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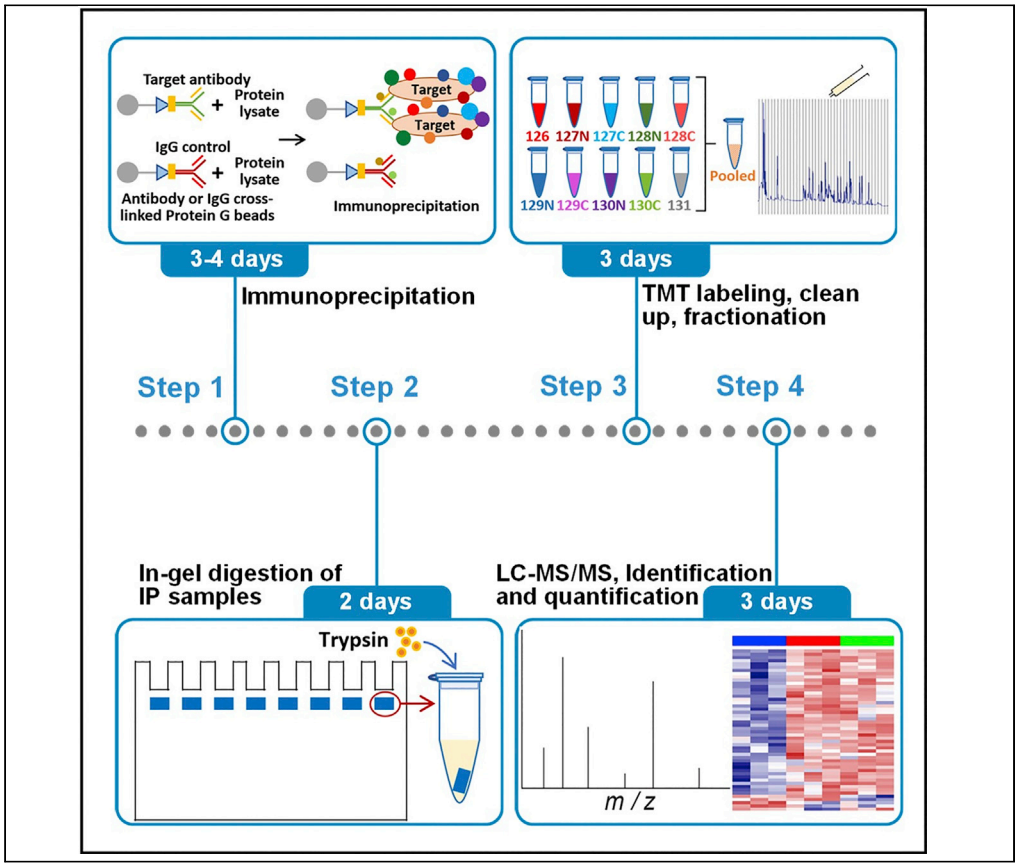
Protocol

Endogenous protein interactomes resolved through immunoprecipitation-coupled quantitative proteomics in cell lines



Immunoprecipitation (IP) of endogenously expressed proteins is one of the most biologically relevant techniques to identify protein-protein interactions. We describe an adaptable IP protocol reliant on a specific antibody to the target protein. We detail a quantitative proteomics workflow for the unbiased identification of co-immunoprecipitating proteins, known collectively as an interactome. This includes protocols for the tryptic digestion, Tandem Mass Tag labeling and fractionation of peptides, and their identification and quantification using liquid chromatography-mass spectrometry including computational and statistical analysis.

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

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Highlights

Identification of endogenous protein-protein interaction networks

All-in-one immunoprecipitation-coupled proteomics workflow

Quantitative analysis of protein interactions using Tandem Mass Tag labels

Adaptable to any endogenously expressed protein with an IP-compatible antibody

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Endogenous protein interactomes resolved through immunoprecipitation-coupled quantitative proteomics in cell lines

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SUMMARY

Immunoprecipitation (IP) of endogenously expressed proteins is one of the most biologically relevant techniques to identify protein-protein interactions. We describe an adaptable IP protocol reliant on a specific antibody to the target protein. We detail a quantitative proteomics workflow for the unbiased identification of co-immunoprecipitating proteins, known collectively as an interactome. This includes protocols for the tryptic digestion, Tandem Mass Tag labeling and fractionation of peptides, and their identification and quantification using liquid chromatography-mass spectrometry including computational and statistical analysis.

For complete details on the use and execution of this protocol, please refer to Johnson et al. (2020).

BEFORE YOU BEGIN

Institutional permissions

Samples and experiments performed for this manuscript were undertaken in accordance with the Declaration of Helsinki, and ethically approved by the Women's and Children's Health Network Human Research Ethics Committee, South Australia, Australia (HREC786-07-2020). All subject information was provided following informed parental consent.

Designing and engineering an experimental approach for IP

Timing: Variable

We describe a protocol used to discover the interacting proteins of an enzyme called USP9X by example. Specifically, we describe an IP-coupled quantitative proteomics experiment used to reveal how disease-causing missense mutations in USP9X altered its interactome in a quantitative fashion. For our purpose, we utilized human dermal fibroblast (HDF) cell lines derived/cultured from skin biopsies of either a healthy control individual, or from individuals harboring unique disease causing USP9X missense mutations (Johnson et al., 2020). Importantly, USP9X is endogenously expressed

in these cells. We have applied the IP protocol to other cell lines (e.g., HEK293T, Neural Stem Cells), tissues (e.g., mouse brain) and against other proteins (e.g., CTNNB1, AXIN, APC, NUMB, RAPTOR) (Bridges et al., 2017; Homan et al., 2014; Premarathne et al., 2017). Adapting the protocol for different proteins requires that (1) the target is endogenously expressed in the cells or tissue of interest (2) a highly specific antibody to the protein of interest is available and (3) that the lysis buffer conditions (in particular salt and detergent levels) are conducive to IP of the target protein and its interacting partners with the specific antibody. These conditions should be systematically optimized in pilot studies. If the protein is not endogenously expressed, or if a high-affinity antibody is not available, alternative experimental designs can be used, for example, introduction of in-frame epitope tags at endogenous loci, or within exogenous open reading frame expression vectors. Indeed, we have applied the IP protocol to several epitope tagged proteins (e.g., FLAG or Myc tagged) following loci engineering or transfection of expression vectors into cell lines (Pederick et al., 2018; Pham et al., 2017).

Designing an experiment suitable for Tandem Mass Tag proteomics

Timing: 1 day

It is important to understand the power and limitations of the Tandem Mass Tag (TMT) based quantitative proteomic end assay before designing the experiment (Chen et al., 2021). TMT allows for multiplexing of samples to enable concurrent acquisition of proteomic data, reducing required instrument time. As peptides in each sample are quantified using reporter ions, the relative amounts are quantified simultaneously ensuring coverage in each of the multiplexed samples. This lends itself to 2D proteomics whereby samples are fractionated to decrease peptide complexity in individual fractions leading to increased sensitivity and proteomic depth with retained quantitative information across all samples. Begin experimental design by exacting the scientific question being investigated to devise appropriate experimental controls, number of experimental groups and replicates, and hence total number of samples (experimental groups x replicates) to permit robust statistical quantification of proteins of biological relevance. The number of total samples should be considered in reference to the capacity of 10-plex TMT kits (or 16-plex TMT kits which recently become available). For example, sample numbers 10 or less are ideally suited for 10-plex TMT, as they can be directly compared within the one experiment. Thus, it may be beneficial to trade off the number of replicates to conform to single TMT-kit designs (Ordureau et al., 2020; Rose et al., 2016). Experiments with samples greater than 10 require use of multiple TMT-kits, and in these circumstances use of inter-TMT batch controls is recommended (for example a composite sample run across all TMT-batches) for quantitative consistency. However, multi-batch TMT experiments can often become limited by batch-to-batch data missingness due to the data-dependent acquisition methods, where lower abundant signals are selected in one batch from fragmentation (and hence reporter ion quantitation) but not another. Samples greater than 30 (>3xTMT kits) may be better suited to alternative quantitative approaches such as SWATH (sequential window acquisition of all theoretical fragment ion spectra) (Ludwig et al., 2018). The experimental plan will depend on power, capacity, budget, and other resources, and should be carefully considered. In the experiment we performed (Johnson et al., 2020), we sought to first identify what proteins interacted with USP9X in HDF. For this we identified proteins that were enriched in target USP9X IP samples over control IgG IP samples in the HDF derived from the healthy individual. We also sought to compare how USP9X mutations altered these interactions. Thus, we compared how USP9X-interacting proteins (USP9X IP samples) were altered between different cell lines. All experiments were done in triplicate. In total we had 6 conditions: (1) Healthy HDF with control IgG IP; (2) Healthy HDF with USP9X IP; (3–6) USP9X Mutant A-D HDF with USP9X IP (= 18 samples in total). This equated to 2× 10-plex TMT kits, and we included a single composite sample across each TMT experiment as an inter-batch TMT control (Johnson et al., 2020). Ideally, the composite sample is created by combining small equal amounts of all other samples, but it can be any sample replicated in both batches for the purpose of controlling inter-batch variation.

Cell culture

Timing: 4–5 days

Cell culture methods are cell type specific. In the example of Johnson et al. (2020), we cultured HDFs. One confluent T75 culture flask containing ~3 × 10^6 cells was used for each IP.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti USP9X	Bethyl Laboratories	A301-350A
Rabbit IgG	Sigma-Aldrich	I5006
Chemicals, peptides, and recombinant proteins		
Phosphate Buffered Saline	Thermo Fisher Scientific	14190144
Iodoacetamide (IAM)	Sigma-Aldrich	I1149
Dithiothreitol (DTT)	Sigma-Aldrich	D0632
Sequencing Grade Modified Trypsin	Sigma-Aldrich	11418475001
TMT 10plex Isobaric Label Reagent	Thermo Fisher	90111
Acetonitrile, Anhydrous	Sigma-Aldrich	271004
Acetonitrile, LC-MS grade	Sigma-Aldrich	1000291000
Hydroxylamine solution	Sigma-Aldrich	438227
Formic acid, ~98%, for mass spectrometry	Sigma-Aldrich	5.33002
Formic acid, >95%, lab reagent grade	Sigma-Aldrich	F0507
cOmplete EDTA-free Protease Inhibitor Cocktail	Roche	11873580001
1 M Triethylammonium bicarbonate (TEAB)	Sigma-Aldrich	T7408
25% ammonium solution	BDH	010011.0500
Methanol, HPLC grade (>99.9%)	Sigma-Aldrich	494291
QC Colloidal Coomassie Stain	Bio-Rad	161-0803
Acetic acid, glacial	Sigma-Aldrich	537020
0.05% Trypsin-EDTA Tissue Culture Solution	Thermo Fisher Scientific	25300054
Protein G Sepharose beads	GE Healthcare	17-0618-01
Triethanolamine	Sigma-Aldrich	T1377
Ethanolamine	Sigma-Aldrich	A7177
Dimethyl pimelimidate	Thermo Fisher Scientific	21667
Tween 20	Sigma-Aldrich	P1379
Bicine	Sigma-Aldrich	B3876
Bis-Tris	Sigma-Aldrich	B9754
UltraPure Sodium Dodecyl Sulfate	Thermo Fisher Scientific	15525-017
EDTA	Sigma-Aldrich	E9884
Triton-X-100	Thermo Fisher Scientific	85111
NuPAGE Antioxidant	Thermo Fisher Scientific	NP0005
NuPAGE 3%–8%, Tris-Acetate, 1.5 mm, Mini Protein Gel, 15-well	Thermo Fisher Scientific	EA03785BOX
NuPAGE Tris-Acetate SDS Running Buffer (20×)	Thermo Fisher Scientific	LA0041
Ponceau S Stain	Sigma-Aldrich	P7170
Clarity Western ECL Substrate	Bio-Rad	1705061
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
Critical commercial assays		
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Pierce™ Quantitative Colorimetric Peptide Assay	Thermo Fisher Scientific	23275
Experimental models: Cell lines		
Human Dermal Fibroblasts	Johnson et al. (2020)	Male Control 1
Software and algorithms		
MaxQuant (Version_1.6.10.43 and above)	https://www.maxquant.org/	Version_1.6.10.43 and above
Other		
Empore™ SDB SPE Disks	Sigma-Aldrich	66886-U

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protein LoBind Tubes 1.5 mL	Eppendorf	0030108442
Sep-Pak Light C18 cartridge	Waters	WAT054955
96-well, 2 mL deep well, round bottom, polypropylene collection plate	Axygen	P-2ML-SQ-C
Zorbax 300 Extend-C18, 2.1 × 150 mm, 3.5 μm, 300A, HPLC column	Agilent	765750-902
Vibra-Cell with a 3-mm tip	Sonics	VCX 130
XCell SureLock Mini-Cell and XCell II Blot Module	Thermo Fisher Scientific	EI0002
Rotator Mixer	Ratek	RSM7DC
ChemiDoc MP Imaging System	Bio-Rad	
BioTrace NT nitrocellulose	Pall Corporation	66485
Whatman Filter Paper (3 mm CHR)	GE Healthcare, Life Sciences	3030-917
MicroSpin G50 columns	Sigma-Aldrich	GE27-5330-01

Alternatives: List of reagents and equipment provided in this table are provided as a guide. Substituting the chemicals with other vendors/suppliers is permitted as long as the chemicals are of highest purity.

MATERIALS AND EQUIPMENT

Equipment for high pH fractionation of TMT labeled peptides

Agilent 1260 HPLC system equipped with quaternary pump and degasser (G1311B), autosampler (G1329B), column oven (G1316A), Multi-Wavelength Detector (G1365C) (set at 210, 214 and 280-nm wavelength), fraction collector (G1364C) and thermostat (G1330B).

Alternatives: If access to an LC system with fraction collector is unavailable, centrifugal HpH columns can be used as an alternative (e.g., Pierce™ High pH Reversed-Phase Peptide Fractionation Kit, Thermo Fisher Scientific, P/N: 84868).

Equipment for LC-MS/MS analysis procedure for TMT labeled peptide fractions

A Quadrupole-Orbitrap mass-spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) coupled to a Nano-flow liquid chromatography (Thermo Scientific™ UltiMate™ 3000 RSLCnano system) consisting of a nanoLC pump (NCS-3500RS) and degasser (SRD-3400) and autosampler (WPS-3000 TPL RS), fitted with an Acclaim™ PepMap™ 100 C18 trap cartridge and an in-house packed column 20 cm × 75 μm, Solidcore Halo 2.7 μm 160 Å ES-C18 (Advanced Materials Technology).

Alternatives: Several other mass spectrometers and liquid chromatography units present valid options for analysis of the TMT samples.

Protein Lysis Buffer

Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	50 mM	2.5 mL
NaCl (5 M)	150 mM	1.5 mL
Triton-X-100 (10%)	0.1%	500 μL
EDTA (0.5 M)	1 mM	100 μL
Sodium Fluoride	50 mM	105 mg
Protease Inhibitors: cOmplete ULTRA Tablets, EDTA-free	1× Protease inhibitor/No EDTA	1 tablet
Na_3VO_4 (200 mM)	0.1 mM	25 μL
ddH_2O	N/A	45.375 mL
Total	**N/A**	**50 mL**

Store single use aliquots at −20°C for up to a year. Thaw and use immediately on ice.

⚠ CRITICAL: Contact with Triton-X-100 and PMSF cause skin and eye irradiation. Wear protective gloves/ protective clothing/eye protection/face protection.

Alternatives: Different lysis buffer formulations may be tailored to protein, cell or tissue of interest. Higher NaCl and/or detergent levels increase the stringency of IP complexes.

IP Low Salt Wash Buffer

Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	20 mM	200 µL
ddH_2O	N/A	9.8 mL
Total	**N/A**	**10 mL**

Use ice-cold. Can be stored at 4°C for up to a year.

IP Elution Buffer

Reagent	Final concentration	Amount
Tris-HCl pH 6.8 (1 M)	62.5 mM	625 µL
Ultra Pure Sodium dodecyl sulfate (SDS)	2%	200 mg
Glycerol	10%	1 mL
Bromophenol blue	0.001%	0.1 mg
ddH_2O	N/A	8.375 mL
Total	**N/A**	**10 mL**

Can be stored at R/T for up to a year.

⚠ CRITICAL: Contact with SDS cause skin and eye irradiation. SDS is a surfactant. Wear protective gloves/protective clothing/eye protection/face protection. Do not inhale SDS.

Alternatives: Alternative formulations exist but need to be considered in the context of compatibility with downstream workflow applications, e.g., in-gel or in solution digestion and TMT labeling procedures.

4× Laemmli Buffer

Reagent	Final concentration	Amount
Tris-HCl pH 6.8 (1 M)	0.25 M	2.5 mL
Ultra Pure Sodium dodecyl sulfate (SDS)	8%	800 mg
Glycerol	40%	4 mL
Bromophenol blue	0.004%	0.4 mg
β-Mercaptoethanol	20%	2 mL
ddH_2O	N/A	1.5 mL
Total	**N/A**	**10 mL**

Store regular use aliquots at R/T but long-term aliquots at −20°C for up to a year.

⚠ CRITICAL: Contact with SDS cause skin and eye irradiation. SDS is a surfactant. Wear protective gloves/ protective clothing/eye protection/face protection. Do not inhale SDS. β-Mercaptoethanol is toxic via contact with skin, eyes, and inhalation. Wear protective gloves/ protective clothing/eye protection/face protection and use fume hood.

Alternatives: Alternative protein loading buffers exist but need to be considered in the context of compatibility with downstream workflow applications i.e., in-gel digestion.

20× Western Transfer Buffer		
Reagent	Final concentration	Amount
Bicine	0.5 M	81.6 g
Bis-Tris	0.5 M	104.6 g
EDTA	20.5 mM	6 g
ddH_2O	N/A	1 L
Total	**N/A**	**1 L**
Can be stored at 4°C for up to a year.		

Western Transfer Buffer		
Reagent	Final concentration	Amount
20× Western Transfer Buffer	1×	50 mL
Methanol	20%	200 mL
ddH_2O	N/A	750 mL
Total	**N/A**	**1 L**
Can be stored at 4°C for up to a week.		

⚠ CRITICAL: Methanol is flammable and toxic via contact with skin, eyes and inhalation. Wear protective gloves/protective clothing/eye protection/face protection and use fume hood.

10× Tris Buffered Saline (TBS)		
Reagent	Final concentration	Amount
Tris-HCl pH 7.6 (2 M)	0.2 M	500 mL
Sodium Chloride	1.5 M	438.3 g
ddH_2O	N/A	4.5 L
Total	**N/A**	**5 L**
As required, dilute 1:10 to make 1 L aliquots of TBS. As required add 0.1% Tween20 to TBS to make TBS-Tween20 (TBST). TBS and TBST can be stored at R/T for a year.		

Destaining Solution (100 mM TEAB/acetonitrile (1:1, vol/vol))		
Reagent	Final concentration	Amount
1 M TEAB	1 M	5 mL
ddH_2O	N/A	45 mL
Acetonitrile	N/A	50 mL
Total	**N/A**	**100 mL**
Destaining solution can be stored at 20°C–22°C for up to one year.		

⚠ CRITICAL: Acetonitrile is a very flammable liquid and considered acutely toxic solvent upon skin exposure and inhalation. Wear appropriate protective gear (lab coat, gloves, eye goggles) when working with it. A chemical fume hood should be used when working with large volumes of this solvent.

10 mM Dithiothreitol (DTT) in TEAB Solution		
Reagent	Final concentration	Amount
DTT	10 mM	15 mg
ddH_2O	N/A	9.50 mL
TEAB	50 mM	500 μL
Total	**N/A**	**10 mL**
Aliquots of DTT solution can be stored at −80°C for up to one year.		

⚠ CRITICAL: DTT causes skin and eye irritation. It may be harmful if absorbed through skin, inhaled or swallowed. Wear appropriate protective gear (lab coat, gloves, eye, and face) and handle large volumes in a well-ventilated chemistry hood.

55 mM Iodoacetamide (IAM) in TEAB Solution		
Reagent	Final concentration	Amount
IAM	55 mM	101.72 mg
ddH_2O	N/A	9.5 mL
TEAB	50 mM	500 μL
Total	**N/A**	**10 mL**
Aliquots of IAM solution can be stored at −80°C for up to one year.		

⚠ CRITICAL: IAM is toxic if in contact with skin, swallowed or inhaled. It may cause an allergic skin reaction. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

Fixing Solution		
Reagent	Final concentration	Amount
Methanol	N/A	10 mL
ddH_2O	N/A	83 mL
Glacial acetic acid	N/A	7 mL
Total	**N/A**	**100 mL**
Fixing solution can be stored at 20°C–22°C for up to one year.		

⚠ CRITICAL: Methanol is flammable and toxic via contact with skin, eyes and inhalation. Wear protective gloves/ protective clothing / eye protection / face protection and use fume hood. Acetic acid is flammable and corrosive. Wear protective gloves/ protective clothing / eye protection / face protection and use fume hood.

Coomassie Wash Solution		
Reagent	Final concentration	Amount
Methanol	N/A	50 mL
ddH_2O	N/A	40 mL
Glacial acetic acid	N/A	10 mL
Total	**N/A**	**100 mL**
Coomassie Wash Solution can be stored at 20°C–22°C for up to one year.		

⚠ CRITICAL: Methanol is flammable and toxic via contact with skin, eyes and inhalation. Wear protective gloves/ protective clothing / eye protection / face protection and use

fume hood. Acetic acid is flammable and corrosive. Wear protective gloves/ protective clothing / eye protection / face protection and use fume hood.

Gel Rehydration Solution		
Reagent	Final concentration	Amount
ddH_2O	N/A	90 mL
Glacial acetic acid	N/A	10 mL
Total	**N/A**	**100 mL**
Gel Rehydration Solution can be stored at 20°C–22°C for up to 6 months.		

⚠ CRITICAL: Acetic acid is flammable and corrosive. Wear protective gloves/ protective clothing / eye protection / face protection and use fume hood.

Trypsin Peptide Digestion Solution		
Reagent	Final concentration	Amount
Sequencing Grade Modified Trypsin	13 ng / µL	20 µg
1 M TEAB	50 mM	75 µL
ddH_2O	N/A	1.425 mL
Total	**N/A**	**1.5 mL**
Trypsin solution cannot be stored, it must be used immediately after preparation. The total volume can be altered depending on the total number of samples to be digested, however the trypsin to protein ration should be kept to 1: 50.		

Peptide Digestion Buffer		
Reagent	Final concentration	Amount
1 M TEAB	50 mM	2.5 mL
ddH_2O	N/A	47.5 mL
Total	**N/A**	**50 mL**
Digestion buffer can be stored at 20°C–22°C for up to one month and up to one year if stored in 4°C.		

Peptide Extraction Buffer		
Reagent	Final concentration	Amount
Acetonitrile, LC-MS Grade	50%	50 mL
Formic acid, >95%, lab reagent grade	5%	1.25 mL
ddH_2O	N/A	23.75 mL
Total	**N/A**	**75 mL**
Peptide Extraction Buffer can be stored at 20°C–22°C for up to one month and up to one year if stored in 4°C.		

⚠ CRITICAL: Formic acid is highly irritating and corrosive. It can cause severe burns to eyes, skin, and the respiratory system. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

HEPES Buffer (pH 8.8)		
Reagent	Final concentration	Amount
HEPES	200 mM	4.766 g
ddH_2O	N/A	100 mL
Total	**N/A**	**100 mL**
Adjust to pH 8.8 with 10 N NaOH. HEPES Buffer can be stored at R/T for 1 year.		

TMT Label Reagent		
Reagent	Final concentration	Amount
TMT 10plex Isobaric Label Reagent	19 μg/L	0.8 mg
Anhydrous acetonitrile	N/A	42 μL
Total	**N/A**	**42 μL**

TMT reagent preparation as indicated in the table is applicable for the preparation of one of the ten TMT reagents. The rest of the reagents must be prepared the same way. Before starting the reconstitution, allow the sealed TMT reagent to sit at 20°C–22°C for at least 30 min. Exposure to moisture can decompose TMTs over time during storage. It is recommended to re-suspend the TMT reagents in anhydrous acetonitrile and freeze the left-over TMT reagents at −80°C. Avoid storing TMTs at R/T for prolonged time or subjecting them to extensive freeze/thaw cycles.

⚠ CRITICAL: TMT 10plex Isobaric Label Reagent may cause eye, skin irritation. It may be harmful when inhaled or swallowed. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

TMT Reaction Quenching Solution		
Reagent	Final concentration	Amount
50% Hydroxylamine	5%	100 μL
ddH_2O	N/A	900 μL
Total	**N/A**	**1 mL**

TMT Reaction Quenching Solution should be prepared just prior to use. It cannot be stored.

⚠ CRITICAL: Hydroxylamine is known to cause skin irritation, allergic skin reaction, eye damage, respiration irritation, and damage to organs and is harmful if it is swallowed. It is very toxic to aquatic life. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care.

Sep-Pak Wash Solution		
Reagent	Final concentration	Amount
Formic acid, >95%, lab reagent grade	1%	1 mL
ddH_2O	N/A	99 mL
Total	**N/A**	**100 mL**

Sep-Pak Wash Solution can be stored at 4°C for several months.

⚠ CRITICAL: Formic Acid is flammable (liquid and vapor). Use concentrated stocks in a fume hood. It is also a strong acid, and should not be stored among oxidizers, caustics, or powdered metals. It is a corrosive chemical that causes severe skin burns and eye damage. It is toxic when inhaled and can irritate and damage the nose, throat and lungs. Ingestion can cause severe injury leading to death. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care.

LC-MS Loading Buffer		
Reagent	Final concentration	Amount
ddH_2O	N/A	999 mL
Formic acid, ~98%, for mass spectrometry	0.1%	1 mL
Total	**N/A**	**1 L**

Can be stored at 4°C for up to one year.

⚠ CRITICAL: Formic Acid is flammable (liquid and vapor). Use concentrated stocks in a fume hood. It is also a strong acid, and should not be stored among oxidizers, caustics, or powdered metals. It is a corrosive chemical that causes severe skin burns and eye damage. It is toxic when inhaled and can irritate and damage the nose, throat and lungs. Ingestion can cause severe injury leading to death. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care.

Sep-Pak Elution Solution		
Reagent	Final concentration	Amount
Acetonitrile, LC-MS Grade	80%	80 mL
Water	19.5%	19.5 mL
Glacial Acetic acid	0.5%	0.5 mL
Total	**N/A**	**100 mL**

Sep-Pak Elution Solution can be stored at 20°C–22°C in the dark for up to one year.

⚠ CRITICAL: Acetic acid is a flammable liquid, upon contact it can cause serious damage to eye and skin corrosion. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood. Methanol is highly flammable liquid, an eye irritant, and considered an acutely and chronically toxic solvent. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

HpH Mobile Phase A Solution		
Reagent	Final concentration	Amount
Ammonium hydroxide, 25% stock	6 mM	200 µL
ddH_2O	N/A	499.8 mL
Total	**N/A**	**500 mL**

HpH Mobile Phase A Solution Mobile must be freshly made to ensure pH levels which are essential. The pH of phase solutions changes with time due to increasing levels of dissolved CO_2 forming carbonic acid over time, which lowers pH.

⚠ CRITICAL: Ammonium hydroxide is harmful if swallowed. Causes severe skin burns and eye damage. Very toxic to aquatic life with long lasting effects. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

HpH Mobile Phase B Solution		
Reagent	Final concentration	Amount
Acetonitrile, LC-MS Grade	90%	400 mL
ddH2O	N/A	99.8 mL
Ammonium hydroxide	6 mM	200 µL
Total	**N/A**	**500 mL**

HpH Mobile Phase B Solution Mobile must be freshly made to ensure pH levels which are essential. The pH of phase solutions changes with time due to increasing levels of dissolved CO_2 forming carbonic acid over time, which lowers pH.

⚠ CRITICAL: Ammonium hydroxide is harmful if swallowed. Causes severe skin burns and eye damage. Very toxic to aquatic life with long lasting effects. Wear appropriate

protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

LC-MS Mobile Phase A Solution		
Reagent	Final concentration	Amount
ddH_20	N/A	999 mL
Formic acid, ~98%, for mass spectrometry	0.1%	1 mL
Total	N/A	1 L
Can be stored at 4°C for several months.		

⚠ CRITICAL: Formic Acid is flammable (liquid and vapor). Use concentrated stocks in a fume hood. It is also a strong acid, and should not be stored among oxidizers, caustics, or powdered metals. It is a corrosive chemical that causes severe skin burns and eye damage. It is toxic when inhaled and can irritate and damage the nose, throat, and lungs. Ingestion can cause severe injury leading to death. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care.

LC-MS Mobile Phase B Solution		
Reagent	Final concentration	Amount
Acetonitrile, LC-MS Grade	N/A	999 mL
Formic acid, ~98%, for mass spectrometry	0.1%	1 mL
Total	N/A	1 L
Can be stored at 4°C for several months.		

⚠ CRITICAL: Formic Acid is flammable (liquid and vapor). Use concentrated stocks in a fume hood. It is also a strong acid, and should not be stored among oxidizers, caustics, or powdered metals. It is a corrosive chemical that causes severe skin burns and eye damage. It is toxic when inhaled and can irritate and damage the nose, throat, and lungs. Ingestion can cause severe injury leading to death. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care. Acetonitrile is flammable (liquid and vapor). Use concentrated stocks in a fume hood. It is toxic when inhaled and can irritate and damage the nose, throat, and lungs. Ingestion can cause severe injury leading to death. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood and dispose with appropriate care.

STEP-BY-STEP METHOD DETAILS

IP of proteins

Timing: 2–3 days

IP of proteins can be broken down into three major steps including conjugating the antibody to beads (typically Protein Sepharose G beads or magnetic beads), generating a protein lysate, and the IP in which the antibody-conjugated beads are incubated with the lysate. The specific interaction between the target protein and the antibody permits capturing of the target protein and its protein-binding partners (the interactome) whilst non-interacting proteins are removed.

1. Conjugate antibodies to Protein G Sepharose beads

a. Incubate 50 μL packed volume of Protein G Sepharose beads with 5–50 μg of antibody or control IgG in phosphate buffered saline (PBS, pH7.2) to a total volume of 1.2 mL in 1.6 mL screw-capped Eppendorf tubes for 16 h on a rotator at 20 rpm at 4°C. In the example of Johnson et al. (2020), the antibodies used were 5 μg of rabbit anti-USP9X (Bethyl Laboratories A301-351A; USP9X-IP samples) and 5 μg of rabbit IgG (Sigma-Aldrich; control IgG samples).
b. Centrifuge tubes at 1,000 × *g* for 1 min and remove the supernatant.
c. Wash beads 3 times with 1 mL of 0.2 M triethanolamine (Sigma-Aldrich), diluted in ddH2O water and pH adjusted to 8.2 with HCl.
    i. Resuspend beads in 1 mL of 0.2 M triethanolamine.
    ii. Centrifuge tubes at 1,000 × *g* for 1 min and remove the supernatant.
    iii. Repeat three times.
d. Resuspend beads in 1 mL of a freshly made solution of 20 mM dimethyl pimelimidate-2HCl (DMP, Thermo Fisher Scientific), 0.2 M triethanolamine, pH 8.2 (5.2 mg DMP per mL of 0.2 M triethanolamine). This solution cross links the antibodies to the beads.
e. Mix tubes on rotator at 20 rpm for 60 min at 20°C–22°C, making sure the beads remain in suspension.
f. Centrifuge tubes at 1,000 × *g* for 1 min and remove the supernatant.
g. Resuspend beads in 1 mL 0.2 M ethanolamine pH 8.0 (dissolve 12 mL ethanolamine in 900 mL water and adjusted pH with conc. HCl).
h. Centrifuge tubes at 1,000 × *g* for 1 min and remove the supernatant.
i. Resuspend beads in 1 mL 0.2 M ethanolamine pH 8.0 and mix tubes on rotator at 20 rpm for 30 min at 20°C–22°C.
j. Centrifuge tubes at 1,000 × *g* for 1 min and remove the supernatant.
k. Wash beads twice with 1 mL of PBS.
l. Store the beads in PBS at 4°C and use within 48 h.

2. Isolate and quantify protein lysates from cell lines.
    a. Culture ~3 × 10^6 cells for each IP. Number of cells required may vary depending on cell line. The aim is to extract >700 μg of protein for each IP.
    b. Collect cells from culture.
        i. Remove media from cells and wash with 10 mL PBS.
        ii. Remove PBS and add 2 mL pre-warmed (37°C) 0.05% Trypsin-EDTA Tissue Culture Solution. Incubate at 37°C until cells detach (~5 min).
        iii. Add 5 mL of culture media to neutralize Trypsin and transfer cells to a 10 mL centrifuge tube.

    Note: If using non-adherent cells, steps 2bi–iii can be omitted.

        iv. Centrifuge cells at 300 × *g* for 5 min.
        v. Remove supernatant and resuspend cells in 10 mL of PBS.
        vi. Centrifuge cells at 300 × *g* for 5 min.
        vii. Remove supernatant and resuspend cells in 1 mL of PBS.
        viii. Transfer cells to a 1.6 mL screw-capped Eppendorf.
        ix. Centrifuge at 300 × *g* for 5 min.
        x. Remove supernatant. Use the cell pellets immediately for isolating protein lysates.
    c. All subsequent steps in the isolation and quantification of protein lysates must be performed in a cold room or on ice. To lyse cells:
        i. Resuspend and lyse cells in 250 μL ice cold Protein Lysis Buffer.
        ii. Sonicate solution using a Vibra-Cell VCX 130 sonicator (or similar device) fitted with a 3 mm tip set at 30% amplitude for 12 s.
        iii. Return tube to ice for 2 min.
        iv. Repeat sonication for 5 s.

d. Following sonication, add 250 μL lysis buffer to each tube and centrifuge at 15,000 × *g* at 4°C for 15 min.
e. Transfer supernatants to new 1.6 mL screw-capped Eppendorf tubes.
f. Quantitate protein concentration using a Bicinchoninic Assay kit (e.g., Pierce™ BCA Protein Assay Kit, Thermo Scientific) as per manufacturer's instructions.

3. Immunoprecipitate target protein complexes from lysates.
   a. Split each lysate into two samples, an Input sample, and an IP sample.
      i. To make the IP Sample, add 600 μg of lysate (of variable volume based on protein concentration) to lysis buffer to a make a final volume of 500 μL.
      ii. The remaining lysate is referred to as the Input Sample. The Input sample can be stored at −80°C at this stage.
   b. Preclear the IP sample. This step removes proteins from the IP samples which bind non-specifically to Protein G Sepharose beads.
      i. Wash a new lot of 50 μL packed volume of Protein G Sepharose beads with 1 mL Protein Lysis Buffer in 1.6 mL screw-capped Eppendorf tubes at 4°C.
      ii. Centrifuge the beads at 1,000 × *g* for 5 min at 4°C and remove the supernatant.
      iii. Add the 500 μL IP sample to the beads and incubate at 4°C for 15 min on a rotator at 20 rpm.
      iv. Centrifuge tubes at 1,000 × *g* at 4°C for 5 min. The supernatant is now considered a 'pre-cleared' IP sample.
   c. Centrifuge the pre-prepared antibody or control IgG-conjugated Protein G Sepharose beads (see step 1 above) at 1,000 × *g* for 5 min.
   d. Remove the supernatant and add the 500 μL pre-cleared IP Sample. Incubate on a rotator at 4°C for 16 h.
   e. Prepare a Microspin G50 columns for each IP sample.
      i. Remove the pre-packed Sephadex G-50 beads, remove fixed snap-off plug from the column tip, and add 1 mL of low-salt wash buffer.
      ii. Centrifuge the columns using a low speed capsulefuge (e.g., Tomy Capsulefuge MC-86P0 Mini Centrifuge or similar).
      iii. Discard the flow through. Transfer each IP Sample (lysates and beads) to the MicroSpin G50 columns.
      iv. Centrifuge using a low speed capsulefuge and discard the flow-through.
   f. Wash the IP beads in the Microspin G50 column 3 times as follows.
      i. Add 500 μL of Protein Lysis Buffer.
      ii. Centrifuge using a low speed capsulefuge.
      iii. Discard the flow-through.
   g. Wash the IP beads in the Microspin G50 column 3 times as follows.
      i. Add 500 μL of IP Low Salt Wash Buffer.
      ii. Centrifuge using a low speed capsulefuge.
      iii. Discard the flow-through.
   h. Elute the IP sample.
      i. Close one end of each column with a snap-off plug, add 40 μL IP Elution Buffer (= 1× Laemmli Buffer without β-Mercaptoethanol) into each column.
      ii. Incubate at 20°C–22°C for 7 min.
      iii. Unplug ends of the columns place them in a collection tube containing 0.3 μL 100 mM Dithiothreitol (DTT).
      iv. Spin using a low speed capsulefuge to collect eluate.
      v. Repeat steps i-iv using 20 μL IP Elution Buffer.
      vi. Pool the two elutions (40 μL and 20 μL from step v) for each IP-Samples from step and store at −80°C.

Pause point: Samples can be stored at −80°C indefinitely.

Western blot analyses of immunoprecipitated proteins

Timing: 2 days

The analysis of immunoprecipitated proteins by western blot at this stage is a key step that validates that the IP was successful. The aim is to detect the protein targeted by the antibody in the target protein IP sample, and not in control IgG IP samples (see troubleshooting). Probing for proteins known to interact with the targeted protein can serve as an additional positive control.

4. Analyze the Input and IP Samples by Western blotting to confirm IP.
   a. Prepare aliquots of both Input and IP Samples for western blotting.
      i. Transfer 8 μg aliquots of each Input Sample, and 2.5 μL aliquots of each IP Sample, into new tubes.
      ii. Add an amount of 20 mM Tris-HCl (pH 7.5) to create aliquot volumes of 7.5 μL each, and then add 2.5 μL of 4× Laemmli Buffer and mix by pipetting. The final volume of each aliquot is 10 μL.
      iii. Incubate aliquots at 95°C for 5 min and then place on ice.
   b. Separate proteins using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). We provide details for the XCell SureLock Mini-Cell system (Thermo Scientific) although alternative systems will achieve similar results.
      i. Assemble the XCell SureLock Mini-Cell system with a pre-caste NuPAGE 3%–8% Tris-Acetate 1.5 mm Mini Protein Gel as per manufactures instruction.
      ii. Add 250 μL NuPAGE Antioxidant to 250 mL of 1× NuPAGE Tris-Acetate SDS Running Buffer and pour into inner buffer chamber.
      iii. Fill the outer chamber of the XCell SureLock Mini-Cell with 1× NuPAGE Tris-Acetate SDS Running Buffer to a level ~3 cm above the bottom of the gel.
      iv. Remove gel comb and wash wells using 1 mL pipette tip.
      v. Load all 10 μL of the Input and IP Sample aliquots using gel loading tips. Also load 5 μL of Precision Plus Protein Dual Color Standards (add 5 μL 1× Laemmli Buffer) in flanking wells.
      vi. Run samples at 100 V for 2 h.
   c. Transfer proteins to nitrocellulose membrane.
      i. Carefully remove the precast gel from cassette and soak in cold Western Transfer Buffer for 5 min.
      ii. Pre-soak 'cut-to-gel' sized nitrocellulose membrane (BioTrace NT), and two Whatman filter papers (3 mm CHR) and blotting pads in cold Western Transfer Buffer.
      iii. Assemble the transfer blot 'sandwich' in the XCell II Blot Module.
      iv. Fill the transfer chamber with cold Western Transfer Buffer.
      v. Transfer at 15 V for 16 h in a 4°C cold room.
      vi. Confirm transfer by incubating the nitrocellulose membrane with Ponceau S Stain for 5 min.
      vii. Repeatedly wash of excess stain using TBST.
      viii. After confirming transfer of protein to nitrocellulose membrane, remove remaining protein-bound Ponceau S Stain using repeated washes with TBST.
   d. Conduct western immunoblotting.
      i. Block the nitrocellulose membrane with TBST containing 10% skim milk for 1 h.

Pause point: Blocked membranes can be stored in TBST for weeks at 4°C.

      ii. Remove blocking solution and incubate the membrane for 16 h at 4°C in TBST containing primary antibody (in the example of Johnson et al., 2020, the primary antibody was rabbit anti-USP9X, 400 ng/mL) and 2% skim milk.
      iii. Wash the membrane with TBST buffer 3× 10 min each wash.

iv. Incubate the membrane at R/T for 2 h with TBST containing 2% skim milk and Horseradish Peroxide (HRP)-conjugated secondary antibodies (in the example of Johnson et al., 2020, this was goat anti-rabbit immunoglobulins/HRP secondary antibody used at 1:1,000 dilution).
v. Wash the membrane with TBST buffer 3× 10 min each wash.
vi. Remove TBST and use a Whatman filter to further remove any excess TBST from the membrane.
vii. Incubate the membrane with Clarity Western ECL Substrate reagent for 2 min.
viii. Remove excess ECL reagent and capture emission using ChemiDoc MP Imaging System (See expected outcomes for an example result).

⚠ CRITICAL: Failure to detect the protein of interest at the end of this step indicates a no-go event and the experiment should be abandoned. See troubleshooting for additional information.

In-gel digestion of immunoprecipitated proteins

Timing: 2 days

Gel electrophoresis, commonly used for the preparation of proteomic samples for mass spectrometric analysis, enables selective capturing of proteins and elimination of agents that interfere with downstream analysis. Following gel separation, an in-gel digestion of proteins is performed. The procedure involves excising gel regions containing the protein, reduction of protein disulphide bonds, alkylation of free unreacted thiols, and proteolytic digestion of the protein samples. Resultant peptides can be used for TMT labeling or subjected to LC-MS/MS for label-free quantitative analysis.

5. In-gel digestion of immunoprecipitated proteins with trypsin.

⚠ CRITICAL: Use fresh gloves and wipe down sample preparation areas with 80% ethanol solution to clean surfaces prior to processing the gels to avoid keratin contamination which interferes with downstream proteomics analysis.

a. Prepare a 35 μL aliquot of each IP sample for SDS-PAGE by adding 12 μL of 4× Laemmli Buffer. Incubate the samples at 95°C return to ice.
b. Prepare the SDS-PAGE apparatus as per step 4. A single IP sample may need to be loaded across multiple wells depending on well volume capacity. Run the gel at 200 V for 10 min.
c. Conduct a Coomassie stain of the proteins.
   i. Carefully remove the precast gels from cassettes.
   ii. Wash gels with Fixing Solution for 5 min.
   iii. Remove Fixing Solution and incubate with Coomassie Stain for 16 h.
   iv. Discard Coomassie Stain and remove any remaining traces using three 5 min incubations in Coomassie Wash Solution with slight agitation.
   v. Remove the solution and rehydrate the gel by incubating in Gel Rehydration Solution for 1 h.
   vi. Remove suspended particulate matter by immersing the entire gel in ddH_2O water for 1 h, while changing the water every 20 min.
d. Excise all gel regions containing protein.
   i. Place the gel on a clean surface.
   ii. Use a scalpel to excise all gel regions containing protein of each sample.
   iii. Pool multiple wells of the same sample together where relevant.

iv. Dice the gel regions further into smaller pieces of 1 × 1 mm and place them inside a Protein LoBind microcentrifuge tube.
v. Centrifuge tubes at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.

e. Wash gel pieces with acetonitrile.
i. Add 500 μL of acetonitrile (LC-MS grade) to each gel sample.
ii. Incubate for 10 min at 20°C–22°C. At this stage, the gel pieces may turn opaque and shrink. To avoid gel pieces sticking together, pulse vortexing is used to separate pieces.
iii. Centrifuge at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.
iv. Discard the supernatant.

f. Add 30 μL of the 10 mM DTT Solution in TEAB to each sample, ensuring that the gel pieces are fully submerged. If necessary, additional 10 mM DTT Solution in TEAB can be added. Incubate the samples in a heating block/incubator for 30 min at 56°C. Cool the sample tubes to 20°C–22°C.

g. Wash gel pieces with acetonitrile.
i. Add 500 μL of acetonitrile (LC-MS grade) to each sample.
ii. Incubate tubes for 10 min at 20°C–22°C.
iii. Centrifuge tubes at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.
iv. Discard the supernatant.

h. Add 30 μL of 55 mM iodoacetamide (IAM) in TEAB solution to the sample ensuring that the gel pieces are fully submerged. If necessary, additional 55 mM IAM in TEAB solution can be added. Incubate the samples in the dark at 20°C–22°C for 20 min.

i. Wash gel pieces with acetonitrile.
i. Add 500 μL of acetonitrile (LC-MS grade) to each sample.
ii. Incubate tubes for 10 min at 20°C–22°C.
iii. Centrifuge tubes at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.
iv. Discard the supernatant.

j. De-stain the gel pieces.
i. Add 500 μL of Destaining Solution to each sample.
ii. Incubate the samples at 20°C–22°C for 20 min. Incubation time can be varied as per the color intensity of the gel pieces. For darker stained gel samples, longer incubation can be performed.
iii. Centrifuge tubes at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.
iv. Discard the supernatant.

k. Wash gel pieces with acetonitrile.
i. Add 500 μL of acetonitrile (LC-MS grade) to each sample.
ii. Vortex samples for 10 s.
iii. Incubate tubes for 10 min at 20°C–22°C.
iv. Centrifuge tubes at 1,000 × *g* for 1 min to collect the gel pieces in the bottom of the tube.
v. Discard the supernatant.

Note: Although it is also not necessary to remove all traces of the Coomassie Stain from the gel, steps 5j and 5k can be repeated if significant amounts of stain remains.

⚠ CRITICAL: Remove all remaining acetonitrile at this step, as it can hinder the proteolytic activity of trypsin. Visually inspect the gel to ensure the absence of acetonitrile along with the gel. If necessary, the left over traces of acetonitrile can be removed by subjecting the gels to brief vacuum centrifugation up to 10 min.

Pause point: The excised and destained gel bands are now ready for digestion. However, if necessary, they can be stored at −20°C for up to 4 weeks.

l. Add 100 μL of Trypsin Peptide Digestion Solution to each sample and incubate on ice or in the fridge at 4°C for 30 min to allow absorption of the solution into the gel. Ensure the gel pieces

are fully submerged. If necessary, additional Trypsin Peptide Digestion Solution can be added.

Note: Addition of trypsin on ice is recommended as it helps in reduction of autolysis of trypsin, thus ensuring efficient digestion of proteins.

m. After the incubation, ensure that all gel pieces have absorbed the Trypsin Peptide Digestion Solution and are completely submerged in the solution, and perform the digestion for 16 h in a heating block at 37°C.

⚠ CRITICAL: Complete digestion is essential as undigested, or partly digested peptides reduce the number of total peptide free *N*-termini through which TMT labeling occurs. To ensure good digestion efficiency, it is important to maintain constant temperature across the sample tube. As the uncovered heating blocks can expose the sample tubes to non-uniform temperatures, ensure the heating block is covered with aluminum foil to minimize the loss of heat and help keep temperatures uniform, or incubate the sample tubes in an enclosed incubators with uniform surface temperatures.

n. Remove the samples from heat block and chill the sample tubes on ice.
o. Centrifuge the samples at 1,000 × *g* for 1 min to gather the solution and gel pieces in the bottom of the tube and carefully transfer the supernatant containing the peptide to a fresh Protein LoBind tube.
p. Extract residual peptides from the remaining gel pieces by adding an additional 100 µL of Peptide Extraction Buffer to the gel pieces and incubate on a heating block for 15 min at 37°C. Repeat steps 5n–o and pool supernatants at step 5o together.

Note: It is advised to retain the extracted gel pieces. The same gel pieces can be re-digested with the same enzyme or any alternative enzyme in case the digestion fails.

q. Dry the final combined sample at step 5o in a SpeedVac vacuum concentrator at 45°C. Under these conditions, samples are typically dried with 2–3 h.

Pause point: The digested, dried peptides can be stored at −20°C or −80°C freezer for up to 4 weeks.

Note: The volumes of reagents used for destaining, digestion and extractions can be varied proportionally, depending on the size of the gel matrix.

Labeling digested peptides with Tandem Mass Tags

Timing: 1 day

In this step, digested peptides are labeled with Tandem Mass Tags (TMTs) and a 'Label Check' is performed to assess labeling efficiency, and to normalize peptide amounts, before the samples are pooled for further analyses.

6. TMT Labeling of Peptides.
   a. Resuspend each of the dried peptide samples.
      i. To each sample, add 105 µL of 200 mM HEPES (pH 8.8).
      ii. Vortex for 10 s.
      iii. Spin the samples down at 1,000 × *g* for 1 min.

⚠ CRITICAL: Reagents/buffers containing primary amines must be avoided, as they competitively bind TMT reagents resulting in poor labeling efficiencies. Buffers such as HEPES or EPPS are recommended. Any residual acid from incomplete drying of samples post-digestion could alter the sample pH, hence it is important use higher concentration of buffers (e.g., HEPES ≥ 200 mM).

b. Measure the peptide concentration using the Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific), as per manufacturer's instructions.
c. Aliquot 100 μg peptide of each sample into to fresh Protein LoBind tubes for labeling in a 10-plex TMT reaction.

Note: If 100 μg is not available for any one sample, make all aliquots equal to the amount of the most limiting sample. A minimum of 20 μg is required. Lower amounts of starting material needs further optimization of amount of TMT label to be added to the sample.

d. Equalize all sample volumes to that of the largest sample volume through addition of 200 mM HEPES (pH 8.8).
e. Prepare TMT 10-plex Isobaric Label Reagents.
   i. Immediately before use, add 42 μL of anhydrous acetonitrile to each 0.8 mg TMT label vial.
   ii. Vortex for 5 min.
   iii. Centrifuge at 1,000 g for 1 min.
f. Add a different TMT Label to each respective peptide sample (up to 10 samples) and incubate at 20°C–22°C for 1 h with occasional vortexing.

Note: Upon optimization, amount of total TMT label used can be reduced.

Note: If more than ten samples are to be analyzed then strategies for multi-batch TMT designs need to be considered including inter-batch controls.

⚠ CRITICAL: TMT labeling cannot be performed in buffers containing primary amines (e.g., Tris-HCl buffer).

g. Quench excess TMT label.
   i. add 8 μL of TMT Reaction Quenching Solution to each sample.
   ii. Vortex for 10 s.
   iii. Incubate at 20°C–22°C for 15 min.

7. Perform TMT Label Check.

To ensure equal amounts of TMT labeled peptides from each individual sample are combined in the final large scale pooled sample subjected to two-dimensional liquid chromatography/mass spectrometry (LC-MS/MS), a small scale pilot study known as a "Label Check" experiment is first performed. In the Label Check experiment, a test sample is created by pooling small, equal volumes of each sample, and assessed by LC-MS/MS, and ion intensities of each sample analyzed. The ion intensities results of the Label Check experiment form the basis of a normalization step that ensures that each TMT labeled sample contributes a similar total reporter ion intensity when pooled for LC-MS/MS.

a. Create a small scale pooled sample.
   i. Combine approximately 1 μg (2 μL) of each sample into a single fresh Protein LoBind tube.
   ii. Vacuum dry the combined sample.
   iii. Desalt the combined sample via the SDB Stage-tip clean up procedure using Empore™ SDB SPE Disks (Rappsilber et al., 2007).
   iv. Dry the desalted sample.

Note: The volume of each sample used to generate the pool needs to be identical, and the remaining sample volume will also be equal.

b. Reconstitute the small scale pooled sample in LC-MS Loading Buffer.
c. Analyze approximately 1 μg of peptide mixture on the Orbitrap mass spectrometer (refer to steps 10-g and 11 for LC-MS/MS settings).
d. Use the resulting data file (.raw format) for performing a database search by MaxQuant (refer to step 11 for search parameters).
e. Calculate the total TMT reporter ion intensity for each individual samples in the small scale pooled sample.
f. Calculate the volume of each TMT labeled peptide sample required to generate the large scale pooled sample for LC-MS/MS: Each sample in this pool must contribute equal levels of total TMT reporter ion intensity. Thus the total TMT reporter ion intensity of each sample is limited by, and must be equal to, that of the sample with the lowest total TMT reporter ion intensity. The volume of each sample to be added to the large scale pool is therefore calculated as follows: Sample volume to be added to pool = (total of TMT reporter ion intensity in the lowest abundant sample / total of TMT reporter ion intensity in the individual sample) * remaining sample volume.
g. Create a large scale pooled sample by combining the samples using volumes calculated in step f.

Note: Store the samples in 4°C until Label Check is complete, as LC-MS/MS analysis can typically take 2–6 h.

h. Dry the final combined sample in a SpeedVac vacuum concentrator.

Pause point: The TMT labeled, dried peptides can be stored at −20°C or −80°C freezer for up to 6 weeks until further processing.

Note: After drying, the sample pellet may look like viscous or oily due to the presence of salts.

Desalting of TMT labeled peptides

Timing: 1 day

Following the TMT labeling procedure, some of the unbound TMT reagents and buffer components may remain in the sample and interfere in downstream processing including, separation of peptides during chromatography, electrospray ionization and mass spectrometry analysis. Hence samples are desalted using Sep-Pak Light C18 cartridge before performing any further sample processing.

8. Desalting TMT labeled peptides by Solid Phase extraction.
   a. Resuspend the dried TMT labeled peptide sample.
      i. Add 1 mL of Sep-Pak Wash Solution to the sample.
      ii. Vortex rigorously for 1 min.
      iii. Centrifuge at 1,000 g for 1 min.
      iv. Check that peptides are fully resuspended and total volume collected at bottom of the tube. Repeat steps 8aii–iv if necessary.
   b. Assemble the Sep-Pak Light C18 cartridge on the vacuum manifold.
      i. Fix a Sep-Pak Light C18 cartridge at the top of the vacuum manifold.
      ii. Attach a 5 mL collection tube under the Sep-Pak Light C18 cartridge in the suction manifold to collect the flow through from the Sep-Pak.
      iii. Connect the entire assembly to a vacuum line.

Note: If access to a vacuum manifold is unavailable, solution can be passed through cartridge using positive air pressure.

c. Wash the Sep-Pak with 100% methanol (HPLC grade; >99.9% purity).
   i. Dispense 1 mL of methanol into the Sep-Pak Light C18 cartridge.
   ii. Start the suction pump and let the methanol pass through the Sep-Pak Light C18 cartridge.

⚠ CRITICAL: Methanol is a highly flammable liquid, an eye irritant, and considered an acutely and chronically toxic solvent. Wear appropriate protective gear (lab coat, gloves, eye, and goggles) and handle large volumes in a well-ventilated chemistry hood.

d. Wash the Sep-Pak Light C18 cartridge with Sep-Pak Elution Solution (80% acetonitrile, 0.5% acetic acid, in ddH2O).
   i. Dispense 1 mL of Sep-Pak Elution Solution into the Sep-Pak Light C18 cartridge.
   ii. Start the suction pump and let the Sep-Pak Elution Solution pass through the Sep-Pak Light C18 cartridge.
e. Wash the Sep-Pak Light C18 cartridge with Sep-Pak Wash Solution to equilibrate the column.
   i. Dispense 1 mL of Sep-Pak Wash Solution into the Sep-Pak Light C18 cartridge.
   ii. Start the suction pump and let the Sep-Pak Elution Solution pass through the Sep-Pak Light C18 cartridge.
   iii. Repeat the equilibration procedure twice more to remove all traces of organic solvent.
f. Place a fresh collection tube under the equilibrated Sep-Pak Light C18 cartridge for the collection of the flow-through.
g. Fill the Sep-Pak Light C18 cartridge with the sample (from step 8a) and let the sample pass through very slowly at approximately 0.5 mL/min.

Note: The final flow through from the sample may be collected and stored for subsequent analysis to check for unbound peptides, if desired.

h. Wash the Sep-Pak Light C18 cartridge with 1 mL Sep-Pak Wash Solution (as per steps 8ei–ii) (collect wash solution if necessary).
i. Elute peptides into a fresh 1.5 mL tube with 1 mL of Sep-Pak Elution Solution (as per step 8d).
j. Dry peptides in a vacuum centrifuge.

Pause point: Desalted, TMT labeled peptides can be store at −80°C until required.

Fractionation of the peptide mixture using high pH reverse phase chemistry

Timing: 1 day

In this section the TMT labeled peptide sample is subjected to fractionation using high pH (HpH) reverse phase chemistry. The fractionation serves as an orthogonal separation method prior to downstream low-pH reversed-phase LC-MS/MS analysis. These two in-line orthogonal fractionation approaches increase the likelihood of peptide detection in mass spectrometers by decreasing the sample complexity thus reducing the ion suppression effect commonly observed in TMT based quantification (Di Palma et al., 2012).

9. High pH Fractionation of TMT labeled peptides.

Note: If access to an LC system with fraction collector is unavailable, centrifugal HpH columns can be used as an alternative (e.g., Pierce™ High pH Reversed-Phase Peptide Fractionation Kit, Thermo Fisher Scientific, P/N: 84868).

a. Reconstitute sample in 600 µL of HpH Mobile Phase A Solution and vigorously vortex.

Table 1. HpH Mobile phase Gradient table

Time intervals (min)	HpH mobile phase A solution (%)	HpH mobile phase B solution (%)
0	97	3
10	97	3
65	70	30
75	30	70
80	10	90
85	97	3
110	97	3

b. Inject the sample onto the column (Zorbax 300 Extend-C18, 2.1 × 150 mm, 3.5 μm, 300A, HPLC column (Agilent), operated at 25°C) and separate the peptides into a total of 96 fractions using an Agilent 1260 HPLC system equipped with quaternary pump, a degasser and a Multi-Wavelength Detector (MWD) (set at 210, 214 and 280-nm wavelength).
c. Elute the peptides off the column using a gradient of HpH Mobile Phase B Solution at a flow rate of 0.3 mL/min according to Table 1.
d. Collect the eluate into a 96-well plate (2 mL deep well, round bottom, polypropylene collection plate; Axygen), using the fraction collector, at 2 min per fraction for the first 16 min and then 1 min per fraction through end of the program at 100 min.
e. Concatenate the 96 well fractions into 20 fractions in the pattern indicated in Table 2.

Note: The fractionation control settings and concatenation pattern may need adjusting depending on LC chromatogram features to ensure that peptides are collected depending on chromatography observed and fraction collection order on individual systems. Under the conditions for this study, Fractions -3 to -1 (first 14 fractions) collect the void volume of the column prior to injection, until the wash-out of unbound material (primarily unreacted TMT label). Subsequent fractions collect peptides eluting from the column, until the end of the gradient run (i.e., high Buffer B). This pattern optimizes the peptide amounts and fractionation in the sample based on the elution pattern observed, but collection times and windows may need adjusting for individual instruments. Factors to consider include the void volume of the instrument so that fraction collection begins as peptides begin to elute, the volume collected in each fraction does not exceed the collection volume available and the collection of fraction should finish as peptides are completely eluted from the column.

f. Place the samples into an evaporative concentrator and concentrate under vacuum at 45°C to dryness.

Pause point: The dried peptide samples are ready for mass-spectrometry analysis and can be stored at −80°C for up to 2 months.

Liquid chromatography mass-spectrometry analysis

Timing: 1 day

This section describes LC-MS/MS analysis procedure for TMT labeled peptide fractions on a Quadrupole-Orbitrap mass-spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) coupled to a Nano-flow liquid chromatography (Thermo Scientific™ UltiMate™ 3000 RSLCnano system). Alternative mass spectrometers and liquid chromatography of other brands represent valid options for analysis of the samples.

10. Liquid chromatography mass-spectrometry LC-MS/MS analysis of TMT labeled peptides.
    a. Reconstitute each of the peptide fractions.

Table 2. Pooling scheme of the fractions of TMT labeled samples

	i	*ii*	*iii*	*iv*	*v*	*vi*	*vii*	*viii*	*ix*	*x*
A	-3	2	3	1	2	17	1	16	7	
B	-3	1	4	17	3	16	2	15	6	
C	-3	-1	5	16	4	15	3	14	5	
D	-3	-1	6	15	5	14	4	13	4	12
E	-2	-1	7	14	6	13	5	12	3	11
F	-2	-1	8	13	7	12	6	11	2	10
G	-2	-1	9	12	8	11	7	10	1	9
H	-2	-1	10	11	9	10	8	9	17	8

Peptides from the wells indicated with the same number can be pooled to form a fraction as indicated in the table. For example, sample -3 can be prepared by pooling Ai, Bi, Ci and Di.

i. Add 6 μL of LC-MS Loading Buffer to each fraction.
ii. Vortex for 10–30 s.
iii. Sonicate for 2–5 min (e.g., using a Vibra-Cell Sonicator with 3 mm tip; Sonics).
iv. Centrifuge the samples at 10,000 × *g* for 2 min at 20°C–22°C to pellet any particulate matter.
v. Transfer the samples into vials compatible with the liquid chromatography system of choice.

⚠ CRITICAL: Any particulate matter that is not removed at this step can block and damage LC columns and instruments.

Note: Exact volume of the sample reconstitution buffer and peptide sample loading volume can differ depending on the total peptide amount that is available and sensitivity of the mass spectrometers. A small amount of the peptide sample can first be injected to test the response, before injecting the samples. Label Check analysis performed in step 7 can be used as a guide to expected intensity.

Pause point: TMT labeled samples, can be stored in MS loading buffer for up to 6 days at 4°C, 6 weeks at −20°C, or 6 months at −80°C.

b. Place the samples in the auto-sampler of the liquid chromatography system.

⚠ CRITICAL: To ensure longer shelf life of the peptide samples, it is important to maintain the auto-sampler system in cooler temperature between 4°C–8°C.

c. Set the column heater temperature to 35°C and apply a voltage of 1.9 kV to the nano-LC electrospray ionization source.
d. Inject each of the individual samples onto the peptide trap column.
e. Desalt the peptide trap column by washing it with 15 μL LC-MS Mobile Phase A Solution for 10 min.
f. Switch the valve in-line with the column.
g. Elute peptides from the peptide-trap into the analytical column with the linear gradients of LC-MS Mobile Phase B Solution with a stepped gradient of 120 min including elution of peptides, wash and re-equilibration of the columns. A recommended mobile phase gradient has been suggested in Table 3.

Note: Shorter gradients can be used for less complex samples.

h. Analyze LC eluent on a Quadrupole-Orbitrap mass-spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) on a MS2-based TMT method as outlined in Table 4.

Table 3. Nano-Flow liquid chromatography gradient used at a 600 nl/min flow rate

Time interval (min)	Gradient (% LC-MS mobile phase B Solution)
0	5
10	5
110	30
118	85
130	5
140	5

Identification and quantification of proteins from the raw mass spectrometry data

Timing: 2 days

In this section the raw data file is analyzed using MaxQuant to produce an output file of protein list and label intensities that can be normalized and used for identification of proteins which are enriched in the IP in comparison to control samples. This data provides identification of the target IP proteins interactome.

11. Analysis of raw mass spectrometry data.
    a. Process the raw data files using MaxQuant (Version_1.6.10.43 and above) (Cox and Mann 2008).

Note: For more details on setting up MaxQuant and conducting a data search, follow steps described in (Tyanova et al., 2016). Latest version of MaxQuant (version v2.0.3.1, May 2022) offers isobaric match between run (MBR) options that is reported to enhance the quantitative depth of the proteome as oppose to the search conducted without MBR (Yu et al., 2020). Alternatively, a whole suite of other software can also be used including Proteome discover (Orsburn 2021) (with embedded Mascot or Sequest search functions), and MSFragger (Kong et al., 2017), among others.

    b. Specify the parameters for searching TMT proteomics data on the MaxQuant software.

Note: Default settings in MaxQuant software are typically optimal to use, with a few exceptions such as defining variable modifications and TMT labeling bias correction value substitution. Typical parameters for MS2-DDA-TMT-MS data are outlined in Table 5.

    c. Input the TMT kit lot specific reporter ion isotopic distribution correction factors to account for natural carbon isotopes (+1, +2) and incomplete stable isotope incorporation (-1, -2).

Note: This value is specific to the kit and can be retrieved from information catalog provided along with the kit.

12. Output and analyze data to assess proteins interacting with the targeted IP protein.
    a. Output search and TMT intensity raw data. This data identifies peptides and protein groups detected in each sample and provides their quantities as reported by TMT-channel intensities at either peptide or protein levels. These intensities provide relative quantitation of peptides and proteins between samples. MaxQuant output provides list of proteins identified (Gene names or FASTA headers), and their associated "Reporter Intensity Corrected" and "Reporter Intensity Count" metrics for each TMT channel. "Reporter intensity corrected" metrics are used for quantification.
    b. Normalize TMT quantitation data by total abundance.
        i. Sum the total ion intensities for each channel.

Table 4. Parameters used for the operation of the mass spectrometer

Setting	Value
Polarity	Positive
Full MS	
Microscans	1
Resolution	60,000
Automatic gain control target	3×10^6 ion counts
Maximum ion time	120 ms
Scan range	350–1,850 *m/z*
dd-MS^2	
Microscans	1
Resolution	45,000
Automatic gain control target	1×10^5
Maximum ion time	85 ms
Loop count	10
Isolation window	0.8 *m/z*
Isolation offset	0
Fixed first mass	100 *m/z*
Normalized collision energy	33
DD settings	
Minimum AGC target	2×10^4 ion counts
Apex trigger	2–4 s
Charge exclusion	Unassigned, 1, ≥6
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	30 s

ii. For each channel, divide the total intensity sum by that of the total intensity sum of the highest total intensity channel. This ratio is used as a normalization factor.

iii. Normalize each individual proteins abundance in a channel by multiplying the channels normalization factor to the protein TMT quantitation data.

c. Use the normalized protein abundance data to identify proteins enriched in target protein IP samples (e.g., USP9X wildtype and mutant IP samples in Johnson et al., 2020) compared to control IgG IP samples.

i. Calculate the mean normalized protein abundance of the triplicate samples of each experimental group (control IgG IP and target protein IP groups).

ii. For each protein, divide target protein IP mean by control IgG IP mean. Proteins with increased normalized abundance (Figure below) in the target protein IP sample compared to the IgG control IP sample can be considered enriched in the target protein IP sample, and as such a part of the target proteins interactome. Similar comparisons can be made between other experimental groups (Figure 1).

d. Conduct t-tests between target protein IP sample groups using normalized data triplicates. Differences between groups with $p < 0.05$, and with fold-change of 1.5 are considered biologically significant.

Note: TMT method will usually have a quantitative value for each channel, though a very low value may be indicative of peptide/protein absence in the sample. Caution should be taken when interpreting very high fold-change results (absolute log2(fold-change) > 4–5), as this may indicate absence of peptides or proteins in sample groups where peptide intensity is very low. Absence/presence of signals may be enough to indicate complex participation and not require statistical analysis. For example, if the protein is confidently detected in at least two out of three replicates in the target protein IP sample and none of the replicates in the control IgG IP, this may be considered sufficient evidence that it is an interacting partner.

Table 5. Typical parameters for searching TMT proteomics data using MaxQuant

Setting	Value
Raw data	
Parameter group	All samples: Group 0
Experiment	Enter unique name, suffixed by biological replicate (e.g., 'Control_1')
Fraction	All samples: 1
PTM	False
Group-specific parameters	
Type	TMT-MS2
Isobaric labels	10plex TMT
Multiplicity	1
Labels	None
Variable modifications	Oxidation (M), Acetyl (protein N-term)
Digestion mode	Specific
Enzyme	Trypsin/P
Maximum missed (cleavages)	2
Global parameters	
FASTA files	Specify protein sequence databases
Fixed modifications	Carbamidomethyl (C)
Second peptide	True
Match between runs	True
Match time window	0.7 min
Alignment time window	20 min
Advanced site intensities	Yes
PSM FDR, Protein FDR	0.01
Min. unique peptides[a]	1

[a]Recommended setting. For increased confidence of identification and quantification of proteins, minimum unique peptide cut off can be set in the search engine.

Note: Visualization of results can be achieved through heat maps filtered for statistically significant proteins (x-axis = Sample or Sample group, y = Protein, fill = Abundance or mean abundance), or using a volcano plot (x = Fold change, y = $-\log_{10}$ (p value from ANOVA).

e. Conduct Gene Ontology enrichment analysis (or other types of pathway enrichment) on lists of differentially expressed proteins. Multiple freely available web-based resources represent convenient tools to perform such analysis, including enrichR (https://maayanlab.cloud/Enrichr/) and DAVID (https://david.ncifcrf.gov/). Using these tools on IP derived proteomic data needs consideration, as interacting pathways are likely to be overrepresented in the data initially.

EXPECTED OUTCOMES

This protocol will enable identification of an endogenous protein's interactome in whole or in part. We provide a step-by-step guide for performing an antibody-based protein complex pull-down (suitable for any protein for which a high-affinity antibody is available) and a typical bottom-up quantitative proteomic approach utilized for identifying and quantifying proteins. The protocol combines IP, gel electrophoresis and controlled in-gel proteolysis, and identification and quantification of proteins using multiplexed TMT labeling coupled with peptide sequencing.

The success of the IP is determined by a western blot probing for the presence of the targeted protein (step 4). The targeted protein should not be present in the IgG control IP, and only present in the target antibody IP. Results for an IP targeting USP9X is shown in Figure 2 (Johnson et al., 2020). Note that if the HRP-conjugated secondary used in the western blotting recognizes the species of

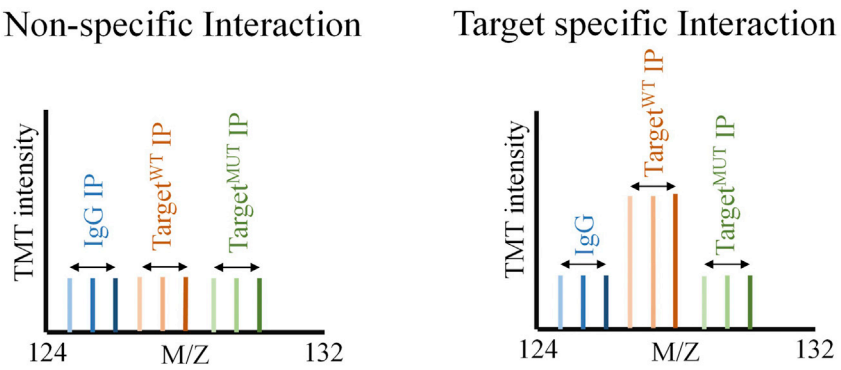

Figure 1. Conceptual representation of data obtained from an IP-coupled TMT based proteomics
In this example, cells express either the wild-type target protein (Target^{WT}) or a mutant version of the target (Target^{MUT}) unable to interact with certain binding partners. A triplicate experiment is shown with all samples run using a single TMT (9-plex). The TMT peaks for a non-specific interactor, and target specific interactor are shown.

antibody used in the IP, then the antibodies heavy and light chain fragments will also be detected (size 50 and 25 kDa respectively).

The use of Sephadex spin columns followed by gel electrophoresis removes all traces of the IP beads which prevents clogging of the injection needle and liquid chromatography's plumbing lines. The use of TMT based multiplexing provides key advantages including (1) reproducibility of analysis as multiple samples are analyzed in the same batch thus minimizing run to run variability (2) boosting detection sensitivity of peptides that are present in relatively lower concentrations in samples (typically the case in IP samples).

Reproducible and robust analysis of protein complexes can be challenging as typical amount of protein starting material yield in a pulled down using antibodies ranges in tens of micrograms depending on nature of the protein complex. In our experience, starting with 500–1,000 μg of protein and 5–10 μg of antibody yielded 10–20 μg of IP proteins which typically leads into identification of 20–50 highly confident interacting proteins. A volcano plot of the biologically important proteins identified in the USP9X interactome ($P<0.05$, $FC>1$), and their quantitative difference in various USP9X mutant cell lines is illustrated (Figure 2).

LIMITATIONS

Access to an IP-quality antibody: The IP protocol is dependent on the availability of a specific and high-affinity antibody to the endogenous protein of interest. If one does not exist or cannot be generated/accessed easily, then engineering of an epitope tag fused to the protein remains the best alternative approach. Highly-specific commercial antibodies for epitope tags such as FLAG, MYC, V5, HA (among others) are available and work efficiently in IP. Such tags can be added in-frame into endogenous loci using CRISPR engineering (Pederick et al., 2018), or fused in-frame to an open reading frame encoding the protein in a vector suitable for exogenous expression (Homan et al., 2014; Pham et al., 2017).

IP occurs under defined buffer conditions: Each protein-protein interaction is optimal under different buffer conditions, including pH, salt type/concentration and detergent. The conditions should be optimized for efficient pull-down of the target protein as the priority, but such conditions may not be conducive (to varying extents) to capture all target protein interactors.

Disruption of spatial information: The IP is conducted in cell lysate, not in-situ. As such some protein-protein interactions may be captured which would normally not occur under physiological

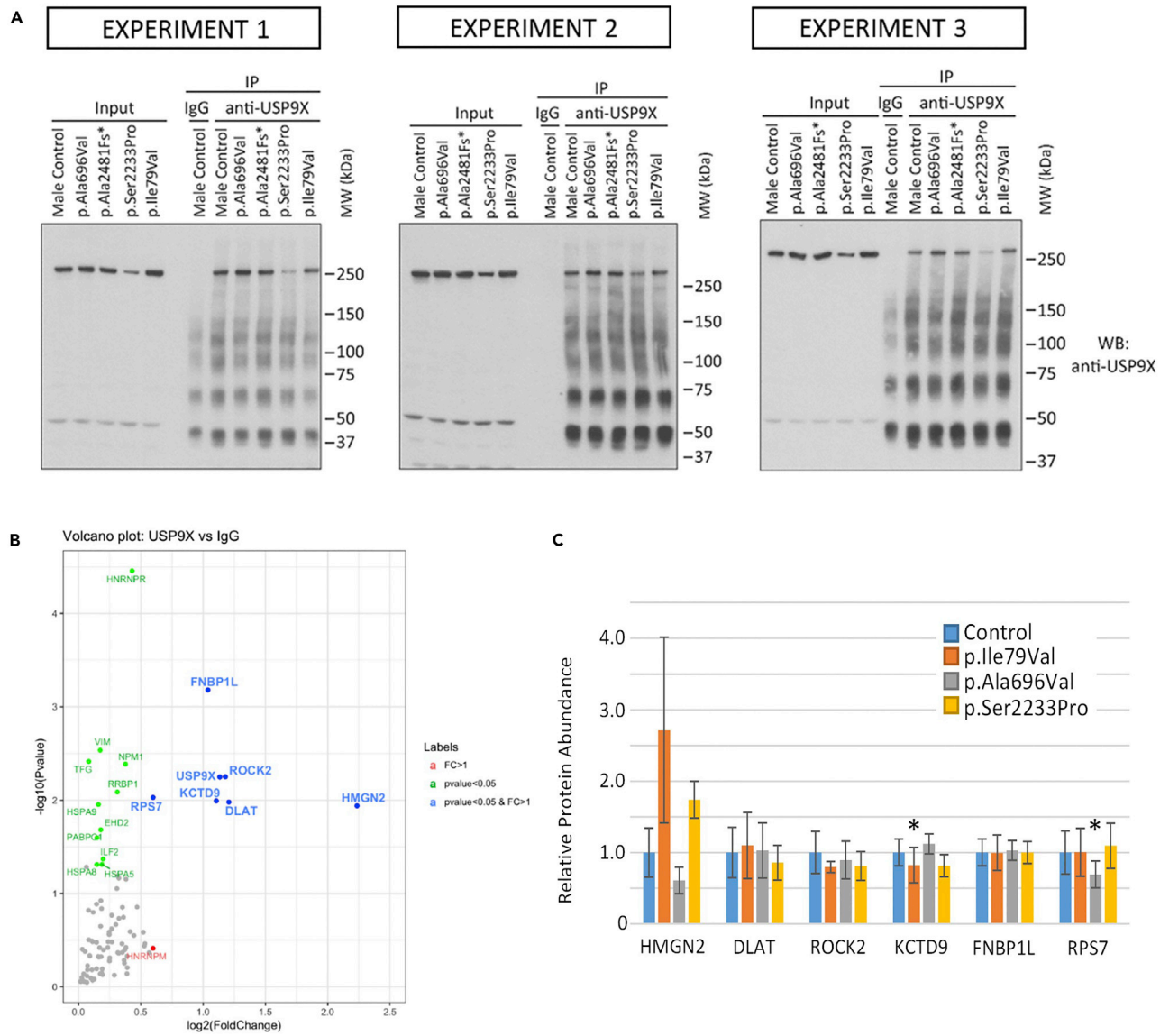

Figure 2. Identification of USP9X interactors in human dermal fibroblast cell lines derived from control individuals and individuals with USP9X mutations

The USP9X mutations (p.Ile79Val, p.Ala696Val and p.Ser2233Pro) are known to cause neurodevelopmental disorders and predicted to disrupt binding of USP9X to different sub-sets of interacting proteins depending on the location of the mutation.

(A) Western blot analysis of 3 replicate USP9X IP experiments. IgG IPs served as negative control samples in subsequent proteomics. USP9X and IgG control immunoprecipitated samples from each experiment subsequently analyzed using TMT LC-MS/MS identification and quantification.

(B) Volcano plot of proteins immunoprecipitated with USP9X from control fibroblasts. Results derived from 3 independent IP experiments in (A). Fold change and statistical values represents comparison to proteins immunoprecipitated with control IgG. p values derived from adjusted Students paired t-test.

(C) Relative protein quantities of significantly enriched USP9X interactors (enriched in USP9X IPs compared to IgG IPs in control cells) in mutant USP9X IP experiments. *p<0.05 paired Student's t-test. Data is represented as mean ± standard deviation. Data is reproduced from Johnson et al. (2020) with permission from Elsevier.

conditions because of physical spatial separation within the cells or other spatially controlled regulatory events. Orthogonal approaches such as co-localization studies should be part of downstream validation of interactions. In-situ interactome techniques such as proximity ligation assay may also be considered as an alternative for validation (Qin et al., 2021).

Inherent technical variation: The bead-based antibody pulldown necessitates a downstream purification technique (in our case gel electrophoresis) to remove the interfering agents. However, the in-gel digestion procedure involves several additional sample handling steps such as destaining, digestion of proteins and elution. Each additional step potentially contributes to technical sample to sample (and batch to batch) variation, introducing noise in downstream quantifications. Alternatively, on-bead digestion of protein can be performed to minimize the steps involved for preparing the samples for protein digestion.

Ratio compression effects: Although the multiplexed quantification using TMT proteomics is advantageous in increasing the reproducibility, the TMT quantification technique is commonly/frequently known to cause ratio suppression effect thus leading to underestimation of the true differences in protein abundances between control and other sample groups. Some of the latest advancement including pre-fractionation of the samples and SPS-MS3 mass spectrometry analysis are known to circumvent this issue to a significant level (Ting et al., 2011).

TROUBLESHOOTING

Problem 1

Target protein does not IP: Target protein is not detected in the IP sample upon western blotting (step 4).

Potential solution

There can be a range of reasons this may occur. (1) The target protein is not expressed in the sample: test the presence of the protein in the lysate by western blot. (2) IP antibody did not conjugate to the beads (step 1): check the isotype of IP antibody is aligned with binding by Protein Sepharose A; (3) IP buffer is to not conducive to IP of the protein (step 3): In the first instance, reduce the stringency of the Protein Lysis Buffer and/or IP Low Salt Wash Buffer by reducing the salt concentrations. However often pilot studies are required to investigate the best buffer for the IP of a particular protein. For example, conditions involving IP of membrane bound proteins may require non-ionic detergents such as NP-40 or Triton-X100, whereas as IP of soluble protein typically do not. The major elements to test are the salt concentrations, detergent levels, and pH of the binding and wash buffers. (4) The IP antibody is not conducive to IP (steps 1 and 3): Not all antibodies are suitable for IP, e.g., some recognize antigens present under denaturing conditions only. It is recommended where possible to use antibodies previously shown to be compliant with IP. If pioneering an IP of protein, polyclonal antibodies typically fair better in IP compared to monoclonal antibodies.

Problem 2

Non-specific target protein enrichment: Target protein is being detected in the control IgG IP samples by western blot (step 4).

Potential solution

This can occur for a few different reasons (1) The target protein may be inherently 'sticky' and binds IgG non-specifically (step 3): Increase the stringency of the IP Binding Buffer increasing the amount of salt and/or detergent. (2) The target protein is binding non-specifically to the Protein Sepharose A beads (step 3). Increase the pre-clearing incubation time at step 3b and/or add a blocking step by incubating antibody conjugated Protein Sepharose A beads with lysis buffer containing 5% Bovine Serum Albumin for 2 h prior to incubating with lysate. If all fails, then the user can consider an alternative to Protein Sepharose A beads such as Dynabeads (Thermofisher).

Problem 3

Low protein yield during antibody pulldown: Protein yields in the pulldown are low and protein bands are not visible in the gel upon staining (step 5 and 6).

Potential solution

Increase the amount of protein in the lysate and/or the amount of antibody used in the IP (steps 1–3). A western-blot can be used to show depletion of the target protein in the flow through lysate. If the target is not significantly depleted by IP, then extra antibody can be used. If the antibody is highly-efficient for IP, then extra lysate can be used. Alternatively, conditions of the IP binding and washing buffers (antibody binding) may need empirical optimization to enhance the target protein-protein interactions. This typically will involve assaying the amount of salt, detergent and pH of the lysis buffer (step 3).

Problem 4

Poor TMT labeling efficiency (steps 6 and 7): Although the measures described in this protocol can assist in maximizing the labeling efficacy of peptides (typically over 99% of total peptides are labeled with TMT tag), occasionally, the process may yield lower labeling efficiency. This is generally caused by upstream inefficient digestion of the peptides (step 5), the presence of primary amines or incorrect pH of the labeling reaction (steps 5 and 6), or degradation of the TMT reagents (step 6).

Potential solution

Primary amine containing buffers must be avoided, and the use of HEPES or EPPS buffer with pH in the range of ~8.0–8.5 is strongly recommended (step 6). Avoid exposing tags to moisture, store the sealed bag of TMT reagents, along with desiccant in freezer below −20°C. Reconstitute the TMT label freshly using dry acetonitrile or ethanol.

Problem 5

Poor protein proteomic depth or low intensity of MS signals (steps 9 and 10). This could be stemming from several parameters, though if intensity of the MS1 signal is high (step 9), it is likely due to MS2 fragmentation settings (step 10). Column and trap degradation, or MS performance quality will also result in lower intensities and decreased protein identification.

Potential solution

As many factors could be responsible for this, the workflow should be thoroughly checked, including the LC-MS/MS analysis method, pattern of chromatographic separation of the peptides and protein FASTA database used for data search (steps 9 and 10). Check the LC-MS/MS method for potential issues, particularly around the optimization TMT reporter ion MS/MS fragmentation. Optimize the chromatographic gradient to maximize the identification of unique MS/MS of peptides. For both purposes, a standard, commercially available TMT labeled peptide mixture can be used (e.g., Pierce™ TMT11plex Yeast Digest Standard, Thermo Fisher) Catalogue number. Column and MS performance can be assessed by analysis of a known complex standard, and maintenance may be needed to improve results.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lachlan Jolly (lachlan.jolly@adelaide.edu.au).

Materials availability

Additional data and materials from this study are available from the authors on reasonable request, subject to compliance with our obligations under human research ethics.

Data and code availability

This protocol does not include the raw or processed proteomics data generated during this study, which is available from the authors on reasonable request, subject to compliance with our obligations under human research ethics. No novel code has been used in the study.

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AUTHOR CONTRIBUTIONS

R.K., K.S.K., and L.C. designed, implemented, and wrote the protocol. L.A.J. conceived, designed and supervised the study, and wrote, reviewed and edited the protocol. J.G. and P.H. reviewed and edited the protocol.

DECLARATION OF INTERESTS

The authors have no competing interests to declare.

REFERENCES

Bridges, C.R., Tan, M.C., Premarathne, S., Nanayakkara, D., Bellette, B., Zencak, D., Domingo, D., Gecz, J., Murtaza, M., Jolly, L.A., and Wood, S.A. (2017). USP9X deubiquitylating enzyme maintains RAPTOR protein levels, mTORC1 signalling and proliferation in neural progenitors. Sci. Rep. *7*, 391.

Chen, X., Sun, Y., Zhang, T., Shu, L., Roepstorff, P., and Yang, F. (2021). Quantitative proteomics using isobaric labeling: a practical guide. Dev. Reprod. Biol. *19*, 689–706.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. *26*, 1367–1372.

Di Palma, S., Hennrich, M.L., Heck, A.J.R., and Mohammed, S. (2012). Recent advances in peptide separation by multidimensional liquid chromatography for proteome analysis. J. Proteomics *75*, 3791–3813.

Homan, C.C., Kumar, R., Nguyen, L.S., Haan, E., Raymond, F.L., Abidi, F., Raynaud, M., Schwartz, C.E., Wood, S.A., Gecz, J., and Jolly, L.A. (2014). Mutations in USP9X are associated with X-linked intellectual disability and disrupt neuronal cell migration and growth. Am. J. Hum. Genet. *94*, 470–478.

Johnson, B.V., Kumar, R., Oishi, S., Alexander, S., Kasherman, M., Vega, M.S., Ivancevic, A., Gardner, A., Domingo, D., Corbett, M., et al. (2020). Partial loss of USP9X function leads to a male neurodevelopmental and behavioral disorder converging on transforming growth factor beta signaling. Biol. Psychiatry *87*, 100–112.

Kong, A.T., Leprevost, F.V., Avtonomov, D.M., Mellacheruvu, D., and Nesvizhskii, A.I. (2017). MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. Nat. Methods *14*, 513–520.

Ludwig, C., Gillet, L., Rosenberger, G., Amon, S., Collins, B.C., and Aebersold, R. (2018). Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. Mol. Syst. Biol. *14*, e8126.

Ordureau, A., Paulo, J.A., Zhang, J., An, H., Swatek, K.N., Cannon, J.R., Wan, Q., Komander, D., and Harper, J.W. (2020). Global landscape and dynamics of parkin and USP30-dependent ubiquitylomes in iNeurons during mitophagic signaling. Mol. Cell *77*, 1124–1142.e10.

Orsburn, B.C. (2021). Proteome discoverer-A community enhanced data processing suite for protein informatics. Proteomes *9*, 15.

Pederick, D.T., Richards, K.L., Piltz, S.G., Kumar, R., Mincheva-Tasheva, S., Mandelstam, S.A., Dale, R.C., Scheffer, I.E., Gecz, J., Petrou, S., et al. (2018). Abnormal cell sorting underlies the unique X-linked inheritance of PCDH19 epilepsy. Neuron *97*, 59–66.e5.

Pham, D.H., Tan, C.C., Homan, C.C., Kolc, K.L., Corbett, M.A., McAninch, D., Fox, A.H., Thomas, P.Q., Kumar, R., and Gecz, J. (2017). Protocadherin 19 (PCDH19) interacts with paraspeckle protein NONO to co-regulate gene expression with estrogen receptor alpha (ERalpha). Hum. Mol. Genet. *26*, 2042–2052.

Premarathne, S., Murtaza, M., Matigian, N., Jolly, L.A., and Wood, S.A. (2017). Loss of Usp9x disrupts cell adhesion, and components of the Wnt and Notch signaling pathways in neural progenitors. Sci. Rep. *7*, 8109.

Qin, W., Cho, K.F., Cavanagh, P.E., and Ting, A.Y. (2021). Deciphering molecular interactions by proximity labeling. Nat. Methods *18*, 133–143.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. *2*, 1896–1906.

Rose, C.M., Isasa, M., Ordureau, A., Prado, M.A., Beausoleil, S.A., Jedrychowski, M.P., Finley, D.J., Harper, J.W., and Gygi, S.P. (2016). Highly multiplexed quantitative mass spectrometry analysis of ubiquitylomes. Cell Syst. *3*, 395–403.e4.

Ting, L., Rad, R., Gygi, S.P., and Haas, W. (2011). MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods *8*, 937–940.

Tyanova, S., Temu, T., and Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat. Protoc. *11*, 2301–2319.

Yu, S.H., Kyriakidou, P., and Cox, J. (2020). Isobaric matching between runs and novel PSM-level normalization in MaxQuant strongly improve reporter ion-based quantification. J. Proteome Res. *19*, 3945–3954.