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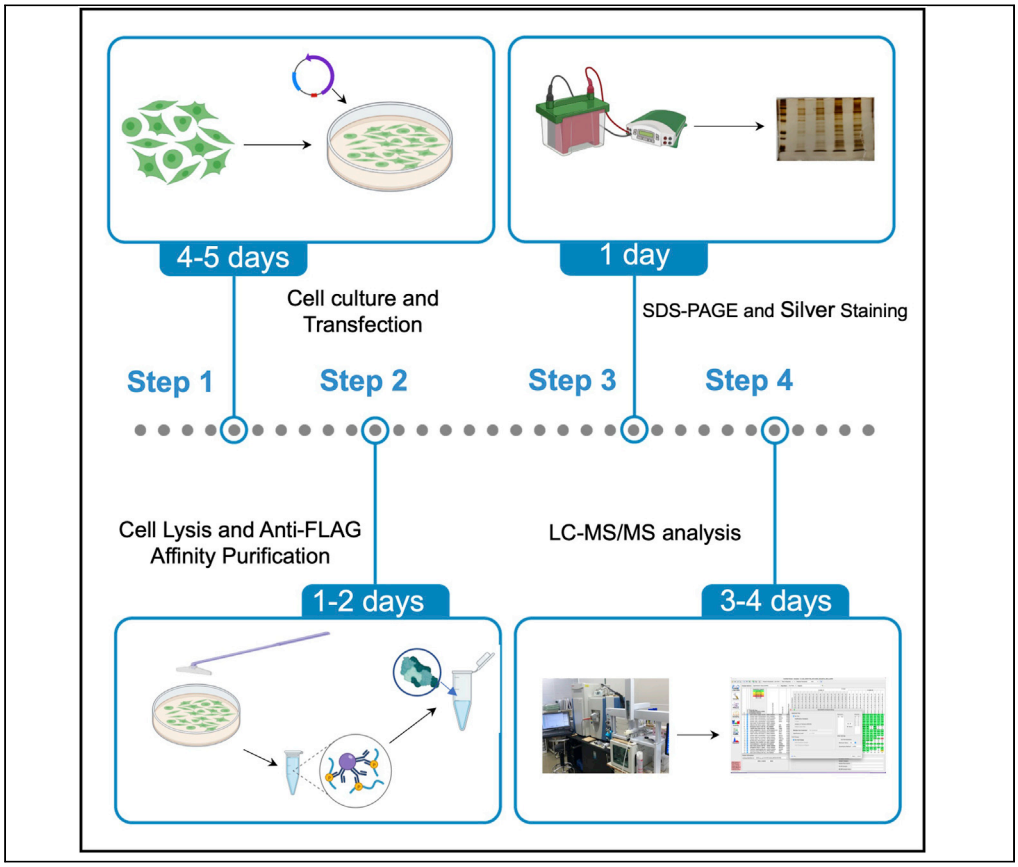
Protocol

Mass spectrometry analysis of affinity-purified cytoplasmic translation initiation complexes from human and fly cells



eIF5-mimic protein (5MP) controls translation through binding to the ribosomal pre-initiation complex (PIC) and alters non-AUG translation rates for cancer oncogenes and repeat-expansions in neurodegenerative diseases. Here, we describe a semi-quantitative protocol for detecting 5MP-associated proteins in cultured human and fly cells. We detail one-step anti-FLAG affinity purification and whole-lane mass spectrometry analysis of samples resolved by SDS-PAGE. This protocol allows for quantitative evaluation of the effect of 5MP mutations on its molecular interactions, to elucidate translational control by 5MP.

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

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Highlights

Anti-FLAG-affinity purification of translation initiation complex via 5MP

Purification of proteins from human and fly cells after simple transfection procedures

Whole-lane mass spectrometry to analyze affinity-purified proteins

emPAI as a proxy for molecular amounts of identified proteins

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Mass spectrometry analysis of affinity-purified cytoplasmic translation initiation complexes from human and fly cells

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SUMMARY

eIF5-mimic protein (5MP) controls translation through binding to the ribosomal pre-initiation complex (PIC) and alters non-AUG translation rates for cancer oncogenes and repeat-expansions in neurodegenerative diseases. Here, we describe a semi-quantitative protocol for detecting 5MP-associated proteins in cultured human and fly cells. We detail one-step anti-FLAG affinity purification and whole-lane mass spectrometry analysis of samples resolved by SDS-PAGE. This protocol allows for quantitative evaluation of the effect of 5MP mutations on its molecular interactions, to elucidate translational control by 5MP.

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$_i^{Met}$ 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). 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).

One-step affinity purification of epitope-tagged eIF or their regulator including 5MP has been proven to be a powerful tool to study interaction with their binding partners in translation initiation (Asano et al., 2000; Kozel et al., 2016; Singh et al., 2021). Epitope-tags, such as FLAG-tag, are introduced to either terminus of the expressed proteins by PCR using oligonucleotides that include the coding sequence of the tags or their complementary sequences (Singh and Asano, 2007). FLAG peptide is an eight amino acid-long peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). M2 antibody against this peptide is frequently used because of the ease of releasing FLAG-tagged proteins by

competition with excess FLAG-peptides, or triple FLAG-peptide, in particular (Uno and Masai, 2011). Here we describe a semi-quantitative method of detecting FLAG-tagged 5MP-associated proteins in cultured human or fly cells, taking advantage of mass spectrometry analysis and exponentially *m*odified *P*rotein *A*bundance *I*ndex (emPAI), an empirical formula to convert peptide counts into molar amounts of the detected proteins (Ishihama et al., 2005). To evaluate conservation of the detected interactions between fly and human cells, we describe protocols to study 5MP complexes formed in both human and fly cells. Before you begin, you must establish a condition to efficiently transfect the expression plasmid (see troubleshooting section).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
ANTI-FLAG® M2 Affinity Gel	Sigma-Aldrich	A2220
Aprotinin	Santa Cruz Biotechnology	SC-3595A
β-Mercaptoethanol	Sigma-Aldrich	M6250-250
Bromophenol Blue	Sigma-Aldrich	B8026-5GM
CHAPS	Thermo Scientific	28300
DTT	Sigma-Aldrich	D9779
EDTA	Fisher Scientific	S311-500
EGTA	Fisher Scientific	32-462-625GM
3×Flag peptide	Sigma-Aldrich	F4799
Glycerol	Fisher Scientific	BP229-1
β-glycerophosphate	Fisher Scientific	35-675-100GM
Leupeptin	Santa Cruz Biotechnology	SC-215242A
Magnesium Chloride (MgCl2)	Fisher Scientific	AC223211000
Penicillin and streptomycin mixture	Thermo Fisher Scientific	15140-122
Pepstatin	Santa Cruz Biotechnology	SC-45036A
Phenylmethanesulfonyl fluoride (PMSF)	Sigma	P-7626
Polyethylenimine MAX (MW 25,000, linear)	Polysciences, Inc	Cat. 24765
Potassium Chloride (KCl)	Fisher Scientific	P330-500
Protein A Sepharose CL-4B	Pharmacia	17-0780-01
Sodium Chloride (NaCl)	Research Prod. Int.	523030-12000
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	L3771-500
Sodium Fluoride (NaF)	Sigma-Aldrich	450022-5G
Sodium Vanadate (Na3VO4)	GoldBio	TCEP25
Critical commercial assays		
PureYield™ Plasmid Midiprep System	Promega	A2492
Sliver stain II kit WAKO	Wako (Fisher Scientific)	NC9535926
Experimental models: Cell lines		
Human embryonic kidney (HEK) 293T	John A. Chiorini	N/A
D. melanogaster cell line S2	Erika Geisbrecht	N/A
Recombinant DNA		
pEF1A-h5MP1; 3×F-h5MP1 under the eEF1A promoter	(Kozel et al., 2016)	Asano lab p1556
pEF1A-h5MP1-BN1; pEF1A-h5MP1carrying *BN1*	(Singh et al., 2021)	Asano lab p1659
pAC-Dme5MP; *Drosophila* Kra under the fly actin promoter	(Kozel et al., 2016)	Asano lab p1708
pAC-Dme5MP-BN1; pAc-Dme5MP carrying *BN1*	(Singh et al., 2021)	Asano lab p1964
Software and algorithms		
Origin software	MicroCal	N/A
Scaffold 4.8.4	Proteome Software Inc.	N/A
Mascot search engine version 2.5-2.7	Matrix Science	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Acetonitrile (for LC/MS)	FUJIFILM Wako Pure Chemical	012-19851
Advance Captive Spray Ionization probe	AMR/Bruker	N/A
Advance UHPLC system	AMR/Michrom Bioresources	N/A
Ammonium bicarbonate (ammonium hydrogencarbonate) for Proteomics	FUJIFILM Wako Pure Chemical	018-21742
TransIT-Insect Transfection Reagent	Mirus	MIR6104
Autoclave	STERIS	Amsco Lab 250
Centrifugal evaporator (Centrifugal concentrator)	TOMY Seiko (TOMY digital biology)	CC-105
Centrifuge	Thermo Scientific	Sorval ST 16R
CO2 incubator series 8000 WJ	Thermo Scientific	TC # IS-497-001
Deionized Water Unit	Thermo Scientific	7119
Distilled Water (for HPLC)	FUJIFILM Wako Pure Chemical	046-16971
HyClone DMEM	Fisher Scientific	SH3028401
FBS	Thermo Fisher Scientific	10082147
Formic Acid (for LC/MS)	FUJIFILM Wako Pure Chemical	067-04531
Glassware/plasticware	N/A	N/A
High Speed Refrigerated Micro Centrifuge MX-107	TOMY Seiko (TOMY digital biology)	MX-107
L-column 2 ODS, 3 μm, 0.1 × 150 mm, PEEK-steeved type	CERI	7711400
L-column 2 ODS, 5 μm, 0.3 × 5 mm, cartridge trap column	CERI	752450
Maximizer MBR-022UP (Shaker)	TAITEC Corp.	MBR-022UP
Methanol (for HPLC)	FUJIFILM Wako Pure Chemical	138-06473
OHP film (film for overhead projectors, A4)	KOKUYO	VF-1
PAL HTS-xt (auto sampler)	AMR/CLC Analytic AG	N/A
Petri dishes	Fisher Scientific	FB0875713
Pierce DTT, No-Weight Format (48 × 7.7 mg)	Thermo Fisher Scientific	20291
Pierce Iodoacetamide, Single-Use (24 × 9.3 mg)	Thermo Fisher Scientific	90034
Protein Electrophoresis Equipment	Bio-Rad	PowerPac Basic
Protein Electrophoresis Running Reservoir	Bio-Rad	MiniProtean Tetra Cell
Protein LoBind micro-tube (1.5 mL)	Eppendorf	No.0030108116
Proteome Discoverer version 1.4	Thermo Fisher Scientific	N/A
Q Exactive mass spectrometer	Thermo Fisher Scientific	N/A
Safety Cabinet	Labconco	Purifier Logic+ Class II Type A2 Biosafety Cabinet
Scalpel (Knife holder No.3, Substitute edge No.11)	FRIGZ, Kai Corp.	Z504-132, 1-8545-12
Schneider medium	Fisher Scientific	BW04-351Q
Silver stain MS kit	FUJIFILM Wako Pure Chemical	No.299-58901
Trifluoroacetic acid (for HPLC)	FUJIFILM Wako Pure Chemical	206-10731
Trypsin/Lys-C Mix, Mass Spec Grad (5 × 20 μg)	Promega	V5073
Ultrapure Water (for LC/MS)	FUJIFILM Wako Pure Chemical	214-01301
Xcalibur 4.027.19/Tune 2.8 SP1	Thermo Fisher Scientific	N/A
−80°C Ultradeep Freezer	New Brunswick Scientific	U535 Innova

MATERIALS AND EQUIPMENT

0.15 M NaCl, 1 L (for transfection)

Reagent	Final concentration	Amount
2 M NaCl	0.15 M	75 mL
diH_2O	N/A	925 mL
Total	**N/A**	**1 L**

Note: Aliquot 100 mL in bottles and autoclave. Store at 4°C for 2 years.

PEI, 25 mL (for transfection)		
Reagent	Final concentration	Amount
PEI	1 mg/mL	0.025 g
diH_2O	N/A	25 mL
Total	**N/A**	**25 mL**

Note: They are dissolved in water, adjusted to pH7.0 and filtered. Aliquot 1 mL in sterile 1.5-mL centrifuge tubes. Store at −20°C for 2 years.

Phosphate Buffered Saline (PBS) (1 Liter)		
Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
ddH_2O	N/A	To make volume 1 L
Total	**N/A**	**1 L**

- In a beaker add 800 mL dH_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to cold dH_2O.
- Adjust pH to 7.4 using HCl.
- Bring volume to 1 liter, transfer to glass bottle, autoclave, and store at 4°C. Good for 1 year.

FLAG-AP Buffer (50 mL)		
Reagent	Final concentration	Amount
1 M Tris HCl (pH7.5)	20 mM	1.0 mL
2 M KCl	100 mM	2.5 mL
2 M MgCl2	5 mM	0.125 mL
0.5 M EDTA	0.1 mM	10.0 µL
100 mM EGTA	5 mM	2.5 mL
1 M DTT*	1 mM	50.0 µL
NaF	20 mM	41.98 µg
100 mM Na3VO4*	0.1 mM	50.0 µL
0.5 M β-glycerophosphate	20 mM	2.0 mL
CHAPS	0.3%	0.15 g
200 mM PMSF*	1 mM	250 µL
1 mg/mL aprotinin*	2 µg/mL	100.0 µL
1 mg/mL leupeptin*	4 µg/mL	200.0 µL
1 mg/mL pepstatin*	1 µg/mL	50.0 µL
diH_2O	N/A	To make volume 50 mL
Total	**N/A**	**50 mL**

- After assembling the above components, filter the buffer with 0.22 µm filter.
- Store at 4°C. Good for 3 days.
- Alternatively, a stock buffer can be made without the reagents marked with asterisk (*) and stored prior to filtration at −20°C for 3 months. Before use, add the additional reagents, filtrate, and use immediately for the assay. Do not use the stock if white precipitates form after thawing.

Note: Three buffers derived from the FLAG-AP buffer are used for the procedure described here; the FLAG-AP buffer, the FLAG-AP stock buffer and the FLAG elution buffer. The FLAG-AP stock buffer is the same as the FLAG-AP buffer except omitting the PIs, Na3VO4

and DTT. FLAG elution buffer is the FLAG AP buffer with 200 μg/mL 3×Flag peptide (see below).

FLAG elution Buffer (200 μg/mL 3×Flag peptide) (2.286 mL) – Make this prior to the purification		
Reagent	Final concentration	Amount
FLAG AP buffer	1×	2 mL
8× 3×FLAG peptide	1.6 mg/mL	286 μL
Total	**N/A**	**2.286 mL**

Note: Make 3×Flag peptide solution by dissolving 6.4 mg peptide into 0.5 mL FLAG-AP buffer. Aliquot and store at −80°C for 1 year.

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
diH_2O	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 1 year.

Note: Add β-mercaptoethanol (final 5%) to aliquot prior to use. Once you add β-mercaptoethanol, store aliquot at −20°C and it is good for 5 years. It is highly recommended to wear goggles and gloves while handling SDS powder, as it is known to cause skin and respiratory irritation.

STEP-BY-STEP METHOD DETAILS

Immuno-affinity purification of FLAG-tagged human 5MP1 (h5MP1) and its binding partners

Timing: 4 days (1 h for step 1; 1 h for step 2; 5 h for step 3)

Day 1

1. Seed HEK293T in 10-cm dishes.
   a. Take a cryogenic vial of HEK293T and thaw at 37°C.
   b. Spin 1,500 rpm for 3 min at room temperature.
   c. Remove supernatant and wash the cells with 5 mL PBS to remove DMSO, which is toxic to many cultured cells.
   e. Pellet down the cells by centrifuging 1,500 rpm for 3 min at room temperature and aspirate the supernatant. Suspend cells with 0.5 mL of DMEM/10% FBS/PenStrep and seed them in a 10-cm dish with 10 mL of the same medium at ~25% confluency which is around 2.5 million cells.
   f. Incubate cells at 37°C, 5% CO2; 4 passages are recommended for the cells to fully recover from cryogenic freezing before performing transfection.

Note: Cryogenic vial of HEK293T cells is typically made from ~80-90% confluent cells/10-cm dish and each vial contains 2.5 million viable cells in 1 mL stock medium (DMEM/10% FBS/PennStrep with 10% DMSO).

Day 2

2. Transfection of HEK293T with pET-h5MP1 or mutant DNA.
   a. Make solution A with 500 μL 0.15 M NaCl and 8 μg DNA.
   b. Make master mix solution B with 500 μL × *n* 0.15 M NaCl and 40 μL × *n* 1 mg/mL PEI (*n* = number of transfection).
   c. Distribute 0.5 mL solution B to each tube with solution A and vortex the tube.
   d. Incubate 20–30 min at room temperature.
   e. Replace the media 2 h prior to transfection with 10 mL of fresh media for cells to be transfected.
   f. Add the mixture to cells in a 10-cm dish with 30%–50% confluent cells which is around 300K–500K cells per mL.
   g. Incubate cells at 37°C, 5% CO2.

Note: It is recommended to have at least one negative control with empty expression vector transfection (n≥2).

Note: DNA is purified from an *E. coli strain* (such as DH5α) carrying the desired plasmid, using a commercially available endotoxin-free mid-scale plasmid preparation kit (such as Pureyield™ Plasmid Midiprep System). See troubleshooting below.

Day 4

3. Anti FLAG-affinity purification.

Harvest cells 42–48 h after transfection.

   a. Prepare the anti-FLAG affinity resin by removing 40 μL of anti-FLAG M2 affinity gel 40 μL per plate and wash with 10× bed volume of FLAG-AP stock buffer twice (spin at 5,000 rpm, 4°C, 1 min).
   b. (*In the culture room and clean bench*) Wash plates with ice-cold 5 mL PBS and remove PBS.
   c. Take out the plates from the clean bench and place them directly onto ice.
   d. Add 0.5 mL FLAG-AP buffer per plate.
   e. Move the materials on ice to the lab.
   f. Collect cells in the lysis buffer with a scraper and pipettman. Move the cells into a ice-cold 1.5-mL microcentrifuge tube and leave it on ice for 10 min.
   g. Spin cells at 15,000 rpm at 4°C for 20 min.
   h. Collect supernatant fraction after the centrifugation as "In-put" fraction. Save ~1% portion for diagnostic analyses. Add the remainder to the tube containing the washed anti-FLAG resin.
   i. Incubate at 4°C for 90 min, rotating.
   j. Spin the tube at 4,000 rpm at 4°C, 1 min.
   k. Remove sup carefully using P200 pipette without disturbing the pelleted resin *Keep sup from empty vector control transfection (c-sup)* for the FLAG eluate clean-up step No. 4 (see next).
   l. Wash with 0.5 mL FLAG-AP buffer (spin as above).
   m. Wash with 0.2 mL FLAG-AP buffer (spin as above).
   n. Elute with 30 μL FLAG elution buffer by incubating at 4°C for 20 min, rotating.
   o. Spin the tube as step i and keep the eluate.
   p. Elute with 20 μL FLAG elution buffer by incubating at 4°C for 10 min, rotating.
   q. Spin the tube as step i and combine the eluate with that from step n.
   r. Immediately proceed to *FLAG eluate cleanup* below.

Note: Diagnostic analysis is meant to check the h5MP1 expression level and molecular weight size confirmation through western blot.

4. FLAG eluate cleanup.

This process removes any contaminating antibodies through incubation with Protein A Sepharose, while keeping original associated proteins in solution. To ensure the latter, the Protein A Sepharose is pre-coated with cell lysate proteins in the supernatant fraction from step 3-j. In order to avoid possible contamination of control proteins, it is recommended to wash the resin twice. The pre-coated step is required to avoid loss of any specific proteins from the experimental samples by binding on the resin.

a. Weigh 0.025 × n g Protein A Sepharose CL-4B in a tube and add 25 × n μL di water.
b. Swirl the resin, rotating, for 5 min at room temperature.
c. Spin the tube at 4,000 rpm at 4°C, 1 min and discard the supernatant.
d. Wash with 100 × *n* μL FLAG AP stock buffer.
e. Add 100 × *n* μL control supernatant (c-sup) from above to the washed total resin.
f. Incubate for 20 min at 4°C, rotating.
g. Wash with 100 × *n* μL FLAG AP stock buffer twice.
h. In the last wash, split the resin to the number of eluates in a 1.5-mL microcentrifuge tube.
i. Add the FLAG eluate from above to the tube with the coated resin.
j. Incubate for 20 min at 4°C, rotating.
k. Spin as above and recover sup as the final product.

Immuno-affinity purification of FLAG-tagged fly 5MP (Kra) and its binding partners

Timing: 4 days (1 h for step 5; 1 h for step 6; 5 h for step 7)

Day 1

5. Seed S2 cells in 6-well plate.
a. Take a cryogenic vial of S2 cell stock and thaw at 28°C.
b. Spin 1,000 rpm for 3 min at room temperature.
c. Remove supernatant.
d. Wash the cells using 5 mL PBS to remove DMSO, which is toxic to many cultured cells.
e. Use same parameter as in step b above for centrifugation.
f. Suspend cells in 0.5 mL of Schneider's *Drosophila* Medium supplemented with 10% heat-inactivated FBS and PennStrep; and seed them in a 6-well plate with 2.5 mL of the same medium at a density of 6×10^5 cells/mL.
g. Incubate cells at 28°C.

Note: Prior to transfection, it is suggested to passage the culture at least 3 times so that the cells reached enough time to recover from the effects of cryogenic storage. Frozen S2 cell stocks are typically made from ~6×10^6 cells/mL grown in a 25 cm^2 flask and each cryovials contains 1 mL of 2×10^6 cells/mL.

Day 2

6. Transfection of S2 with pAC-h5MP1 or mutant DNA.
a. Warm up serum-free S2 medium, *Trans*IT-Insect reagent and plasmids at room temperature. Vortex gently the *Trans*IT-Insect reagent and leave it at the room temperature.
b. Take 250 μL of serum-free medium and add 3 μg of DNA for each transfection and mix by pipetting.
c. Drop slowly 5 μL of *Trans*IT-Insect reagent and mix by pipetting.
d. Incubate for 30 min at room temperature.

e. Add the mixture drop-wise to cells spreading out evenly in each of the 6-well plate.
f. Incubate cells at 28°C for 48 h.

Note: It is recommended to have at least one negative control with empty expression vector transfection ($n \geq 2$).

DNA used for transfections is purified from an *E. coli strain* (such as DH5α) carrying the desired plasmid, using a commercially available endotoxin-free mid-scale plasmid preparation kit (such as Pureyield™ Plasmid Midiprep System). See troubleshooting below.

7. Anti FLAG-affinity purification.

Day 4

The following procedure is followed almost the same as the above HEK293T protocol:

Prepare the anti-FLAG affinity resin:

a. Take anti-FLAG M2 affinity gel 30 μL per well.
b. Wash with 10× bed volume of FLAG-AP stock buffer twice (spin at 5,000 rpm, 4°C, 1 min).

Harvest cells 42–48 h after transfection; at this time the transfection efficiency should be above 80%.

c. (*In the culture room and clean bench*) Wash wells with 3 mL PBS and remove PBS.
d. Take out the plates from the clean bench and place them directly onto ice.
e. Add 0.5 mL FLAG-AP buffer per plate.
f. Move the materials on ice to the lab.
g. Transfer the cells into a 1.5-mL microcentrifuge tube.
h. Spin cells at 15,000 rpm at 4°C for 20 min.
i. Collect supernatant fraction as "In-put" fraction. Save ~1% portion for diagnostic analyses. Add the remainder to the tube containing the washed anti-FLAG resin.
j. Incubate at 4°C for 90 min, rotating.
k. Spin the tube at 4,000 rpm at 4°C, 1 min.
l. Remove sup. *Keep sup from empty vector control transfection (c-sup)* for the FLAG eluate clean-up (see next).
m. Wash with 0.5 mL FLAG-AP buffer (spin as above).
n. Wash with 0.2 mL FLAG-AP buffer (spin as above).
o. Elute with 30 μL FLAG elution buffer by incubating at 4°C for 20 min, rotating.
p. Spin the tube as step v and keep the eluate.
q. Elute with 20 μL FLAG elution buffer by incubating at 4°C for 10 min, rotating.
r. Spin the tube as step c and combine the eluate with that from step h.
s. Immediately proceed to *FLAG eluate cleanup* exactly as step 4 of human 5MP1 complex purification described above.

Whole-lane mass-spectrometry analysis of the affinity-purified products after SDS-PAGE

Timing: 4 days (4 h for step 8; 12 h for step 9; 2 h for step 10; 3-4 days for step 11)

Day 1

8. SDS-PAGE and silver staining.
   a. Analyze 15 μL (30%) of eluted fractions along with control samples (e.g., whole cell lysate or purified proteins) on 15% SDS-PAGE gel.
   b. Silver-stain the gel. The following describes the outline protocol using the Silver Staining kit from Wako. (MS-compatible kit must be used, to avoid glutaraldehyde).
      i. Incubate the SDS-PAGE gel in Fixing Solution 1 for 10 min.
      ii. Continue with the fixing using Fixing Solution 2 for 10 min, and soak the gel in the Enhancing solution for 10 min.

iii. Wash twice with DI water for 5 min each time.
iv. Stain the gel with the provided Staining Solution by shaking for 20 min.
v. Rinse the gel three times for 5 min each.
vi. Add Developing solution and shake for few minutes until the bands appear. Do not overstain the gel.
vii. Use the provided Stopper Solution to avoid overstaining and wash the gel three time using di water for 2 min each (Figure 1A).
viii. The gel now can be stored at 4°C.

Day 2

9. In-gel digestion.
   a. Excise the whole lane of the gel into 12–16 pieces with a razor blade, according to the size standards and staining patterns (Figures 1B and 1C).

   Note: The gel is cut on the fresh sheet of OHP film (KOKUYO). Before using the OHP sheet and scalpel (Knife holder No.3, Substitute edge No.11: FRIGZ, Kai Corp.), wipe off twice with methanol (FUJIFILM Wako Pure Chemical) and distilled water (FUJIFILM Wako Pure Chemical), respectively. OHP film sheet is a normal type without treatment for printing with inkjet.

   Note: It is no problem even if the gel breaks a little (see Figure 1A). It is important to return the broken part to the original position. Then, cut the gel.

   b. Cut the gel piece approximately 1 mm sized cubes, to facilitate in-gel trypsin digestion. The gel pieces (cubes) are transferred to a 1.5-mL Protein LoBind micro-tube (Eppendorf) (Figure 1D).
   c. Add 200 μL ultrapure water, shake for 10 min at room temperature with the Maximizer MBR-022UP (TAITEC Corp.) (Figure 2A).

   Note: Unless otherwise stated, the incubation processes during the pre-processing and subsequent in-gel digestion steps are performed in the Maximizer MBR-022UP at the speed of 900 rpm.

   d. Spin down, and remove the supernatant.
   e. Add 200 μL ultrapure water, and shake for 10 min at room temperature.

   Note: The gel pieces of 1.5 mL tube can store at 4°C for 1 week at this step prior to proceeding to step f.

   f. Spin down, and remove the supernatant.
   g. De-stain the sliver-stained protein band of gel pieces (cubes) with 300 μL mixture of De-staining solution A and De-staining solution B (Silver stain MS kit, FUJIFILM Wako Pure Chemical) in equal quantity for 20 min.
   h. Spin-down, and remove the supernatant.
   i. Wash the gel with 200 μL of ultrapure water for 10 min at 25°C.
   j. Spin down, and remove the supernatant. Repeat washing in steps i-j twice more.
   k. Dehydrate the washed pieces with 150 μL of acetonitrile (FUJIFILM Wako Pure Chemical) for 10 min.
   l. Spin down, and remove the supernatant.
   m. Subsequently, dehydrate the gel pieces with a centrifugal evaporator (Centrifugal concentrator CC-105: TOMY Seiko, TOMY digital biology) for 15 min (Figures 2B and 2C).
   n. Reduce proteins in the gel pieces with 200 μL of 10 mM DTT (Thermo Fisher Scientific) in 25 mM ammonium bicarbonate (FUJIFILM Wako Pure Chemical) for 60 min at 56°C.
   o. Spin down, and remove the supernatant.

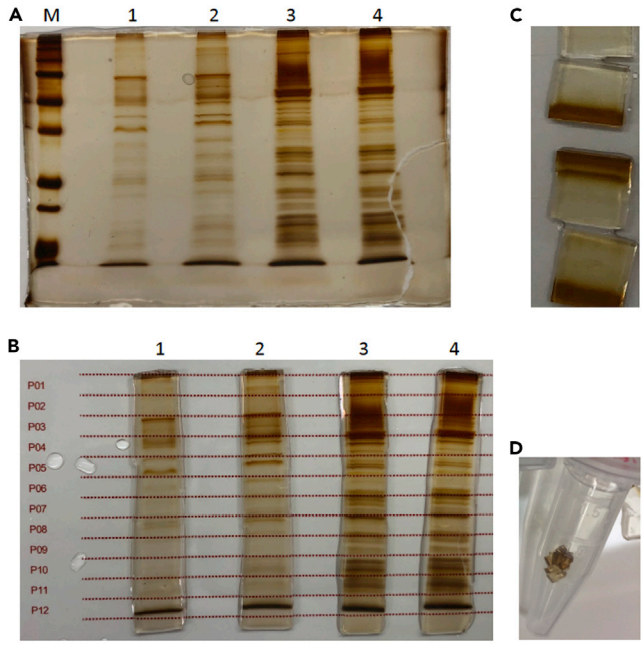

Figure 1. To excise the whole lane of the gel into 12 pieces

(A) Original gel. M, a size standard; 1 and 2, vector controls; 3 and 4, WT h5MP1.

(B) The excised gel strips separated from each lane. Lanes 1–4, same as in panel A.

(C) The cut gel pieces.

(D) Gel pieces were diced into cubes with approximately 1 mm dimension.

p. Wash the gel pieces with 100 μL of 25 mM ammonium bicarbonate for 10 min at 25°C.

q. Spin down, and remove the supernatant.

r. Alkylate proteins of the gel pieces with 95–100 μL of 55 mM iodoacetamide (Thermo Fisher Scientific) in 25 mM ammonium bicarbonate for 45 min at 25°C in the dark (protect from light with aluminum foil) (Figure 2C).

s. Spin down, and remove the supernatant.

t. Wash the gel pieces with 100 μL of 25 mM ammonium bicarbonate for 10 min at 25°C.

u. Spin down, and remove the supernatant.

v. Dehydrate with 200 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min (Figure 3A).

w. Spin down, and remove the supernatant. Repeat steps v-w once more.

x. Subsequently, the gel pieces are dehydrated with a centrifugal evaporator for 15 min (Figure 3B).

y. *In-gel digestion*: Add 30 μL of 20 ng/μL trypsin and lysyl endopeptidase (Promega) in a buffer containing 40 mM ammonium bicarbonate, pH 8.0. Incubate for 1–1.5 h on ice (Figure 3C).

Note: Wait for the gel pieces to swell with a solution of trypsin and lysyl endopeptidase. If the gel does not swell, add small amounts of 40 mM ammonium bicarbonate buffer to the gel pieces and wait for them to swell. If necessary, repeat this several times until the gel swells.

z. Supplement the gel pieces (cubes) with 40 mM ammonium bicarbonate buffer.

aa. Incubate overnight at 37°C, at the speed of 600 rpm.

Figure 2. In-gel digestion and dehydration

(A) Samples are consistently shaken by the Maximizer MBR-022UP in the pre-processing for in-gel digestion.

(B) Dehydration by a centrifugal evaporator.

(C) The maximizer MBR-022UP is covered with aluminum foil to avoid light in step r.

Day 3

10. Post-digestion processing.
    a. Extract the digested peptides from gel pieces with 50 μL of 50% acetonitrile, 0.1% formic acid (FUJIFILM Wako Pure Chemical) for 30 min at 25°C and incubate the reaction in the Maximizer MBR-022UP at 1,300 rpm.
    b. Spin down, and transfer the supernatant to a fresh 1.5 mL Protein LoBind micro-tube.
    c. Extract once more with 50 μL of 70% acetonitrile, 0.1% formic acid and incubate for 30 min as in step bb.
    d. Spin down, and combine the supernatant with the supernatant from step cc.
    e. Concentrate the combined supernatants to 15 μL with a centrifugal evaporator.

Note: The concentrated samples can store at 4°C–5°C for 1 week until LC-MS/MS analysis. Before applying to LCMS analysis, the concentrated sample was centrifuged at the speed of 12,000 rpm (13,000 × *g*) for 10 min at 4°C with High Speed Refrigerated Micro Centrifuge MX-107(TOMY Seiko, TOMY digital biology) for preventing small gel fragments from getting into the HPLC system in subsequent steps.

11. LC-MS/MS analysis.
    a. Dilute the concentrated samples 2-fold with 2% acetonitrile, 0.1% trifluoroacetic acid (FUJIFILM Wako Pure Chemical), and then transfer to a fresh autosampler's vial.
    b. Apply the diluted sample to LC-MS/MS analysis (10 μL/injection) (Figure 4A).
    c. Analyze on an Advance UHPLC system (AMR/Michrom Bioresources) coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) (Figure 4B).
        i. The injected sample is trapped on a pre-column (L-column 2 ODS, 5 μm, 0.3 × 5 mm, cartridge trap column, CERI), and then de-salted and concentrated. Equilibration, de-salt and concentration were performed with 2% acetonitrile, 0.1% trifluoroacetic acid.

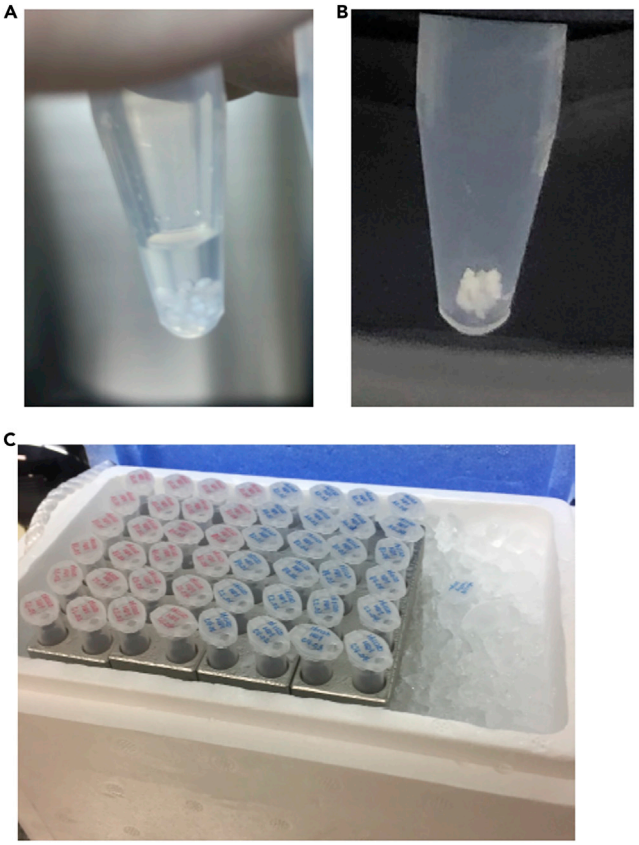

Figure 3. Dehydration of gel pieces and in-gel digestion

(A–C) The gel pieces dehydrated with (A) 50% acetonitrile followed by (B) a centrifugal evaporator were subjected for in-gel digestion on ice (C).

ii. The concentrated peptides are separated on micro-ODS column (L-column 2 ODS, 3 μm, 0.1 × 150 mm, PEEK-steeved type, CERI), at 40°C, flow rate 500 nL/min.

iii. Mobile phase A is (ultrapure water, 0.1% formic acid), while mobile phase B is (acetonitrile, 0.1% formic acid).

iv. The eluent gradient is set to 5%–45% Phase B/0–20 min, 45%–95% Phase B/20–25 min, 95% Phase B/25–35 min (wash), 95-5% Phase B/35–36 min, and 5% Phase B/36–45 min (equilibration).

v. Setting for MS and MS/MS acquisitions: MS range is m/z 350–2,000, resolution 70,000, positive mode. MS/MS spectra are acquired according for using a Top-10 DDA (data-dependent acquisition), resolution 17,500, high-energy collision dissociation value 27 (Figure 4C). ESI spray: Advance Captive Spray Ionization probe (AMR/Bruker, Figure 4D), 1,400 V, 250°C, 500 nL/min.

vi. Perform continuous injection with auto-sampler (PAL HTS-xt, AMR/CLC Analytic AG), which links to a Xcalibur/Tune (Thermo Fisher Scientific) operating software to process the raw LC-MS/MS data (mass spectra) (Figure 4E).

d. Analyze the raw LC-MS/MS data using Proteome Discoverer version 1.4 (Thermo Fisher Scientific) with the Mascot search engine version 2.5–2.7 (Matrix Science) (Figure 5A).

i. Protein database: SwissProt or Uniprot restricted to *Homo sapiens or Drosophila melanogaster.*

ii. Parameter for analysis: carbamidomethylation of Cys is selected as fixed modification. Oxidation of Met, deamidation of Asn, and deamidation of Gln are selected as variable

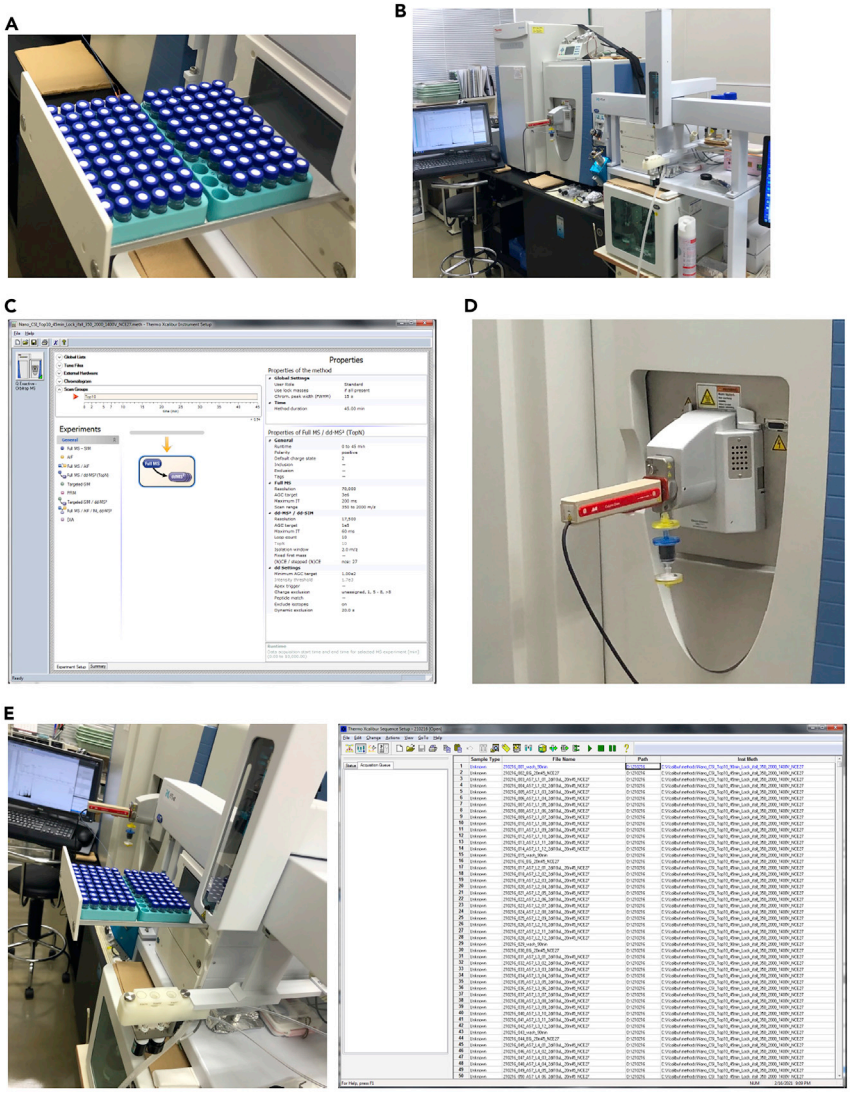

Figure 4. LC-MS/MS analysis

(A) Set sample vials on autosampler for LC-MS/MS analysis.

(B) The overview of Advance UHPLC system coupled to a Q Exactive mass spectrometer.

(C) Profile of Top-10 DDA on Xcalibur/Tune.

(D) Advance Captive Spray Ionization probe.

(E) PAL HTS-xt autosampler and time table of continuous injection/acquisition.

modification. A precursor mass tolerance is 10 ppm and a fragment ion mass tolerance is 0.02 Da. Max missed cleavages: 2. Digestion enzyme: Trypsin.

iii. A decoy database comprised of either randomized or reversed sequences in the target database is used for false discovery rate (FDR) estimation, and Percolator algorithm is used to evaluate false positives. Search results are filtered against 1% global FDR for high confidence level.

e. Further analyze using Scaffold 4.8.4 (Proteome Software Inc.) for calculating emPAI values (Figure 5B).

i. Quantitative Analysis Setup: not to use normalization; Quantitative method, emPAI; Protein Threshold; 1.0%FDR; Min#peptides, 2; Peptide Threshold, 95%.

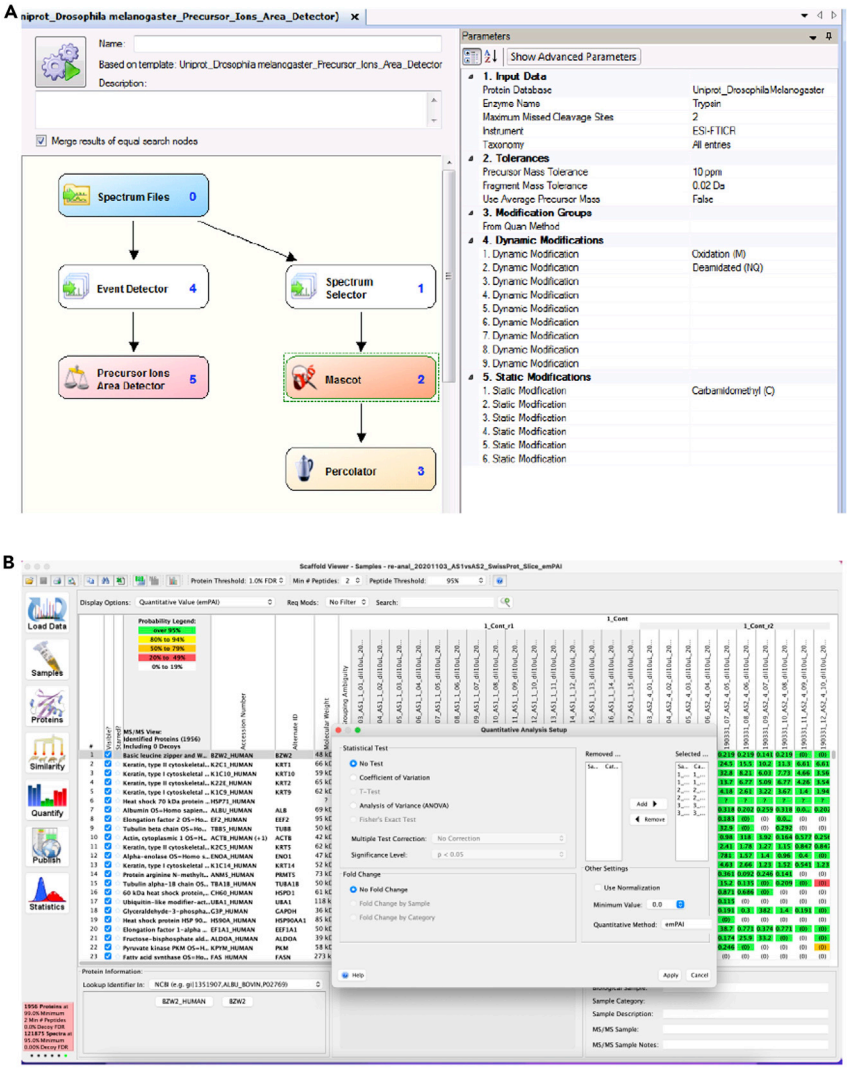

Figure 5. Data analysis

(A) Work-flow of data analysis with Proteome Discoverer.

(B) Quantitative analysis setting on Scaffold 4.8.4.

Note: The emPAI is defined by (10^{PAI} – 1) where PAI, or Protein Abundance Index, is the number of observed peptides divided by the number of observable peptides per protein. If we use normalization in step e, the sum of emPAI values computed from each gel slice does not match the emPAI value computed from the sum of peptide count from each gel slices. On the other hand, these values match well if we do not use normalization. Thus, do not use normalization in the setup.

EXPECTED OUTCOMES

This method enables semi-quantitative measurement of every protein molecule found in anti-FLAG affinity purified fractions. A typical example from the original study with human 5MP1 (Singh et al., 2021) is listed in Table 1, after statistical analysis was conducted (see problem 5). We observed h5MP1 association with eIFs 1, 1A, 2, 2B, 3, 4G1 and NAT1/DAP5/eIF4G2 as well as the 40S ribosomal subunits. All of these interactions depended on the basic surface of h5MP1 altered by the five amino-acid substitution termed *BN1*. Based on the emPAI value, a proxy for protein molarity, we were able to partition these interactions into three complexes, the major h5MP1:eIF2

association, the minor h5MP1:eIF2:eIF2B and h5MP1:eIF1:eIF1A:eIF2:eIF3:eIF4G1:40S complexes. The last complex is proposed as the 48S ribosomal pre-initiation complex (PIC) loaded with h5MP1 in place of eIF5. We also observed similar complexes when fly 5MP was expressed in S2 cells. Thus, 5MP association with 48S PIC appears to be conserved between humans and flies.

LIMITATIONS

Here, the emPAI value is computed as a proxy for relative molar amount of identified proteins (Ishihama et al., 2005). This is defined as $10^{PAI} - 1$, where PAI is defined as the ratio of observed peptide count from the identified protein to the total count expected for the specific protein molecule. PAI (rather than emPAI) should theoretically correlate with protein molarity, but a systematic survey using known amounts of standard proteins showed that emPAI reproducibly correlates with the molarity, hence emPAI proposed as the measurement of molecular amount.

The caveat of this approach is the large deviation of emPAI values obtained with the standard proteins (Ishihama et al., 2005). Adding to this, emPAI values can be perturbed by the variability in peptide extraction from the gel. Therefore, it is not appropriate to discuss a small difference in emPAI values unless it is proven statistically significant. Multiple experiments must be conducted to establish statistical significance of the difference ($p<0.05$, students' Ttest). For the same reason, it is not appropriate to discuss a small difference in subunit composition for multi-protein complexes such as eIF2 or eIF3 between the two comparisons. There is a report claiming that the base of emPAI is 6.5 (Kudlicki, 2012), instead of 10. Since emPAI is based on empirical correlation, it is likely that the correlation is affected by individual settings. It is therefore reasonable to conduct PAI-protein amount correlation experiments to determine the appropriate logarithmic base for your individual setting. This may improve statistics for differences of interest (see troubleshooting).

TROUBLESHOOTING

Problem 1

Transfection does not work with our cultured mammalian cells.

Potential solution

We normally check transfection by testing a portion of the same cells in a spare Petri dish with a plasmid expressing green fluorescent protein (GFP). We anticipate ~80%< cells expressing GFP under a fluorescence microscope. Once we establish transfection conditions, then we move ahead with western blotting test for the protein of interest expressed from a vector plasmid. Yet, a GFP plasmid is always used as a control as the healthy cell growth condition may be perturbed for any reason.

In this protocol, we used PEI for transfection with HEK293T. While this is an economic choice for transfection of cultured mammalian cells (Uno and Masai, 2011), it does not work with all kinds of the cells. For example, we verified that it worked with HeLa cells, while it did not work with HT1080, a fibrosarcoma cell line (Kozel et al., 2016). Another reason for a failure to transfect might be due to plasmid DNA purification. Some bacteria express endotoxin; the endotoxin contamination is a frequent cause of ill transfection. Thus, DNA is recommended to be purified with a method verified to be ''endotoxin- free''.

Problem 2

Transfection does not work with our cultured fly S2 cells.

Potential solution

S2 cells are easy to grow and expand between 22°–28°C, but for the best transfection efficiency, it is highly recommended to grow them at 28°C using high quality plasmids. The plasmids used for transfections in the protocol were purified using Pureyield™ Plasmid Midiprep System to obtain endotoxin-free plasmids (see problem 1). The use of Penicillin-Streptomycin in the growth media also greatly reduces the transfection efficiency so the cells were grown in antibiotic free Schneider's

Table 1. emPAI values obtained for translation initiation factors and the ribosomal proteins associated with human 5MP1, its BN1 mutant or vector control samples

			1_vec1	2_vec2	3_5MP1_1	4_5MP1_2	5_BN1_1	6_BN1_2	Average (Exp 1, 2)		
Protein	Accession Number	M. W.	Lane1	Lane4	Lane2	Lane5	Lane3	Lane6	Vec	WT	BN1
h5MP1	BZW2_HUMAN	48 kDa	4.1965	0.39041	1,004.50	1,002.0	1,004.0	1,001.40	2.2935	1,003.25	1,002.70
h5MP2	BZW1_HUMAN	48 kDa	0	0	1.3606	0.5880	0	0.48648	0	0.97	0.24
eIF1A	IF1AX_HUMAN	16 kDa	0.7506	0	15.44	0	0.2052	0	0.3753	7.72	0.10
eIF1	EIF1B_HUMAN	13 kDa	0.2670	0	5.641	0	0.6053	0	0.1335	2.82	0.30
eIF2									0.9985	480.28	202.52 (p=0.05, n=6)
eIF2α	IF2A_HUMAN	36 kDa	2.1184	0.30012	999.88	999.61	246.56	841.57	1.2093	999.75	544.07
eIF2β	IF2B_HUMAN	38 kDa	0.6385	0	478.35	209.49	50.954	39.587	0.3193	343.92	45.27
eIF2γ	IF2G_HUMAN	51 kDa	2.9341	0	144.58	49.516	22.931	13.543	1.4671	97.05	18.24
eIF3									3.2116	13.822 (p=0.005, n=26) (p=0.005, n=13)	0.48447 (p=0.003, n=26) (p=0.003, n=13)
eIF3a	EIF3A_HUMAN	167 kDa	4.1693	0.10145	10.199	3.4288	0.50058	0.08037	2.1354	6.81	0.29
eIF3b	EIF3B_HUMAN	92 kDa	6.2074	0.2311	34.483	2.999	0.80231	0.14867	3.2193	18.74	0.48
eIF3c	EIF3C_HUMAN	105 kDa	2.3849	0.030953	5.0407	5.2277	0.48629	0	1.2079	5.13	0.24
eIF3d	EIF3D_HUMAN	64 kDa	5.6594	0.051161	19.981	3.4677	0.56684	0	2.8553	11.72	0.28
eIF3e	EIF3E_HUMAN	52 kDa	15.511	0.62849	95.713	3.3187	0.35633	0	8.0697	49.52	0.18
eIF3f	EIF3F_HUMAN	38 kDa	6.5445	0.65733	19.723	2.8467	1.5249	0.18341	3.6009	11.28	0.85
eIF3g	EIF3G_HUMAN	36 kDa	4.3787	0.092588	5.4208	1.6486	0.70114	0	2.2356	3.53	0.35
eIF3h	EIF3H_HUMAN	40 kDa	5.6863	0.17156	43.707	3.1581	0.48564	0.08239	2.9289	23.43	0.28
eIF3i	EIF3I_HUMAN	37 kDa	13.584	0.83151	47.92	5.6984	2.3544	0.54071	7.2078	26.81	1.45
eIF3j	EIF3J_HUMAN	29 kDa	1.9414	0	1.9414	2.6497	0.24082	0.38218	0.9707	2.30	0.31
eIF3k	EIF3K_HUMAN	25 kDa	3.4535	0.28268	8.3984	5.4696	0.86334	0.64526	1.8681	6.93	0.75
eIF3l	EIF3L_HUMAN	67 kDa	6.8261	0.21093	9.4286	4.5986	1.1502	0.04901	3.5185	7.01	0.60
eIF3m	EIF3M_HUMAN	43 kDa	3.7817	0.077361	8.3505	4.5502	0.45146	0	1.9295	6.45	0.23
eIF4G1	IF4G1_HUMAN	175 kDa	0.0961	0.07617	0.2237	1.1222	0.11641	0.41723	0.0861	0.67	0.27
NAT1/eIF4G2	IF4G2_HUMAN	102 kDa	0.0318	0.03183	1.902	1.6416	0.03183	0.50285	0.0318	1.77	0.27
eIF5	IF5_HUMAN	49 kDa	0.2139	0	0	0.1379	0	0.06674	0.1069	0.07	0.03
eIF2B									0.2231	52.6535	3.91806 (p=0.004, n=10) (p=0.02, n=5)
eIF2Bα	EI2BA_HUMAN	34 kDa	0.4528	0.92246	49.482	49.482	1.7929	6.1051	0.6876	49.48	3.95
eIF2Bβ	EI2BB_HUMAN	39 kDa	0.0845	0.3835	109.71	48.174	4.4971	11.376	0.2340	78.94	7.94
eIF2Bδ	EI2BD_HUMAN	58 kDa	0.1172	0	137.41	35.625	4.5693	3.9852	0.0586	86.52	4.28
eIF2Bε	EI2BE_HUMAN	80 kDa	0	0	9.064	14.594	1.0474	2.0486	0.0000	11.83	1.55
eIF2Bγ	EI2BG_HUMAN	50 kDa	0.1350	0.13506	53.081	19.913	1.4273	2.3317	0.1351	36.50	1.88
40S ribosome									46.722	69.786 (p=0.003, n=62) (p=0.08, n=31)	1.9030 (p=0.03, n-62) (p=0.02, n=31)
40S rp S10	RS10_HUMAN	19 kDa	7.3879	0.38708	15.138	1.6687	1.266	0.17774	3.8875	8.40	0.72
40S rp S11	RS11_HUMAN	18 kDa	294.93	0.95312	249.33	19.337	4.3312	0	147.94	134.33	2.17

(*Continued on next page*)

Table 1. *Continued*

			1_vec1	2_vec2	3_5MP1_1	4_5MP1_2	5_BN1_1	6_BN1_2	Average (Exp 1, 2)		
Protein	Accession Number	M. W.	Lane1	Lane4	Lane2	Lane5	Lane3	Lane6	Vec	WT	BN1
40S rp S12	RS12_HUMAN	15 kDa	122.63	0	122.63	0	4.3419	0	61.315	61.32	2.17
40S rp S13	RS13_HUMAN	17 kDa	11.194	0	16.431	1.0433	1.0433	0	5.5970	8.74	0.52
40S rp S14	RS14_HUMAN	16 kDa	74.231	0	158.49	0	8.5277	0	37.116	79.25	4.26
40S rp S15	RS15_HUMAN	17 kDa	24.931	0	6.3113	0	1.4702	0	12.466	3.16	0.74
40S rp S15a	RS15A_HUMAN	15 kDa	136.1	0	167.29	0	1.2708	0	68.050	83.65	0.64
40S rp S16	RS16_HUMAN	16 kDa	127.1	0.45251	827.24	1.1098	5.4655	0	63.776	414.17	2.73
40S rp S17	RS17_HUMAN	16 kDa	27.342	0	135.75	0	4.874	0	13.671	67.88	2.44
40S rp S18	RS18_HUMAN	18 kDa	361.55	0.682	180.24	7.004	3.0013	0.41433	181.12	93.62	1.71
40S rp S19	RS19_HUMAN	16 kDa	943.75	0	999.72	0	10.869	0	471.88	499.86	5.43
40S rp S2	RS2_HUMAN	31 kDa	7.2439	0.22251	15.654	3.0809	0.65245	0.10567	3.7332	9.37	0.38
40S rp S20	RS20_HUMAN	13 kDa	14.319	0	23.142	0	2.914	0	7.1595	11.57	1.46
40S rp S21	RS21_HUMAN	9 kDa	126.63	0	65.86	0	17.348	0	63.315	32.93	8.67
40S rp S23	RS23_HUMAN	16 kDa	9.2704	0	21.325	0.2142	2.2047	0	4.6352	10.77	1.10
40S rp S24	RS24_HUMAN	15 kDa	6.2473	0	9.7698	0.2190	0.48605	0	3.1237	4.99	0.24
40S rp S25	RS25_HUMAN	14 kDa	10.313	0.55439	33.079	0.5544	0.55439	0.24675	5.4337	16.82	0.40
40S rp S26	RS26_HUMAN	13 kDa	1.011	0.26223	5.4431	0.5932	0.26223	0	0.6366	3.02	0.13
40S rp S27	RS27_HUMAN	9 kDa	78.849	0	108.18	0	3.7795	0	39.425	54.09	1.89
40S rp S28	RS28_HUMAN	8 kDa	81.963	0	38.725	0	12.163	0	40.982	19.36	6.08
40S rp S29	RS29_HUMAN	7 kDa	4.5316	0	4.5316	0	0.5336	0	2.2658	2.27	0.27
40S rp S3	RS3_HUMAN	27 kDa	108.74	2.64	196.42	53.238	8.3147	0.79905	55.690	124.83	4.56
40S rp S30	RS30_HUMAN	7 kDa	1.3519	0	4.5316	0	0.5336	0	0.6760	2.27	0.27
40S rp S3a	RS3A_HUMAN	30 kDa	151.88	1.5678	391.58	47.281	5.5938	0.11047	76.724	219.43	2.85
40S rp S4	RS4X_HUMAN	30 kDa	55.822	0.7016	77.168	23.274	2.9832	0.11217	28.262	50.22	1.55
40S rp S5	RS5_HUMAN	23 kDa	18.969	1.2628	29.038	12.275	1.9707	0.97485	10.116	20.66	1.47
40S rp S6	RS6_HUMAN	29 kDa	14.452	0.11573	70.583	8.9711	0.54966	0	7.2839	39.78	0.27
40S rp S7	RS7_HUMAN	22 kDa	11.586	0.15108	43.649	6.169	2.5476	0	5.8685	24.91	1.27
40S rp S8	RS8_HUMAN	24 kDa	27.526	0.13755	60.811	13.976	1.1668	0	13.832	37.39	0.58
40S rp S9	RS9_HUMAN	23 kDa	6.9287	0	8.1022	3.5647	0.73695	0	3.4644	5.83	0.37
40S rp SA	RSSA_HUMAN	33 kDa	16.722	1.1525	30.494	6.4811	2.8251	0.46713	8.9373	18.49	1.65

Columns 1–3, protein name, its accession number and molecular weight. Columns 4–9, emPAI values from individual experiment. Columns 10–12, average emPAI values across Exp 1 and 2: Average emPAI values for protein complexes (eIF2, eIF3, eIF2B and 40S) are presented above the values for their subunits, with p values in parentheses for difference between vec vs WT (column 11) and WT vs BN1 (column 12).

Drosophila medium. Cell viability of more than 95% should be maintained for any S2 transfection. The transfection reagent, *Trans*IT-Insect was obtained from Mirus and it gave the best result.

Problem 3

The protein of interest does not interact with its established binding partners in the method provided.

Potential solution

The method described here is optimized for the purification of cytoplasmic translation initiation complexes. In other words, it is not optimized for the purification of protein complexes located in other cytoplasmic compartments, such as endosomes or nuclei. The purification of nuclear proteins typically includes detergents, which sometimes disrupt native protein complexes. Thus, cross-linkers such as formaldehyde (Reibarkh et al., 2008), or disuccinimidyl suberate d0/d12 (DSS) (Erzberger et al., 2014), more recently used in chemical crosslinking coupled to mass spectrometry, can be used to retain intact protein-protein interactions. Alternatively, proteinase inhibitors may help preventing the degradation of the binding partners and hence assist in their detection in the protein complexes. While we used typically selected protease inhibitors, commercially available protease-inhibitor cocktails may be additionally supplemented with the lysis buffer (Singh and Asano, 2007). Another reason for not detecting expected protein-protein interactions is the artefacts due to competition with their interaction with the affinity resin. For example, eIF4B is found abundantly in the vector control purified complex, suggesting that eIF4G is depleted from its natural complex by attaching the affinity resin. Interestingly, eIF4B was not found in the FLAG-5MP1-containing complexes (also see problem 4). Under these conditions, it is difficult to analyze and interpret interactions involving eIF4B.

Problem 4

The vector control sample contains numerous proteins.

Potential solution

As shown in Figure 1A, lanes 1 and 2, we observed a myriad of proteins in vector control samples from mock-treated cells. Our goal was to identify proteins that co-purify specifically with FLAG-h5MP1, and the specificity of the interaction was estimated based on statistical significance for differences in emPAI values. Therefore, the presence of numerous proteins in the vector control samples was not a problem. In order to purify the complex further, however, we recommend performing double-affinity purification using additional tags – typically hexahistidine (His_6)-tag. Since the h5MP1 construct we used was also tagged with a His_6-tag at its N-terminus, we performed double-affinity purification firstly with nickel-column (to purify through the His6-tag) and then with αFLAG-column (to purify through the FLAG-tag). While we could eliminate most of the proteins in the vector control samples, we failed to detect h5MP1 interaction with eIF3 and hence the 40S subunit (Kozel et al., 2016). We reasoned that h5MP1 binding to eIF3 was disrupted during the course of nickel affinity purification. In agreement with this assessment, the disruption was observed for eIF5 binding to eIF3 in humans (Kozel et al., 2016), and eIF5 and 5MP bind the c subunit of eIF3 through their conserved basic surfaces (Singh et al., 2021; Yamamoto et al., 2005). However, this is in contrast to yeast eIF5 complexes, for which eIF5 binding to eIF3 and the 40S subunit was retained after nickel-αFLAG double-affinity purification (Asano et al., 2000). It should be noted that His_6-tag was added to yeast eIF2β N-terminus in this experiment, instead of eIF5 N-terminus (as in the case of human eIF5 and 5MP1).

MS analysis of our vector control samples indicated that they contain abundant proteins that are not found in h5MP1-co-purifying fractions (Kozel et al., 2016). They include PRMT5 (73 kDa) and MET50 (37 kDa), which form a tight hetero-octameric complex catalyzing protein arginine methylation (Antonysamy et al., 2012), and eIF4B (80 kDa). These proteins are consistently observed as visible bands in silver-stained gels. This control-"specific" binding can be explained by their

Table 2. Average emPAI values obtained for eIF3 subunits and the 40S subunit proteins (RPS) in two independent sets of experiments using HEK293T

	Vector control	WT h5MP1	*P* (vec vs WT)	h5MP1-*BN1*	*P* (WT vs *BN1*)
eIF3, Expt 1	6.16	23.9	0.016	0.81	0.008
eIF3, Expt 2	0.26	3.65	1.83 E-07	0.19	9.1 E-08
eIF3, total	3.21	13.8	0.005	0.48	0.003
40S, Expt 1	93.1	132.8	0.12 (N. S.)	3.69	0.003
40S, Expt 2	0.36	6.78	0.008	0.11	0.008
40S, total	46.7	69.8	0.08 (N. S.) 0.003*	1.9	0.002

N. S., not significant. n=13 (eIF3) or 31 (40S) for all *P* values, except for n=62 for asterisk.

binding to the αFLAG column at different (perhaps slower binding) kinetics and their outcompetition by more rapid and specific binding of the FLAG-tagged proteins and their authentic binding partners.

Problem 5

The emPAI values determined for affinity-purified proteins vary from experiment to experiment.

Potential solution

As mentioned earlier, the emPAI values display large deviations, when tested by known amounts of the standard proteins (Ishihama et al., 2005). Therefore, it is common to observe some deviations (up to 10-fold) in emPAI values for a single protein across the purified fractions (e.g., eIF1 and eIF1A in Table 1). Thus, this method is more appropriate to establish statistical significance for specific association of multi-subunit proteins such as eIF2, eIF2B, eIF3 and the 40S ribosome (Table 1). Nevertheless, we observed significant variation of eIF3 (made of 13 subunits) and the 40S ribosome (with 31 ribosomal proteins) values in two independent sets of experiments with human proteins, as described in Table 2. This is clearly due to more non-specific binding of these components to the affinity column in Expt 1; Expt 2 achieved specific binding at the expense of detecting robust interactions. Despite these differences, *BN1* almost completely eliminated h5MP1 association with eIF3 and the 40S in both the experiments (Tables 1 and 2), indicating that the interaction is specific and depends on the basic surface of h5MP1 altered by *BN1*. Thus, subtle changes in sample handling can affect the degree of non-specific binding of some of the critical components. Yet, the quantification by emPAI values allows precise evaluation of relative abundance of various complexes resulting from detected interactions, as well as the quality of individual sets of experiments.

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., N.T., A.N., and K.A. wrote the original draft. K.A. and C.R.S. edited and completed the manuscript.

DECLARATION OF INTERESTS

Authors declare no conflict of interests.

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