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Protocol for mapping differential protein-protein interaction networks using affinity purification-mass spectrometry

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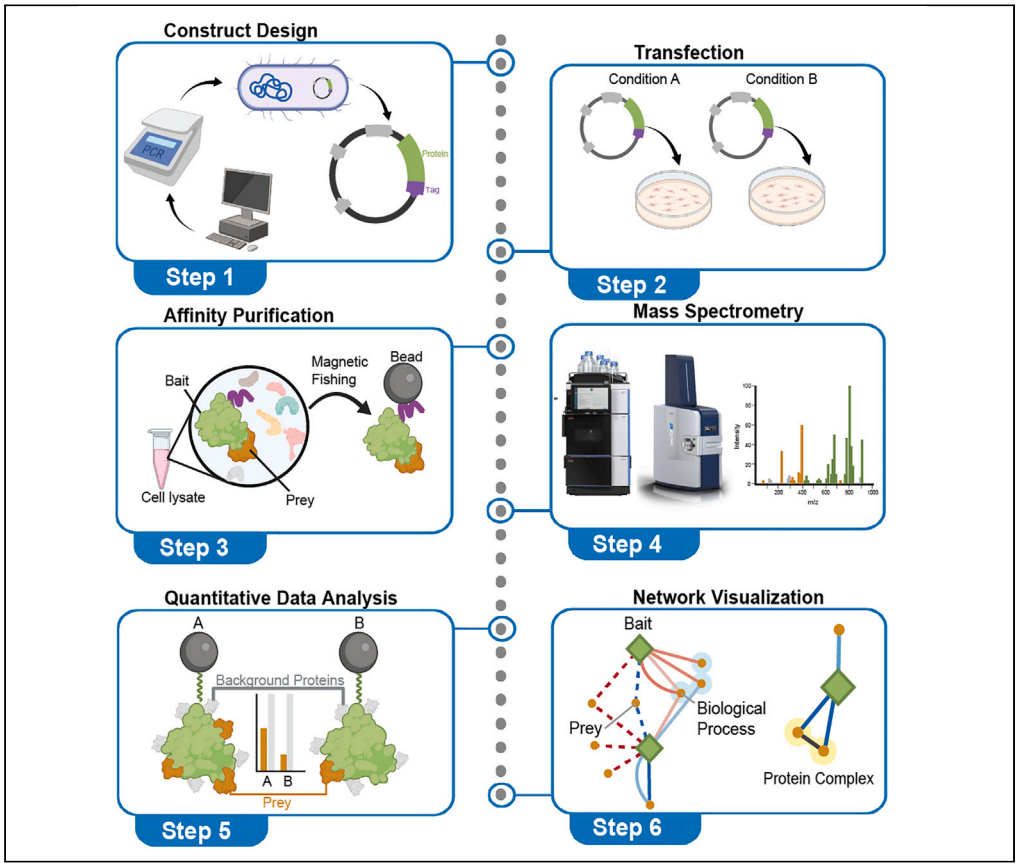

Proteins congregate into complexes to perform diverse cellular functions. Protein complexes are remodeled by protein-coding mutations or cellular signaling changes, driving phenotypic outcomes in health and disease. We present an affinity purification-mass spectrometry (APMS) proteomics protocol to express affinity-tagged "bait" proteins in mammalian cells, identify and quantify purified protein interactors, and visualize differential protein-protein interaction networks between pairwise conditions. Our protocol possesses general applicability to various cell types and biological areas.

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

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Highlights

Steps for construct design, transfection, APMS, and data quality control

Approach for protein-protein interaction (PPI) scoring to remove background

Cross-run normalization and statistical comparison between pairwise conditions

Visualization of quantitative changes in PPI networks

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Protocol for mapping differential protein-protein interaction networks using affinity purification-mass spectrometry

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SUMMARY

Proteins congregate into complexes to perform diverse cellular functions. Protein complexes are remodeled by protein-coding mutations or cellular signaling changes, driving phenotypic outcomes in health and disease. We present an affinity purification-mass spectrometry (AP-MS) proteomics protocol to express affinity-tagged "bait" proteins in mammalian cells, identify and quantify purified protein interactors, and visualize differential protein-protein interaction networks between pairwise conditions. Our protocol possesses general applicability to various cell types and biological areas.

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

BEFORE YOU BEGIN

The protocol described in this paper has been previously employed to define changes in virus-host PPI networks between SARS-CoV-2 variants of concern (VOC) and their corresponding wave one (W1) viral protein forms using affinity purification-mass spectrometry (AP-MS) analysis in HEK293T cells. This approach can also be adapted to other cell types as long as target cells enable the introduction of (i.e., transfection or transduction), and protein expression from, a DNA construct.

Institutional permissions

Timing: variable

Researchers should obtain permission from the relevant institutions before conducting any BSL-2 level research with recombinant nucleic acid constructs and mammalian cell lines.

Define groups for quantitative comparisons

Timing: variable

1. Begin by defining two or more experimental conditions for comparison.

   Note: Our study compared SARS-CoV-2 W1 viral protein forms to their corresponding mutated variant of concern (VOC) forms. One may also choose to compare the same construct in the context of different cellular perturbations (e.g., drug administration), which may also regulate PPIs.

2. Define the negative controls that are relevant to your study.

   Note: These typically include green fluorescence protein (GFP) and empty vector (EV) constructs.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
SARS-CoV-2 VOC viral protein plasmids	Bouhaddou et al.[1]	N/A
Stellar competent cells	Takara	Cat#636766
Antibodies		
Mouse monoclonal anti-Strep Tag antibody (used at 1:1,000)	QIAGEN	Cat#34850; RRID:AB_2810987
Experimental models: Cell lines		
HEK293T cells	ATCC	Cat#CRL-3216
Chemicals, peptides, and recombinant proteins		
PolyJet DNA *in vitro* transfection reagent	SignaGen	Cat#SL100688
S.O.C. medium	Invitrogen	Cat#15544034
LB broth (powder)	Fisher	Cat#BP1427
NucleoBond Xtra Midi kit	Macherey-Nagel	Cat#740410.50
Dulbecco's modified Eagle's medium (DMEM)	Corning	Cat#10-013-CV
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
Fetal bovine serum (FBS)	Gibco	Cat#16140071
Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium	Gibco	Cat#14190144
EDTA (0.5 M), pH 8.0, RNase-free	Invitrogen	Cat#AM9260G
NP-40 Surfact-Amps detergent solution	Thermo Scientific	Cat#85124
cOmplete, mini, EDTA-free protease inhibitor cocktail	Roche	Cat#11836170001
PhosSTOP phosphatase inhibitor tablets	Roche	Cat#4906845001
Bovine serum albumin standard pre-diluted set	Thermo Scientific	Cat#23208
Tris-HCl, 1 M buffer solution, pH 7.4	Thermo Scientific	Cat#J60202.K2
Tris-HCl, 1 M buffer solution, pH 8.0	Thermo Scientific	Cat# BP1758
NaCl (5 M), RNase-free	Invitrogen	Cat#AM9759
Urea	Sigma-Aldrich	Cat#U5128
Dithiothreitol (DTT) solution, BioUltra, ~1 M in water	Sigma-Aldrich	Cat#43816
Iodoacetamide (IAA)	Sigma-Aldrich	Cat#I6125
Sequencing-grade modified trypsin	Promega	Cat#V5111
Trifluoroacetic acid (TFA), LC-MS grade	Thermo Scientific	Cat#85183
Formic acid (FA), LC-MS grade	Fisher Scientific	Cat#PI85178
Sep-Pak C18 1 cc vac cartridge (50 mg sorbent per cartridge)	Waters	Cat#WAT054955
Water, Optima LC/MS grade	Fisher Scientific	Cat#W64
Acetonitrile, Optima LC/MS grade	Fisher Scientific	Cat#A955
Water with 0.1% formic acid (v/v), Optima LC/MS grade	Thermo Scientific	Cat#LS118

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
80% Acetonitrile, 20% water with 0.1% formic acid (v/v), Optima LC/MS grade	Thermo Scientific	Cat#LS122500
MagStrep "type3" XT beads	IBA Lifesciences	Cat#2-4090-010
Buffer BXT (10×)	IBA Lifesciences	Cat#2-1042-025
6× Laemmli sample buffer	Thermo Scientific	Cat#AAJ61337AC
Critical commercial assays		
Bradford protein assay kit	Thermo Scientific	Cat#23200
Software and algorithms		
SnapGene® software	Dotmatics	https://www.snapgene.com/
MaxQuant	Cox and Mann[2]	https://www.maxquant.org/
R statistical programming language	R CRAN	https://www.r-project.org/
RStudio IDE	Posit	https://posit.co
MSstats	Choi et al.[3]	https://bioconductor.org/packages/release/bioc/html/MSstats.html
artMS	Jimenez-Morales et al.[4]	http://bioconductor.org/packages/artMS/
Cytoscape	Shannon et al.[5]	https://cytoscape.org/
Spectronaut (Biognosys)	Bruderer et al.[6]	https://biognosys.com/software/spectronaut/
DIA-NN	Demichev et al.[7]	https://github.com/vdemichev/DiaNN
SAINTexpress	Teo et al.[8]	https://saint-apms.sourceforge.net/Main.html
MiST	Jäger et al.[9]	https://github.com/kroganlab/mist
CompPASS	Sowa et al.[10]	https://bioplex.hms.harvard.edu/comppass/
clusterProfiler	Wu et al.[11]	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
Adobe Illustrator	Adobe, Inc.	https://adobe.com/products/illustrator
Deposited data		
AP-MS proteomics data	Bouhaddou et al.[1]	PRIDE : PXD036798
Other		
Round bottom polystyrene test tube	Corning	Cat#352001
Tissue culture dishes (15 cm)	Fisher Scientific	Cat#FB012924
Falcon 15 mL conical centrifuge tubes	Fisher Scientific	Cat#14-959-53A
Protein LoBind tubes (1.5 mL)	Eppendorf	Cat#022431081
Digital dry bath/heating block	Fisher Scientific	Cat#88-870-001
Paper pH strips	Fisher Scientific	Cat#13-640-508
ThermoMixer C	Eppendorf	Cat#2231001005
ThermoTop	Eppendorf	Cat#2231001048
DynaMag-2 magnet	Invitrogen	Cat#12321D
NanoDrop One spectrophotometer	Thermo Scientific	Cat#ND-ONE-W
Vacuum concentrator	Labconco	Cat#7315021
Solid phase extraction vacuum manifold	Biotage	Cat#121–2015ML
Laser-based micropipette puller	Sutter Instrument	Cat#P-2000
ReproSil-Pur 120 C18Aq media by Dr. Maisch GmbH	ESI Source Solutions	Cat#r119.aq.
Pressure injection cell	Next Advance	Cat#PC77
EASY-nLC 1200 system	Thermo Scientific	Cat# LC140
Orbitrap Exploris 480 mass spectrometer	Thermo Scientific	Cat#BRE725539

MATERIALS AND EQUIPMENT

Note: Always use the highest available quality reagents that are annotated as mass spectrometry grade.

IP Buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA)

Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.4)	50 mM	5 mL
NaCl (5 M)	150 mM	3 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
EDTA (0.5 M)	1 mM	200 μL
Water	N/A	91.8 mL
Total	**N/A**	**100 mL**

Note: Store at 4°C for up to one month.

IP Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1× protease/phosphatase inhibitors)		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.4)	50 mM	500 μL
NaCl (5 M)	150 mM	300 μL
EDTA (0.5 M)	1 mM	20 μL
NP-40 (10% in water)	0.5%	500 μL
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail tablet	1×	1 tablet/10 mL
PhosSTOP Phosphatase inhibitor tablet	1×	1 tablet/10 mL
Water	N/A	~8.5 mL
Total	**N/A**	**10 mL**

Note: Always prepare fresh. Use immediately and discard the unused buffer.

IP Wash Buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.05% NP-40)		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.4)	50 mM	500 μL
NaCl (5 M)	150 mM	300 μL
EDTA (0.5 M)	1 mM	20 μL
NP-40 (10% in water)	0.05%	50 μL
Water	N/A	9.13 mL
Total	**N/A**	**10 mL**

Note: Store at 4°C for up to one month.

DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin mix		
Reagent	Final concentration	Amount
DMEM	N/A	445 mL
FBS	10%	50 mL
Penicillin-Streptomycin mix (10,000 U/mL)	100 U/mL	5 mL
Total	**N/A**	**500 mL**

Note: Store at 4°C for one to two months. Check for mycoplasma contamination regularly.

Denaturation-Reduction Buffer (2 M Urea, 1 mM DTT, 50 mM Tris-HCl)		
Reagent	Final concentration	Amount
Urea	2 M	0.12 g
Dithiothreitol (DTT, ~1 M in water)	1 mM	1 μL
Tris-HCl (1 M, pH 8.0)	50 mM	50 μL
Water	N/A	~945 μL
Total	**N/A**	**1 mL**

Note: Always prepare fresh. Use immediately and discard the unused buffer.

Alkylation solution (0.1 M IAA in water)		
Reagent	Final concentration	Amount
Iodoacetamide (IAA)	0.1 M	18.5 mg
Water	N/A	~970 µL
Total	**N/A**	**1 mL**

⚠ CRITICAL: Always prepare fresh and protect from light. Use immediately and discard the unused solution.

Trypsin solution (0.5 µg/µL in 50 mM acetic acid)		
Reagent	Final concentration	Amount
Sequencing grade modified Trypsin	0.5 µg/µL	20 µg
Acetic acid (50 mM)	50 mM	40 µL
Total	**N/A**	**40 µL**

Note: Prepare aliquots for one-time use and store at −80°C for several months. Avoid multiple freeze-thaw cycles.

C18 activation solution (80% ACN, 0.1% TFA in water)		
Reagent	Final concentration	Amount
Acetonitrile	80.0%	8.0 mL
Water	19.9%	1.99 mL
Trifluoroacetic acid (100%)	0.1%	10 µL
Total	**N/A**	**10 mL**

Note: This solution can be stored at RT for several months.

C18 equilibration/wash solution (0.1% TFA in water)		
Reagent	Final concentration	Amount
Water	99.9%	9.99 mL
Trifluoroacetic acid (100%)	0.1%	10 µL
Total	**N/A**	**10 mL**

Note: This solution can be stored at RT for several months.

C18 elution solution (50% ACN, 0.25% FA in water)		
Reagent	Final concentration	Amount
Acetonitrile	50.0%	5.0 mL
Water	49.75%	4.975 mL
Formic acid (100%)	0.25%	25 µL
Total	**N/A**	**10 mL**

Note: This solution can be stored at RT for several months.

Table 1. Amino acid sequence of 2x-Strep tag for insertion at N- and C-terminus of protein

2x-Strep Tag location	Amino acid Sequence
N-term	MWSHPQFEKGGGSGGGSGGGSWSHPQFEKGGGGS
C-term	GGGGSWSHPQFEKGGGSGGGSGGGSWSHPQFEK[a]

[a]indicates a stop codon.

STEP-BY-STEP METHOD DETAILS

Plasmid generation

Timing: Between 1 week and 1 month

This section describes steps to generate a plasmid construct with a Strep affinity tag. We describe how to design the construct and how to use bacterial cells to transform, grow, and purify them.

1. Obtain protein sequences of interest in a donor DNA plasmid.

Note: In our study,[1] we performed site-directed mutagenesis on wave one (W1) SARS-CoV-2 gene plasmids to generate variant isoforms (GenScript Biotech). For example, we mutated the SARS-CoV-2 Orf6 aspartic acid (D) at position 61 to a leucine (L), a mutation present within the Omicron BA.2 and BA.4 variants. Quantitative comparison of the PPI network between this mutant and the W1 (non-mutated) form revealed disruption of the Orf6-nuclear pore interaction,[1] which is known to be important for Orf6-mediated innate immune inhibition.

Note: This approach is not limited to comparing changes in amino acid sequences but is amenable to the comparison of other conditions, such as cellular perturbations (e.g., drug treatment). In principle, this approach is used when minor changes in PPIs are expected, which is the case given small changes in protein sequence[12] or when analyzing the same protein under different conditions, such that one would expect quantitative (i.e., not binary) changes in PPIs to occur.

Note: We recommend that sequences are codon-optimized for expression in mammalian cells using either IDT codon-optimization (https://www.idtdna.com/codonopt) or GenScript Biotech tools. The degree of codon optimization is highly construct-specific and is not always required for efficient protein expression. However, we have often noticed improved protein expression following codon optimization and recommend it whenever possible.

2. Insert two copies of the Strep tag (2×-Strep; Table 1) at either the N- or C-terminus of the protein into the plasmid.
   a. Insert short linker sequences, containing glycines (G) and serines (S), between the 2×Strep and the protein as well as between each Strep tag.

Note: We recommend starting by adding the tag on the C-terminal end; however, if the expression is not adequate as determined by Western blotting (evaluated below), we then attempt an N-terminal tag. See the notes below for a discussion on alternative affinity tags and tag location selection strategies.

Note: Other affinity tags, including 3x-Flag[13] and 3xHA,[14] may also be used. Tag selection should be optimized based on the protein of interest; some tags may work better than others for different proteins and cellular contexts. Another idea to consider is including an internal tag within the protein sequence (as opposed to N- or C-term), which may have fewer adverse effects on protein function. However, the efficacy of these strategies, involving an assessment of how introduction of the tag impacts protein folding and function, must be evaluated on a case-by-case basis.

Note: To guide our tagging strategy, we used GPS-Lipid to predict protein lipid modification of the termini (http://lipid.biocuckoo.org/webserver.php), DeepTMHMM v.1. to predict transmembrane/hydrophobic regions (https://services.healthtech.dtu.dk/services/DeepTMHMM-1.0/), and SignalP v. 6.0 to predict signal peptides (https://services.healthtech.dtu.dk/services/SignalP-6.0/). N-terminal signal peptides are often cleaved, precluding an N-terminal tag, which would be lost. The proximity of tags to lipid-modified residues and/or transmembrane domains may affect proper protein function or localization and thus should be avoided.

3. Clone the DNA sequence containing the protein of interest conjugated to an affinity tag into a plasmid with high expression in target cells.

Note: In our study,[1] we cloned our DNA sequences into the lentiviral constitutive expression vector pLVX-EF1alpha-IRES-Puro (Takara Bio), driving constitutive protein expression under an eIF1α promoter in mammalian cells. Many alternative expression vectors exist and can be substituted here, even those with inducible expression (e.g., pLVX-TetOne-IRES-Puro).

a. Perform transformation using Stellar competent *E. coli* cells.
   i. Thaw Stellar competent cells on ice and add 50 μL of homogenized cell suspension to a new 1.5 mL Eppendorf tube. Do not vortex.
   ii. Add no more than 5 ng of DNA and place the tubes on ice for 30 min.
   iii. Heat shock the cells for 45 s at 42°C.
   iv. Place tubes back on ice for 1–2 min.
   v. Add S.O.C medium (thawed at RT) to reach a final volume of 500 μL.
   vi. Incubate the cells on a shaker for 1 h at 200 rpm.
   vii. Streak 100 μL of transformed cells onto a 10 cm agar plate.
   viii. Grow at 37°C for 24–28 h.

Note: Different bacteria and/or constructs may require a lower temperature, such as 30°C. Consult the manufacturer's instructions.

b. Pick individual colonies and confirm transformation efficacy via plasmid sequencing. Include the donor DNA plasmid as well to verify the absence of erroneous mutations in the donor plasmid and unwarranted mutations introduced during cloning.

Note: To confirm transformation efficiency, compare the sequencing results (receiver plasmid) with the expected sequence (donor plasmid) using a tool such as SnapGene Viewer. If there are any mutations, we suggest re-performing the transformation and/or trying different bacteria or culture temperatures.

c. Grow transformed bacterial cells in 10 mL sterile Lennox formulation (LB) broth containing 100 μg/mL ampicillin or other antibiotic (corresponding to the encoded resistance gene) for 16 h at 37°C with shaking.

Note: Scale up the culture volume according to the amount of plasmid required.

Note: Optimal shaking speed and temperature are construct-specific; thus, various conditions should be evaluated. Previously, we have shaken at speeds ranging from 140 rpm to 230 rpm, depending on the specific constructs, at 30°C–37°C.

d. Perform plasmid purification.

Note: We use the NucleoBond Xtra Midi kit (Macherey-Nagel) and recover ~1 mg of plasmid per construct from 200-250 mL of culture medium.

i. Centrifuge the overnight culture and pellet the bacterial cells.
ii. Add 8 mL of resuspension buffer and thoroughly resuspend the pelleted cells.
iii. Add 8 mL of lysis buffer and gently invert five times.
iv. Incubate for 5 min at RT.
v. Equilibrate the column with 12 mL of equilibration buffer.
vi. Allow the column to empty using gravity flow.
vii. Add 8 mL of neutralization buffer to the lysate. Gently invert until the lysate turns completely colorless.
viii. Add lysate to the equilibrated column.
ix. Allow the column to empty by gravity flow.
x. Wash the column with 5 mL of equilibration buffer. Discard the filter.
xi. Wash with 8 mL of wash buffer.
xii. Place a suitable elution tube under the column to collect the flowthrough.
xiii. Elute with 5 mL of elution buffer.
xiv. Desalt with 70% isopropanol and air dry.

⚠ CRITICAL: When considering experimental design, it is important to include both a tagged GFP construct and an empty vector (no protein or tag) construct as negative controls. These controls are important when performing the SAINT PPI scoring following mass spectrometry analysis (see below). Clone these into the same backbone expression plasmid as your proteins of interest, which is important to properly capture construct-specific background proteins. Briefly, SAINT[15] analysis is a PPI scoring algorithm that statistically assesses the abundance of each prey in the experimental condition relative to background levels in the negative controls (i.e., empty vector and GFP). For more information, see the section scoring protein-protein interactions (Step 29).

Transfection of affinity-tagged genes

Timing: 1 week

This section describes steps to transfect the Strep tag containing plasmids into HEK293T cells.

4. Confirm the expression of tagged protein and determine the quantity of DNA needed for optimal expression of each protein. Each construct may require a different amount of DNA input to achieve adequate protein expression levels due to differences in translation efficiency and protein stability. To determine the optimal quantity of DNA for each construct,
   a. Transfect HEK293T cells at 80% confluency in a 24-well format with 0.4 μg DNA per construct, including GFP and empty vector (EV) controls.
   b. Determine the total amount of plasmid DNA needed to see greater than 80% of cells expressing GFP.
   c. Perform a Western blot with a mouse monoclonal anti-Strep tag antibody (1:1000 dilution).

Note: It is important to notice a clearly demarcated band at the appropriate molecular weight (see Figure 1 for an example of clearly demarcated bands) for each protein of interest. Use GFP as a positive expression control.

Note: Low protein expression due to insufficient transfection efficiency, low construct expression, or high rates of protein degradation will hinder the recovery of interacting proteins. Optimize DNA quantity to maximize protein expression in the absence of overt cytotoxicity.

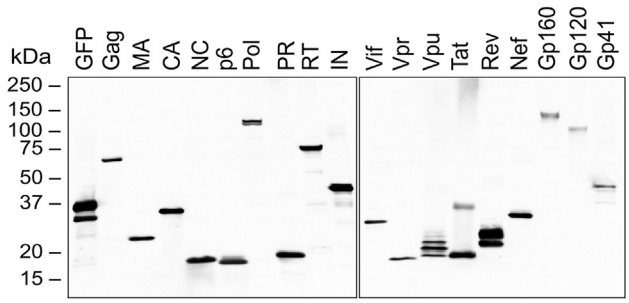

Figure 1. Western blot analysis of 18 HIV proteins.
Anti-FLAG Western blot analysis of cell lysates of 18 HIV-SF proteins after being transiently transfected into HEK293 cells. Figure is reproduced from Jäger et al.[9]

d. Adjust DNA input depending on Western blot results, ideally remaining between 0.1 and 0.4 μg DNA per well in a 24-well format. This will enable proper conversion of quantities to the 15-cm dish format.
e. Following optimization, scale the amount of input DNA by the number of cells from a 24-well format to a 15-cm dish.

Note: GFP usually has the highest expression in HEK293T cells, and only 1.0–2.0 μg of plasmid is transfected in a 15-cm dish. Empty vector plasmid should be added such that the total transfected plasmid per dish equals 15 μg (see Step 6). Up to 15 μg of your construct can be transfected for poorly expressed proteins (i.e., no empty vector). We recommend further optimizing the amount of construct needed for each condition in a 15-cm format, varying inputs in 2.5 μg increments until a clearly demarcated band at the appropriate molecular weight is achieved via western blot (see Figure 1).

Note: Below, we describe how to perform cross-run normalization of prey intensities, which is critical for label-free AP-MS proteomics approaches. However, working to equalize bait expression between conditions is essential to achieve a reliable comparison between conditions. When comparing two baits, we recommend their expression falls within 2-fold as assessed by densitometry measurements; if outside of this range, we recommend optimizing the transfection conditions (i.e., increasing/decreasing DNA concentration) to further equalize expression. Densitometry measurements can be performed using the freely available ImageJ software as well as several other commercially available software.

5. Once the optimal DNA quantity has been determined for each construct, seed 5–10 million HEK293T cells in 30 mL DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a 15-cm cell culture plate.

Note: To perform differential statistical analysis (below), it is important to include three separate replicates per experimental condition.

⚠ CRITICAL: Allow cells to reach approximately 50–70% confluence before proceeding with transfections, which typically occurs within 16–24 h of cell seeding. Transfecting too few cells can lead to excessive cell death and/or insufficient protein recovery.

6. Sixteen to 24 h after seeding (or once cells have reached 50–70% confluence), perform DNA transfection.
a. Prepare Tube A. Combine plasmid DNA with serum-free DMEM.

Table 2. Volumes of PolyJet and DMEM to create a master mix (Tube B) for 15 μg DNA

	Single plate	For three plates	Total + ~10% error
PolyJet (μL)	45	135	148
DMEM (μL)	455	1365	1502
Total (μL)	500	1500	1650

Note: When performing multiple transfections, create a master mix of Tube B in a larger tube (e.g., 15 mL Falcon tube).

i. Aliquot each DNA plasmid in separate 2 mL tubes. Use a separate tube for each replicate. Use 15 μg total plasmid per 15-cm dish. Determine the mass of plasmid DNA required for optimal expression (above), then add empty vector DNA (i.e., backbone construct) to a total of 15 μg DNA (gene + empty vector = 15 μg total).
ii. Add DMEM media to each tube with aliquoted DNA to a total of 500 μL.
iii. Vortex each tube briefly to mix.

b. Prepare Tube B. Prepare mastermix of PolyJet transfection reagent with serum-free DMEM.
i. Vortex PolyJet transfection reagent well before use.
ii. Aliquot PolyJet into a tube large enough to fit 500 μL multiplied by the number of total samples (i.e., replicates) as a single mastermix will be used for all samples. Use 3 μL of PolyJet transfection reagent per 1 μg of plasmid (e.g., for 15 μg of plasmid, use 45 μL of PolyJet).
iii. Add serum-free DMEM to PolyJet to a total of 500 μL per sample. Scale PolyJet and serum-free DMEM mix accordingly (Table 2).
iv. Vortex the tube briefly to mix.

c. Add 500 μL of Tube B (PolyJet/DMEM) to each Tube A (DNA/DMEM), resulting in a total volume of 1 mL per tube. Invert each tube 3–4 times to mix gently. Incubate for 15 min at room temperature (RT, 21°C–23°C) to allow PolyJet-DNA complexes to form.

d. Following incubation, carefully add transfection complexes dropwise to cells. Mix well by carefully tilting dishes back-and-forth and side-to-side several times.

e. Incubate cells for 48 h post transfection at 37°C/5% CO_2 prior to harvesting for affinity purification.

Note: Alternative transfection reagents, such as Lipofectamine 3000 (Thermo Scientific) or Polyethylenimine "Max" (PEI MAX) (Polysciences), can also be used. We have found that PolyJet provides efficient transfection and adequate protein expression with our SARS-CoV-2 constructs in HEK293T cells and is a cost-effective solution for large-scale experiments. We generally observe more than 80% transfection efficiency (checked with flow cytometry) using eGFP as a measure. Alternative transfection reagents may be more effective for other cell types and plasmids. Transfection conditions should be optimized on a case-by-case basis.

Cell harvest

Timing: 30 min to 4 h

This section outlines the cell harvest method after transfection.

Note: The cell harvest procedure described was developed for HEK293T cells, which detach from the cell culture dish in 10 mM EDTA. Cell harvest may need to be performed differently for different cell types. We recommend avoiding the use of trypsin to detach cells from the culture plate since this will result in the loss of the peptides from proteins on the cell surface. If using adherent cells that do not detach under 10 mM EDTA conditions, consider adding the lysis buffer directly to the dish and scraping the cells before transferring them to a cold 1.5 mL tube.

7. Carefully aspirate the supernatant from 15-cm dishes using a vacuum line fitted with a P200 pipette tip. Add 10 mL Dulbecco's phosphate-buffered saline (DPBS), without calcium and magnesium, to each plate, supplemented with 10 mM EDTA.
8. Incubate dishes for 5–10 min at RT until cells mostly detach from the monolayer. Gently shake and tap the side of the dish to detach any remaining cells.
9. Transfer each plate of detached cells to individual cold 15 mL Falcon tubes on ice. Store the cell suspension on ice until the final step of the harvest. Centrifuge the cell suspension at 400 × *g* for 5 min at 4°C. Carefully aspirate the supernatant using a vacuum line fitted with a P200 pipette tip.
10. Wash the cells by gently resuspending the cell pellet in 10 mL DPBS. Centrifuge the cell suspension at 400 × *g* for 5 min at 4°C. Carefully aspirate the supernatant using a vacuum line fitted with a P200 pipette tip. Repeat the wash step once more.
11. Using a P1000 pipet tip, resuspend the cell pellet in 1 mL ice-cold DPBS and transfer cell suspension to a cold 1.5 mL protein LoBind tube on ice. Centrifuge at 400 × *g* for 5 min at 4°C in a microcentrifuge. Carefully aspirate the supernatant using a vacuum fitted with a P200 pipette tip. Ensure that all DPBS is removed from the surface of the cell pellet.

⚠ CRITICAL: Be careful to not accidentally aspirate the cell pellet. Fitting a P200 pipette at the end of the vacuum line should enhance precision during DPBS aspiration.

12. Snap freeze cell pellets immediately on dry ice or liquid nitrogen and store at −80°C until ready to proceed to subsequent steps.

Note: Once snap frozen on dry ice and placed at −80°C, cell pellets can be stored for several months before subsequent steps without substantial loss in protein integrity.

Strep tag affinity purification

Timing: 2 days

This section describes the procedure for the affinity purification of the Strep tag containing proteins of interest from the cell lysate and sample preparation for further mass spectrometry analysis.

13. Thaw frozen cell pellets on ice for 15–20 min and resuspend in 1 mL IP Lysis Buffer (IP Buffer supplemented with 0.5% NP-40 and 1× protease/phosphatase inhibitors).

Note: A freeze-thaw cycle can be added here in order to improve lysis efficiency. Lysates should be frozen on dry ice for at least 10 min (up to 12–16 h) and then thawed on ice for 15–20 min. Multiple freeze-thaw cycles can be implemented to improve lysis efficiency further, up to a maximum of three cycles. However, performing too many additional freeze-thaw cycles may also increase protein degradation and reduce protein recovery.

14. Centrifuge at 13,000 × *g* for 15 min at 4°C to clarify the lysate and pellet debris. Protein will remain in the supernatant.

Optional: To save samples for Western blot analysis, aliquot 50 µL of clarified lysate and dilute with 4–6× Laemmli Sample Buffer (SB). Store in PCR strip tubes or 1.5 mL protein LoBind tubes and proceed to Western blot analysis. Optionally, freeze lysates in the Laemmli SB for several weeks at −80°C until ready to run the Western blot.

15. Prepare MagStrep "type3" XT beads on ice.
    a. Pipet beads up and down using a wide-bore pipet tip to resuspend stock bead slurry.
    b. Prepare one 1.5 mL protein LoBind tube per sample.

c. Aliquot 30 μL slurry into each tube.

Note: Beads settle down quickly, resulting in unwanted sample-to-sample variability. Pipetting up and down to mix the slurry after every few samples ensures consistent aliquoting of the beads across the samples.

d. Wash MagStrep "type3" XT beads 2-times with 1 mL IP Wash Buffer (IP Buffer with 0.05% NP-40) using a magnetic rack.
   i. Place beads on a magnetic rack and remove the solution, leaving the beads behind.
   ii. Add 1 mL IP Wash Buffer, remove tubes from the magnetic rack, and vortex briefly to mix.
   iii. Place tubes back on the magnetic rack, remove the solution, leaving beads behind, and continue to the next wash.
e. After the second wash, resuspend beads in 0.3 mL IP Buffer.

16. Add the remaining protein lysate (1 mL minus any put aside for Western blot) to the beads and incubate for 2 h at 4°C on an end-over-end tube rotator.
17. Wash beads to reduce non-specifically bound proteins to the beads.
    a. Centrifuge at 600 × *g* for 30 s to pellet the beads.
    b. Place tubes on the magnet and discard the supernatant.
    c. Resuspend the beads in 1 mL of IP Wash Buffer.
    d. Rotate the tubes on an end-over-end tube rotator for 5 min at 4°C.
    e. Repeat Steps 17a-d two additional times.
    f. Resuspend beads in 1 mL of IP Buffer.

Optional: Protein can also be eluted from the beads to check the expression of the target Strep tagged protein and any interactors by performing a silver stain following SDS-PAGE. To do this,

    g. Move 200 μL (20%) of mixed bead slurry to a new 1.5 mL protein LoBind tube.
    h. Collect beads on the magnetic rack and discard the solution, leaving beads behind.
    i. Add 30 μL of 1× Buffer BXT (dilute 10× Buffer BXT 1:10 with water) and gently agitate on an electronic shaker (e.g., Eppendorf ThermoMixer C) for 30 min at RT.
    j. Collect beads on the magnetic rack and transfer eluates to a fresh 0.5–1.5 mL protein LoBind tube.
    k. Add Laemmli Sample Buffer and proceed with SDS-PAGE and silver stain. To perform the silver stain, follow the protocol described by Chevallet et al.[16]

Pause point: Eluates can be stored in Laemmli Sample Buffer for several weeks at −80°C.

18. Perform an "on-bead digestion," of proteins bound to the magnetic beads,
    a. Briefly collect the remaining beads on the magnetic rack and discard the solution, leaving beads behind.
    b. Add 50 μL Denaturation-Reduction Buffer and incubate for 30 min at 37°C, 1100 rpm, on an electronic mixer (e.g., Eppendorf ThermoMixer C with a heated lid like ThermoTop).
    c. Add 1.5 μL of alkylation solution (final concentration of IAA is 3 mM) and incubate for 45 min at RT, 1100 rpm on the electronic mixer.

⚠ CRITICAL: Protect samples from light during Step 18c by covering them with aluminum foil.

    d. Add 1.6 μL of 0.1 M DTT (final concentration is 3 mM) and incubate for 10 min at RT, 1100 rpm on the electronic mixer.
    e. Add 15 μL of 50 mM Tris-HCl (pH 8.0) to offset evaporation to each sample. Skip this step if using a heated lid, which should prevent condensation.

f. Add 1.5 μL of sequencing grade trypsin solution (0.5 μg/μL) and incubate for 4–6 h at 37°C, 1100 rpm on the electronic mixer.
g. Add an additional 0.5 μL of stock trypsin solution and incubate at 37°C for 1–2 h at 1100 rpm on the electronic mixer.
h. Briefly collect beads on the magnetic rack and transfer the digest in the supernatant to a new protein LoBind tube.
i. Resuspend the beads in 50 μL of 50 mM Tris-HCl (pH 8.0). Centrifuge and pool with the supernatant from the previous step.

Note: A KingFisher Flex (KFF) purification system can automate the protocol steps. For affinity purification, place KFF in a cold room and allow it to equilibrate to 4°C 12–16 h before use. Use a slow mix speed and the following mix times: 30 s for equilibration and wash steps, 2 h for binding, and 1 min for final bead release. Use three 10 s bead collection times between all steps. A KFF protocol file for Strep tag affinity purification in pdf format is provided in supplemental information (Methods S1).

19. To purify samples prior to mass spectrometry analysis, use one C18 Sep-Pak cartridge containing 50 mg sorbent (suitable for up to 500 μg of peptides),
a. Add 6 μL of 10% TFA in water to the digested peptides to a final concentration of 0.5% and check pH with a pH strip. It should be less than 3. Continue to add 10% TFA as needed to reach the desired pH.
b. Place C18 Sep-Pak columns on a solid phase extraction vacuum manifold.
c. Activate the C18 Sep-Pak column with 1 mL of C18 activation solution. Discard the flowthrough.
d. Equilibrate the column with 1 mL of C18 equilibration/wash solution. Repeat two additional times. Discard the flowthrough.
e. Add the sample to the column. Discard the flowthrough.

Optional: Flowthrough from Step 19e can be collected to assess sample loss during desalting.

f. Wash peptides with 1 mL of C18 equilibration/wash solution. Repeat two additional times. Discard the flowthrough.
g. Elute bound peptides with 400 μL of C18 elution solution. Repeat this step an additional time.
h. Dry peptides in a vacuum concentrator.

⚠ CRITICAL: Do not allow the Sep-Pak column sorbent to dry at any step. It may significantly decrease the peptide recovery.

Pause point: Dried peptides can be stored at −80°C for several months.

Mass spectrometry data acquisition and database search

Timing: 1–2 days

This section describes the liquid chromatography and mass spectrometry (LC-MS) analysis of AP-MS samples. It also includes details for the analysis of the raw data.

20. Resuspend dried peptides in 50 μL of water with 0.1% formic acid (v/v) and estimate the peptide concentration using a UV spectrophotometer (like Thermo Scientific NanoDrop One) which measures absorbance at 280 nm wavelength (A280).

21. Use an ultra-high-pressure liquid chromatography (LC) system paired with a mass spectrometer (MS) to separate peptides on a reverse-phase C18 column on a gradient of mobile phase A (water with 0.1% formic acid (v/v), LC/MS grade) and mobile phase B (80% acetonitrile, 20% water with 0.1% formic acid (v/v), LC/MS grade).

Note: In our study,[1] we used an EASY-nLC 1200 liquid chromatography system (Thermo Scientific).

a. Inject approximately 500 ng of sample on a C18 reverse phase column (25 cm length x 75 μm I.D.) packed with ReproSil-Pur 120 Å, 1.9 μm C18 particles (Dr. Maisch GmbH) in-house.

Note: Follow the steps detailed in Jami-Alahmadi et al.[17] for in-house packing of C18 columns.

Optional: Commercial columns of similar dimensions can also be used.

Note: The amount of peptides to inject is MS instrument-dependent, as some instruments may require more or less peptides to achieve the same sensitivity.

b. Equilibrate the column with mobile phase A and separate peptides using a gradient of mobile phase B from 2% to 7% over 1 min, followed by an increase to 36% B over 53 min, then hold at 95% B for 13 min, then reduce back down to 2% B for 11 min at a flow rate of 300 nL/min.

Note: The LC gradient may require slight adjustments in an LC and MS instrument-specific manner.

Note: A trap-and-elute setting can be used in place of direct injection to perform an additional online sample clean-up.

22. For the mass spectrometry analysis of peptides, use a high-resolution mass spectrometer in either data-dependent acquisition (DDA) or data-independent acquisition (DIA) mode.

Note: In our study,[1] we used an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) coupled to an EASY-nLC 1200 liquid chromatography system (Thermo Scientific) with a Nano-spray Flex nanoelectrospray source (Thermo Scientific).

a. For data-dependent acquisition (DDA) mode, perform a full scan over an m/z range of 300–1500 in the Orbitrap at >50,000 resolving power with an AGC target of 1e6 and RF lens setting of 40%. Set dynamic exclusion to 45 s and exclusion width to 10 ppm. Fragment top 20 peptides, within charge state 2–6, with high-energy collision dissociation (HCD) or collision-induced dissociation (CID) at 20 MS/MS scans per cycle and a resolving power of 17,500.
b. For acquiring data in data-independent acquisition mode (DIA), perform MS scan at 60,000 resolving power over a scan range of 350–1100 m/z at a normalized AGC target of 300% and an RF lens setting of 40%. Perform DIA scans at 15000 resolving power, using 20 m/z isolation windows over 350–1100 m/z at a normalized HCD collision energy of 30%.

Note: Aliquots from each set of three biological replicates can be pooled and acquired in DDA mode to build a spectral library.

Note: Other high-resolution mass spectrometers designed for proteomics can be substituted in Step 22 for DDA and DIA analyses.

23. Search the raw MS data,
    a. For DDA data analysis, analyze raw files using MaxQuant[2] with default settings (or another search engine of your choice) to search the data against relevant proteomes. In our study, we used *Homo sapiens* and SARS-CoV-2 proteomes.
        i. Go to the 'Raw data' tab, add the raw files, and set the experimental details.
        ii. Go to the 'Group-specific parameters' tab, under 'Label-free quantification', change the 'Label-free quantification' value to 'LFQ', and leave all values default.
        iii. Go to the 'Global parameters' tab, and under 'Sequences', add the fasta file of interest.
        iv. Set the number of threads according to the number of cores available and hit 'Start'.
    b. For DIA data analysis, use Spectronaut[6] or DIA-NN[7] in the library-free mode to process the raw files. For DIA-NN,
        i. Select the raw files of interest.
        ii. Add the fasta file and select the 'Reannotate' and 'Contaminants' options.
        iii. Under the 'Precursor ion generation' section, enable 'FASTA digest for library-free search/library generation' and 'Deep learning-based spectra, RTs and IMs prediction' options.
        iv. Use all other parameters with default settings.

    Note: For DIA data analysis with Spectronaut,

        a. Go to the 'Analysis' tab and use the 'directDIA' pipeline.
        b. Load the raw and fasta files of interest.
        c. Use the 'BGS factory settings (default)' workflow.
        d. Set the conditions under the 'Run Conditions' window.
        e. Leave all other parameters set to default values.

    ⚠ CRITICAL: For all searches, set methionine oxidation as a variable modification and carbamidomethyl cysteine as a static modification. Filter results to a final 1% false discovery rate (FDR) at the peptide spectrum match (PSM), peptide, and protein levels. For either analysis, remove cross-run normalization (which will be performed later in MSstats, see Step 32) and imputation of missing values.

    Note: Users can also attempt to first build an experiment-specific spectral library from the DDA data using Spectronaut or DIA-NN (or another search engine of your choice). Then, search the DIA data using the spectral library generated in the previous step. To do this, keep the setting described in Step 23b and enable the 'Generate spectral library' option. In Spectronaut, go to the 'Library' tab and select 'Generate Library from Pulsar / Search Archives' pipeline to generate a spectral library from DDA and/or DIA data using the Pulsar search engine with 'BGS Factory Settings (default)' settings.

Data quality control

Timing: 1 day

This section includes various measures to check the AP-MS data quality.

24. Evaluate peptide intensity correlations between replicates of the same condition. We recommend using a square matrix heatmap-based visualization approach, where each replicate populates the rows and columns in the same order. Each cell will be colored and labeled according to the Pearson's r correlation coefficient.

    Note: Correlation coefficients between biological replicates should be greater than 0.8 to preserve them; if they fall below this number, discard the problematic replicate prior to subsequent analyses.

25. Evaluate peptide intensity pattern consistency between replicates using principal components analysis (PCA). Visualize the first and second principal components on the x and y-axes, respectively.

Note: If a replicate does not appear to cluster with the others, discard this replicate.

26. Evaluate differences in bait expression by comparing the abundance of bait peptides across runs. To do this, first, for each pair of conditions, identify a set of bait peptides that are detected across all biological replicates and sum their intensities. Ensure that the resulting summarized bait protein intensities are within 2-fold between each pair of conditions being compared.

Note: If bait expression levels are greater than 2-fold between conditions, we recommend optimizing the transfection conditions (i.e., increasing/decreasing DNA concentration) to further equalize expression and redoing the experiment.

27. Evaluate the sum of all peptide intensities for each sample. Some samples may result in higher overall peptide intensities. Here, consistency should be evaluated between the biological replicates.

Note: If there is greater than a 2-fold difference between biological replicates of the same condition, discard the outlying replicate prior to subsequent analysis.

28. Evaluate peptide and protein counts per sample.

Note: If a sample possesses a greater than 2-fold difference in peptide or protein counts relative to other replicates of the same condition, discard that replicate prior to subsequent analyses.

Note: We used the artMS R package[4] to generate figures that were interpreted to perform quality control analyses.

Scoring protein-protein interactions

Timing: 1 day

This section describes using SAINT express, MiST, and compPASS algorithms to score protein-protein interactions.

29. Perform PPI scoring using the software SAINTexpress.[8] This scoring algorithm assesses the abundance of prey relative to the negative controls (i.e., empty vector and GFP). We consider a prey significant if its false discovery rate (i.e., Bayesian false discovery rate "BFDR") is less than 0.05.
    a. First, generate SAINT input files. For each file, columns should be separated by a tab.
        i. The interactions file should be set up to contain four columns: (1) purification names, (2) bait names, (3) prey names, and a (4) quantitative measure, such as spectral counts or intensities. The column names can be modified, but the order must be preserved. The purification and bait names are set by the user. If the bait name is equivalent across purifications, it is treated as a replicate by SAINT. Prey names are typically protein accession numbers consistent with the mass spectrometry searching software output. If using Spectronaut, for example, the quantitative measure can be extracted from the 'PG.Quantity' column from the MSstats Report generated by the program.
        ii. The bait file should be set up to contain three columns: (1) purification names, (2) bait names, and (3) an identifier for target/control. The purification names and bait names must match with the interactions file. The last column contains the identifier 'T' for target data and 'C' for control data.

iii. The prey file should be set up to contain three columns: (1) prey names, (2) protein length, and (3) prey gene names. The prey names should match with those defined in the interactions file.

b. To use SAINTexpress in the command line in a Linux environment:
   i. First, install SAINTexpress from the following site: https://sourceforge.net/projects/saint-apms/files/.
   ii. Before compilation, install the GNU Scientific Library from the following site: https://www.gnu.org/software/gsl/
   iii. Open the Linux command line and change the directory to the one in which the program is installed, then run the following command:

```
> make
```

   iv. Run the program utilizing the following command:

```
> SAINTexpress-spc [interaction file name] [prey file name] [bait file name]
```

   v. The output file will be generated in the terminal's working directory.

c. To use SAINTexpress through a web-based deployment:
   i. Go to *http://apostl.moffitt.org/*. Navigate to SAINTexpress through the APOSTL Tools page.
   ii. Input the three input files generated above.
   iii. Push 'execute' and keep default settings.

d. The generated SAINT output file has 17 columns providing a statistical readout for every bait-prey interaction. Notably, the SaintScore column provides a SAINT score, ranging from 0 to 1, demonstrating the prey-bait interaction specificity. The BFDR column provides the Bayesian false discovery rate (BFDR) per interaction.

30. Additionally perform PPI scoring using MiST[9] or compPASS.[10] This scoring algorithm assesses prey specificity across the baits in your experiment. For example, if a prey binds to many baits in your sample, it is likely background. This pattern of non-specific binding results in a score of lower confidence. For MiST, we consider a prey to be significant if the MiST score is greater than 0.7. For compPASS, we convert the WD score into a percentile and consider a prey to be significant if the WD percentile is greater than 98%.

a. Performing PPI scoring using MiST:
   i. Prepare the input files for MiST: data and keys. The data file should contain four columns: (1) sample identifier, (2) protein identifier, (3) observed peptide frequency, and (4) protein molecular weight. The column names can be modified, but the order must be preserved. The sample identifier is defined by the user per condition. The protein identifier is a unique identifying code, such as the UniProt accession code. The observed peptide frequency column can be a variety of quantitative measures, such as spectral counts or intensities. The keys file contains two columns: (1) sample identifier and (2) bait name. The sample identifier should match with the data file. The bait name can be the UniProt accession code or any easily identifiable name. Input files should be tab-delimited.
   ii. Install MiST from the following GitHub repository: https://github.com/kroganlab/mist.git. The program can be installed either by downloading the zip file from the site directly or through the terminal by executing the following code in the terminal, given that Git (https://git-scm.com/) is already installed.

```
> git clone https://github.com/kroganlab/mist.git.
```

iii. Perform data pre-processing by altering the YML config file located in the /tests/small/ directory. The file is named "mist_small_test.yml." The config file includes options to remove contaminants and carryover. Importantly, copy over the column names defined in the data file for options id_colname (sample identifier), prey_colname (protein identifier), pepcount_colname (peptide frequency), and mw_colname (protein molecular weight).

iv. To execute the program, run the following command in the terminal, ensuring that the set directory leads to the MiST files. "main.R" is the R script provided with the installation of MiST.

```
> main.R --config [path to YML file]
```

The program will output a results file containing an entry for observed bait-prey pairs, providing a MiST score readout for each interaction.

b. Performing PPI scoring using compPASS:

i. To prepare the input file, create a tab-delimited text file with the following 5–6 columns: (1) experiment ID, (2) replicate, (3) experiment type (optional), (4) bait, (5) prey, and (6) spectral count. The column names can be modified, but the order must be preserved. The experiment ID is defined by the user and is shared by replicates, whereas the replicate column provides a unique ID for replicates. Prey and bait IDs can be various identifying symbols, such as UniProt accession codes or gene symbols. The spectral count column specifies the number of spectra found for every bait-prey pair in an experimental replicate.

ii. To utilize compPASS through R, install the cRompass package by executing the following code in R console:

```
> install.packages("devtools")

> library("devtools")

> install_github("dnusinow/cRomppass")
```

iii. Run the following command in R console where "input_file" should be replaced with the file path to the stored input file. "norm.factor" can be replaced with the quantile (between 0 and 1) for normalization. If left empty, it defaults to 0.98, which is recommended.

```
> compass(input_file,norm.factor))
```

Note: To utilize compPASS through a web-based deployment, navigate to https://bioplex.hms.harvard.edu/comppass/. Upload the input file to the "Experiments" button. The program will automatically process and produce the output file.

iv. The generated output dataset will contain seven columns providing a statistical readout of studied bait-prey interactions. Notably, compPASS calculates a z-score for the bait-prey interactions and a WD score highlighting the uniqueness of each interaction.

Note: The MiST score threshold should be evaluated separately for each dataset as it is affected by the dataset size. In the updated MiST algorithm (version 1.5), the MiST score now automatically scales to dataset size.

Note: Traditionally, we have used MiST scoring for virus-host interaction datasets and compPASS for host-host interaction datasets. Historically, MiST has been used to score

virus-host interactions because it was originally trained on the HIV-host PPI dataset, containing internal weights from this initial training, suggesting it may be better suited to score virus-host interaction datasets. On the other hand, compPASS was developed for host-host interactions, suggesting it may be better suited to evaluate these interactions. However, PPI scoring results should be assessed on a case-by-case basis and evaluated relative to gold standards; thus, MiST and compPASS should be run for both virus-host and host-host interaction datasets and the results investigated for the recovery of gold standards.

Note: Specificity scoring algorithms like MiST and compPASS work best when many different conditions are included, since they work by comparing across different conditions. If only two conditions are included (the minimum), consider using SAINTexpress alone. Furthermore, MiST and compPASS work best when a dataset includes different bait proteins; in our study, this equated to including all SARS-CoV-2 proteins. Include exclusionary criteria for similar baits (including for pairs of conditions being compared if the baits contain high sequence similarity). This ensures specificity algorithms will not adversely penalize preys that are discovered across similar baits, which is biologically expected.

31. To create our final set of high confidence preys, we use a combination of abundance (e.g., SAINTexpress) and specificity (e.g., MiST or compPASS) cutoffs. In the past, we have required MiST>0.7 (or compPASS WD percentile > 98%) & SAINTexpress BFDR<0.05. We additionally require that each prey possesses an average spectral count (among biological replicates) of at least 2. The value of specific thresholds used should be tailored to each dataset, especially for their ability to identify any known interactors balanced against their inclusion of common contaminants or non-specific binders.[9]

Quantitative data analysis

Timing: 1 day

This section describes the cross-run normalization and statistical analysis of AP-MS data using MSstats.

32. Perform quantitative statistical analysis of prey protein abundance changes between conditions using peptide ion fragment data from the full datasets (not yet filtered by high-confidence preys).
    a. Export peptide ion fragment data from your search algorithm of choice and analyze using MSstats workflows. Peptide ion fragment data is organized such that each row contains quantitative information about each MS2 fragment ion detected per sample (i.e., data for each sample is stored in ''long-format'', therefore they are stacked on top of each other). MSstats has several built-in pipelines to convert evidence files from MaxQuant and others to MSstats format.
        i. Define contrasts, which denote comparisons between conditions of interest.
        ii. Convert MaxQuant evidence files to MSstats format using MaxQtoMSstatsFormat with settings: D =''Leading.razor.protein'', useUniquePeptides = FALSE, summaryforMultipleRows = sum, removeFewMeasurements = FALSE, removeOxidationMpeptides = FALSE, and removeProtein_with1Peptide = FALSE.
        iii. Run the dataProcess function with featureSubset = ''all'', normalization = ''equalizeMedians'', MBimpute = FALSE, and summaryMethod =''TMP''. In essence, this performs a cross-run normalization by median equalization, does not impute missing values, and summarizes multiple peptide ion or fragment intensities into a single intensity for their protein group.

iv. Perform statistical tests of differences in intensity between conditions of interest (defined by contrasts, above) using defaults for MSstats for adjusted P values, even in cases of n = 2. By default, MSstats uses the Student's t-test for P value calculation and the Benjamini–Hochberg method of FDR estimation to adjust P values. This analysis results in $\log_2$ fold changes (log2FC) and p-values per interaction between pairwise comparisons (in our prior study,[1] corresponding mutant and wave 1 baits).

Note: As described above, we suggest starting by performing MSstats normalization using global median equalization, no imputation of missing values, and median smoothing to summarize multiple peptide ion or fragment intensities into a single intensity for their protein group. However, an alternative approach is to normalize by bait expression. Global median equalization essentially normalizes to the background, correcting for differences in overall peptide intensities injected into the mass spectrometer for each run, important to correct for variability in input quantity and sample handling. Bait-based normalization assumes prey intensities scale linearly with bait abundance, an assumption originating from the law of mass action. However, bait abundance could be at saturating intensities, such that further increases in bait abundances do not correspond to increases in prey abundances. Thus, the choice of normalization should be evaluated and applied in a dataset-specific fashion. Typically, we do not expect large differences in protein-protein interactions between conditions[12]; for example, we often compare proteins with a single amino acid change. In such cases, the prey distribution of $\log_2$ fold changes should be centered around zero, without a major skew in either direction (i.e., all or most preys increase or decrease). If this is observed, we recommend altering the normalization approach. There are exceptions to cases like these, if, for instance, all increasing preys are known to be part of a complex.

Note: If normalizing by bait abundance, we recommend using the overlapping set of bait peptides identified in all replicates of the two conditions being compared so as not to artificially skew bait, and the resulting prey, abundances. Additionally, we recommend performing normalization using a custom-build pipeline prior to running MSstats (with normalization turned off).

Note: Although cross-run normalization is a critical aspect of label-free AP-MS-based proteomics, working to equalize bait expression between conditions is essential to achieve a reliable comparison between conditions. As mentioned above, when comparing two baits, we recommend their expression falls within 2-fold; if outside of this range, we recommend optimizing the transfection conditions (i.e., increasing/decreasing DNA concentration) to further equalize expression and redoing the experiment.

33. To define significantly different protein-protein interactions, or "differential protein-protein interactions",
    a. Define differential interactions based on two criteria:
        i. The prey must be a high-confidence interaction in either condition being compared (see scoring thresholds above), AND the prey must be changing in abundance between the conditions being compared with an absolute value log2FC > 1 and $p < 0.05$.

Note: Thresholds for differential interactions should be tailored for each dataset.

Data visualization

Timing: 1 day to 1 week

This section describes the steps for visualizing statistically significant differentially interacting proteins through heatmaps and networks.

34. Visualize differentially interacting proteins as a heatmap,
    a. Create a heatmap with distinct bait comparisons (i.e., mutant versus wild-type) along the rows and preys along the columns (for an example, see Figure 2A). The heatmap can be made to include all preys that are high confidence in either condition, many of which will not significantly change between conditions; however, we only include preys that are significantly differentially interacting (based on criteria outlined in Step 33) for at least one of the comparisons tested. We recommend coloring each cell with the $\log_2$ fold change between conditions for each prey or gray if not detected in either condition. Visually indicate if a prey is only detected in one of the conditions (i.e., mutant only or wild type only), thus possessing an infinite $\log_2$ fold change; we annotate these using white hatches. Lastly, annotate significantly different interactions (i.e., $p < 0.05$); we have used a black bounding box to indicate $p < 0.05$ (see Figure 2A).
35. Visualize differentially interacting proteins as a network,
    a. Create two text files, one called "edges" and one called "nodes". The edges file contains the pairwise interactions between bait and prey proteins, as well as any other edge annotations. The node file contains a list of proteins and annotation as to whether they are a bait or a prey.
    b. To make the edges file,
        i. Extract a network of differentially interacting proteins and their baits. Typically, this takes the form of a table with four columns: (1) bait, (2) prey, (3) $\log_2$ fold change, (4) p-value, and (5) data source. The $\log_2$ fold change refers to the magnitude of change of prey abundance in the affinity purification between conditions. Data source refers to either "AP-MS" or "CORUM", where AP-MS refers to edges derived from the experimental mass spectrometry measurement and CORUM refers to edges added from the CORUM database[18] (see below).
        ii. Add edges from the CORUM database. First, download a table of interactions from the online resource.[17] Next, merge protein complex interactions from CORUM that exist between any two preys bound to the same bait.

Note: To simplify network visualization, we typically draw CORUM edges between preys that are bound to the same bait and not between preys bound to different baits. However, it is also possible to include edges between preys bound to different baits. Furthermore, additional protein-protein interaction databases can be integrated; we recommend CORUM because it contains high-confidence protein complexes with well-studied functions.

    c. To make the nodes file,
        i. Collapse the unique proteins in the bait and prey columns in the edges table into a column called "nodes". Add an additional column called "is_bait", which is given a TRUE if the protein is a bait and FALSE if the protein is a prey.
    d. Visualize differential interaction network using Cytoscape.[5]
        i. Import edges file using the "Import Network from File System" button. Select source and target nodes as bait and prey columns, respectively. All other columns will default to "edge attributes".
        ii. Import nodes table using the "Import Table from File" button. Make sure to import into the loaded network.
        iii. Set edge thickness or color to the data source or $\log_2$ fold change columns. Optionally you could set the thickness of the line proportional to the -log10(p).
        iv. Change the node shape according to the "is_bait" column in the nodes file. We set baits to be diamonds and preys to be circles. We additionally make bait nodes bigger than prey nodes.
        v. Arrange nodes manually in a visually aesthetic manner. One strategy is to start with a Circular Layout and arrange bait-prey interactions in a circular format (see Figure 2).

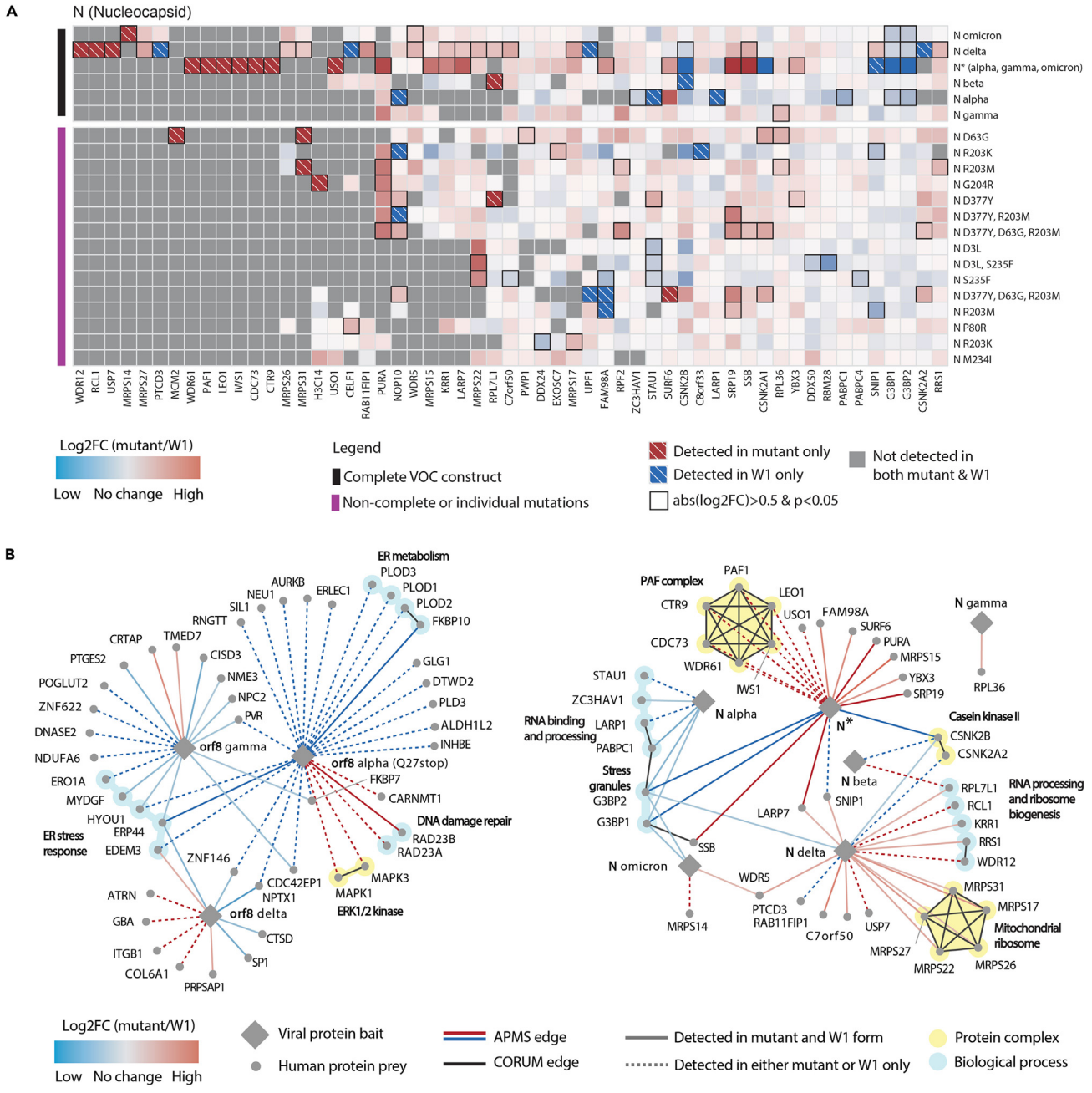

Figure 2. Visualization of differential protein-protein interactions

(A) Heatmap of virus-host protein-protein interactions, reproduced from Bouhaddou et al.[1]. Rows contain nucleocapsid (N) viral protein mutants from each SARS-CoV-2 variant of concern. Columns contain interacting host prey proteins that are high-confidence (see PPI scoring) for either condition being compared (i.e., mutant or wave one). Colors map to the $\log_2$ fold changes (log2FC) between mutant and wave one (W1) protein forms. Red indicates increased binding to mutant, and blue indicates decreased binding to mutant. Black boxes represent significant ($p < 0.05$) changes. White-dashed boxes indicate a prey detected in only the mutant (red) or W1 (blue).

(B) Network of differential virus-host PPIs. Only significantly different interactions (|log2FC|>0.5 & $p < 0.05$) that are high-confidence in either condition being compared (i.e., mutant or wave one) are shown.

Note: Change prey protein labels to be centered to the outside of the node. These can be adjusted in graphics software (e.g., Adobe Illustrator, see below) to not overlap with any other nodes or edges.

e. Once nodes are arranged in Cytoscape, export as PDF and import into Adobe Illustrator for final aesthetic adjustments.
   i. Add colorful circles for protein complexes and biological processes. Protein complexes are evident from CORUM edges incorporated into the network. Biological process terms must be manually refined from a GO Biological Process gene overrepresentation enrichment analysis using the clusterProfiler[11] package in R, for which several online tutorials are available. Specifically, perform the enrichment analysis on the group of preys from each bait separately.
   ii. Next, manually group preys into shared biological processes. We recommend only annotating preys with a biological process term if their primary function is associated with said term. To accomplish this, it is important to read about the known function(s) of all genes prior to finalizing an annotation. If a gene has multiple known functions or its connection to a certain biological process is unclear, avoid annotating this gene. We recommend annotating biological processes within the preys for each given bait, and not between preys of different baits, which we feel simplifies the interpretation of the results. However, this decision is dataset dependent and should be decided on a case-by-case basis.

EXPECTED OUTCOMES

This protocol provides researchers with an AP-MS proteomics pipeline integrated with a quantitative and statistical computational analysis to quantify and visualize differential PPIs. For instance, users may wish to understand how protein-coding mutations or experimental perturbations impact protein interaction affinities. The expected outcome is a list of significantly changing protein interactions and protein complexes, which can be visualized as a heatmap and/or differential interacting networks. Subsequently, differential PPIs can be used to understand how specific alterations in protein sequences or biological contexts impinge on disease etiology and/or fundamental biological processes. In our original study,[1] we used this protocol to generate PPI networks for SARS-CoV-2 VOCs and showed that compared to other VOCs, Omicron BA.1 possessed altered regulation of interferon stimulated genes (ISGs), which correlated with altered SARS-CoV-2 Orf6-nuclear pore interaction affinities. Specifically, using the pipeline outlined in this protocol, we pinpointed Orf6-nuclear pore interactions to be regulated by a specific residue (Orf6 D61), which, when changed to a leucine (L), reduced the Orf6-nuclear pore interaction, resulting in reduced innate immune antagonist potency of the Orf6 protein. This result allowed us to pinpoint the biochemical mechanism at the crux of reduced Orf6-mediated innate immune antagonism during Omicron BA.1 infection. Importantly, our pipeline possesses general applicability to the comparison of pairs of proteins (i.e., mutants, isoforms, etc.) from any organism or between specific conditions/treatments of interest to study a range of diseases and biological processes.

LIMITATIONS

A limitation of this protocol is that it captures high-affinity interactions representing highly stable protein complexes rather than transient interactions, such as kinase-substrate interactions. Proximity labeling approaches, like TurboID,[19] are better suited to capture transient interactions. Additionally, this approach does not identify whether a PPI is direct or indirect. For example, interactions may occur through a protein or nucleic acid intermediate, such as RNA. Methods to remove RNA-dependent interactions can be attempted in such cases (e.g., benzonase). Moreover, cell line/type variability in transfection/construct expression efficiency may render this approach difficult to implement in specific cell types/cell lines. In place, a viral transduction or electroporation approach to deliver the construct(s) may be utilized to enhance cell distribution.

TROUBLESHOOTING

Problem 1

Insufficient target protein expression (Steps 1–6).

Potential solution

- Ensure the plasmid is codon optimized.
- Confirm the bait identity by plasmid sequencing.
- Always use DNase-free tubes or reagents and maintain sterile conditions while purifying plasmid from bacterial culture.
- While preparing the plasmid/transfection reagent mixture (other than PolyJet), the tubes should be gently mixed by inverting them. Vigorous shaking may lead to plasmid disintegration and low transfection efficiency.
- Optimize the total plasmid amount and plasmid-to-transfection reagent ratio. Excess plasmid and/or transfection reagent may have cytotoxic effects, reducing transfection efficiency.

Problem 2

Insufficient enrichment of Strep tagged target proteins (Steps 14–16).

Potential solution

- Before binding the sample to the MagStep 'type-3' XT beads, clear the cell lysate by centrifugation to remove any cell debris. Avoid re-use the MagStep 'type-3' XT beads.
- Increase the starting cell lysate amount and optimize the protein-to-bead ratio.

Problem 3

High number of non-specific interactors in the background (Step 17).

Potential solution

To minimize the carryover of background proteins between washes, increase the beads' washing steps with the IP Wash Buffer and change the tubes in between steps.

Problem 4

No/low peptide hits for target protein in the mass spectrometry data (Steps 21–23).

Potential solution

- Check the purity of the sample via SDS-PAGE followed by Coomassie or silver stain. The enriched sample should have a relatively larger target protein band than the empty vector sample.
- Use LC-MS-grade chemicals for the proteomics sample preparation and run a quality check (like HeLa protein digest) to ensure good LC-MS performance before analyzing the sample.
- Depending on the LC and MS instrument configuration, increase the total injected peptide amount for adequate sequence coverage.
- Use DIA-based data acquisition in place of DDA to minimize missing data values.

Problem 5

Network visualization appears chaotic (Step 35).

Potential solution

A few simple strategies that can improve the aesthetic quality and interpretability of a network diagram are detailed below.

- Move bait and prey names from the center of the node to open black space adjacent to the node (see Figure 2B). This can be finalized within a software such as Adobe Illustrator, involving manually moving each label such that it does not directly touch any edge, node, or other label.

- Incorporate additional prey-prey protein-protein interaction data from high confidence databases with manually curated complexes, such as CORUM. Avoid using databases with too many edges as this can increase the visual chaos of the network.
- When annotating protein complexes and biological processes, bring nodes that participate in the same biological entity in close proximity and surround them with a colorful halo (see Figure 2B). We recommend moving the halo to the background so as not to obscure protein names or edge colors.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mehdi Bouhaddou (bouhaddou@ucla.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contacts, Prashant Kaushal (prashantkaushal@ucla.edu) or Mehdi Bouhaddou (bouhaddou@ucla.edu).

Materials availability

Plasmids used in this study are all available on Addgene. All cell lines, reagents, and instruments needed for this protocol are available commercially.

Data and code availability

A KingFisher Flex protocol for Strep tag affinity purification is provided in pdf format Methods S1. Additional data are available from the lead contact upon reasonable request. This study did not generate new code.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.K., N.J.K., and M.B.; research design and methodology, P.K., M.R.U., G.M.J., J.X., B.P., Y.Z., E.S., M.E., R.K., D.L.S., L.Z.-A., N.J.K., and M.B.; data analysis and interpretation, P.K. and M.B.; writing – review and editing, P.K., M.R.U., G.M.J., Y.D., S.K.M., K.A., D.M.W., S.F.B., J.X., B.P., Y.Z., E.S., M.E., L.Z.-A., R.K., D.L.S., N.J.K., and M.B.

DECLARATION OF INTERESTS

The Krogan Laboratory has received research support from Vir Biotechnology, F. Hoffmann-La Roche, and Rezo Therapeutics. N.J.K. has a financially compensated consulting agreement with Maze Therapeutics. N.J.K. is the President and is on the Board of Directors of Rezo Therapeutics, and he is a shareholder in Tenaya Therapeutics, Maze Therapeutics, Rezo Therapeutics, and Interline Therapeutics. M.B. is a scientific advisor for GEn1E LifeSciences.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103286.

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