. Load samples into four NuPage 12% Bis-Tris gels, 17-well, 1.0 mm in 1× MES SDS running buffer. Run at 100 V constant for about 2 h.

Note: The amount of protein to be loaded for gel electrophoresis will depend on the efficacy of the primary antibody. For FLAG-tagged proteins, we have had success with loading 10–15 μL of the protein/LDS/β-ME mixture.

20. Transfer 2 gels to Trans-Blot Turbo Transfer Packs containing 0.2 μm Midi nitrocellulose membranes. Use the Trans-Blot Turbo "Mixed MW" program. Repeat for the other 2 gels.
21. Use a total protein stain, such as Ponceau S staining solution, to confirm that treating protein lysates with increasing temperatures leads to decreased total protein on the immunoblot.
22. Block membranes with 5% milk (w/v) in TBST for 1 h at room temperature (22°C).
23. Incubate membranes in primary antibody (anti-FLAG at 1:1000 in 5% milk (w/v)/TBST) 16–20 h at 4°C while shaking.
24. Wash membranes 3 times with TBST at room temperature (22°C), 15 min each with rocking.
25. Incubate membranes in secondary antibody (goat anti-mouse at 1:10,000 in 5% milk (w/v)/TBST) for 1 h at room temperature (22°C) on rocker.
26. Wash membranes 3 times with TBST at room temperature (22°C), 15 min each with rocking.
27. Incubate all 4 membranes with 1–2 mL SuperSignal West Femto substrate per blot at the same time for 5 min.
28. Place all blots in a ChemiDoc Imaging System and develop until bands for all temperatures are visible.

Note: Samples exposed to the highest temperatures (e.g., greater than 60°C) may not show visible bands.

Performing the ITDR

Timing: 2.5 h

Although the absolute kinetics of ligand-substrate binding cannot be determined using CETSA (see limitations), differences in concentrations between multiple compounds to obtain the same thermal stability can be determined using ITDR. ITDR involves treating protein lysates with increasing compound concentrations at one temperature. The temperature used for ITDR can be determined from CETSA experiments and should be chosen based on the greatest thermal difference between control and treatment conditions. As an example, we will describe the ITDR protocol for R24 to test its dose-dependent thermal stabilization of *C. elegans* NHR-86.

29. Repeat **Steps 1–6** and dilute protein lysates to 5–10 mg/mL. Keep protein lysates on ice.
30. Prepare several concentrations of compound(s) to be tested at 2× concentration.
    a. R24 is a compound that can be solubilized in DMSO. We have found that a final DMSO concentration of up to 3% is acceptable and will not lead to variable results.
    b. Prepare 8 concentrations of R24 in PBS at 2× concentration (see preparing ligands).
    c. Prepare 4% DMSO in PBS (2× concentration).
31. In an 8-tube PCR strip, transfer 25 μL protein lysate into each tube.
32. Allow both protein lysate and compound(s) to equilibrate to room temperature (22°C) for 10 min.
33. Transfer 25 μL of 2× compound or solvent control to each PCR tube containing 25 μL of protein lysate.
34. Incubate at room temperature (22°C) for 15 min.

35. While incubating, set up a thermocycler and set to "Incubate" at 60°C for ∞ (i.e., for infinity).

Note: Determine the optimal temperature for ITDR by identifying the CETSA condition that produces the largest difference in temperatures between control and treatment conditions.

36. Transfer the tubes onto the preheated blocks.
    a. Incubate protein lysates treated with compound at 60°C for 3 min with the heated lid closed.
    b. Remove tubes from block after 3 min and allow to cool to room temperature (22°C) for 3 min.
    c. Place on ice.
37. Transfer each mixture into ice-chilled 1.5 mL microcentrifuge tubes. Centrifuge at 20,000 × *g* for 20 min at 4°C.
38. Prepare labeled 8-tube PCR strips for gel electrophoresis.
    a. Label 1 PCR tube for each sample.
    b. In each PCR tube, transfer 10 μL 4× NuPage LDS Sample Buffer and 2 μL β-mercaptoethanol (β-ME) (5% total volume).
39. Carefully transfer 28 μL of the supernatant (without disturbing the pellet) from **Step 37** into the labeled PCR tubes of **Step 38** and mix gently. Pellets may or may not be visible depending on the temperature.
40. Incubate at 70°C for 10 min.
41. Perform gel electrophoresis and immunoblot (see immunoblot analysis).

Note: To optimize consistency between samples, run gel electrophoresis, immunoblot transfer, and immunoblot development/visualization for all samples together in one run.

EXPECTED OUTCOMES

Binding between a protein of interest (e.g., nuclear hormone receptor) and ligand will yield a thermal shift in protein stability towards higher temperatures. By quantifying the protein bands after immunoblotting, one can assess whether the protein in the soluble fraction is more stable at increased temperatures than solvent control alone.

We used CETSA to determine if NHR-86 binds to the phenazine metabolite PCN or the synthetic immunostimulatory molecule R24. We observed that both PCN and R24 thermally stabilized NHR-86 (Figure 1, please also refer to Figures 3C–3E from Peterson, Tse et al.[1]). We also observed that PCA, a close analog of PCN, does not thermally stabilize NHR-86. Hence, the binding between NHR-86 and its ligands is specific.

Here, we performed an ITDR to demonstrate an additional technique to study protein-ligand binding and to further characterize the thermal stabilization of NHR-86 by R24. An ITDR experiment between *C. elegans* 3xFLAG::NHR-86 protein lysates and R24 (Figure 2) revealed that increasing doses of R24 led to increased thermal stability of NHR-86.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of immunoblots

Densitometry of immunoblots can be completed using ImageJ (Fiji). For 4 treatment conditions (i.e., DMSO, PCN, PCA, R24) and 8 temperatures, a total of 32 samples will need to be analyzed. Since we developed the 4 immunoblots in 4 quadrants on the ChemiDoc (Bio-Rad), we will first need to align all 4 blots into one line before we can assess the densitometric band intensities. This can be done on Adobe Illustrator, ImageJ, or other image processing software. Once all 4 blots are aligned, quantify the band intensities of each condition.

42. Use the "Rectangle" tool to draw a rectangle around the first band and press "1" on the keyboard to set the first lane.

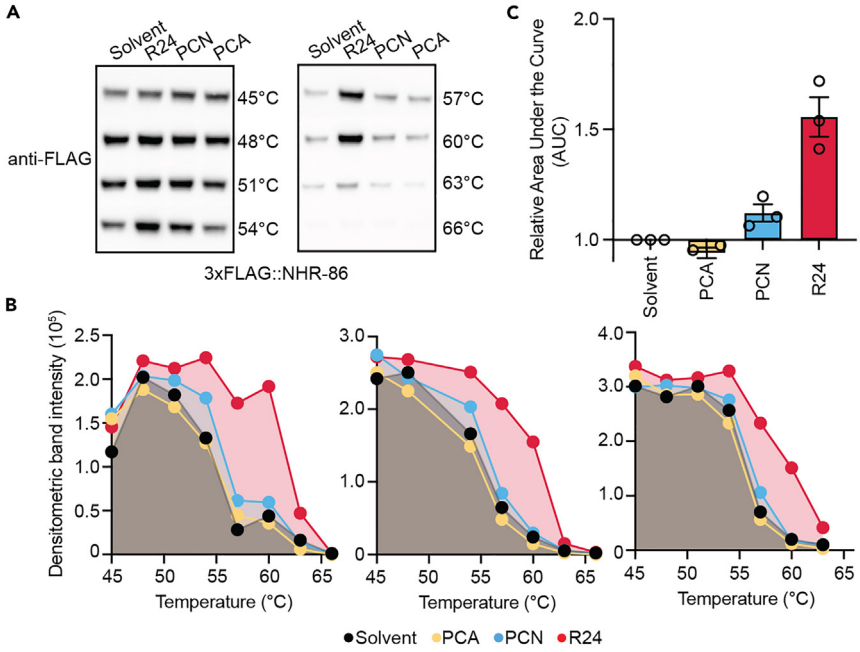


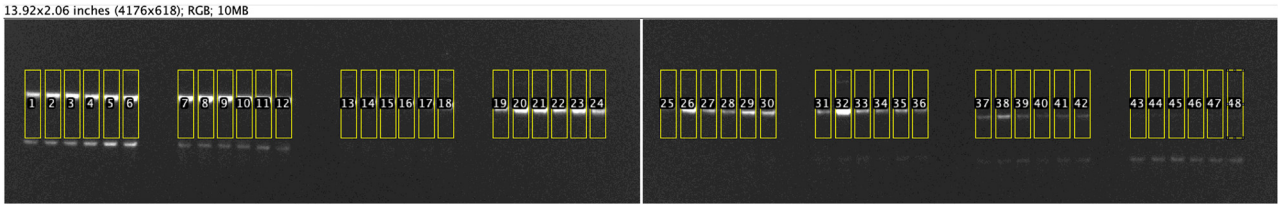
Figure 1. CETSA on *C. elegans* 3xFLAG::NHR-86 protein lysates

(A) Representative immunoblot of CETSA on *C. elegans* 3xFLAG::NHR-86 protein lysates treated with solvent control (2% DMSO), R24 (70 μM), PCN (450 μM) or PCA (450 μM).

(B) Immunoblots from three independent CETSA experiments were quantified by densitometry and plotted over increasing temperatures.

(C). Quantification of the area under the curves (AUC) of the three independent experiments in (B). Error bars represent SEM. All data in this figure have been previously published[1] and permission to use these data here have been granted from all authors.

43. Move the rectangle to the next band and press "2" on the keyboard to identify the next sample. Repeat until a rectangle surrounds every band.



44. When all rectangles are completed, press "3" on the keyboard. Graphs of the bands' intensities will be shown. Using the "Straight" line tool, draw a line at the base of the peak for each band.
45. Use the "Wand (tracing) tool" to highlight the area under the peaks by clicking within the peak of each band. Densitometric quantification of the peaks will begin to appear in a new window. An example dataset is shown in Table 1.
46. CETSA: Graph the raw densitometric values using a graphing software such as GraphPad Prism.
    a. Calculate the area under the curve (AUC) for each condition.
    b. Normalize the AUC for each treatment condition to the control condition. The AUCs for Figure 1C are shown in Table 2.

Table 1. Densitometric quantification of CETSA

	Treatment condition			
Temperature (°C)	2% DMSO	PCA, 450 μM	PCN, 450 μM	R24, 70 μM
45	11700.095	15493.388	16000.267	14545.338
48	20203.288	18798.631	20331.045	22080.702
51	18205.045	16843.995	19859.995	21229.874
54	13302.388	12726.409	17837.167	22442.602
57	2839.983	4478.933	6160.832	17263.924
60	4390.225	3583.569	5970.882	19166.874
63	1637.134	613.87	1303.77	4715.933
66	120.435	0	126.728	125.899

Note: We have found that calculating the AUC for each condition produced more consistent results when comparing the thermal stability of proteins in separate experiments for the same conditions.

Alternatives: Rather than calculating the AUC for each condition, a temperature at which 50% of protein aggregates can be calculated (T_{agg}) by fitting a Boltzmann Sigmoidal curve to the normalized data, as described previously.[2,3]

47. ITDR: Set the lowest value to "1" and highest value to "100". Plot the data points on GraphPad Prism and fit a curve using the "Sigmoidal, 4PL" model.

Note: Because ITDR assays are not reversible, the reactions are not in equilibrium.[2] Thus, it is not possible to determine binding kinetics of a protein and its ligand in this assay (see limitations). However, determining the ITDR for several different compounds can help to further confirm data obtained by CETSA. Further specificity can be obtained by using an endogenously tagged strain of *C. elegans* for another nuclear hormone receptor (*i.e., C. elegans* NHR-12::3xFLAG is not stabilized by R24 or PCN).

LIMITATIONS

The majority of nuclear hormone receptors in *C. elegans* remain orphaned. CETSA and ITDR can be applied to *C. elegans* samples to quickly determine whether there is potential binding between nuclear hormone receptors and compounds of interest.

Successful CETSA and ITDR experiments are dependent on performing consistent immunoblots.[3] Immunoblots are inherently variable (e.g., loading during gel electrophoresis, bubbles during transfer, variability during chemiluminescence development). Thus, it is imperative to maintain consistency throughout the experiments.

A major limitation to CETSA and ITDR in de-orphaning *C. elegans* nuclear hormone receptors is the inability to discern the difference in ligand binding for nuclear hormone receptors with multiple isoforms. For example, NHR-66 has 10 predicted isoforms. These 10 isoforms could theoretically have 10 different binding partners. Unless each isoform can be individually tagged using CRISPR, tagging

Table 2. AUC quantification for CETSA

	Treatment condition			
Replicate	2% DMSO	PCA, 450 μM	PCN, 450 μM	R24, 70 μM
1	194376	238582	342707	194376
2	225696	278511	388332	225696
3	310379	346436	459729	310379

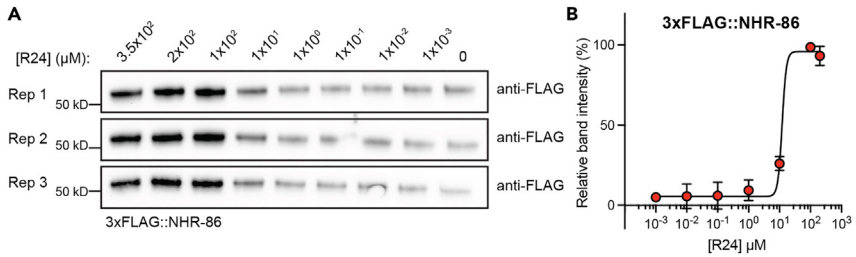


Figure 2. ITDR for *C. elegans* 3xFLAG::NHR-86 protein lysates treated with different concentrations of R24

(A) Three replicates of the immunoblot for *C. elegans* 3xFLAG::NHR-86 protein lysates treated with decreasing concentrations of R24 at 60°C for 3 min.

(B) Relative band intensities from (A) fitted to a "Sigmoidal, 4PL" model. Densitometric values were normalized to solvent control (DMSO). Data points are the average for each of three biological replicates. Error bars represent SD. The data point for 350 μM R24 was not graphed because R24 visibly precipitated out of solution at this concentration.

a nuclear hormone receptor at a terminus shared by all isoforms may not allow for the specific determination of isoform-specific ligands. It is possible, however, to express specific isoforms on an extrachromosomal array.

It is important to perform additional biophysical assays to confirm that a protein and ligand bind. We suggest that these studies be performed using protein expressed and purified from *E. coli*. Because CETSA experiments are performed on whole cell lysates of *C. elegans*, these confirmatory experiments are essential to prove a direct interaction between a ligand and the protein of interest.

Because CETSA and ITDR depend on the irreversible precipitation of protein at higher temperatures, the reaction will never be in equilibrium. Hence, binding constants cannot be determined using these assays. Assays such as isothermal titration calorimetry (ITC),[8] microscale thermophoresis,[9] and intrinsic tryptophan fluorescence assays,[1,10] can all be used to determine binding between substrate and ligand as orthologous methods.

Binding between a protein and ligand theoretically leads to increased thermal stability. However, previous publications have found that direct binding could also destabilize proteins and lead to lowered thermal stability.[11] Hence, additional confirmatory assays need to be performed to determine whether protein and ligand bind, as described above.

TROUBLESHOOTING

Problem 1

Low protein yield (related to Step 6).

Potential solution

- Insufficient worm lysis – Homogenize for additional rounds, transferring the tubes onto ice every 10 strokes to minimize heat.
- Not enough worms – Prepare additional plates, particularly for strains that are developmentally delayed, or consider liquid culture. Although the physiology of worms in liquid culture can be quite different than when grown on solid media, many more worms can be grown.

Problem 2

No difference in protein thermal stability between different conditions for CETSA (related to Immunoblot Analysis).

Potential solution

- Ligand does not bind protein of interest.
- Compound concentrations need to be optimized – Perform the CETSA on multiple compound concentrations using fewer temperatures (e.g., every other temperature) to determine optimal conditions.
- Protein concentrations need to be optimized – Some ligands may theoretically bind to their substrates more efficiently at different protein concentrations. Perform the CETSA on multiple protein concentrations using ~2 ligand concentrations to determine optimal conditions.
- Incubation time needs to be optimized – We describe a protocol wherein protein is exposed to ligand for 15 min at room temperature (22°C). Incubation times as short as 5 min or up to 1 h have produced differences in thermal stabilization of protein and thus should be optimized for specific nuclear hormone receptors.
- CETSA temperatures need to be adjusted – We used temperatures from 45°C–66°C at 3°C increments for NHR-86. The temperature increment and/or temperature range may need to be optimized for different nuclear hormone receptors.

Problem 3

Higher compound concentrations lead to less thermal stability of protein in ITDR experiments compared to lower concentrations (related to Immunoblot Analysis).

Potential solution

- Compounds at high concentrations may not be soluble in PBS, as was observed in Figure 2 for R24 at 350 μM. Ensure that compounds are completely soluble in solution before performing ITDR.
- Binding between ligand and protein of interest may decrease, rather than increase, thermal stability of protein as demonstrated previously.[11] Confirm that ligand and protein bind via orthologous methods (see limitations).

Problem 4

Protein of interest does not appear to be aggregating out of solution (i.e., band intensities are similar from temperature to temperature) by immunoblot (related to Immunoblot Analysis).

Potential solution

- Ensure band is specific for your protein.
- When transferring the supernatant to a new tube (related to Step 17), ensure that the pipette tip does not disturb the pellet or touch the sides of the tubes
- The highest temperature tested may not fully aggregate protein. Expose protein lysates to a higher temperature.

Problem 5

No specific bands visible by immunoblot (related to Immunoblot Analysis).

Potential solution

- Insufficient lysis (see problem 1).
- Transfer did not work (related to step 20) – Although the Trans-Blot Turbo System works well for NHR-86, other proteins might benefit from a wet or semi-dry transfer.
- Temperature range used for CETSA too high (related to step 13) – If the start of the temperature range is too high, the protein will aggregate out of the soluble fraction. Start at a lower temperature.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Read Pukkila-Worley (Read.Pukkila-Worley@umassmed.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

This study did not generate datasets or codes.

ACKNOWLEDGMENTS

We would like to thank Megan Orzalli for use of her laboratory equipment. This work was supported by R01 AI130289 (to R.P.-W.), R01 AI159159 (to R.P.-W.), R21 AI163430 (to R.P.-W.), an Innovator Award from the Kenneth Rainin Foundation (to R.P.-W.), the Dan and Diane Riccio Fund for Neuroscience (to R.P.-W.), F30 DK127690 (to S.Y.T.), T32 AI095213 (to S.Y.T.), T32 GM107000 (to S.Y.T.), and G.E.R.M. Program Research Award from Infectious Disease Society of America (to S.Y.T.).

AUTHOR CONTRIBUTIONS

Conceptualization, S.Y.T., R.P.-W.; investigation, S.Y.T.; visualization, S.Y.T.; funding acquisition, R.P.-W.; project administration, R.P.-W.; supervision, R.P.-W.; writing – original draft, S.Y.T.; writing – review & editing, S.Y.T., R.P.-W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Peterson, N.D., Tse, S.Y., Huang, Q.J., Wani, K.A., Schiffer, C.A., and Pukkila-Worley, R. (2023). Non-canonical pattern recognition of a pathogen-derived metabolite by a nuclear hormone receptor identifies virulent bacteria in C. elegans. Immunity *56*, 768–782.e9. https://doi.org/10.1016/j.immuni.2023.01.027.

2. Martinez Molina, D., Jafari, R., Ignatushchenko, M., Seki, T., Larsson, E.A., Dan, C., Sreekumar, L., Cao, Y., and Nordlund, P. (2013). Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. Science *341*, 84–87. https://doi.org/10.1126/science.1233606.

3. Jafari, R., Almqvist, H., Axelsson, H., Ignatushchenko, M., Lundbäck, T., Nordlund, P., and Martinez Molina, D. (2014). The cellular thermal shift assay for evaluating drug target interactions in cells. Nat. Protoc. *9*, 2100–2122. https://doi.org/10.1038/nprot.2014.138.

4. Dokshin, G.A., Ghanta, K.S., Piscopo, K.M., and Mello, C.C. (2018). Robust Genome Editing with Short Single-Stranded and Long, Partially Single-Stranded DNA Donors in Caenorhabditis elegans. Genetics *210*, 781–787. https://doi.org/10.1534/genetics.118.301532.

5. Ghanta, K.S., Ishidate, T., and Mello, C.C. (2021). Microinjection for precision genome editing in Caenorhabditis elegans. STAR Protoc. *2*, 100748. https://doi.org/10.1016/j.xpro.2021.100748.

6. Booth, W.T., Schlachter, C.R., Pote, S., Ussin, N., Mank, N.J., Klapper, V., Offermann, L.R., Tang, C., Hurlburt, B.K., and Chruszcz, M. (2018). Impact of an N-terminal Polyhistidine Tag on Protein Thermal Stability. ACS Omega *3*, 760–768. https://doi.org/10.1021/acsomega.7b01598.

7. Gerstein, M.B., Lu, Z.J., Van Nostrand, E.L., Cheng, C., Arshinoff, B.I., Liu, T., Yip, K.Y., Robilotto, R., Rechtsteiner, A., Ikegami, K., et al. (2010). Integrative Analysis of the Caenorhabditis elegans Genome by the modENCODE Project. Science *330*, 1775–1787. https://doi.org/10.1126/science.1196914.

8. Velazquez-Campoy, A., and Freire, E. (2006). Isothermal titration calorimetry to determine association constants for high-affinity ligands. Nat. Protoc. *1*, 186–191. https://doi.org/10.1038/nprot.2006.28.

9. Romain, M., Thiroux, B., Tardy, M., Quesnel, B., and Thuru, X. (2020). Measurement of Protein-Protein Interactions through Microscale Thermophoresis (MST). Bio. Protoc. *10*, e3574. https://doi.org/10.21769/bioprotoc.3574.

10. Yammine, A., Gao, J., and Kwan, A.H. (2019). Tryptophan Fluorescence Quenching Assays for Measuring Protein-ligand Binding Affinities: Principles and a Practical Guide. Bio. Protoc. *9*, e3253. https://doi.org/10.21769/bioprotoc.3253.

11. Tillman, M.C., Khadka, M., Duffy, J., Wang, M.C., and Ortlund, E.A. (2019). Structural characterization of life-extending Caenorhabditis elegans Lipid Binding Protein 8. Sci. Rep. *9*, 9966. https://doi.org/10.1038/s41598-019-46230-8.