



1 ▼

Feb 11, 2022

🌐 Crosslinking assay to study a specific cargo-coat interaction through a transmembrane receptor in the secretory pathway V.1

Javier Manzano-Lopez †¹, Sofia Rodriguez-Gallardo †¹,
Susana Sabido-Bozo¹, Ana Maria Perez-Linero¹, [Rafael Lucena](#)¹,
Antonio Cordones-Romero¹, Sergio Lopez¹, Auxiliadora Aguilera-Romero¹,
[Manuel Muñiz](#)¹

¹Department of Cell Biology, Faculty of Biology, University of Seville and Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41012, Seville, Spain.

Javier Manzano-Lopez †: †These authors contributed equally to this work. *Corresponding author.

Sofia Rodriguez-Gallardo †: †These authors contributed equally to this work.

Auxiliadora Aguilera-Romero: *Corresponding author.

Manuel Muñiz: *Corresponding author.

1



dx.doi.org/10.17504/protocols.io.bytapwie

Manuel Muñiz

Intracellular trafficking through the secretory organelles depends on transient interactions between cargo proteins and transport machinery. Cytosolic coat protein complexes capture specific luminal cargo proteins for incorporation into transport vesicles by interacting with them indirectly through a transmembrane adaptor or cargo receptor. Due to their transient nature, it is difficult to study these specific ternary protein interactions just using conventional native co-immunoprecipitation. To overcome this technical challenge, we have applied a crosslinking assay to immobilize the transient and/or weak protein interactions. Here, we describe a protocol of protein cross-linking and co-immunoprecipitation, which was employed to prove the indirect interaction in the endoplasmic reticulum of a luminal secretory protein with a selective subunit of the cytosolic COPII coat through a specific transmembrane cargo receptor. This method can be extended to address other transient ternary interactions between cytosolic proteins and luminal or extracellular proteins through a transmembrane receptor within the endomembrane system.

DOI

dx.doi.org/10.17504/protocols.io.bytapwie

Javier Manzano-Lopez †, Sofia Rodriguez-Gallardo †, Susana Sabido-Bozo, Ana Maria Perez-Linero, Rafael Lucena, Antonio Cordones-Romero, Sergio Lopez, Auxiliadora Aguilera-Romero, Manuel Muñiz 2022. Crosslinking assay to study a specific cargo-coat interaction through a transmembrane receptor in the secretory pathway. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bytapwie>



Crosslinking, DSP, COPII, receptor, p24 complex, GPI-APs

protocol ,

Oct 05, 2021

Oct 07, 2021

53826

Recipes

Growth media

1. SD agar plates: synthetical minimal medium containing 2% glucose, 0.67% yeast nitrogen base, 0.5% ammonium sulfate, and 2% bacteriological agar, supplemented with the appropriate nutritional requirements (0.012% except for adenine 0.03%) to complement auxotrophies.
2. SD medium: synthetical minimal medium containing 2% glucose, 0.67% yeast nitrogen base and 0.5% ammonium sulphate, supplemented with the appropriate nutritional requirements (0.012% except for adenine 0.03%) to complement auxotrophies.
3. YPD medium: rich medium containing 2% glucose, 1% yeast extract, and 2% peptone, supplemented with 0.2% adenine and uracil.

Buffers

1. 1000x PI (Protease inhibitor cocktail): antipain 0.1%, leupeptin 0.1% and pepstatin 0.1% in DMSO
2. 100x PMSF: 100mM phenylmethylsulphonyl fluoride in isopropanol
3. B88: 20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM Mg(OAc)₂, 1 mM PMSF, 1x PI
4. PBS (Phosphate Buffered Saline): 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄ pH 7.4, 1 mM PMSF, 1x PI
5. PBS-D 0.2%: PBS containing 0.2% (w/v) digitonin
6. PBS-D 1%: PBS containing 1% (w/v) digitonin
7. PBS-D 5%: PBS containing 5% (w/v) digitonin
8. 10x DSP fresh stock solution (10mM DSP in DMSO anhydrous). Dissolve DSP in DMSO anhydrous immediately before use.

8. 10x glycine stock solution: 250 mM glycine pH 7.5
9. SB 2x (Sample buffer): 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8. Add 2-mercaptoethanol immediately before use.
10. TBS (Tris-Buffered Saline): 20 mM Tris-HCl pH8, 150 mM NaCl
11. TBS-T; TBS containing 10% Tween-20
12. TBS-T+ 5% milk: TBS-T containing 5% skimmed milk powder
13. Stripping buffer: TBS-T + 100 mM 2-mercaptoethanol + 1 ml SDS 20%

Equipment

1. Incubator shaker (Eppendorf, model M1299-0092)
2. Micropipettes (Gilson, Pipetman, models: P20, P200, P1000)
3. Spectrophotometer or microplate reader (any brand with 600 nm wavelength) or equipment to count cell density
4. Bead beater (Fastprep, MP Biomedicals, model: Fastprep-24).
5. Centrifuge (Thermo scientific, model: Heraeus Fresco 17; Eppendorf, model: centrifuge 5818R)
6. Orbital wheel (any brand)
7. Thermoshaker (Thermo Fisher, model 13687711)
8. Electrophoresis system (Mini PROTEAN, Bio-Rad catalog number: 1658005)
9. Power supply (Bio-Rad, catalog number: 1645052)
10. Western Blot Transfer system (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad catalog number: 1703930)
11. Orbital shaker (any brand)

Materials

1. Budding yeast cells (W303) genomically expressing Lst1-mCherry with a centromeric plasmid expressing GFP-tagged Gas1 under control of its own promoter (pRS416-GAS1-GFP). This yeast strain can be obtained upon request from our laboratory.
2. Toothpicks sterilized before use (any brand)
3. Yeast extract (Pronadisa catalog number: 1702.00)
4. Peptone (Panreac catalog number: A2210,0500)
5. Glucose (Panreac catalog number: 131341.0914)
6. Yeast nitrogen base without amino acids (Difco Laboratories, catalog number: 291940)
7. Nutritional supplements:
 - a. Uracil SIGMA catalog number: U0750
 - b. L-Leucine SIGMA catalog number: L8000
 - c. L-Tryptophan SIGMA catalog number: T0254
 - d. Adenine SIGMA catalog number: A8626
 - e. L-Histidine SIGMA catalog number: H8000
 - f. L(+)-Lysine SIGMA catalog number: L5501
 - g. L-Methionine SIGMA catalog number: M9625)
8. Agar (Oxoid catalog number: LP0011)
9. 50 ml Falcon tubes (any brand)
10. 1,5 ml screw-cap microcentrifuge tubes (any brand)
11. Glass beads (SIGMA catalog number: G9268-500G)
12. 1,5 ml UltraClear microcentrifuge tubes (Axygen catalog number: MCT-175-L-C)
13. DSP (dithiobis[succinimidylpropionate]) Lomant's Reagent (Thermo Fisher Scientific catalog

- number: 22585)
18. DMSO (Dimethyl sulfoxide) anhydrous (Sigma catalog number: 276855-100ML)
 13. Digitonin (Panreac AppliChem catalog number: A1905,0005)
 16. Naked Bab Agarose beads (ChromoTek, catalog number: bab-20)
 14. GFP-Trap Agarose beads (ChromoTek, catalog number: gta-20)
 15. Protease Inhibitors (PI):
 - a. Antipain (SIGMA catalog number: A6191)
 - b. Leupeptin (SIGMA catalog number: L2023)
 - c. Pepstatin A (SIGMA catalog number: P4265)
 19. PMSF (Amresco catalog number: 0754-5G)
 20. Glycine (Amresco number catalog: 0167-1KG)
 21. PBS (Phosphate Buffered Saline) (Amresco catalog number: 0780-50L)
 21. Tris (Amresco catalog number: 0497-1KG)
 22. NaCl (Panreac catalog number: 131659.1211)
 23. EDTA (Amresco catalog number: 0322-500G)
 24. HCl 5mol/l (Panreac catalog number: 182109.1211)
 25. Acetic Acid Glacial (Panreac catalog number: 141008.1211)
 26. 10% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl (Bio-Rad catalog number: 4561023)
 30. 2-Mercaptoethanol (Merck catalog number: M3148-25ML)
 31. Nitrocellulose membrane (Amersham™ Protran® Western blotting membranes, nitrocellulose, Merck catalog number: GE10600002)
 22. Tween-20 (polyoxyethylene sorbitan) (Amresco catalog number: 0777-1L)
 23. Skimmed milk powder (any brand)
 24. RFP antibody [6G6] (ChromoTek, catalog number: AB_2631395)
 25. Non-commercial antibodies to Sec24, Emp24, Pgc1, and GFP were generous gifts from the Riezman lab (University of Geneva, Switzerland).

Yeast growth and culture

- 1 Transform the yeast strain genomically expressing Lst1-mCherry with a centromeric plasmid expressing GFP-tagged Gas1 under control of its own promoter (pRS416-GAS1-GFP).
- 2 Pick up a transformed colony and streak it on an SD agar plate with appropriate amino acid supplements (SC-URA) and incubate them at 24°C for 2-3 days.
- 3 Inoculate transformed yeast cells in 3ml of SC-URA in a 12ml sterile tube and grow them to the early-to-mid logarithmic phase at 24°C with shaking at 250 rpm.
- 4 Dilute yeast cells in 200 ml of YPD medium and grow them overnight at 24°C with shaking at 250 rpm until reaching 1.5×10^7 cells/ml. Note: YPD medium is required for correct cell surface expression of Gas1-GFP.

- 5 Collect 120×10^7 cells per sample condition and centrifuge at $3000 \times g$ for 5 min at 4°C .
- 6 Discard the supernatant and resuspend by vortexing the cell pellet in 1.5 ml of B88 buffer precooled at 4°C . Transfer the cell suspension to a 2 ml screw-cap tube.
- 7 Centrifuge at $13,000 \times g$ for 1 min at 4°C , discard supernatant and freeze the pellet at -80°C .

Cell lysis

- 8 Quick thaw the cell pellets and immediately place on ice.
- 9 Resuspend each cell pellet with 1.5 ml of prechilled B88 buffer.
- 10 Add 300 μl of glass beads per tube and lyse mechanically the cells using a bead beater system. For Fastprep device: 3 pulses of 20 s at 5 m/s. Rest on ice for 3 min between pulses.
- 11 Spin down glass beads and cell debris at $1000 \times g$ for 10 min at 4°C .
- 12 Collect 1 ml of supernatant into a 1.5 ml ultraclear tube.

Crosslinking

- 13 During the 10 min centrifugation at 4°C at $1000 \times g$ (step 11), prepare a fresh 10x DSP stock solution (10mM DSP in DMSO anhydrous).
- 14 Add 110 μl of 10 x DSP stock solution to the cleared cell extract (step 12) and incubate for 20 min at 20°C in a thermoblock.

- 15 Quench the cross-linking reaction by adding 150 µl of 10x glycine stock solution and incubate for 5 min at 20°C.
- 16 Spin down cellular membranes at 13,000 x g for 15 min at 4°C to enrich the pellet with ER membranes.
- 17 Discard the supernatant and resuspend each membrane pellet in 1 ml of PBS at 4°C.

Co-immunoprecipitation of crosslinked proteins

- 18 Add 250 µl of PBS-D 5% digitonin to the tubes with the crosslinked samples from step 17 to obtain a solution with a final concentration of 1% digitonin. Mix with end-over-end rotation for 1 h at 4°C.
- 19 Centrifuge the cell suspension at 17,000 x g for 20 min at 4°C to remove insoluble membranes.
- 20 Transfer the supernatant to a 1.5 ml ultraclear tube and add 100 µl of a 15% solution of naked agarose beads (Bab beads) and rotate for 1 h at 4°C to remove unspecific protein binding.
- 21 Centrifuge at 5,000 x g for 30 s and transfer the supernatant to a 1.5 ml ultraclear tube.
- 22 Save a 20 µl aliquot of each supernatant for the analysis of protein expression. These will be the total lysate input samples.
- 23 Add 100µl of GFP-Trap agarose beads (30% slurry) to the remainder of the supernatant (step 21) and rotate overnight at 4°C.
- 24 The next day centrifuge at 5,000 x g for 30 s and remove supernatant.

Resuspend the beads pellet with 1 ml of PBS-D 1% and transfer to a new 1.5 ultraclear tube.

25

26 Centrifuge at 5,000 x g for 30 s and remove the supernatant.

27 Add 1 ml PBS-D 0.2%. Repeat steps 25 and 26 twice more.

28 Centrifuge at 5,000 x g for 30 s and remove the supernatant. Centrifuge again to remove the remaining liquid and dry the beads using a white gel-loading micropipette tip.

29 Elute proteins from the beads and dissociate the crosslinked protein complexes by adding 40 µl of 1x SDS-PAGE sample buffer containing fresh 5% 2-mercaptoethanol at 95°C for 10 min.

30 Vortex and spin down the beads to the maximum speed at room temperature for 1 min and collect the supernatant (IP sample).

31 Add 20 µl of 2x SDS-PAGE sample buffer containing fresh 5% 2-mercaptoethanol to the tubes containing 20 µl of the total lysate input (T sample) from step 21, vortex and incubate at 95°C for 10 min.

Western blotting

32 Load the IP samples and their respective T samples onto a 10 % acrylamide gel, separate the proteins by SDS-PAGE gel electrophoresis and transfer them to a nitrocellulose membrane.

33 Check protein transfer by incubating the nitrocellulose membrane with Ponceau Staining Solution for 1min and washing it with distilled water.

34 Block the unspecific antibody binding by incubating the membrane in TBS-T + 5% milk for 30 min with shaking.

35 Blot the crosslinked proteins with anti-RFP (1:500), anti-Sec24 (1:1000), anti-Emp24 (1:1000), anti-GFP (1:500), and anti-Pgk1 (1:5000) antibodies sequentially with membrane stripping

between blots to detect Lst1-mCherry, Sec24, Pgk1, and Gas1-GFP respectively.

Stripping

- 36 Wash the membrane 3x 3 min in TBS-T.
- 37 Incubate 15 min at room temperature in TBS-T + 100 mM mercaptoethanol + 1 ml SDS 20% (heat only the TBS-T and add the mercaptoethanol and the SDS).
- 38 Wash 3x 5 min in TBS-T.
- 39 Incubate 30 min at room temperature in stripping buffer (20 mM glycine-HCl, pH 2% and 1% SDS).
- 40 Wash the membrane 6x 5 min in TBS-T. The stripped membrane is now ready for reprobing with another antibody. The stripped membrane can be also kept overnight in TBS-T + 5% milk + azide at 4°C. In this case, wash the membrane 3x 3 min in TBS-T before reprobing with the antibody.

Controls

- 41 Carry out appropriate binding specificity controls:
 - A) Non-expressing Gas1-GFP yeast cells.
 - B) Without crosslinker.
 - C) Detection of a non-specific protein such as the cytosolic protein Pgk1.