Protocol

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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
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SUMMARY
eIF5-mimic protein (5MP) controls translation through its interaction with eukaryotic translation initiation factor (eIF) 2 and eIF3 and alters non-AUG translation rates for oncogenes in cancer and repeat expansions in neurodegenerative disease. To precisely evaluate the effect of 5MP mutations on binding affinity against eIFs, here we describe two label-free protocols of affinity measurement for 5MP binding to eIF2 or eIF3 protein segments, termed isothermal titration calorimetry (ITC) and bio-layer interferometry (BLI), starting with how to purify proteins used. For complete details on the use and execution of this protocol, please refer to Singh et al. (2021).

BEFORE YOU BEGIN
Translation initiation in eukaryotes is a complex process involving 5'-terminally capped mRNA, ribosome, Met-tRNA\textsuperscript{Met} and eukaryotic translation initiation factors (eIF) 1, 1A, 2, 3, 4F, 5 and 5B (Asano, 2014; Asano et al., 2001; Hinnebusch et al., 2007). As a molecular mimic of the C-terminal part of the eIF5, eIF5-mimic protein regulates translation initiation (Singh et al., 2011). Of note, recent studies highlight its ability to modulate translation initiation frequencies from near-cognate (non-AUG) start codons for oncogenes in cancer (Sato et al., 2019; Tang et al., 2017) and for repeat-expansion in neurodegenerative disease (Singh et al., 2021). Here we describe two methods of label-free affinity measurement for 5MP binding to its binding partners in the \( \beta \) subunit of eIF2 and the c subunit of eIF3, using isothermal titration calorimetry (ITC) and bio-layer interferometry (BLI). To dissect the interaction of human 5MP1 (h5MP1), one of the two copies of 5MP found in Homo sapiens, we used two mutations altering acidic and basic surfaces of its C-terminal domain termed 7A (Singh et al., 2021) and BN1 (Singh et al., 2021). We also used the minimal segments of eIF2\( \beta \) (Luna et al., 2012) and eIF3c (Singh et al., 2021) termed h\( \text{eIF2\( \beta \)}_{53-136} \) and h\( \text{eIF3c}_{20-102} \), respectively, determined to be responsible for their binding to h5MP1.

In ITC, highly purified molecules (proteins, nucleic acids or ligands) are mixed together, and the interaction between the molecules in the system is determined by the amount of heat released...
(Pierce et al., 1999). The isothermal titration calorimeter, or the ITC machine, has two cells, one for reference and the other with samples. A fixed amount of a molecule is loaded into the sample cell, to which increasing amounts of the other molecule is added for a titration experiment. The output profile, a thermogram, is expected to display a sigmoidal curve, which allows the calculation of the dissociation constant (K_D) for the binding affinity and stoichiometry (N) between the two molecules.

Workflow of the ITC assay:

1. Dilute highly purified proteins to a fixed concentration (typically between 1 μM to 1 mM, depending on K_D of their binding).
2. Wash Cell & Syringe of the ITC instrument (MicroCal ITC200) thoroughly with deionized water followed by size-exclusion buffer.
3. Fill sample cell with 200 μL of a protein and pipette/injector with 40 μL its binding partner.
4. Titrate samples at an equilibrium temperature of 25°C under a program setting.
5. Process data sets and plotted the results using Origin software.

The BLI (e.g., the BLItz system from ForteBio) detects in real-time the dynamic changes in reflectance interference wave pattern due to the change in protein mass on the biosensor tip (Rich and Myszka, 2007). Among the many methods of immobilizing a binding molecule to the biosensor tip, the anti-GST biosensor was chosen due to the convenience of one-step immunoaffinity-purification of glutathione-S-transferase (GST)-fusion proteins.

Workflow of the BLI assay (onscreen instructions were prompted by the software):

6. Take Initial Baseline using 200 μL of appropriate binding buffer for 30 s.
7. Load the appropriate GST fusion protein on the Anti-GST Biosensor in 4 μL for 120 s as you observe loading curve in real time.
8. Take another Baseline measurement using 200 μL of BLI binding buffer for 30 s.
9. Perform association with the analytes (binding partner) using 4 μL of known concentration of the analytes for 120 s; it provides the binding/association curve.
10. Conduct dissociation step in 250 μL of BLI binding buffer for 120 s so that the dissociation curve can be generated.

Successful experiments critically depend on the quality (in most cases, purity) of the proteins used. Below we describe the preparation of the materials established in our labs in detail, which were used for these label-free assays reported in (Singh et al., 2021).

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bacterial and virus strains | E. coli BL21 (DE3), chemically competent cells | EMD Biosciences | 69451 |
| Chemicals, peptides, and recombinant proteins | Agar | Fisher Scientific | BP1423-500 |
| | Ampicillin | GoldBio | A-301-100 |
| | Ammonium persulfate | Sigma-Aldrich | A-6761 |
| | β-Mercaptoethanol | Sigma-Aldrich | M6250-250 |
| | Bromophenol Blue | Sigma-Aldrich | B8026-5GM |
| | BSA | Pierce Thermo Scientific | 23209; 2 mg/mL |
| | DTT | Sigma-Aldrich | D9779 |
| | EDTA | Fisher Scientific | S311-500 |

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### Protocol

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Glycine             | Sigma-Aldrich | G7126-5KG |
| Glutathione Sepharose 4B | Sigma-Aldrich | Cytiva 17-0756-01 |
| Ethanol             | Decon Labs, Inc. | 2701 1 GAL |
| Glycerol            | Fisher Scientific | BP229-1 |
| HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) | GoldBio | H-401-500 |
| Hydrochloric Acid   | Fisher Scientific | A-1445-500 |
| Imidazole           | Fisher Scientific | O3196-500 |
| Isopropanol (IPA)   | GoldBio | 12481C-50 |
| Kanamycin monosulfate | GoldBio | K-120-25 |
| Luria Bertini (LB) media | Fisher Scientific | BP-1426-2 |
| Sodium chloride (NaCl) | Research Products International | S23030-12000 |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich | L3771-500 |
| Sodium hydroxide (NaOH) | Fisher Scientific | SS255-1 |
| TCEP                | GoldBio | TCEP25 |
| TEMED               | Bio-Rad | 161-0800 |
| TGX-stain free fast cast Acrylamide 12% kit | Bio-Rad | 1610185 |
| Tris Base           | Research Products International | T60040-1000 |
| Tween 20            | Fisher Scientific | BP337-500 |

**Recombinant DNA**

| pET28 a/b/c (+) | EMD Biosciences | 69864 |
| pET30 GBFusion1 | Gerhard Wagner | N/A |
| pET-hSMP1      | (Hirashi et al., 2014) | Asano lab p1479 |
| pET-hSMP1-7A   | (Singh et al., 2021) | Asano lab p1957 |
| pET-hSMP1-BN1  | (Singh et al., 2021) | Asano lab p1958 |
| pGEX-hSMP1     | (Singh et al., 2011) | Asano lab p1535 |
| pGEX-hSMP1-7A  | (Singh et al., 2011) | Asano lab p1116 |
| pGEX-hSMP1-BN1 | (Singh et al., 2021) | Asano lab p1818 |
| pGB-heIF2b-K2K3 | (Luna et al., 2012) | Asano lab p1336 |
| pGB-heIF3c12-His | (Singh et al., 2021) | Asano lab p1837 |

**Software and algorithms**

| Origin software | MicroCal | N/A |
| BLItz Pro software | ForteBio | N/A |

**Other**

| Amicon Ultra Centrifugation Unit | Millipore | UFC901024 |
| Anti-GST biosensors | ForteBio | 18-5096 |
| Autoclave | STERIS | Amsco Lab 250 |
| Baffled Shaker Flasks, 2 Liters | KIMAX Kimble | 25630 |
| Bottle Top Filter | CELLTREAT Scientific Products | 229716 |
| BLItz | ForteBio | 45-5000 |
| Centrifuge | Thermo Scientific | Sorval ST 16R |
| Centrifuge Bottles (1 Liter) | Beckman Coulter | 366751 |
| Centrifuge Tubes (50 mL) | Genesee Scientific | 28-108 |
| Deionized Water Unit | Thermo Scientific | 7119 |
| Disc filter membrane | Millipore | GPWP04700 |
| Dialysis membrane tube | Thermo Scientific | 68100 |
| Dialysis membrane clips | Thermo Scientific | 68011 |
| FPLC | Cytiva (Previously GE) | Akta Pure |
| Fraction collection tubers | Fisher Scientific | 14-96-27 |
| Glassware/plasticware | N/A | N/A |
| Hamilton Syringe | Hamilton | Syn50018P |
| High speed centrifuge | Beckman Coulter | Avanti J-26XP |
| High speed centrifuge rotor | Beckman Coulter | JLA 8.1 |
| High speed centrifuge rotor | Beckman Coulter | JA 30.5 |
| HiLoad 16/600 Superdex 75 pg Column | Cytiva (Previously GE) | 289893-33 |

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MATERIALS AND EQUIPMENT

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Incubator           | Labline Instruments Inc | Imperial III |
| Incubator Shaker    | New Brunswick Scientific | Excella E24 |
| Incubator Shaker    | New Brunswick Scientific | I2500 Series |
| Isothermal Titration Calorimetry Instrument | Malvern | MicroCal iTC200 |
| Nano Drop UV Spectrophotometer | Thermo Scientific | NanoDrop One |
| Loop/Spreader       | N/A    |            |
| Oak Ridge PSF tubes | Nalgene | 3115-0050 |
| Petri dishes        | N/A    |            |
| Protein Electrophoresis Equipment | Bio-Rad | PowerPac Basic |
| Protein Electrophoresis Running Reservoir | Bio-Rad | MiniProtein Tetra Cell |
| Protindex Hibond Ni-NTA | Marvelgent Biosciences | 11-025-050 |
| Agarose 6 Fast Flow Resin | Marvelgent Biosciences |            |
| Sonicator           | Misonix | S-4000     |
| Sonicator Stainless Steel container | N/A | N/A        |
| Spectrophotometer   | Amersham Biosciences | Ultraspec 10 |
| Syringe Filter      | Sarstedt | 83.1826.001 |
| –80°C Ultradeep Freezer | New Brunswick Scientific | U535 Innova |

**LB Media:**

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| LB Media Miller  | N/A                 | 25 g   |
| diH2O            | N/A                 | 1 liter|
| Total            | N/A                 | 1 liter|

**LB Agar Plates**

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| LB Media Miller  | N/A                 | 25 g   |
| Agar             | 1.5% w/v            | 7.5 g  |
| diH2O            | N/A                 | 1 liter|
| Total            | N/A                 | 500 mL |

Antibiotic Stock: Ampicillin 200 mg/mL; Kanamycin 25 mg/mL.

- Weigh required amount of LB (25 grams X n, where n liter of media) and dissolve in deionized water (n liters).
- Pour 1 liter using a measuring cylinder in 2 L Erlenmeyer flask and autoclave (using the 20 min sterilization cycle at 121°C and 20 psi).
- Store at room temperature if used within 12 h; otherwise, store at 4°C for maximum duration of 1 year.

Petri plates/dishes: 20–25.

- Weigh required amount of LB and agar powder. Dissolve in deionized water.
- Pour 1 liter in 1-liter autoclavable bottle and autoclave (using the 20 min sterilization cycle).
- Let the media cool to 50°C, add appropriate antibiotic, mix (Final concentrations: Ampicillin 100 µg/mL; Kanamycin 50 µg/mL).
- Pour 20–25 mL in petri plates.
- Let it set and store at 4°C in dark for maximum duration of 3 months.
Note: Let the media cool down to ~55°C before adding antibiotics to it. LB Agar Ampicillin or Kanamycin plates can be stored at 4°C for the maximum duration of 2–3 weeks.

Note: Prepare LB agar plates (with appropriate antibiotics) & LB media in advance.

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**10× SDS-PAGE Running Buffer (1 Liter)**

| Reagent      | Final concentration | Amount   |
|--------------|---------------------|----------|
| Tris base    | 0.25 M              | 30.3 g   |
| Glycine      | 1.924 M             | 144.4 g  |
| SDS          | 1% w/v              | 10 g     |
| ddH₂O        | N/A                 | To make volume 1 liter |
| Total        | n/a                 | 1 liter  |

- Fill beaker with 800 mL of ddH₂O and place on stir plate with stir bar.
- Weigh Tris base & Glycine and add with stirring, allow to dissolve.
- Weigh SDS and add slowly to mix thoroughly.
- Bring to 1-liter final volume with ddH₂O.
- Store in a glass bottle at room temperature for maximum duration of 1 year.

Note: The pH of buffer should be 8.1–8.3 and there’s no need of pH adjustment. 10× running buffer can be stored at room temperature for long time. Dilute to 1× using ddH₂O prior to use for running gels.

Note: Mix-in SDS slowly while stirring to avoid frothing.

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**5× Protein Loading Buffer (Laemmli Buffer) (10 mL)**

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| 1 M Tris pH 6.8 | 0.25 M              | 2.5 mL   |
| Glycerol      | 50% w/v             | 5 mL     |
| SDS           | 10% w/v             | 1 g      |
| Bromophenol Blue | 0.25% w/v         | 25 mg    |
| β-mercaptoethanol | 25% v/v           | 2.5 mL   |
| ddH₂O         | N/A                 | To make volume 10 mL |
| Total         | n/a                 | 10 mL    |

- Add Tris and glycerol in a beaker and place on stir plate with stir bar.
- Mix in slowly SDS and let it dissolve.
- Add Bromophenol blue to the solution.
- Aliquot and store at room temperature or 4°C for maximum duration of 1 year.

Note: Add β-mercaptoethanol (final 5%) to aliquot prior to use. Once you add β-mercaptoethanol, store aliquot at −20°C.

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**8× Binding Buffer (1 Liter)**

| Reagent      | Final concentration | Amount   |
|--------------|---------------------|----------|
| Tris base    | 160 mM              | 19.38 g  |
| NaCl         | 500 mM              | 29.22 g  |
| imidazole    | 40 mM               | 2.72 g   |
| ddH₂O        | N/A                 | To make volume 1 liter |
| Total        | n/a                 | 1 liter  |
• In a beaker add 800 mL dH₂O and place on stir plate with stir bar.
• Weigh all chemicals and add to dH₂O.
• Bring volume to 1 liter, transfer to glass bottle and store at room temperature for maximum duration of 1 year.

Note: Prior to use, dilute to 1× with cold dH₂O, add TCEP (1 mM final concentration) and adjust pH to 8.0. Filter with 0.22 μm filter and store at 4°C.

### 4× Elution Buffer (1 Liter)

| Reagent     | Final concentration | Amount  |
|-------------|---------------------|---------|
| Tris base   | 160 mM              | 19.38 g |
| NaCl        | 500 mM              | 29.22 g |
| imidazole   | 4 M                 | 272.32 g|
| dH₂O        | N/A                 |         |
| Total       | N/A                 | 1 liter |

• In a beaker add 800 mL dH₂O and place on stir plate with stir bar.
• Weigh all chemicals and add to dH₂O.
• Adjust pH to 7.5 using HCl or NaOH.
• Bring volume to 1 liter, transfer to glass bottle and store at 4°C for maximum duration of 1 year.

Note: All chemical can be prepared in advanced but TCEP should be added before use with adjustment of solution pH.

### Size Exclusion Buffer (1 Liter)

| Reagent   | Final concentration | Amount  |
|-----------|---------------------|---------|
| HEPES     | 10 mM               | 2.38 g  |
| NaCl      | 125 mM              | 7.3 g   |
| TCEP      | 1 mM                | 0.28 g  |
| dH₂O      | N/A                 |         |
| Total     | N/A                 | 1 liter |

• In a beaker add 800 mL dH₂O and place on stir plate with stir bar.
• Weigh all chemicals and add to cold dH₂O.
• Add TCEP (1 mM final concentration) and adjust pH to 8.0.
• Filter with 0.22 μm filter and store at 4°C.

### Thrombin Dialysis Buffer

| Reagent | Final concentration | Amount  |
|---------|---------------------|---------|
| Tris base | 20 mM               | 2.42 g  |
| NaCl    | 150 mM              | 8.77 g  |
| TCEP    | 1 mM                | 0.28 g  |
| dH₂O    | N/A                 |         |
| Total   | N/A                 | 1 liter |

• In a beaker add 800 mL dH₂O and place on stir plate with stir bar.
• Weigh all chemicals and add to dH₂O.
• Bring volume to 1 liter, transfer to glass bottle and store at room temperature for maximum duration of 1 year.

Note: Dilute to 1× with cold dH₂O, add TCEP (1 mM final concentration) and adjust pH to 8.0. Filter with 0.22 μm filter and store at 4°C.
• In a beaker add 800 mL dd H2O and place on stir plate with stir bar.
• Weigh all chemicals and add to cold dd H2O.
• Adjust pH to 8.0 using HCl.
• Bring volume to 1 liter, transfer to glass bottle and store at 4°C.

| Phosphate Buffered Saline (PBS) (1 Liter) | Reagent          | Final concentration | Amount |
|----------------------------------------|------------------|---------------------|--------|
| NaCl                                   | 137 mM           | 8 g                 |
| KCl                                    | 2.7 mM           | 0.2 g               |
| Na2HPO4                                | 10 mM            | 1.44 g              |
| KH2PO4                                 | 1.8 mM           | 0.24 g              |
| ddH2O                                  | N/A              | To make volume 1 liter |
| Total                                  | N/A              | 1 liter             |

• In a beaker add 800 mL dH2O and place on stir plate with stir bar.
• Weigh all chemicals and add to cold dH2O.
• Adjust pH to 7.4 using HCl.
• Bring volume to 1 liter, transfer to glass bottle and store at 4°C. Good for 1 year.

| BLI Binding Buffer (PBS-based) (10 mL) - Make this prior to the assay | Reagent | Final concentration | Amount |
|---------------------------------------------------------------------|---------|---------------------|--------|
| 1x PBS                                                              | 1x      | 10 mL               |
| Tween 20                                                            | 0.05%   | 5 µL                |
| BSA (2 mg/mL)                                                       | 0.0005% | 0.25 µL             |
| 1 M DTT                                                             | 0.1 mM  | 10 µL               |

STEP-BY-STEP METHOD DETAILS

⊙ Timing: 5 days for h5MP1 protein purification
⊙ Timing: 1.5–2 h for Day 1
⊙ Timing: 16–18 h for Day 2
⊙ Timing: 5 h for Day 3

Expression & purification of human 5MP1 (h5MP1) and its binding partners helF2β53-136 and helF3c20-102.

1. h5MP1 protein purification.
   Day 1.
   a. Transformation of BL21 (DE3) E. coli cells with pET-h5MP1 (Wild Type/BN1/7A) and helF2β53-136 and helF3c20-102.
   i. Take a vial of competent cells and thaw on ice.
   Note: Make sure to thaw competent cells on ice and handled gently as sudden changes of temperature affects cells viability.

   ii. Add <100 ng of plasmid to the competent cells.
iii. Incubate cells-DNA mixture on ice for 30 min.
iv. Heat shock competent cells at 42°C for 30 s.
v. Add 300 µL of sterile LB broth to the tube and incubate at 37°C in a shaker incubator at 220 rpm for 1 h.
vi. Plate 60 µL of cells onto LB agar plates with 50 µg/mL Kanamycin and place the plates in an incubator overnight (16–18 h) at 37°C.

Day 2.
b. Pre-culture for h5MP1, helF2β53-136 and helF3c20-102.
i. Inoculate a single transformed colony into 100 mL LB media with Kanamycin (50 µg/mL) and incubated in shaker at 220 rpm at 37°C overnight.

day 3.
c. Large scale cultures.
i. Inoculate 20 mL of the overnight pre culture into 1 L LB media with Kanamycin (50 µg/mL) and incubate in shaker at 220 rpm at 37°C till OD600=0.6.
ii. Induce cell culture for T7 RNA polymerase expression with 0.5 mM IPTG final concentration and incubate further for 3–4 h.
iii. Harvest cells by centrifugation at 14000 g (Beckmann JLA 8.1 Rotor) for 15 min at 4°C and store pellets in the −80°C freezer.

Note: Maintain sterile condition while working with bacterial culture to avoid any contamination. Use aseptic conditions for all transfer, clean bench and surfaces with 70% ethanol.

Day 4.
d. Purification of h5MP1, helF2β53-136 and helF3c20-102.
i. Thaw bacterial pellets at room temperature and resuspended in 35 mL/1 liter culture binding buffer (this buffer may contain protease inhibitors, see troubleshooting Problem 1). Lyse cells by sonication (10 s on; 20 s off cycle for a total of 3.5 min processing time at 95% amplitude) in ice-water mixed bath.

Note: Take 20 µL aliquot of cell lysate before centrifugation, add 5 µL 5× protein loading dye and boil for 2–3 min at 98°C. Label it as whole cell lysate and keep it aside to run on SDS-PAGE later.

iii. Equilibrate Ni-NTA column (5 mL resin bed volume) with binding buffer to at least 5 CV (25 mL). Load clear lysate to the column at 2 mL/min. Collect the flow-through.

Note: Take 20 µL aliquot of flow-through, add 5 µL 5× protein loading dye and boil for 2–3 min at 98°C. Label it as flow through and keep it aside to run on SDS-PAGE later.

iv. Wash the column with binding buffer until the absorbance at 280 reaches a plateau. This is followed by a second wash step with 5% of elution buffer. A peak should elute containing non-specific binding proteins. Wash until absorbance decreases and reaches a plateau. Collect all washes.

Note: Take 20 µL aliquot of washes, add 5 µL 5× protein loading dye and boil for 2–3 min at 98°C. Label it 5% wash and keep it aside to run on SDS-PAGE later.

v. Elute protein in 9 mL fractions with 50% elution buffer.
vi. Analyze eluted fractions along with whole cell lysate, flow-through and washes on 12% SDS-PAGE gel, and pool together the fractions containing purified proteins.
vii. Remove the His-tag was by adding Thrombin (Hemotech) 10 Units/mg to protein and dialyze overnight at 4°C against Thrombin Dialysis buffer.

viii. Analyze the digestion on a 12% SDS-PAGE. If the tag has been removed, add benzamidine to a final concentration of 1 mM. Load the protein solution onto a Ni-NTA column pre-equilibrated with binding buffer to remove any remanent non-cleaved protein. Collect flow-through.

Note: After His-tag removal, protein does not bind to column and will come in flow-through. Still check 5% wash for any non-specific binding of protein to column.

ix. Concentrate protein using Centrifugal Filtration Unit (10 kDa MW Cut off) to 3 mL and load onto a HiLoad 16/60 Superdex 75 preparation-grade column pre-equilibrated with size-exclusion buffer (10 mM HEPES pH 7.5, 125 mM NaCl, 1 mM TCEP) for further polishing and removal of the small amount of thrombin.

x. Analyze the eluted fractions on a 12% SDS PAGE for purity. Pool fractions with protein and concentrate to 2 mg/mL final concentration, aliquote and store at −80°C freezer for the range of duration between 3 to 12 months (depending on protein purification fractions). See Figure 1, lanes 1–3 for the final products.

Note: All buffers for purification need to be prepared with cold deionized water, filter with 0.22 μm filter and store at 4°C. All columns used for purification need to be stored at cold temperature.

Note: All protein purification steps need to be done over ice or at 4°C.

2. Preparation of lysates containing GST-h5MP1.

The lysates containing GST-h5MP1 proteins (WT, 7A or BN1 mutant) for their immobilization to the anti-GST biosensors in BLI were prepared as described in 1) up to step d-ii, except that BL21(DE3) transformants carrying pGEX-h5MP1 or its derivative were IPTG (0.5 mM) -induced for 1 h at 30°C and lysed in PBS containing 1% Triton X-100. Intact full-length GST-fusion product was analyzed as follows:

a. 250 μL of the cell lysates is added to 4 μL bed-volume of Glutathione Sepharose 4B and incubated for 30 min at room temperature on a rocker.

b. Wash the resins three times with 0.2 mL ice-cold PBS.

c. Add 3 μL of 5X Protein Loading Buffer and heat the tubes at 95°C for 2 min to elute the bound GST fusion proteins.

d. Analyze the supernatant by SDS-PAGE, followed by Coomassie Blue staining.

Note: GST-fusion protein concentration is expected to be ~20 μg/mL.

** Isothermal titration calorimetry (ITC)**

© Timing: 1–1.5 h each run
Experiments are performed on an MicroCal ITC200 instrument. This assay uses untagged proteins.

3. Setting up ITC run.
   a. All proteins used in the ITC must be buffer exchanged into size exclusion buffer using HiLoad 16/60 Superdex 75 column.
   b. Prepare all solutions of h5MP1 proteins (WT or BN1/7A mutants) to 20 mM final concentration. helF2b53-136 is at 200 mM final concentration.
   c. Wash Cell & Syringe of MicroCal ITC200 instrument thoroughly with deionized water followed by size-exclusion buffer.
   d. Fill cell with 200 µL of h5MP1 using a Hamilton syringe making sure no bubbles are left in the chamber by gently agitating the chamber with the syringe.
   e. Fill the pipette/injector with 40 µL of helF2b53-136 using the automated injection fill step. Move pipette/injector into the filled cell chamber.

4. Running ITC titration.
   a. The program will wait for a stable baseline at an equilibrium temperature of 25 °C.
   b. The program setting consists of 20 injections with first unique injection of 0.5 µL and remaining 19 injections of 2 µL each with 2n injection time where n = injection volume and 150 s spacing between each injection. The agitation speed was set to 750 rpm.

5. Data Analysis.
   a. Analyze all datasets (Figure 2, top) to obtain the binding constant, stoichiometry and binding enthalpy using the single site model within the ITC data analysis package of the Origin software. The software automatically determines the fitting parameters such as the baseline, the range to integrate the injection peaks and the initialization of the fitting parameters upon calculation of the heat of reactions (Figure 2, bottom).

6. Conclusions.

Repeat experiments under same condition for a total of 3 runs. The ITC200 Origin software calculates all the thermodynamic parameters based on the initial concentrations of the proteins, these included ΔH, ΔG, ΔS, Kd and stoichiometry.

Figure 2. Representative single ITC titrations
The raw data of the titrations is shown in top while the non-linear least square fit of the integrated heats as a function of the molar ratio of the different protein pairs.
(A–C) (A) h5MP1-WT/helF2b53-136; (B) SMP1-BN1/helF2b53-136 and (C) SMP1-7A/helF2b53-136.
Biolayer interferometry (BLI)

**Timing:** 3 h each run

Experiments were performed on a BLItz Label-Free Protein Analysis System from ForteBio. This assay uses GST-5MP1 as ligand; and purified heIF3c20-102 and GB-heIF2b53-136 as analytes.

7. Biosensors activation and setting up the BLItz system (Figure 3A).
   a. Six anti-GST biosensors were first hydrated using 200 µL BLI binding buffer for 10 min in a tube. BSA and Tween-20 were added to avoid any non-specific binding. The biosensors were kept hydrated in PBS buffer until binding experiments were ready.

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**Figure 3. Operation of the BLItz system**

(A) Operation interface and parameters.

(B) Chronological steps of operations. 1, Initial baseline; 2, Loading; 3, Baseline; 4, Association; 5, Dissociation.
b. Advanced kinetic module was chosen as experiment type to perform the affinity binding assay with 2000 rpm shaker speed. GST-5MP1 was first loaded on the anti-GST biosensor followed by analyte association and dissociation.

c. The parameter for Initial Baseline, Loading, Baseline, Association and Dissociation are set at 30, 120, 30, 120 and 120 s respectively.

d. Bacterial cell lysates containing GST-5MP1, GST-5MP1-BN1 and GST-5MP1-7A proteins were used as ligands to immobilize on the anti-GST biosensors. The two proteins, heIF3c20-102 and GB-heIF2β53-136 purified for ITC experiment were used as analytes. Their concentrations were measured using Protein Bradford Assay and verified with SDS-PAGE. Prior to setting the experiments multiple simple binding assays were performed to check the binding pattern of ligand-analyte interaction.

Note: The initial preliminary runs provide information for deciding the different concentrations to be used in the experiment. Accordingly, different concentrations (0 μM reference, 0.031 μM, 0.0625 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1.0 μM, 2.0 μM) of the analyte were freshly prepared and equilibrated at the room temperature.

Note: We used GST proteins in the lysates instead of purified ones, because the former contains other bacterial proteins which act as good blocking agents on the biosensor. The anti GST biosensor is very specific for GST tagged proteins; even if there are non-specific traces bound, they were cleared up in the subsequent washing step.

8. Ligand binding using Anti-GST biosensor (Figure 3B, steps 7–9).
   a. Conduct Initial Baseline for 30 s prior to the GST-5MP1 or GST-5MP1-BN1 or GST-5MP1-7A loading on the chip using the drop holder.
   b. Perform Loading step using 4 μL lysate of GST tagged proteins. Set the Loading duration for 120 s with shaker speed at 2000 rpm.
   c. Conduct a 30 s baseline step to remove unbound GST-5MP1 proteins using tube position. After every step, wash the drop holder three times using double distilled water.

9. Analytes association and dissociation steps through Advanced Kinetic assay module (Figure 3B, steps 10 and 11).
   a. Perform the Association step for different concentration of the purified analytes, heIF3c20-102 and GB-heIF2β53-136, starting with 0 μM concentration as reference to be incorporated during the data analysis. Set the parameter for this step at 120 s and 2000 rpm and generate an association curve.
   b. Perform the Dissociation step for 120 s at 2000 rpm. Do this measurement using 200 μL of BLI binding buffer to get the dissociation curve.
   c. Repeat the sequential steps of Initial Baseline to Dissociation for each concentration of the GB-heIF3c20-102 or GB-heIF2β53-136 by replacing new Anti-GST biosensors.
   d. Complete 6 runs of an assay for every ligand and analyte combination, during which, the run data are closely monitored.
   e. If the sensogram comes out irregular with erratic curves for various reasons, repeat the step by additional runs. Generally it is caused by tiny air-bubbles introduced while pipetting the solution. Tiny air-bubbles can affect the refractive index and thus the interference pattern. Exclude The while analyzing the data.

10. Analysis of data (Figure 4).
    a. Perform data analysis with the BLItz Pro software. Following the runs, carefully observe every measurement curve for any erratic patterns. After the erratic runs, analyze the data using Global fitting (1:1) to display curve fits and results (Figure 4A).
    b. Enable step corrections for both for the start of association and the start of dissociation; Set the fitting parameter to Global.
    c. Record final results (Figure 4B) and export them into an excel file for additional statistical analysis (e.g., by 2-tailed students’ Ttests).
11. Conclusion.

Repeat experiments for at least 3 runs for every combination of ligand and analyte. Following the assay analysis, BLItz Pro software could generate association rate constant, $k_a$ (1/Ms), dissociation rate constant, $k_d$ (1/s), and affinity constant, $K_D$ (M). In addition, maximum response ($R_{\text{max}}$), standard error in $R_{\text{max}}$ and response at equilibrium ($R_{\text{equilibrium}}$) could also be provided.

EXPECTED OUTCOMES

For protein purification, we expect a single protein species with expected molecular weight of >95% purity on SDS-PAGE. If a protein with unexpected size is observed in addition to or in place of the expected product, it is either its degradation product (if shorter than expected) or unrelated co-purifying E. coli protein and cannot be used in the downstream experiment (see below troubleshooting).

In ITC, it is expected to obtain dissociation constant ($K_D$) for the binding affinity and stoichiometry ($N$) between the two molecules. In BLI, association and dissociation rate constants ($k_a$ and $k_d$) are obtained besides $K_D$. However, the interpretation of the obtained values needs careful consideration and examination (see below limitations and troubleshooting).

LIMITATIONS

$K_D$ for interaction between given two molecules can vary widely depending on the methods used to measure it. For example, $K_D$ for interaction between h5MP1 and eIF2β33-136 was 0.12 ± 0.01 μM in BLI and 5.0 ± 0.15 μM in ITC (Singh et al., 2021). The difference may be due to that in the conformation of interacting molecules, such as due to attachment of different peptide tags (such as GST or GB1) or buffer conditions. Ideally, the most trustworthy values should be obtained when the proteins of interest have a functional conformation as found in vivo. Additionally, the difference may arise due to purely technological reasons. For example, $K_D$ determination by ITC can be easily hindered if one or both of the interacting molecules change(s) conformation, thereby releasing or absorbing heat. In
agreement with this, we were not able to obtain $K_D$ for interaction between 5MP1 and helF3c20-102 in ITC, even though $K_D$ of $0.20 \pm 0.04 \, \mu M$ was obtained for the same interaction in BLI. Given these technical limitations, it is more important to use these methods in mutational analyses to determine the magnitude of the effect of mutations introduced to a protein of interest. Then, the result is carefully compared to that obtained by functional assays in fully or partially reconstituted systems, or ultimately in cells or in vivo. In this way, one can dissect the interaction and thereby determine the physiological relevance of the interaction under the study. Likewise, other parameters obtained by these approaches, such as the stoichiometry number, $N$, by ITC, must be carefully interpreted in comparison with the results from other functional interaction assays or structural biology approaches (Obayashi et al., 2017).

**TROUBLESHOOTING**

**Problem 1**
The yield of purified protein is low.

**Potential solution**
The protein may be unstable under the growth conditions. The stability of protein is determined by the balance in the rates of its production and degradation. Thus, changes in growth conditions can sometimes lead to changes in protein stability and hence its yield. Typical alternative temperatures to be tested are 30°C and 18°C (if a refrigerated incubator is not available, flasks are shaken in an open platform shaker at the room temperature of 20°C set in a well air-conditioned room). Our favorite low temperature alternative is the growth at 20°C for overnight. To do so, grow bacteria at 37°C till $A_{600}=0.5$. Then the flask containing the culture is cooled quickly in ice water mixture to ~20°C and begins to be shaken after adding IPTG at a lower concentration (e.g., 0.05 mM). Alternatively, other media such as 2xYT or different concentration of IPTG (as low as 0.05 mM) can be tested. If none of these works, alternatively designed constructs, such as solubilization-enhancement tagging (Reibarkh et al., 2008; Zhou et al., 2001) or mutations to increase protein solubility, or different expression systems (such as baculovirus expression system) must be sought.

**Problem 2**
There are many other protein bands in purified protein fraction.

**Potential solution**
Even with the high-resolution preparatory column, you may not be able to separate the protein of interest with co-purifying proteins or its degradation products. In this case, it is advised to change the bacterial growth conditions to minimize expression of the co-purifying protein or suppressing protein degradation. The latter may be alternatively achieved by adding protease inhibitors to the lysis buffer, such as phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin and pepstatin, or simply adding commercially available protease-inhibitor cocktail (Singh and Asano, 2007).

**Problem 3**
ITC does not detect interaction.

**Potential solution**
Heat content changes caused by protein conformational changes can compensate for those caused by protein-protein interaction, hence hindering its detection through ITC. Alternatively, the high concentration of the proteins under the experimental conditions may allow them to precipitate, physically blocking their interaction. This can be solved by switching the protein component in the sample cell (if the concentration of the protein in the cell is different from that of the injectant protein), or by increasing the solubility of the proteins through changing buffer conditions or introducing a solubilization-enhancement tag (Zhou et al., 2001) or mutations to increase protein solubility (e.g., by substituting surface-exposed hydrophobic amino acids with polar amino acids).
**Problem 4**
The $K_D$ values obtained by ITC vary from experiment to experiment.

**Potential solution**
If the high concentration of the proteins under the experimental conditions causes them to precipitate and the rate of precipitation varies between experiments, this can result in variation in the ITC outputs. Again, this can be solved by increasing the solubility of the proteins through changing buffer conditions or introducing a solubilization-enhancement tag (Zhou et al., 2001) or mutations to increase protein solubility.

**Problem 5**
The kinetic parameters obtained by BLI vary from experiment to experiment.

**Potential solution**
In an optical biosensor experiment such as BLI, affinity can be overestimated through avidity effects, i.e., through one dimer (or multimer) binding to two (or more) immobilized molecules. These effects have been documented frequently in surface plasmon resonance (Ladbury et al., 1995). Avidity effects can also influence reproducibility of BLI outputs, as they are computed from a series of titration experiments and as multimer formation may likely depend on concentration of the protein forming multimers. Of the common protein tag added for convenience of purification, GST is known to form dimers (Singh and Asano, 2007). Thus, we avoided avidity effects in our BLI approach through immobilizing GST-h5MP1 fusion into the sensor. By the same token, if a protein under the study is known to form dimer or multimer, one way to avoid/minimize avidity effects and thereby gain reproducible results is to attach this protein to the biosensor chip.

Similar to ITC, values obtained from BLI assays must be interpreted carefully in reference to the results from complementary interaction or functional assays.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katsura Asano, kasano@ksu.edu.

**Materials availability**
Plasmids generated in this study are available upon request (materials transfer agreement may be necessary).

This study did not generate new unique reagents.

**Data and code availability**
The published article (Singh et al., 2021) includes all datasets/codes generated or analyzed during this study.

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**AUTHOR CONTRIBUTIONS**
C.R.S., R.J., C.E., and K.A. wrote the original draft. K.A. edited and completed the manuscript.
DECLARATION OF INTERESTS
Authors declare no conflict of interests.

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