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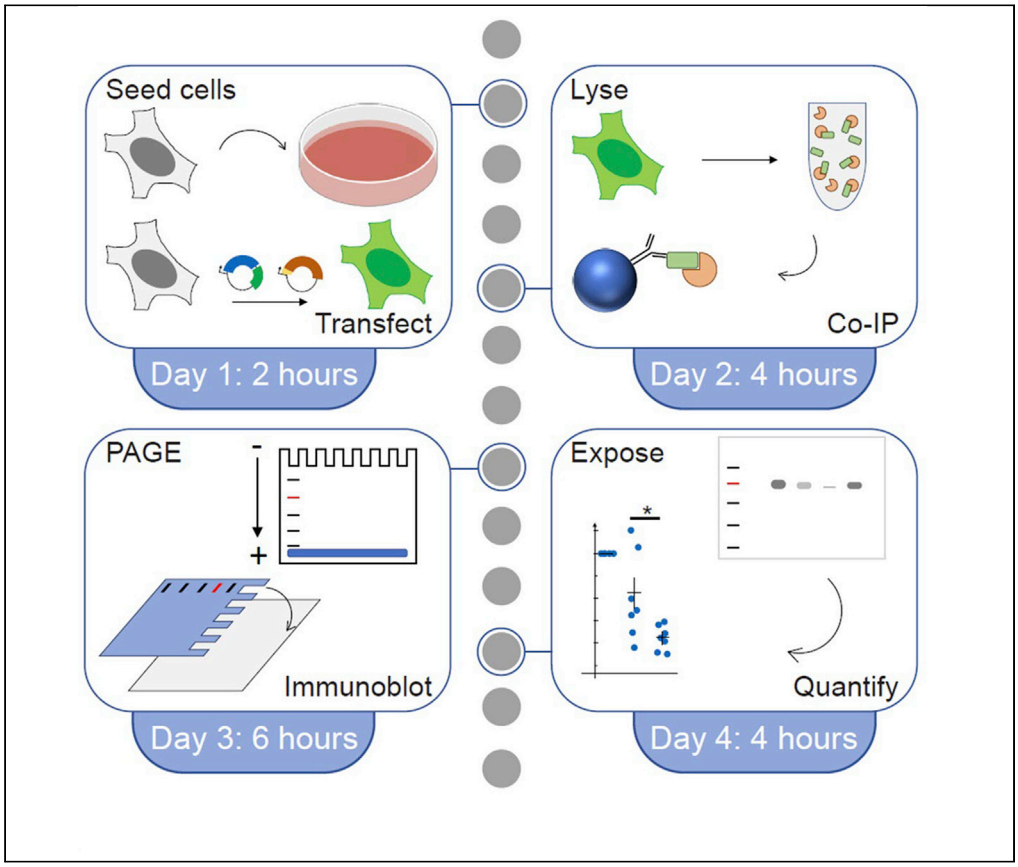
Co-immunoprecipitation and semi-quantitative immunoblotting for the analysis of protein-protein interactions



Co-immunoprecipitation (co-IP) of protein complexes from cell lysates is widely used to study protein-protein interactions. However, establishing robust co-IP assays often involves considerable optimization. Moreover, co-IP results are frequently presented in non-quantitative ways. This protocol presents an optimized co-IP workflow with an analysis based on semi-quantitative immunoblot densitometry to increase reliability and reproducibility.

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Highlights

Co-immunoprecipitation protocol to quantify protein interactions in cell lysates

Semi-quantitative immunoblot densitometry for statistical assessment of perturbations

The optimized workflow gives results in four days with standard lab equipment

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Co-immunoprecipitation and semi-quantitative immunoblotting for the analysis of protein-protein interactions

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SUMMARY

Co-immunoprecipitation (co-IP) of protein complexes from cell lysates is widely used to study protein-protein interactions. However, establishing robust co-IP assays often involves considerable optimization. Moreover, co-IP results are frequently presented in non-quantitative ways. This protocol presents an optimized co-IP workflow with an analysis based on semi-quantitative immunoblot densitometry to increase reliability and reproducibility.

For complete details on the use and execution of this protocol, please refer to Burckhardt et al. (2021).

BEFORE YOU BEGIN

Design the experiment and establish the expression plasmids

Timing: 1–2 weeks

1. Layout the experiment and include positive and negative controls and the perturbations that are used to test a specific hypothesis (Figures 1A–1C). Design the plasmid constructs for bait and prey proteins accordingly.
2. For this protocol fuse the bait protein to green fluorescent protein (GFP) and pull down with a GFP antibody (Figure 1). The prey proteins are fused to high affinity short peptide tags such as influenza hemagglutinin (HA) tag, FLAG tag or V5 tag. If multiple prey proteins are expressed use different tags, particularly if more than one prey protein is expected to be pulled down by the bait.
3. Subclone the DNA into suitable plasmids for transient expression in human cells. Prepare sufficient amounts of plasmid DNA for the transfection.
4. Use high quality plasmid DNA from a midiprep or maxiprep scale purification at concentrations of 0.5–1 μg/μL for the transfection.

Get human embryonic kidney cells in culture and expand the cells

Timing: 1 week

5. On day 1 of the protocol, use $5*10^6$ human embryonic kidney (HEK) cells per co-IP sample. Expand the cells accordingly ahead of time.

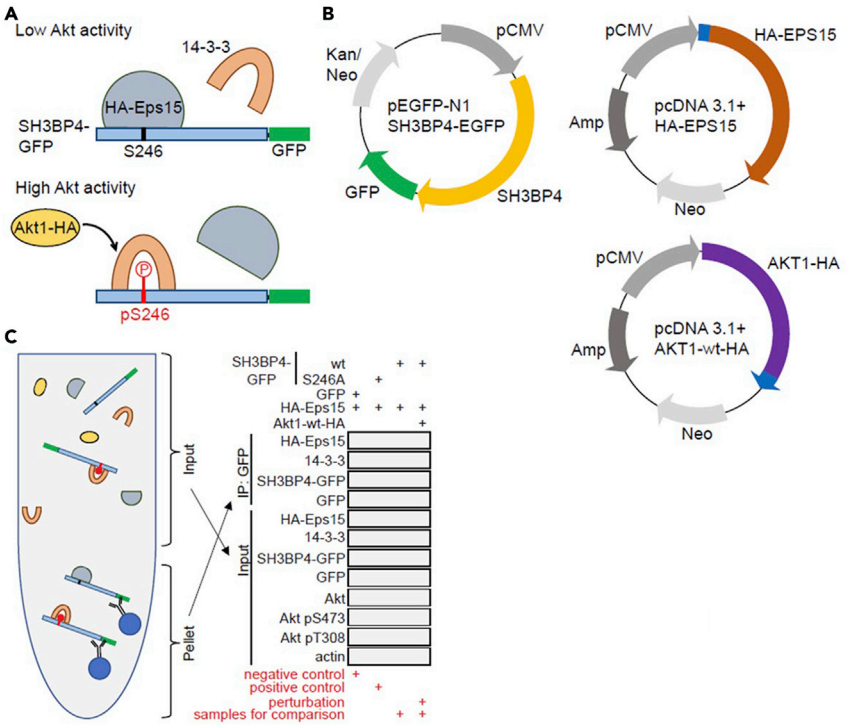

Figure 1. Configuration of a co-IP experiment

(A) In the experiment that is presented to illustrate this protocol, the hypothesis is tested whether overexpression of Akt reduces Eps15 binding to SH3BP4. Akt phosphorylates SH3BP4 at S246 which leads to 14-3-3 adapter binding. Since the S246 is proximal to the Eps15 binding sites, 14-3-3 adapters are thought to interfere with Eps15 attachment (Burckhardt et al., 2021). In this co-IP experiment, SH3BP4 is fused to a green fluorescent protein tag (GFP), while Eps15 and Akt1 are labeled with the influenza virus hemagglutinin (HA) tag (HA-Eps15 and Akt1-HA, respectively). Importantly, if multiple prey proteins are overexpressed, they should be labeled with different tags. When the prey proteins are of similar molecular weight and when multiple prey proteins are expected to be pulled down, the unequivocal confirmation of the protein identity is particularly important. Alternatively, the overexpressed proteins can be probed with specific antibodies (Figure 3B). Moreover, the highly expressed endogenous 14-3-3ϵ is detected with an isoform specific antibody.

(B) Plasmid maps for pEGFP-N1 SH3BP4-GFP, pcDNA 3.1(+) HA-EPS15 and pcDNA 3.1(+) AKT1-wt-HA. The CMV promoter (pCMV) and Kanamycin (Kan), Neomycin (Neo) and the ampicillin (Amp) resistance genes are indicated. The HA-tag is represented in blue. Wildtype, wt.

(C) Schematic illustration of the co-IP pellet and the input fractions and how they yield the various immunoblots. Immunoprecipitation (IP) and expression in the cell lysate (input, in this protocol collected before the co-IP). In this experiment, binding of HA-Eps15 to SH3BP4-GFP is monitored and four samples are analyzed. In the first sample, GFP is expressed as a control for unspecific HA-Eps15 binding. In the second sample the SH3BP4 S246A phosphorylation deficient mutant that strongly binds Eps15 is used as a positive control. In the third and fourth samples wt SH3BP4-GFP and HA-Eps15 are expressed in the absence and presence of Akt1-HA overexpression, respectively. Concurrently, the binding of endogenous 14-3-3ϵ to SH3BP4-GFP is monitored. The 14-3-3ϵ adapter protein binds SH3BP4 that is phosphorylated at S246, while it doesn't bind the S246A mutant. The Akt activity is assessed with antibodies specific for Akt phosphorylated at S473 and T308. Actin is used as a loading control. The graphics are adapted from Burckhardt et al., (2021), with permission.

Prepare the stable buffers

Timing: 2–4 h

6. Many of the buffers that are needed for this protocol are common. The first table lists the approximate volumes that are needed per sample or per immunoblot. Prepare the missing buffers and solutions as described below.

Just in time preparation of lysis buffer

Timing: 15 min

7. On day 2, prepare fresh cell lysis buffer as described below. Make sure that there is enough lysis buffer for the cell lysis (day 2) and the wash steps (day 3) available.

Just in time preparation of enhanced chemiluminescence solution

Timing: 5 min

8. On day 4, just in time for the exposure of the immunoblots (also referred to as western blots, both terms are used interchangeably in this protocol), prepare the enhanced chemiluminescence solution as described below.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Green fluorescent protein (GFP), mouse monoclonal	Roche	11814460001 RRID: AB_390913
Influenza virus hemagglutinin (HA)-tag, rabbit polyclonal	Sigma	SAB4300603, RRID: AB_10620829
Green fluorescent protein (GFP), rabbit polyclonal	Abcam	ab290 RRID: AB_303395
Eps15, rabbit polyclonal	Schmid Lab, UTSW	NA
Pan-Akt, rabbit polyclonal	Cell Signaling Technology	Cat #4691, RRID: AB_915783
Phospho-Akt-Ser473, rabbit polyclonal	Cell Signaling Technology	Cat #4060, RRID: AB_2305337
Phospho-Akt-Thr308, rabbit polyclonal	Cell Signaling Technology	Cat #2965, RRID: AB_10695743
14-3-3ϵ, mouse monoclonal	Santa Cruz Biotechnology	Cat# sc23957, RRID: AB_626619
Actin, mouse monoclonal	Sigma	A1978, RRID: AB_476692
Vimentin, mouse monoclonal	Sigma	V6630, RRID: AB_477627
GAPDH, rabbit polyclonal	Cell Signaling Technology	5174, RRID: AB_10622025
Goat polyclonal anti-mouse-HRP	Jackson ImmunoResearch	Cat# 115-035-003, RRID: AB_10015289
Goat polyclonal anti-rabbit-HRP	Jackson ImmunoResearch	Cat# 111-035-003, RRID: AB_2313567
Chemicals, peptides, and recombinant proteins		
Polyethyleneimine (PEI, Linear, MW 25 K)	Polysciences	23966
NP-40 (alternative to the original NP-40 that was discontinued)	Sigma	492016
Tween-20	Fisher Scientific	BP337
Sodium chloride	Research Products International (RPI)	S23020
Glycine	Fisher Scientific	BP381-5
Tris Base	Fisher Scientific	BP152
Di-sodium hydrogen phosphate (Na_2HPO_4)	RPI	S23100
Monobasic potassium phosphate (KH_2PO_4)	Fisher Scientific	BP362
Potassium chloride	Fisher Scientific	BP366
Hydrochloric acid (fuming, 37%)	Sigma	1003171011
Sodium hydroxide	Sigma	S8045
cOmplete Protease Inhibitor Cocktail Tablets	Roche	11697498001
Sodium fluoride	Fisher Scientific	S299
Sodium orthovanadate	Fisher Scientific	S454
Sodium pyrophosphate decahydrate	Sigma	221368
β-Glycerophosphate disodium salt hydrate	Sigma	G5422

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bromophenol Blue	Bio-Rad	161-0404
Glycerol	Fisher Scientific	BP229
Sodium dodecyl sulfate (SDS)	Thermo Scientific	28365
β-Mercaptoethanol (BME)	Fisher Scientific	BP176
Hydrogen peroxide solution (H_2O_2, 37%, w/w)	Alfa Aesar	L14000
Ethanol	Pharmco	111000190
Methanol	Pharmco-Aaper	339000000CSGL
40% Acrylamide/Bis Solution, 37.5:1	Bio-Rad	1610148
N, N, N′, N′-tetramethylethylenediamine (TEMED)	Bio-Rad	161-0800
Ammonium persulfate (APS)	Thermo Scientific	17874
Isopropanol	Fisher Scientific	BP2618500
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
Bovine serum albumin	Equitech-Bio	BAH65
p-Coumaric acid	Sigma	C9008
Luminol	Sigma	A8511
Dimethyl sulfoxide (DMSO)	Fisher Scientific	BP231-100
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco	11995-065
Fetal Bovine Serum (FBS)	Sigma	F0926
Antibiotic-Antimycotic (AA, 100×)	Gibco	15240062
0.5% Trypsin-EDTA Solution	Gibco	15400-054
Trypan Blue Solution, 0.4%	Gibco	15250061
Critical commercial assays		
SuperSignal West Femto ECL reagent	Thermo Scientific	34095
Experimental models: cell lines		
Human embryonic kidney cells: HEK Lenti-X 293T	Takara	632180
Recombinant DNA		
pEGFP-N1	Clontech/Takara	6085-1
pcDNA3.1 (+)	Thermo Scientific	V79020
Software and algorithms		
ImageJ/Fiji	NIH	https://imagej.github.io/ RRID:SCR_002285
Image Studio Lite	Li-COR	https://www.licor.com/bio/image-studio-lite/download
Excel	Microsoft	RRID:SCR_016137
MATLAB	MathWorks, Inc	RRID:SCR_001622
Other		
Cold room (4°C)	N/A	N/A
Sonicator with tip probe	Fisher Scientific	FB505
Water bath (at 37°C)	N/A	N/A
Tube rotator	N/A	N/A
Platform shaker	N/A	N/A
Cell culture incubator with temperature and CO_2 control	N/A	N/A
Automated cell counter	Nexcelom	Cellometer Auto 1000
Cell counting slides	Nexcelom	CHT4-SD100-514
Refrigerated microcentrifuge	N/A	N/A
Benchtop pH meter	N/A	N/A
Lubricating (vacuum) grease	N/A	N/A
Milli-Q water purification system	Millipore	C85358
Gel casting and electrophoresis equipment	Bio-Rad	1658001FC
Wet-blot transfer equipment	Bio-Rad	1703930
Power supply for gel electrophoresis and blotting	Bio-Rad	1645050
Gel imaging system with digital image acquisition	N/A	N/A
0.5 mL Microcentrifuge tubes	N/A	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
1.5 mL Microcentrifuge tubes	N/A	N/A
2 mL Microcentrifuge tubes	N/A	N/A
Cell culture dishes, 10 cm, round	N/A	N/A
Fluorescence microscope compatible with plastic cell culture dishes and equipped with filters for GFP	N/A	N/A
Aluminum cooling block for 2 mL plastic tubes	N/A	N/A
Protein G Agarose Resin	ABT	4RRPG-5
Heating block for up to 95°C	N/A	N/A
Whatman paper, 3MM-CHR	GE Healthcare	3030-917
PVDF membrane, 0.45 μM pore size	Millipore	UFC40HV00
Dry milk	N/A	N/A
Razor blades	N/A	N/A
Plastic casserole dish, around 25 × 25 × 7 cm	N/A	N/A
Plastic containers for western blot membranes, /size medium (for mini-protean gel size)	GenHunter Corporation	PerfectWestern B101
Sterile single use vacuum filter units	Fisher Scientific	5650010
Cell lifters	Fisher Scientific	08-100-240

MATERIALS AND EQUIPMENT

Materials

The following approximate amounts of buffers and solutions are needed per sample or per immunoblot.

Reagent	Amount per sample	Amount per gel and blot
PBS, 1×	20 mL	
PEI transfection solution	30 μL	
Lysis buffer	5 mL	
Tris, 1 M pH 7.5	250 μL	
NaCl, 5 M	150 μL	
6× sample buffer	60 μL	
20% SDS solution		70 μL
Tris 1.5 M, pH 8.8		4.5 mL
Tris 0.5 M, pH 6.8		0.7 mL
Running buffer, 1×		2 L for up to 4 gels
Transfer buffer, 1×		2 L for up to 2 blots
TBST 1×		500 mL
Coumarin stock solution		25 μL
Luminol stock solution		50 μL
Tris, 1 M pH 8.5		1 mL

Polyethyleneimine (PEI) transfection solution		
Reagent	Final concentration	Amount
Polyethyleneimine (PEI, 25 K)	1 mg/mL	100 mg
HCl (from 1 M solution)	n/a	apr
NaOH (from 1 M solution)	n/a	apr
ddH_2O	n/a	Fill to 100 mL
Total	**n/a**	**100 mL**

Note: The protocol for the PEI transfection solution was adapted from Polysciences (Warrington, PA). As per requirement, apr.

Dispense 100 mg of PEI into a 200 mL glass beaker and add 90 mL ultrafiltered, deionized water (ddH_2O, milli-Q, Millipore). Add a stir bar and gently stir the solution. Measure the pH with a benchtop pH meter. Add hydrochloric acid (from a 1 M stock solution) in small volumes with a pipette to adjust the pH to < 2.0. Cover the beaker and stir for up to 3 h until the powder is fully dissolved. Add sodium hydroxide (from 1 M stock solution) in small volumes until the pH is 6.9–7.1. Add PEI solution to a graduated cylinder and fill with ddH_2O to a total volume of 100 mL. Sterile filter the solution through a sterile vacuum filter unit with 0.2 μm pore size. Under sterile conditions, aliquot PEI in 1.5 mL microcentrifuge tubes.

Note: Store aliquots at −80°C. Frozen aliquots can be kept for up to one year. Thawed aliquots can be stored at 4°C for up to two weeks. Avoid re-freezing and additional freeze-thaw cycles.

1 M Tris with pH 6.8, 7.5 and 8.5, respectively	
1 M Tris	120 g of Tris base, dissolve in 900 mL ddH_2O in a 1 L sterile glass bottle. Adjust the pH to the respective value by adding corresponding volumes of concentrated HCl (37%). Make sure all Tris is dissolved before the pH adjustment is completed. Fill up to 1 L with ddH_2O.

Note: store at 20°C–24°C for up to one year.

⚠ CRITICAL: The HCl fumes are toxic and cause respiratory and skin irritation. Open the concentrated HCl (37%) in the fume hood and perform the pipetting steps in the fume hood. Wear personal protective equipment.

5 M NaCl	
5 M NaCl	292 g of NaCl, fill up to 1 L with ddH_2O in a glass bottle. Place the bottle in a 37°C water bath to dissolve the NaCl. If necessary, readjust ddH_2O to 1 L.

Note: The solution can be stored at 20°C–24°C for up to one year.

Lysis buffer

Reagent	Final concentration	Amount
NaCl (5 M stock)	150 mM	1.5 mL
Tris, pH 7.5 (1 M stock)	50 mM	2.5 mL
NP-40	0.5%	250 μL
cOmplete protease inhibitor cocktail	n/a	One tablet
Sodium fluoride	1 mM	2 mg
Sodium orthovanadate	1 mM	9 mg
Sodium pyrophosphate decahydrate	1 mM	22 mg
β-Glycerophosphate disodium salt hydrate	1 mM	11 mg
ddH_2O	n/a	45.75 mL
Total	**n/a**	**50 mL**

Mix Tris, NaCl and 40 mL ddH_2O at 20°C–24°C in a 50 mL conical tube and add NP-40. Dissolve NP-40 by rotating the conical tube end-over-end for 10 min at 20°C–24°C. Fill tube with ddH_2O to 50 mL. Add protease and phosphatase inhibitors and dissolve on ice. Thoroughly mix buffer before use and make sure the detergent and inhibitors are dissolved.

Note: The lysis buffer can be stored 1–2 days at 4°C.

Note: Prepare fresh lysis buffer on day 2. Each experimental co-IP sample requires up to 5 mL of buffer (1 mL for cell lysis and 3 × 1 mL to wash the pulldown beads). The leftover lysis buffer should be stored at 4°C for the washing steps on day 3.

Note: To reduce protein denaturation by detergents, non-ionic detergents such as NP-40, Tween-20 or Triton-X100 are preferred. Typically, detergent concentrations in the range of 0.1%–2% are used. However, some epitopes may be most accessible in the absence of detergent. In this event the cells are homogenized by mechanical force, e.g., in a Dounce homogenizer (Simpson, 2010).

6× sample buffer (Laemmli buffer) (Laemmli, 1970)		
Reagent	Final concentration	Amount
Tris, pH 6.8 (1 M stock)	375 mM	9.375 mL
Glycerol	50%	12.5 mL
Sodium dodecyl sulfate	9% (w/v)	2.25 g
Bromophenol blue	0.03% (w/v)	15 mg
ddH_2O	n/a	Fill to 25 mL
Total	**n/a**	**25 mL**

⚠ CRITICAL: SDS causes respiratory irritation and should be weighed in the fume hood. Personal protective equipment including a face mask should be used.

Weigh SDS in a 50 mL conical tube. Add Tris, Glycerol and Bromophenol blue and fill to 25 mL with ddH_2O. Place the tube in a 37°C water bath for 30 min to dissolve the SDS. After dissolution of the SDS check again the volume and adjust to 25 mL with ddH_2O. Aliquot in 2 mL microcentrifuge tubes.

Note: Store aliquots at −20°C. The sample buffer can be stored up to one year. To obtain reducing buffer conditions, add β-mercaptoethanol to 10% (v/v) before use.

Separation gel buffer (Tris 1.5 M, pH 8.8)	
Tris 1.5 M	181 g of Tris base, add 800 mL ddH_2O, adjust pH to 8.8 with concentrated HCl (37%). Fill with ddH_2O to 1 L.

Note: Store at 20°C–24°C. The separation gel buffer can be stored up to one year.

⚠ CRITICAL: see above comments regarding toxic HCl fumes.

Stacking gel buffer (Tris 0.5 M, pH 6.8)	
Tris 0.5 M	60 g of Tris base, add 800 mL ddH_2O, adjust pH to 6.8 with concentrated HCl (37%). Fill with ddH_2O to 1 L

Note: Store at 20°C–24°C. The stacking gel buffer can be stored up to one year.

⚠ CRITICAL: see comments regarding toxic HCl fumes above.

20% SDS solution	
20% SDS	20 g of SDS in a 200 mL bottle. Add ddH_2O to 90 mL. Place the bottle in a 37°C water bath to dissolve the SDS. Once the SDS is dissolved, fill ddH_2O up to 100 mL.

Note: If the SDS precipitates over time place the bottle in the 37°C water bath until the SDS is redissolved.

Note: The SDS solution can be stored at 20°C–24°C for up to one year.

⚠ CRITICAL: SDS causes respiratory irritation, see comment above.

10× Running buffer		
Reagent	Final concentration (10×)	Amount
Tris base	250 mM	120 g
Glycine	2 M	576 g
Sodium dodecyl sulfate (SDS)	1% (w/v)	40 g
ddH_2O	NA	Fill to 4 L
Total	**NA**	**4 L**

⚠ CRITICAL: SDS causes respiratory irritation, see comment above.

Weigh Tris base, SDS and glycine and add to a graded 5 L plastic beaker. In the fume hood, place beaker on a stir plate, add a large stir bar. Add 2.5 L ddH_2O and avoid generating dust from the SDS powder. Stir until the solution is homogenous. Fill beaker to 4 L with ddH_2O. Store 10× running buffer in a plastic carboy with spigot at 20°C–24°C. To obtain 1× working solution for polyacrylamide gel electrophoresis (PAGE) dilute 1:10 with ddH_2O.

Note: The pH of this buffer is expected to be 8.3 and no pH adjustments should be needed.

Note: Store 1× buffer at 20°C–24°C for up to one month. The reuse of running buffer is discouraged since the background signals of the immunoblots increase with the accumulation of proteins in the buffer.

10× Western blot transfer buffer		
Reagent	Final concentration (10×)	Amount
Tris base	250 mM	120 g
Glycine	2 M	576 g
ddH_2O	n/a	Fill to 4 L
Total	**n/a**	**4 L**
Weigh Tris base and glycine and dissolve in 2.5 L ddH_2O in a beaker on a stir plate. Fill with ddH_2O to 4 L.		

Note: Store the 10× stock solution in a plastic carboy in the cold room at 4°C for up to one month. For 1 L of 1× transfer buffer fill in a 1 L glass bottle 100 mL from the 10× transfer buffer stock, add 100 mL ethanol and fill to 1 L with ice cold ddH_2O.

Note: Cool down the 1× transfer buffer to 4°C before use. The reuse of transfer buffer is discouraged since the background signal of the immunoblot increases with the accumulation of proteins in the buffer.

⚠ CRITICAL: Ethanol is toxic and flammable and transfer buffer should therefore be disposed following appropriate waste procedures.

Note: Some protocols for protein transfer use methanol instead of ethanol for the transfer buffer. In our experience ethanol-based transfer buffer worked consistently with transfer onto PVDF membranes.

10× Tris buffered saline with Tween (TBST)		
Reagent	Final concentration (10×)	Amount
NaCl	1.5 M	352 g
KCl	27 mM	8 g
Tris base	250 mM	120 g
Tween 20	0.5%	20 mL
ddH_2O	n/a	Fill to 4 L
Total	**n/a**	**4 L**

Add NaCl, KCl and Tris base in a 5 L plastic beaker and fill 2.5 L ddH_2O and dissolve on a stir plate. Adjust pH to 7.4 with HCl (37%). Add Tween and fill with ddH_2O to 4 L. Continue to stir until the solution is homogenous. Sterile filter the solution through a 0.2 μm cell culture vacuum unit into 1 L glass bottles.

Note: Store at 20°C–24°C for up to one month. Dilute 1:10 with ddH_2O before use.

10× Phosphate buffered saline (PBS)		
Reagent	Final concentration (10×)	Amount
NaCl	1.37 M	320 g
KCl	27 mM	8 g
Na_2HPO_4	100 mM	57.6 g
KH_2PO_4	18 mM	9.6 g
ddH_2O	n/a	Fill to 4 L
Total	**n/a**	**4 L**

Dissolve salts in 2.5 L ddH_2O on a stir plate. Adjust the pH to 7.4. Fill to 4 L with ddH_2O. Dilute 1:10 in ddH_2O to obtain 1× working solution. Autoclave the 1× PBS in sterile glass bottles for cell culture applications.

Note: Store at 20°C–24°C for up to one month.

Coumarin stock solution (400×)		
Reagent	Final concentration	Amount
p-Coumaric acid	90 mM	148 mg
DMSO	n/a	10 mL
Total	**n/a**	**10 mL**

Weigh p-Coumaric acid and add to a 15 mL conical tube. Add 10 mL of DMSO and dissolve at 20°C–24°C. Aliquot 100 μL volumes in 0.5 mL microcentrifuge tubes.

Note: store at −20°C for up to one month.

Luminol stock solution (200×)		
Reagent	Final concentration	Amount
Luminol	244 mM	433 mg
DMSO	n/a	10 mL
Total	**n/a**	**10 mL**

Weigh Luminol and add to a 15 mL conical tube. Add 10 mL of DMSO and dissolve at 20°C–24°C. Aliquot 100 μL volumes in 0.5 mL microcentrifuge tubes.

Note: store at −20°C for up to one month.

Enhanced chemiluminescence (ECL) solution		
Reagent	Final concentration	Amount
Luminol (200× stock)	1.2 mM	50 µL
Coumarin (400× stock)	2.5 mM	25 µL
Tris 1 M, pH 8.5	100 mM	1 mL
H_2O_2 (35% w/w)	0.01%	3 µL
ddH_2O	n/a	9.9 mL
Total	**n/a**	**10 mL**

Thaw the Luminol and p-Coumaric acid stock solutions. In a 15 mL conical tube mix Tris, Luminol, p-Coumaric acid, ddH_2O and H_2O_2. Always prepare fresh ECL solution just in time for the immunoblot exposure. The solution can be used for multiple blots.

Note: Do not store the solution.

Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and antibiotic-antimycotic		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	n/a	500 mL
Fetal bovine serum (FBS)	10%	50 mL
antibiotic-antimycotic (100×, AA)	1×	5 mL
Total	**n/a**	**555 mL**

Add FBS and AA to the DMEM.

Note: The complete DMEM medium can be stored at 4°C for up to one month.

STEP-BY-STEP METHOD DETAILS

Day 1: Cell seeding and transfection

Timing: 2 h

Note: The HEK cells are seeded at the beginning of the day and transfected with plasmid DNA around 7–8 h later.

Note: Various co-IP and IP protocols have previously been outlined in detail (Bonifacino et al., 2001; DeCaprio and Kohl, 2017b; DeCaprio and Kohl, 2020; Lin and Lai, 2017; Takahashi, 2015; Markham et al., 2007). Here, we present an adapted step-by-step guide for co-IP experiments from lysates of cultured cells.

Note: For this protocol, the HEK cells are grown in cell culture plastic dishes with 10 cm diameter. At confluency a dish can yield around 9 million cells. For the transfection, the cells are seeded at 5 million cells per dish. Before the experiment, expand sufficient amounts of cells.

1. Day 1, morning. Aspirate the cell culture medium and add 5 mL of sterile PBS to wash the cells.
2. Aspirate the PBS and add 1 mL trypsin/EDTA solution. Incubate at 37°C in the cell culture incubator for around 2 min or until the cells detach.
3. Add 8 mL of PBS and resuspend the cells. Collect the cell suspension in a 50 mL conical tube.

4. Spin down the cells at 180 × g for 2 min.
5. Aspirate the supernatant and resuspend cells in 5–10 mL PBS.
6. Mix 20 μL cell suspension with 20 μL trypan blue solution in a 1.5 mL microcentrifuge tube and pipette into a cell counting slide.
7. Count live cells manually with a hemocytometer or with an automated cell counter to determine the cell density. Trypan blue stained dead cells are excluded. Per 10 cm dish $5*10^6$ cells are needed. Calculate the required volume of cell suspension.
8. Prepare cell master mix. Per dish fill 10 mL of DMEM/10% FBS/AA medium in a 50 mL conical tube.
9. Add the corresponding volume of cell suspension to the conical tube. Mix thoroughly by inverting the tube.
10. Add 10 mL of cell master mix per 10 cm dish.
11. Place the dishes in the incubator for 7–8 h.

Breakpoint: the cells can be grown longer (e.g., 9–14 h) but they should be transfected within 12 h after seeding. If the cells are not in the exponential growth phase and grown to high densities the transfection efficiency may be reduced (Tom et al., 2008).

12. Day 1, afternoon. Thaw a fresh aliquot of PEI transfection solution.

Reagent	Tube 1	Tube 2
DMEM medium (no additives)	490 μL	470 μL
PEI transfection solution		30 μL
Plasmid nr 1, bait protein (concentration 1 μg/μL). GFP fusion construct.	5	
Plasmid nr 2, prey protein (concentration 1 μg/μL). HA-tag fusion construct.	5	
Total	500 μL	500 μL

To prepare the transfection mix, use DMEM medium without serum or antibiotics.

Note: Here PEI transfection is used for transient protein expression in HEK cells (Longo et al., 2013). However, alternative transfection approaches including calcium phosphate transfection or viral gene delivery may be considered.

Note: If more than two plasmids are used, adjust the amount of DNA to not exceed 10 μg in total.

13. Add DMEM to two microcentrifuge tubes. Add plasmid DNA to tube 1, and add PEI to tube 2. Pipette the PEI suspension from tube 2 dropwise to tube 1 and mix by tipping the tube. Let stand for 15 min at 20°C–24°C.

Note: Do not extend beyond the 15 min incubation time. The transfection efficiency decreases with prolonged incubation.

14. Add the transfection mix dropwise to the HEK cells across the 10 cm dish. Slightly rotate the dishes to mix the medium and transfection mix. Put the dishes back into the incubator.

Day 2: Cell collection, lysis, and incubation with antibodies and protein-G agarose beads

Timing: 4 h

Note: Prepare fresh lysis buffer with protease and phosphatase inhibitors as described above. Cool down the lysis buffer and PBS on ice. Cool down cell lifters and 2 mL microcentrifuge tubes in the cold room. Cool down the refrigerated table top centrifuge to 4°C. Prepare a 1 mL pipette and pipette tips in the cold room. Cool down an aluminum cooling block for 2 mL tubes in an ice bucket in the cold room.

15. Day 2. At 12–15 h after transfection, check the GFP expression in a fluorescence microscope that fits plastic cell culture dishes and is equipped with filters for GFP.

Note: HEK cells are receptive to PEI transfection and after 12–15 h most cells should show GFP expression. If the expression is low, wait up to 18 h post transfection. Upon prolonged culture the number of cells increases. To maintain efficient cell lysis and protein and lipid solubilization, the volume of lysis buffer should be adjusted accordingly. See troubleshooting comment below (problem 2).

16. Transfer the cell culture dishes to the cold room. Aspirate the medium. Wash cells once with 5 mL ice cold PBS. Aspirate the PBS and remove the remaining liquid with a pipette.

Note: HEK cells are weakly adherent and may detach when washed. Be observant to add the PBS slowly to the edge of the dish.

Note: Cell collection, sonication, solubilization, immunoblotting and the long-term incubations are performed in a cold room at 4°C.

17. Detach the cells with a cold cell lifter and collect cells in one spot of the dish. With a 1 mL pipette add 500 µL of PBS and collect the cell suspension in a 2 mL tube. Place the plastic tube in an aluminum cooling block on ice.
18. Spin down the cells in a refrigerated table top centrifuge at 4°C at 400 × *g* for one minute. Aspirate the supernatant. Resuspend the cells in 1 mL of ice-cold lysis buffer.

Note: Adjust the centrifugation speed, some cells may require more gentle spins. Also, the composition of the lysis buffer can be adjusted depending on the nature of the protein and the cell line (DeCaprio and Kohl, 2017a).

Note: In this protocol the bait and prey proteins are strongly overexpressed in HEK cells. If low abundant endogenous protein complexes are analyzed by co-IP, the volume of lysis buffer may be reduced. With smaller volumes of lysis buffer, however, make sure that the cells are still properly lysed and that the membrane lipids are solubilized.

19. Disrupt the cells with a probe-type sonicator for 10 s at 20% amplitude in the cold room. To solubilize proteins and lipids, rotate the tubes in the cold room for 30 min.

Note: Sonication rapidly disrupts the cells, shears nucleic acids and generates homogenous cell lysates. Adjust the sonication conditions to reduce the generation of foam and splashes.

20. To remove cell debris, spin down the cell lysate in a table top centrifuge at maximum speed for 10 min. Collect the supernatants in fresh ice cold 1 mL microcentrifuge tubes. Discard the pellets.
21. For each sample, collect 50 µL cell lysate in an ice-cold 1.5 mL tube and store at 4°C in the fridge. These samples will be used to address the protein expression before co-IP.

Note: Here it is assumed that for all samples equal amounts of cells are lysed in equal volumes of buffer. However, the overexpression of certain proteins may reduce or enhance the growth

rate of the cells. To further adjust for potential variabilities, the protein concentration should be determined (e.g., by a BCA assay) and samples should be adjusted for equal amount and concentration of lysate. Moreover, the protein concentration of the lysate should be assessed by probing the expression of a housekeeping protein by immunoblotting. Frequently used housekeeping proteins are actin, tubulin, and GAPDH (Figure 3B).

22. To the remaining 950 µL cell lysate add 2 µL GFP antibody (mouse monoclonal, Roche, 11814460001, corresponds to 0.8 µg of antibody).

Note: In this protocol a mouse monoclonal GFP antibody is used for the immunoprecipitation and detection of the bait protein and an influenza hemagglutinin (HA) tag antibody is used for the detection of the prey protein. There are other tags and corresponding high affinity antibodies available, including FLAG and V5. Depending on the species and the immunoglobulin isotype protein-A or protein-G coupled agarose is used. Resources for the selection can be found in previously published co-IP protocols (Bonifacino et al., 2016).

Note: Antibody affinities vary and the antibodies are stored at different protein concentrations. Consult the information provided in the datasheet on how to best use the antibody of interest for co-IP experiments.

Note: Some proteins display an affinity to agarose beads. To reduce non-specific binding, the cell lysates can be pre-cleared with protein-G agarose beads in the absence of antibody. To this end, protein-G agarose (for volumes and preparation see below) is incubated with the cell lysate rotating for 30 min at 4°C. The beads are spun down at 10 × *g* and the supernatant is collected and processed as described above.

23. For each co-IP sample 30 µL of protein-G agarose slurry is needed. Thoroughly mix and resuspend the protein-G agarose stock solution to get a homogenous slurry.
24. To facilitate the pipetting of the beads, cut 2 mm from a 1 mL pipette tip and transfer the corresponding total volume of slurry to a 2 mL tube. Fill the tube with ice cold lysis buffer and mix.
25. Spin down the slurry at 10 × *g* in a refrigerated table top centrifuge at 4°C. Repeat the wash step two more times to remove the storage solution from the beads.
26. Resuspend the beads in 100 µL lysis buffer per sample.

Note: Slowly pipette the bead solutions to avoid damaging the agarose beads.

Note: Alternatively, magnetic beads coated with antibody can be used. See problem 4 in the troubleshooting section for more detail.

Note: To assure equal amounts of protein-G agarose beads in all samples, the beads can be distributed to the corresponding number of microcentrifuge tubes and the protein concentration per tube can be determined using a BCA assay. Once the volume of beads is equilibrated across all the tubes, the beads are spun down, the supernatant is removed and the cell lysates are added to the beads.

27. Add 100 µL bead suspension per cell lysate sample.

Note: In this protocol the interaction of two exogenously overexpressed proteins is probed. As a negative control, GFP without a fused bait protein is used. In an experiment where endogenous protein complexes are being pulled down, antibody isotype controls can be used to rule out non-specific binding. Optimized isotype control antibodies are available for many species. Alternatively, knockout cells lacking expression of the protein of interest can be used as a negative control.

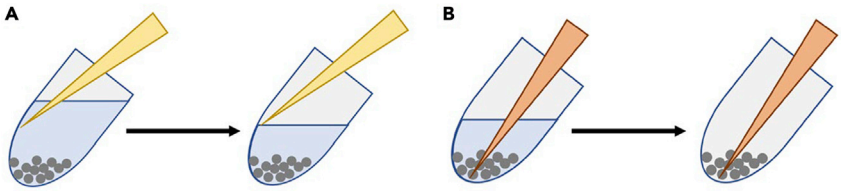

Figure 2. Critical agarose bead washing procedures

(A) For the washing steps 100–200 μL lysis buffer are left behind in the microcentrifuge tube at each washing step to keep the pellet intact. The 200 μL pipette tips (yellow) are placed well apart from the beads at the wall of the tube.

(B) After the last washing step, the entire liquid is removed from the beads via a 20 μL tip (red) placed at the bottom of the tube. Multiple rounds of aspiration may be needed to remove the liquid.

Note: Here, unconjugated antibodies specific for GFP are used for the co-IP. Alternative experimental setups use antibodies covalently coupled to agarose beads or to magnetic beads or biotin conjugated antibodies that are pulled down with streptavidin conjugated beads.

28. Rotate the samples in the cold room at 4°C for at least 9–12 h.

Day 3: Gel electrophoresis, protein transfer, and incubation with primary antibodies

Timing: 6 h

Note: Polyacrylamide gel electrophoresis (PAGE) has been described in detail and step-by-step protocols and video instructions are available (Chrambach and Rodbard, 1971; Smith, 1994; Brunelle and Green, 2014).

Before you start: cool down the table top centrifuge. Thaw an aliquot of 6× sample buffer and warm to 37°C to dissolve precipitated detergent. Add 10% β-mercaptoethanol (BME) to the 6× sample buffer to establish reducing buffer conditions.

29. Day 3. Spin the co-IP samples at 10 × *g* in the table top centrifuge for 1 min at 4°C to pellet the agarose beads.
30. Collect the supernatant in a separate 1.5 mL tube for potential follow up analyses. To avoid perturbations of the bead pellet, leave behind around 100–200 μL liquid in the tube (Figure 2A).
31. Add 1 mL ice-cold lysis buffer to wash the agarose beads. Flip the tube upside-down to wash the beads. Spin the tubes at 10 × *g* for one minute at 4°C. Aspirate and discard the supernatant. Repeat the wash step for two more times.
32. After a total of three wash steps use a 20 μL pipette to remove the remaining liquid from the beads. Place the tip at the bottom of the tube and aspirate the liquid until the beads are dry (Figure 2B).

Note: alternatively, a syringe with a narrow needle can be used to aspirate the remaining liquid.

33. Add 40 μL of 6× sample buffer/10% BME to the beads. Add 10 μL 6× sample buffer/10% BME to the 50 μL lysate samples, which were collected on day 2.
34. Heat all co-IP and lysate samples at 95°C for 5 min.
35. Mix samples by tapping the tubes and spin down for 30 s at 10 × *g* at 20°C–24°C in a table top centrifuge.

Note: Here the agarose beads remain in the co-IP sample. Alternatively, to exclude the beads, elute the proteins with 0.1 M glycine pH 2–3 for 1 min. Spin down the samples for 1 min at

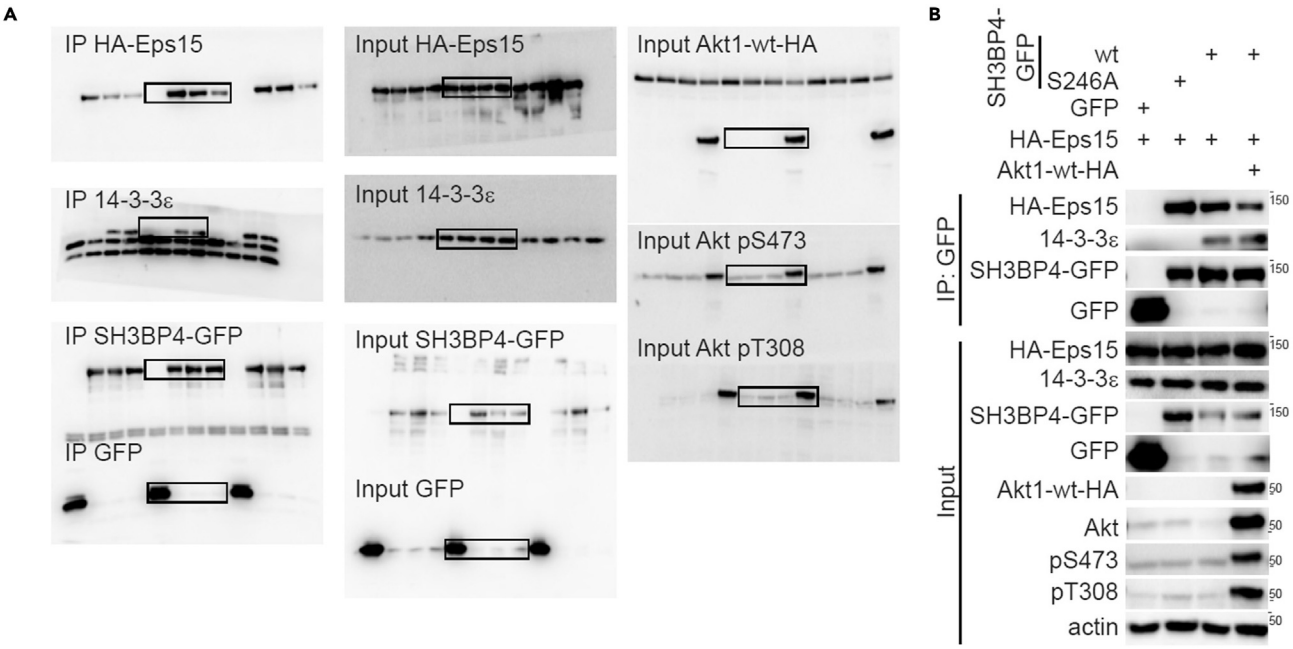

Figure 3. Representative immunoblot data for the experiment laid out in Figure 1

(A) Uncropped original immunoblots are shown. They were obtained from an experiment as it is outlined in Figure 1. Immunoprecipitation (IP) and input (Input) samples are shown. The cropped regions are indicated by boxes. For these immunoblots, three repeats of the same experiment were loaded, the replicate in the middle was chosen for the figure panel in (B).

(B) Cropped immunoblots from (A) are assembled into the figure panel. Most blots are shown in full size in (A). Data is reproduced with permission from Burckhardt et al. (2021).

10 × *g* and collect the supernatant and neutralize with 1 M Tris pH 8.5. Repeat elution 2–3 times, pool the supernatant and add sample buffer. Elution with glycine is advantageous for co-IP reactions where the antibody is covalently coupled to the beads (Kaboord and Perr, 2008). In this situation the antibody is not eluted into the sample. However, elution with glycine increases the sample volume and thus dilutes the co-IP sample. Alternatively, to reduce antibody elution, the antibodies can be crosslinked to the beads prior to the co-immunoprecipitation (Iqbal et al., 2018).

36. Plan the number of gels that will be needed.

Note: For the example experiment that is presented here (Figure 1C), two blots for the co-IP are analyzed (GFP, HA/14-3-3ε where the membrane is cut according to molecular weight) and six blots are used to analyze the input lysate (GFP, HA, 14-3-3ε, Akt, pS473, pT308), resulting in a total of eight blots.

37. Assemble the glass plates on the gel casting system and add tap water to test for potential leaks. Reassemble if needed or seal the corners of the plates with grease.
38. After the leak test, remove and drain the water from the space between the plates.

Note: In our experience, fresh self-cast polyacrylamide gels give the most reproducible results. However, the use of pre-cast gels may be more time effective.

39. Prepare a small volume of fresh 10% (w/v) ammonium persulfate (APS) stock solution. Per gel, weigh 10 mg APS and add 100 μL ddH_2O.

Note: Leftover 10% APS solution can be stored at 4°C for a few days. But the APS solution is not stable and loses activity.

40. For a 1 mm thick 10% polyacrylamide separation gel (mini-gel format, Bio-Rad) around 4.5 mL of separation gel solution are needed. Use the following volumes:

Reagent	Final concentration	Amount (1 gel)
40% Acrylamide/Bis (37.5:1)	10%	2.5 mL
separation gel buffer (1.5 M Tris pH 8.8)	375 mM	2.5 mL
20% SDS	0.2%	50 µL
10% APS	0.1%	50 µL
TEMED	0.1%	5 ul
ddH_2O	n/a	4.9 mL
Total		**5 mL**

41. Mix the Acrylamide/Bis solution, Tris, ddH_2O and TEMED in a 15 mL conical tube at 20°C–24°C.
42. Add the APS and quickly mix the solutions and start pouring the gels.
43. With a 1 mL pipette add the separation gel solution between the glass plates to a level 5 mm below the reach of the comb.
44. Overlay the separation gel with 1 mL of isopropanol to smoothen the upper surface of the gel. The polymerization of the separation gel may take 5–10 min. Follow the polymerization state of the leftover gel in the conical tube.

Note: For one co-IP experiment up to 6–10 gels may be needed. Prepare a gel master mix in a 50 mL conical tube and cast the gels in one run.

Note: The Acrylamide/Bis solution and the TEMED are stored at 4°C.

45. When the leftover gel in the conical tube is polymerized, remove the isopropanol from the top of the separation gel by inverting the gel.
46. Rinse the surface of the gel once with ddH_2O and remove and drain the residual water.

Note: Depending on the molecular weight of the proteins of interest, higher or lower-percentage gels may be needed. Consult gel separation charts for best separation results. Charts are provided by various vendors of electrophoresis equipment and reagents.

47. For the 5% stacking gel use the following volumes:

Reagent	Final concentration	Amount
40% Acrylamide/Bis (37.5:1)	5%	0.25 mL
Stacking gel buffer (0.5 M Tris pH 6.8)	375 mM	0.625 mL
20% SDS	0.2%	12.5 µL
10% APS	0.1%	12.5 µL
TEMED	0.1%	2.5 ul
ddH_2O	n/a	1.6 mL
Total		**2.5 mL**

48. Mix the solutions in a 15 mL conical tube in the same order as for the separation gel and add the APS right in time to cast the gels.
49. Add the stacking gel solution on top of the separation gel and fill to the top of the glass plates.

50. Slowly place the comb for either 10 or 15 wells. Follow the polymerization status of the leftover gel solution in the conical tube.
51. Once the leftover gel is polymerized, assemble the electrophoresis unit, place it in the buffer tank, remove the comb and add running buffer to the top of the chamber.
52. With a 20 μL pipette flush the wells with running buffer to remove unpolymerized gel solution from the wells.
53. Let the electrophoresis chamber stand for a couple minutes and check for leaks. Reassemble the unit or refill with running buffer if needed.
54. Fill the unit with running buffer until the marks. Remove the air bubbles at the bottom end of the gel with a pipette.
55. Load the molecular weight standard according to the instructions on the datasheet. The amount of molecular weight standard needed depends on the sensitivity of the gel imaging system.
56. Load 15-20 μL of protein sample for 10-well gels and 12–15 μL of sample for 15 well gels (volumes for 1 mm thick gels).

Note: Store leftover samples at −20°C in case some of the immunoblots need to be repeated or additional proteins need to be probed.

57. Connect the electrophoresis unit to the power supply. Run the gel at constant 100 V for 90 min and regularly check the fill level of the electrophoresis chamber and the progress of the samples. If necessary, refill the unit with running buffer.
58. Run the samples until adequate protein separation is reached.

Note: Gels can be run at higher voltage but the higher running speed may affect the quality of the protein separation.

59. Prepare 2 L (per 2 gels) of ice cold 1× transfer buffer with 10% ethanol.
60. Cut the PVDF membrane to cover the gel region of interest and add 5 mm extra membrane. Cut the Whatman papers to extend 1 cm beyond the gel.
61. Disassemble the electrophoresis unit and remove one glass plate from the gel. Use a razor blade to cut away the stacking gel.
62. Add 1 L of transfer buffer to the transfer unit. Add 1 L of transfer buffer to a square plastic casserole dish (25 × 25 × 7 cm). Use enough buffer to submerge all components of the immunoblot sandwich.
63. Label the PVDF membrane with a pencil (e.g., with date and experiment ID). To wet and activate the PVDF membrane, add 10 mL methanol to a small plastic container and soak the PVDF membrane for 2 min in methanol.
64. Assemble the immunoblot sandwich in the casserole dish with the white or clear side of the blotting cassette facing down.
65. Add one sponge and two Whatman papers. Place the PVDF membrane on top of the Whatman papers in the casserole.
66. Remove the gel from the glass plate and place the gel on the membrane.
67. Arrange the gel, membrane and Whatman papers and press down the stack to remove bubbles.
68. Keep the stack submerged and add two more Whatman papers and another sponge and close the cassette with the black lid, while keeping the sandwich submerged in transfer buffer.
69. Place the immunoblot sandwich in the transfer unit with the latch facing up and the black lid facing the black side of the unit.
70. Move the transfer unit to the cold room and place it in a plastic tray to contain potential spillovers. Fill the unit to the top with transfer buffer.
71. Add the lid and place some extra weight on the lid to prevent connectivity issues (e.g., use a bottle with leftover buffer) and connect with the power supply.
72. Run the transfer at a constant current of 400 mAmp for 1 h at 4°C. Check for the emergence of gas bubbles on the outside of the electrophoresis unit as an indicator for transfer activity.

⚠ CRITICAL: Methanol and ethanol are toxic and flammable and are considered hazardous waste. See comments above.

Note: Immunoblotting has been described in detail (Gallagher et al., 2008; Litovchick, 2020). The transfer conditions can be adjusted according to the size and properties of the protein of interest. Here wet-blotting is described, however, semidry blotting can be used too.

Note: Some combinations of proteins and antibodies may work best on nitrocellulose membranes. Consult the literature as per the antibody of interest.

Breakpoint: the transfer can be run over night in the cold room with appropriately adjusted transfer conditions and timing. Colling packs may be inserted into the transfer unit to avoid overheating.

73. Check the antibody datasheet on how to best block the membrane. Most antibodies are used in combination with membranes that were blocked with 10% milk or 10% bovine serum albumin (BSA) solution.
74. Prepare 50 mL of 10% dry milk in TBST and pour the solution into a small plastic container.
75. Once the transfer is completed, take out the transfer cassette and blotting sandwich, recover the PVDF membrane and place it in the milk solution.
76. Block the PVDF membrane for 20 min at 20°C–24°C on a shaker.

Note: Rinse the transfer unit, the sandwich cassette and the sponges with deionized water. Over time proteins will accumulate in the sponges and may transfer on the membrane and increase the background signal. The sponges can be soaked in methanol to remove residual protein. Afterwards, rinse the sponges with deionized water and let dry. Discard the methanol following hazardous waste protocols.

77. Prepare the primary antibody incubation solutions. For small plastic containers an incubation volume of 4–5 mL is sufficient.

Note: For the selection of the antibody incubation solution consult the datasheet. Frequently used solutions are 5% BSA in TBST, 5% dry milk in TBST or proprietary antibody incubation solutions.

78. Dilute the antibody according to the instructions and your prior optimization. Incubate the PVDF membranes with primary antibody for 9–12 h on a shaker in the cold room at 4°C.

Note: the primary antibodies described in this protocol can be used at 1:1000 dilution.

Breakpoint: depending on the antibody, the incubation time can be reduced to a couple hours.

Note: In order to reduce the antibody incubation volume to save reagents, the membranes can be sealed in plastic wrap. A volume of 1–2 mL antibody solution may sufficiently cover the membrane.

Note: Antibody solutions can be stored at −20°C for reuse. Label the tube to keep track of the usage. Antibodies will be depleted with each use.

Note: The membrane can be cut into smaller portions to probe for multiple proteins of different sizes. Make sure each membrane fragment is clearly labeled.

Day 4: Incubation with secondary antibody, exposure, and signal quantification

Timing: 4 h

79. Day 4. Get the membranes from the cold room and collect the primary antibody solution in a 15 mL conical tube. Label the tube and store at −20°C for reuse.
80. Add 25 mL TBST to rinse the membrane, and discard the solution. Repeat for a total of three rinse steps. Then wash the membrane in TBST on the shaker for 20 min at 20°C–24°C. Repeat the rinse and wash cycle for a total of three times.
81. Prepare the secondary horseradish peroxidase (HRP) coupled antibodies. Here, the HRP coupled antibodies are diluted 1:5000 in a 5% milk/TBST solution.
82. Check the instructions on the datasheet and use the recommended incubation solution and antibody dilutions. Incubate the membrane with the HRP-coupled antibodies for 1 h shacking at 20°C–24°C.

Note: To reduce the signal from immunoglobulin light and heavy chains, secondary HRP coupled antibodies are available that detect only the properly folded primary antibody that is used for the immunodetection. These secondary antibodies do not detect denatured immunoglobulins from the co-IP step and thus reduce or eliminate the signals from immunoglobulin heavy and light chains from the blots.

83. Rinse and wash the membrane as described above. Do three cycles.
84. Prepare fresh ECL solution.
85. Incubate the membrane in 10 mL ECL solution in a plastic container for 30–60 s at 20°C–24°C.
86. Drain the membrane and move to a fresh plastic container and place in the blot imaging station. Adjust the position of the membrane and start the image acquisition.
87. Record images until the chemiluminescence signal is clearly visible and stop before the signal reaches saturation.

Note: For particularly weak signals, commercial high sensitivity ECL reagents can be used (e.g., SuperSignal West Femto, Thermo Fisher).

88. At the end of the imaging series, acquire an image of the molecular weight standard on the immunoblot membrane. This image will be used to estimate the molecular weight of the protein bands.
89. Once image acquisition is completed, extract the images in 16-bit TIF format for the densitometry and quantification.
90. For the immunoblot densitometry, open Image Studio Light. Open the image of interest in 16-bit TIF format.

Note: Various free and commercial software packages for immunoblot densitometry are available (e.g., ImageJ/Fiji, the software at your imaging station, etc.).

91. Make sure that no pixels in the region of interest are saturated. Otherwise chose images with shorter exposure times.
92. Draw boxes around the protein bands of interest (Figure 4A).
93. Copy the resulting background subtracted intensity values into a spread sheet (Figure 4B, Table S1A).

Note: The intensity values from the co-IP of 14-3-3ϵ are found in the cells D4 to D6. In this example the value of D4 was negative and was therefore set to zero. The intensity values of the protein bands from the co-IP immunoblot for SH3BP4-GFP are found in the cells D10-D12 (Figure 4B).

A

B

	A	B	C	D	E	F
1	co-IP SH3BP4-GFP vs 14-3-3e (endogenous), HA-Eps15 with OX Akt1-wt					
2						
3	co-IP 14-3-3e			Intensity	normalized with IP GFP	normalized with wt
4	pEGFP SH3BP4-GFP S246A		HA-Eps15	0	0.00	0.00
5	pEGFP SH3BP4-GFP wt		HA-Eps15	2860000	0.36	1.00
6	pEGFP SH3BP4-GFP wt	Akt1 wt-HA	HA-Eps15	2260000	0.50	1.40
7						
8						
9	co-IP GFP					
10	pEGFP SH3BP4-GFP S246A		HA-Eps15	8700000		
11	pEGFP SH3BP4-GFP wt		HA-Eps15	7990000		
12	pEGFP SH3BP4-GFP wt	Akt1 wt-HA	HA-Eps15	4510000		

C

	A	B	C	D	E	F	G	H	I	J	K
1	co-IP SH3BP4-GFP vs 14-3-3e (endogenous), HA-Eps15 with OX Akt1-wt										
2											
3	co-IP HA-Eps15			Day 1, nr1	Day 1, nr2	Day 1, nr3	Day 2, nr1	Day2, nr2	Day 2, nr3	Day 3	average
4	pEGFP SH3BP4-GFP S246A		HA-Eps15	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5	pEGFP SH3BP4-GFP wt		HA-Eps15	1.06	0.29	0.42	0.55	0.47	1.19	0.13	0.59
6	pEGFP SH3BP4-GFP wt	Akt1 wt-HA	HA-Eps15	0.38	0.29	0.10	0.24	0.20	0.39	0.10	0.24
7											
8	co-IP 14-3-3e (endogenous)										
9	pEGFP SH3BP4-GFP S246A		HA-Eps15	0.00	0.00	0.00	0.01	0.00	0.00	0.38	0.06
10	pEGFP SH3BP4-GFP wt		HA-Eps15	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
11	pEGFP SH3BP4-GFP wt	Akt1 wt-HA	HA-Eps15	3.21	1.40	1.87	0.74	1.21	1.12	1.14	1.53

D

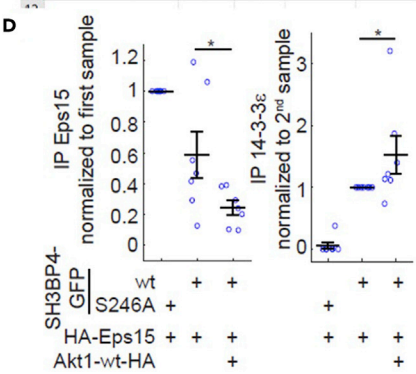

Figure 4. Densitometry of immunoblot data for semi-quantitative analysis

(A) Example immunoblot data is shown with representative boxes used for the densitometry of the protein signal by the Image Studio Light software. For an experiment as outlined in Figure 1, representative immunoblots for the co-Immunoprecipitation (IP) of 14-3-3ϵ and Eps15 are shown.

(B) Example of the quantification of 14-3-3ϵ co-immunoprecipitation (IP) signal after normalization with the SH3BP4-GFP IP signal (see Table S1A). Negative intensity values were set to zero. The intensity values are normalized with the middle sample.

(C) Example quantification of the experiment outlined in Figures 1 and 3. The data from seven repeats that were performed on three separate days was combined. The function AVERAGE in Excel was used to calculate mean values (see Table S1B).

(D) The co-IP data and statistical analysis of SH3BP4-GFP binding to HA-Eps15 and to endogenous 14-3-3ϵ are shown. The data is from the experiment described in Figures 1, 3, and 4A–4C. For statistical analysis the Wilcoxon rank sum test was used and mean and +/− standard error of the mean are shown, * $P < 0.05$. The data is reproduced with permission from Burckhardt et al., (2021).

94. Next, normalize the signal of the prey proteins with the signal from the GFP-tagged bait protein.

Note: Here, the intensity of the co-IP of 14-3-3ϵ is divided by the intensity of the co-IP of SH3BP4-GFP. Thus, in the spreadsheet, cell E5 reads "=D5/D11" (Figure 4B, Table S1A).

95. Next, use the intensity of the control sample of the experiment to normalize the samples with perturbations.

Note: In this experiment, the second sample with the co-IP of SH3BP4-GFP wt in the absence of Akt1-wt-HA overexpression (cell E5) is considered the control. Consequently, the cell F5 is

set to one. The intensity values for the other samples are divided by the intensity value of the control. To this end, the cell F4 reads "=E4/E5' and cell F6 reads "=E6/E5" (Table S1A).

96. Combine the normalized values from 3-5 experiments in the spread sheet (Figure 4C, Table S1B).

Note: Here, the data from seven experiments that were performed on three separate days were combined (Figure 4C). The values from Figure 4B cells F4 to F6 were transferred to the spreadsheet in Figure 4C in the cells E9 to E11. Next the arithmetic mean values are calculated. Hence for the co-IP of 14-3-3ϵ vs. SH3BP4-GFP wt in the presence of Akt1-wt-HA overexpression (row 11) the mean is calculated in the spreadsheet by the function "=AVERAGE(D11:J11)" in the cell K11 (Figure 4C, Table S1B).

97. Use a spreadsheet or statistics software package to calculate mean, error bars and statistical significance and to plot the data (Figure 4D).

Note: In this plot, the values from the cells D5 to J5 are plotted for the second sample of the co-IP of Eps15 (Figure 4D). And the mean value for this sample is obtained from the cell K5 in Figure 4D and Table S1B.

Note: Densitometry has been used to quantify immunoblots and the protocols and limitations have been described in detail (Taylor and Posch, 2014; Gassmann et al., 2009). Critical factors include sample preparation, the validity of the antibodies, the linear range of the assay and the setup of the detection system (Pillai-Kastoori et al., 2020). Moreover, here a fix point reference sample is used (Figures 4B and 4C). However more advanced approaches for sample normalization have been proposed and can be implemented (Degasperi et al., 2014).

98. Process the immunoblot images to assemble the figure panel. Open the TIF image in ImageJ/Fiji (or your image processing software of choice).
99. Scale the image intensity, crop the region of interest and save in a format that is compatible with the software used for the assembly of the figures (e.g., Illustrator, PowerPoint, etc.).
100. Assemble the figure according to the guidelines from the journal of interest.

EXPECTED OUTCOMES

A successful co-IP experiment should contain a negative control sample with no co-IP signal (background control) and a clearly detectable but not saturated signal for the positive control sample. The co-IP bait signals should show equal pulldown. The supernatant samples for both bait and prey should show equal protein expression levels. Finally, the loading control should indicate equal protein levels for all samples (Figures 3A and 3B).

For strong protein-protein interactions the immunoblot signals likely are distinct and perturbations may induce pronounced changes in the binding capacity. This may lead to more than 5-fold differences in protein binding, which may be statistically highly significant. For weaker protein-protein interactions, however, the immunoblot signals are more heterogeneous and the effects of perturbations may be less obvious. Two to four-fold differences are typically observed. Given this variability, with 5–7 repeats from three independent experiments, significance levels may end up in the range of 0.01–0.05 (Figure 4D) (Burckhardt et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

From the immunoblots semi-quantitative data can be derived by densitometry. The results from multiple experimental repeats should be combined and statistical tests should be used to assess the significance of the observed differences (Pollard et al., 2019) (Figures 4C and 4D). Here we are

proposing to outweigh some of the aforementioned limitations of co-IP experiments by increasing the number of repeats to 3–5 or more, if necessary. If the data points follow a normal distribution the Student T-test can be used to address significance levels. If the data doesn't follow a normal distribution, alternative approaches such as the Wilcoxon rank-sum test should be used.

LIMITATIONS

As highlighted throughout the protocol, co-IP experiments have considerable limitations (Takahashi, 2015) and each individual step along the protocol is prone to artifacts. The protocols that are referenced above provide in-depth analyses of these pitfalls. However, a couple experimental and analytical limitations of this co-IP protocol should be addressed in more detail. First, proteins are transiently overexpressed at non-physiological levels which may prevent accurate protein folding, mis-localize proteins, prevent or induce posttranslational modifications or lead to proteolytic cleavage. When overexpressed, proteins may artificially interact with one another in ways that do not occur at endogenous expression levels. Furthermore, overexpression may colocalize proteins that under physiological conditions would be separated into different cellular compartments. Additionally, small and large peptide tags are introduced to facilitate the co-IP and immunodetection. The tags may interfere with protein structure and function. Additionally, the lysis buffer dissociates membranes and de-compartmentalizes proteins and dilutes binding partners. Consequently, transient interactions that depend on membrane binding, high local crowding by compartmentalization or have high off-rates may be difficult to detect. Moreover, additional co-factors may be expressed at low levels and may become limiting. For all the above reasons, it is important to validate the findings from co-IP experiments with purified and characterized proteins in-vitro and with orthogonal assays under physiological conditions in-vivo.

Frequently, co-IP experiments are presented as a series of immunoblots without a quantification of the protein signals. And most often, data from the experimental repeats are not combined for statistical analysis. Here, we are proposing to combine semi-quantitative immunoblot densitometry and statistical assessments to add numbers to the data. Importantly, this protocol can be implemented in labs equipped with standard digital gel imaging systems and it does not require specialized infrastructure. There are, however, multiple limitations to this approach. First, depending on the nature of the protein interaction, the overexpression of bait and prey proteins may favor complex formation to the extent that regulatory mechanisms are overrun. Therefore, the protein overexpression should be adjusted to remain within the dynamic range of the complex formation. Second, the chemiluminescence based protein detection amplifies signals in a non-linear way. This issue is particularly apparent when super-sensitive ECL reagents are used. Fluorescently labeled antibodies that are detected by fluorescence scanners should be considered to obtain a more quantitative readout. Also, the protein detection should be validated to be in a linear range by serial dilution experiments (Janes, 2015). Lastly, to obtain a quantitative understanding of protein interactions, systematic measurements with purified proteins and more sophisticated modalities are needed. Experiments where the bound and unbound ligand can be measure in a concentration dependent manner allow for the quantification of binding parameters. Such methods include enzyme-linked immunosorbent assay (ELISA), Förster resonance energy transfer (FRET) or surface plasmon resonance (SPR) biosensors. Alternatively, isothermal titration calorimetry (ITC)(Velazquez-Campoy et al., 2004) can be used to determine the thermodynamics of protein-protein interactions. In conclusion, the protocol presented here allows for a cost and time effective interrogation of protein complex formation in cell lysates and adds a semi-quantitative measurement and statistical assessment. However, due to the limitations of the protocol, the results should be validated by quantitative experimentation with purified proteins and under physiological settings in-vivo.

TROUBLESHOOTING

Problem 1

Weak protein overexpression (step 15).

Potential solution

Use strong promoters that are active in your cell line (e.g., SV40, CMV, EF1A etc.).

Increase the amount of plasmid for the protein with the lowest expression.

Transfect HEK cells a second time on day 2 and delay the following steps. Change the medium after each transfection to not accumulate the PEI. If the protocol is delayed, adjust the cell number used for lysis or the volume of lysis buffer (see below).

Large proteins above 200 kDa tend to express at lower levels. Consider transient expression by an adenovirus system. Alternatively, establish stable expression by lentiviral transduction and selection via resistance markers or via flow cytometry sorting for high GFP expression.

Consider expressing protein fragments that contain the interaction domain rather than the full-length protein.

Problem 2

The cellular lipids are not solubilized. After sonication, solubilization and the maximum speed spin, lipids may remain floating at the surface of the cell lysate. Particularly membrane proteins tend to associate with these lipids and may contaminate the pull-down experiment (step 19).

Potential solution

Reduce the number of cells. Alternatively, increase the volume of lysis buffer and/or increase the concentration of the detergent up to 2%.

Problem 3

Absent or weak prey protein co-IP (step 87).

Potential solution

Include an established binding partner as a positive control either as prey or bait. If there is no established binding partner, use a pair of characterized interaction partners as a positive control for the troubleshooting.

Protein-protein interactions are often regulated by post translational modifications (including phosphorylation, ubiquitination, etc.), conformational changes, enzymatic activities, intramolecular inhibition and activation, and many more. Explore whether a mutant or truncated version of the protein of interest could be more active in the binding assay.

The protein-protein interaction may be weak and transient with a high off-rate. To stabilize protein complexes, include 5–10% glycerol in the lysis buffer.

For transmembrane and membrane associated proteins try different mild non-ionic detergents like Tween-20 or Triton-X-100 (Helenius et al., 1979; Helenius and Simons, 1975) to stabilize protein complexes.

If the protein of interest is known to complex substrates, ions, nucleotides etc., make sure they are included in the lysis buffer. Consult the literature regarding tailored buffer conditions for specific proteins.

Problem 4

Unspecific protein binding to beads and immunoglobulins (step 87).

Potential solution
If only one of the proteins of interest is sticking to the beads, swap the tags and put the tag for immunoprecipitation on the sticky protein.

Non-solubilized lipids could promote contamination of the co-IP, see above. Increase the amount of detergent or reduce the number of cells.

Pre-adsorb the cell lysate with agarose beads as described above.

Use a different type of beads or adjust the pull-down approach (e.g., biotin-streptavidin, magnetic beads).

Problem 5
Degradation of overexpressed proteins (step 87).

Potential solution
Include sufficient amounts of protease inhibitor in the lysis buffer and keep samples on ice and refrigerated at all times.

Use a more protease resistant and flexible linker for the GFP fusion protein (e.g., $[GGGS]_n$).

Place the GFP on the other end of the protein or even in internal flexible loops.

For small proteins the fusion with a relatively large 26 kDa GFP moiety may reduce protein stability. Swap tags and use the GFP on the other protein.

Problem 6
HEK cells do not tolerate the PEI transfection. At high concentrations PEI shows cytotoxic effects (step 15).

Potential solution
Reduce the amount of PEI. Alternatively, increase the number of seeded cells.

Let the cells attach and spread longer before the PEI transfection.

Refresh the medium a couple of hours after the transfection to remove the excess of PEI that did not associate with the cells.

Problem 7
The agarose beads disappear during the washing steps (step 31).

Potential solution
Start with a larger volume of beads, e.g., 40 μL instead of 30 μL.

Leave behind more liquid in the tube after the washing steps.

Pipette slowly to not perturb the agarose beads.

Problem 8
The immunoblot signal is saturated, or the bands are smeared out or the signal appears quenched at the center of the bands. Due to the strong over expression the supernatant samples may be overloaded (step 87).

Potential solution

Avoid the super sensitive ECL reagents and use a less sensitive reagent (as described above).

Dilute the samples 1:2, 1:3 or 1:10 for the PAGE.

Problem 9

Strong signals from the immunoglobulin light chain (25 kDa) and heavy chain (50 kDa) interfere with the signal from the proteins of interest (step 87).

Potential solution

Probe the immunoblot with an antibody derived from a different species.

Change the percentage of the gel to establish better separation between the protein of interest and the Immunoglobulin signal.

To reduce the elution of antibodies, they can be crosslinked to the beads prior to the immunoprecipitation (Iqbal et al., 2018). Amine reactive crosslinker such as DSSO disuccinimidyl sulfoxide (DSS, Thermo Fisher Scientific), disuccinimidyl suberate (DSS) or dithiobis (succinimidyl propionate) (DSP) can be used.

Use conformation specific secondary antibodies that do not detect the denatured immunoglobulins (as described above).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gaudenz Danuser (Gaudenz.Danuser@utsouthwestern.edu).

Materials availability

Materials are available upon reasonable request.

Data and code availability

Data is available upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100644.

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AUTHOR CONTRIBUTIONS

C.J.B. conceived and designed the work and executed experiments and analyses with overall scientific observation by G.D.; C.J.B. wrote the manuscript; J.D.M. provided scientific guidance and critical insights.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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