Aug 23, 2022

Immunoblotting analysis of samples from GolgiTAG (TMEM115-3HA) immunoprecipitation

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asap

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ABSTRACT

Analysis of the expression of organelle-specific markers is essential to verify the efficiency of any Golgi immunoprecipitation (IP) protocol. Here, we describe our immunoblotting method to assess efficient enrichment of Golgi proteins in Golgi immunoprecipitation products compared to whole cell lysates, as well as the purity of the immunoprecipitated Golgi by monitoring the expression of other organelles' markers. This method can also be used to verify the expression of GolgiTAG (TMEM115-3HA) in cells that are transiently or stably transfected.

Note: For a detailed description of our method for Golgi immunoprecipitation, refer to dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1 (Introducing GolgiTag to Cells and Immunoprecipitation of Golgi).

Note: This protocol was adapted from dx.doi.org/10.17504/protocols.io.bsgrnbv6 (Quantitative Immunoblotting Analysis of LRRK2 Signalling Pathway).

ATTACHMENTS

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DOI

dx.doi.org/10.17504/protocols.io.bp2l61oxdvqe/v1

PROTOCOL CITATION

Rotimi Fasimoye, Dario R Alessi 2022. Immunoblotting analysis of samples from GolgiTAG (TMEM115-3HA) immunoprecipitation. **protocols.io** https://protocols.io/view/immunoblotting-analysis-of-samples-from-golgitag-t-cfbytipw

FUNDERS ACKNOWLEDGEMENT

Aligning Science Across Parkinson's (ASAP) initiative
Grant ID: ASAP-000463

KEYWORDS

GolgiTAG, Immunoprecipitation, LRRK2 Signalling Pathway

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CREATED

Aug 16, 2022

LAST MODIFIED

Aug 23, 2022

OWNERSHIP HISTORY

Aug 16, 2022 madhavi.d

Aug 18, 2022 Dario R Alessi

PROTOCOL INTEGER ID

68696

MATERIALS TEXT

Reagents:

Lysis buffer:

A	B
HEPES-KOH pH 7.5	50 mM
Triton X-100	1% (v/v)
EGTA	1 mM
Na3VO4**	1 mM
NaF	50 mM
β-glycerophosphate	10 mM
Sodium pyrophosphate	5 mM
Sucrose	0.27 M
cOmpleteTM	
EDTA-free Protease Inhibitor Cocktail (Roche, 11836170001)**	
Microcystin-LR (Enzo Life Sciences, ALX-350-012)**	1 µg/ml

**: To be added fresh before use.

☒ cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog ##11836170001)**

☒ Microcystin-LR **Enzo Life Sciences Catalog #ALX-350-012**

☒ Pierce™ BCA Protein Assay Kit **Thermo**

- **Fisher Catalog #23227**
- **4X Loading buffer:** Invitrogen™ NuPAGE™ LDS Sample Buffer, cat no NP0007;

4X SDS loading buffer:

A	B
Tris-HCl pH 6.8	250 mM
SDS	8% (w/v)
Glycerol	40% (v/v)
Bromophenol blue	0.02% (w/v)

Note: Supplement with 5% (v/v) beta-mercaptoethanol before use.

☒ NuPAGE 4–12% Bis–Tris Midi Gel **Thermo Fisher**

- **Scientific Catalog #WG1403BOX** or a self-cast 10% Bis-

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Tris gel.

SDS-PAGE buffer:

For NuPAGETM Bis-Tris gels:

NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #NP000102**

For self-cast Bis-Tris gels:

A	B
MOPS	50 mM
Tris	50 mM
SDS	0.1% (w/v)
EDTA	1 mM

Protein transfer buffer:

A	B
Tris-base	48 mM
Glycine	39 mM
Methanol (freshly supplemented)	20% (v/v)

TBS-T:

A	B
Tris–HCl pH 7.5	50 mM
NaCl	150 mM
Tween 20	0.1% (v/v)

- Membrane blocking solution: 5% (w/v) non-fat milk powder in TBS-T.
- Antibody dilution buffer: 5% (w/v) bovine serum albumin (BSA) in TBS-T.
- Primary antibodies and near-infrared fluorescent IRDye secondary antibodies (See Table 1 and Table 2).

GM130 (D6B1) XP® Rabbit mAb **Cell Signaling Technology Catalog #12480**

Golgin-97 (D8P2K) Rabbit mAb **Cell Signaling Technology Catalog #13192**

HA **Sigma Catalog #11867423001**

HSP60 (D6F1) XP® Rabbit mAb **Cell Signaling Technology Catalog #12165**

VDAC **Contributed by users Catalog #4661**

Calreticulin (D3E6) XP® Rabbit mAb **Cell Signaling Technology Catalog #12238**

LAMP1 (C54H11) Rabbit mAb **Cell Signaling Technology Catalog #3243**

Alpha-tubulin **Cell Signaling Technology Catalog #3873S**

Table 1:

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A	B	C	D	E
Antibody Target	**Company**	**Cat.Number (RRID)**	**Host species**	**Dilution**
GM130 (Golgi marker)	Cell Signalling Technology	12480 (RRID:AB_2797933)	Rabbit	1:1000
GOLGIN97 (Golgi marker)	Cell Signalling Technology	13192 (RRID:AB_2798144)	Rabbit	1:500
ACBD3 (Golgi marker)	Sigma Atlas	HPA015594 (RRID:AB_1844491)	Rabbit	1:1000
HA	Sigma	11867423001 (RRID:AB_390918)	Rat	1:1000
HSP60 (mitochondrial marker)	Cell Signalling Technology	12165 (RRID:AB_2636980)	Rabbit	1:1000
VDAC (mitochondrial marker)	Cell Signalling Technology	4661 (RRID:AB_10557420)	Rabbit	1:1000
Calreticulin (ER marker)	Cell Signalling Technology	12238 (RRID:AB_2688013)	Rabbit	1:1000
LAMP1 (lysosomal marker)	Cell Signalling Technology	3243 (RRID:AB_2134478)	Rabbit	1:1000
CSTC (lysosomal marker)	Santa Cruz	Sc-74590 (RRID:AB_2086955)	Mouse	1:1000
alpha-tubulin (cytoplasmic marker)	Cell Signalling Technology	3873S (RRID:AB_1904178)	Mouse	1:5,000

IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody **Licor Catalog #926-32212**

IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody **Licor Catalog #926-32213**

Goat anti-Rat IRDye 800CW **LI-COR Catalog #926-32219**

Table 2:

A	B	C
Secondary Antibodies	**Company**	**Cat. Number (RRID)**
Donkey anti-mouse IRDye 800CW	LI-COR	926-32212 (RRID:AB_621847)
Donkey anti-rabbit IRDye 800CW	LI-COR	926-32213 (RRID:AB_621848)
Goat anti-Rat IRDye 800CW	LI-COR	926-32219 (RRID:AB_1850025)

Equipment:

- Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).
- Plate reader for Protein quantification (BioTek Epoch, or equivalent).
- Digital Dry Bath/Block Heater, 2 block configuration, CN, EU, UK plugs, 200-240V **Thermo Fisher Catalog #88870005**
- SureLock Midi-Cell Electrophoresis System (if using Invitrogen NuPAGE precast midi gels), or equivalent gel electrophoresis apparatus.
- Protein transfer apparatus: Trans-Blot® Cell (Bio-Rad), or equivalent wet transfer system.

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- See-saw rocker (VWR SSL4, or equivalent).
- Odyssey CLx Imaging System paired with Image StudioTM Software.

Preparation of IP samples or whole cell lysates samples:

1 For a detailed description of our method for Golgi immunoprecipitation, refer to dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1 (Introducing GolgiTag to Cells and Immunoprecipitation of Golgi). This also includes a description of how to prepare whole cell lysates samples.

Preparation of samples for immunoblot analysis:

2 Determine the protein concentration of IP or cell lysate samples by BCA Protein Assay Kit according to the manufacturer's instructions, performing measurements in triplicate.

Note: Ensure the concentration of the samples is in the linear range for the BCA Protein Assay. If it is not, prepare appropriate dilutions in water of each lysate. Generally, protein concentrations of IP samples should range from [M]**0.1 µg/µL** to [M]**0.3 µg/µL** . For near confluent cells lysed as described above, protein concentration should range from [M]**0.5 µg/µL** to [M]**5 µg/µL** (depending on cell type).

3 Prepare samples for immunoblotting to achieve the same protein concentration for all samples (ideally, [M]**0.1 µg/µL** - [M]**2 µg/µL** , depending on the sample at the lowest concentration).

3.1 Dilute with lysis buffer as appropriate.

3.2 

Add a quarter of a volume of 4X SDS/LDS loading buffer freshly supplemented with beta-mercaptoethanol (i.e. for **7.5 µL** of lysate/lysis buffer mix, add **2.5 µL** of loading buffer).

3.3 

Mix by vortexing.

4  5m

Incubate samples for **00:05:00** at **70 °C** heating block before immunoblot analysis.

Note: if blotting for TMEM115, do not heat sample. We noticed that boiling leads to the TMEM115 not going into the resolving gel during electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

5 

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Load samples (**2 µg** for IP samples or **10 µg** for whole cell lysate samples) onto a NuPAGE 4–12% Bis–Tris Midi Gel (ThermoFisherScientific, Cat#WG1402BOX or Cat#WG1403BOX), or a self-cast 10% Bis-Tris gel, alongside pre-stained molecular weight markers (ranging from 10 kDa to 250 kDa). Rinse wells carefully with running buffer before loading samples.

- For optimal signal, the amount of protein loaded for IP samples is **2 µg** while for whole cell lysate sample ranges from **2 µg** - **20 µg** .
- Be aware of maximum loading capacity of each well as per manufacturer's instructions and take care not to overload wells.
- If multiple gels are used for each set of experimental samples, an internal loading control should lso be included for subsequent data normalization.

6 Electrophorese samples at 120V with MOPS SDS running buffer for **02:00:00** or until the blue dye runs off the gel. 2h

Protein transfer (Wet electroblotting):

7 Equilibrate the gel, one piece of nitrocellulose membrane (GE Healthcare, Amersham Protran Supported **0.45 µm** NC) and two pieces of filter paper (Whatman™ 3MM Chr Chromatography Paper, or equivalent) (all the same size as the gel) by pre-soaking them in transfer buffer.

8 Assemble the gel and membrane transfer stack in a tray filled with transfer buffer to ensure that all components are submerged during the assembling.

8.1 Place one sponge pad inside the cassette holder (on the side that will be facing the cathode).

8.2 Place one piece of filter paper on top of the sponge pad, followed by the gel, nitrocellulose membrane, another piece of filter paper and another sponge pad.

Note: Carefully remove any air bubbles between layers using a roller after adding each layer.

9 Carefully close the cassette holder and insert it in the transfer tank. Fill the tank with transfer buffer.

10 Electrophoretically transfer proteins from gel onto a nitrocellulose membrane at 90 V (constant voltage) for **01:30:00** **On ice** using a wet transfer system. 1h 30m

11 After transfer, stain membranes with Ponceau solution to assess transfer efficiency and general quality of the samples.

If an image is required for record, the Ponceau-stained membraned can be scanned.

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12

Each membrane can be divided into two sections by one horizontal cut (just above the 50 kDa ladder band): 'top section' (from the top of the membrane to the 50 kDa marker) and 'bottom section' (from the 50 kDa marker to the bottom of the membrane) (Figure 1), to be incubated with primary antibodies as described in step 14.

Notes: When immunoblotting using anti-Tubulin, it is preferable not to cut the membrane as the protein is 50 kDa.

Membrane blocking and antibody incubation:

13 15m

Destain membranes from Step 2 by washing with TBS-T and incubate in blocking solution for at least **00:15:00** at **Room temperature** on a see-saw rocker.

14

Rinse the membrane in TBS-T and incubate **Overnight** at **4 °C** with primary antibodies (diluted in 5% (w/v) BSA in TBS-T to their working concentration – Table 1), as follows:

Notes: Table 1 lists the primary antibodies (and suggested working dilution) mostly used in our lab to study the GolgiTAG and Golgi proteins and to check for contamination from other organelles. The selectivity and specificity of the antibodies suggested in Table 1 have been extensively validated using appropriate controls. All antibodies listed in Table 1 react with samples from human cells.

15

After incubation with primary antibodies, wash membranes in TBS-T (3 washes, 5-10 minutes each, on a see-saw rocker).

15.1 15m

Wash membranes in TBS-T for **00:05:00** - **00:10:00** on a see-saw rocker(1/3).

15.2 15m

Wash membranes in TBS-T for **00:05:00** - **00:10:00** on a see-saw rocker(2/3).

15.3 15m

Wash membranes in TBS-T for **00:05:00** - **00:10:00** on a see-saw rocker(3/3).

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16 1h

Incubate membranes with near-infrared fluorescent dye-labelled secondary antibodies (diluted to the working concentration: 1:20,000) for **01:00:00** at **Room temperature** on a see-saw rocker.

Note: Table 2 lists the near-infrared fluorescent dye-labelled secondary antibodies used in our lab.

17 25m

Extensively wash membranes in TBS-T (4 washes, **00:10:00** - **00:15:00** each, with agitation).

17.1 25m

Wash membranes in TBS-T for **00:10:00** - **00:15:00** with agitation(1/4).

17.2 25m

Wash membranes in TBS-T for **00:10:00** - **00:15:00** with agitation(2/4).

17.3 25m

Wash membranes in TBS-T for **00:10:00** - **00:15:00** with agitation(3/4).

17.4 25m

Wash membranes in TBS-T for **00:10:00** - **00:15:00** with agitation(4/4).

Image acquisition and Analysis:

18 Acquire the protein bands via near infrared fluorescent detection using the Odyssey CLx Imaging System and the signal intensity quantified using the Image Studio Software.

Note: To control for inter-gel variability, the signal intensity of each band can be normalised against the control sample loaded in each gel of a set of experiments.

19 Analyse immunoblotting data using a software for statistical analysis (GraphPad Prism, or equivalent).

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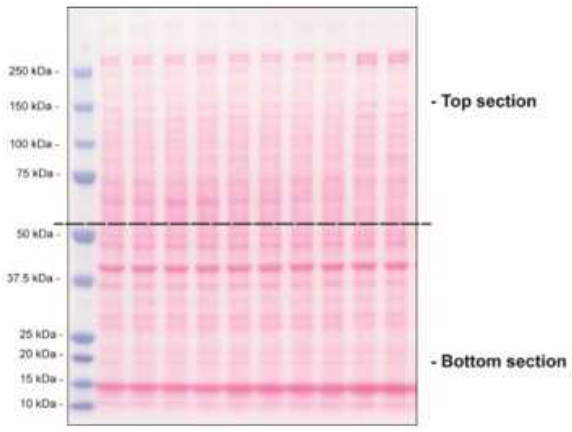

Figure 1: Representation of how to divide Ponceau-stained membranes into two separate halves for subsequent incubation with primary antibodies. Each membrane is cut into two halves just above the 50kDa marker (along the dotted line). The top section is incubated with primary antibodies against GM130, GOLGIN97, ACBD3, HSP60, LAMP1 and Calreticulin while the bottom section is incubated with primary antibodies against HA (could also be incubated with VDAC and CTSC).

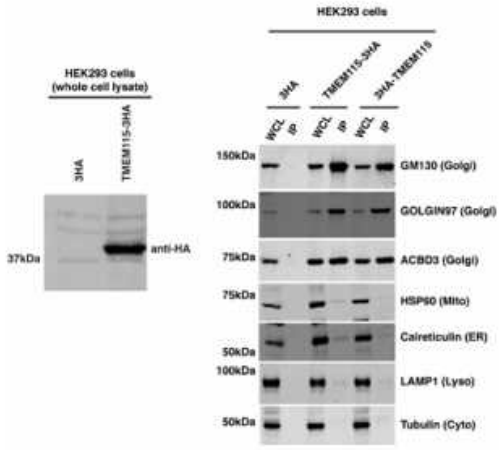

Figure 2: Representative results of immunoblotting analysis of samples from whole cell lysate and Golgi IP. Left panel shows the detection of C-terminal HA-tagged TMEM115 using an anti-HA antibody. Right panel shows the detection of different organelle markers from whole cell lysates and IP samples. Samples were prepared according to the protocol described in doi.... (Introducing GolgiTag to Cells and Immunoprecipitation of Golgi).). WCL: whole cell lysate; IP: Golgi immunoprecipitation samples; ER: endoplasmic reticulum; Mito: mitochondria; Lyso: lysosome; Cyto: cytoplasm. Note: VDAC and CTSC blots are not shown.

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