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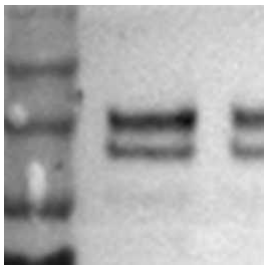
Evaluating GPNMB ACD mutants by Western Blotting and immunofluorescence.



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We use this protocol and it's working

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Abstract

This protocol describes the evaluation of cellular processing of GPNMB mutants by Western Blotting and Immunofluorescent imaging in a HEK293 overexpression model.

Materials

General

- HEK293T cells (ATCC CRL-3216)
- Fugene HD transfection reagent (E2311, Promega)
- pcDNA3.1-GPNMB-EGFP pcDNA3.1-GPNMB-EGFP.png 104KB
- PBS, pH 7.4: #14190250, ThermoFisher Scientific

Western Blotting

- Lysis buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% (v/v) Triton X-100

NOTE: add protease and phosphatase inhibitors fresh each time (eg Halt Protease and Phosphatase Inhibitor Cocktail (100X), #78440, ThermoFisher Scientific)

- Loading buffer: NuPAGE LDS sample buffer (#NP0007, ThermoFisher Scientific)
- Sample reducing agent: NuPAGE sample reducing agent (#NP0009, ThermoFisher Scientific)
- 4-12% Bis-Tris NuPAGE gels (eg #NP0321BOX, ThermoFisher Scientific)
- SDS-PAGE running buffer: MES running buffer (#NP0002, ThermoFisher Scientific)
- Trans-Blot® Turbo™ PVDF Transfer Packs: eg #1704157, BioRad
- TBS-T: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20.
- Blocking and antibody dilution buffer: 5 % (w/v) non-fat milk powder in TBS-T
- Primary and secondary antibodies (see table 1 & 2 for antibody suggestions)

Immunofluorescence

- 4 % (v/v) PFA/PBS: Dilute 16 % Paraformaldehyde Aqueous Solution (#15710, Electron Microscopy Sciences) to 4 % in PBS
- Blocking and antibody dilution buffer: 0.3 % Triton X-100, 5 % (v/v) FCS in PBS
- DAPI staining solution: 300 nM DAPI in PBS (#D1306, ThermoFisher Scientific or similar)
- Mounting medium: DAKO Fluorescence Mounting medium, # S3023, Agilent or similar
- Coverslips #1.5 (eg 631-0150, VWR)
- Slides (eg SuperFrost Plus, J1800AMNZ, Epredia)
- Primary and secondary antibodies (see table 3 & 4 for antibody suggestions)

Troubleshooting

Seed cells

1 Seed HEK293 cells.
A) For Western Blotting, we recommend seeding 2.5 × 10^5 cells per well of a 12-well culture plate.
B) For Immunofluorescence, we recommend seeding 1.2 × 10^5 cells per well of a 24-well culture plate. Seed cells on Poly-D-Lysine coated coverslips.
Note: we routinely culture HEK293T cells in DMEM containing 10% FCS.
Incubate in a tissue culture incubator Overnight .

HEK 293 cell transfection 10m

2 This protocol uses a DNA: Fugene ratio of 1:3. Prepare the transfection complexes as follows:

	A	B	C	D
		DNA	**Fugene**	**Serum-free DMEM**
	12 well	1000 ng	3 ul	100 ul
	24 well	500 ng	1.5 ul	50 ul

Preparation of transfection complexes (quantities are per well)

2.1 Add the required amount of plasmid DNA to serum-free DMEM. Mix briefly.

2.2 Add the required amount of Fugene HD Transfection Reagent.

2.3 Vortex and incubate for 00:10:00 at Room temperature . 10m

2.4 In the meantime, change the medium on the cells to fresh DMEM containing 10 % FCS

2.5 Add 50 µL per 24-well or 100 µL per 12-well drop-wise to the cells and incubate Overnight

Western Blotting

25m

3 This section describes the sample preparation and analysis for Western Blotting.

3.1 Wash the cells gentle with PBS

3.2 Immediately add 100 µL per well of ice-cold cell lysis buffer and place cells on ice.

3.3 Scrape cells with a cell scraper and harvest cell lysate into a 1.5 ml Eppendorf tube.

3.4 Incubate the cells On ice for 00:10:00 , vortex occasionally 10m

3.5 Clear the cell lysate by spinning down at 16200 x g, 4°C for 00:15:00 15m

3.6 Transfer the post-nuclear supernatant to a fresh 1.5 ml Eppendorf tube and store at -20 °C .

3.7 Prepare the cell lysates for Western Blotting by adding LDS sample buffer and denaturing agent and denature the samples at 80 °C for 00:08:00 . 8m

3.8 Run samples on 4-12 % Bis-Tris SDS-page. (approx. 00:35:00 at 160 V const.) 35m

3.9 Transfer proteins onto a PVDF membrane using the Turbo transfer system (BioRad) or similar.

3.10 Block membranes in 5% milk/TBS-T for 01:00:00 at Room temperature . 1h

3.11 Incubate the membranes with primary antibodies at 4 °C Overnight .

	A	B	C	D	E
	Target	**Cat #**	**Supplier**	**Raised in**	**Dilution**
	GPNMB (N-terminal)	AF2550	R&D Systems	Goat	1:1000
	GFP	MA5-15256	ThermoFisher Scientific	Mouse	1:1000
	Actin	A1978	Sigma	Mouse	1:5000

Table 1: Primary antibodies for Western Blotting.

3.12 Wash the plots in TBS-T for 00:05:00 at Room temperature. Repeat this step twice for a total of three washes. 5m

3.13 Dilute the secondary antibody in 5% milk/TBS-T and incubate the membranes with secondary antibodies at Room temperature for 00:45:00. 45m

	A	B	C
	Antibody suggestion		**Dilution**
	anti-mouse-Peroxidase	eg, A3682, Sigma	1:10000
	anti-goat-Peroxidase	eg, A5420, Sigma	1:10000

Table 2: Secondary antibodies for Western Blotting.

3.14 Develop the blots using an appropriate developer. Full-length GPNMB-EGFP is detected as a double band at ~125 kDa. A cleaved C-terminal GPNMB fragment can be detected with the anti-GFP antibody at ~35 kDa.

Immunofluorescence 2h 20m

4 Gently wash coverslips with PBS and fix in 4 % PFA/PBS for 00:15:00 min. 15m

4.1 Gently wash the cells with PBS. Replace the PBS and add the permeabilisation/blocking. Incubate coverslips for a minimum of 00:20:00 min at Room temperature. 20m

4.2 In the meantime, place a piece of Parafilm onto your bench and label if required. This will act as a flat surface to stain the coverslips on.

4.3 Prepare the antibody staining solution in Blocking and staining buffer. Find a suggestion of antibodies for counterstaining below:

	A	B	C	D	E
	Target	**Cat #**	**Supplier**	**Raised in**	**Dilution**
	LAMP-1	H4A3	DSHB	Mouse	1:100
	TGN46	13573-1-AP	Proteintech	Rabbit	1:100

Table 3: Primary antibodies for immunofluorescence.

4.4 Pipette a 45 µL drop of the antibody staining solution onto the Parafilm and invert the coverslip onto the staining solution so that the cells face downwards.

4.5 Incubate for 01:00:00 hr in the dark. 1h

4.6 Wash the coverslips three times with PBS.

4.7 Prepare a staining solution containing the secondary antibody:

	A	B
	Antibody suggestion	**Dilution**
	anti-mouse-AF647	1:1000
	anti-rabbit-AF586	1:1000

Table 4: Secondary antibodies for Immunofluorescence.

4.8 Pipette a 45 µL drop of the antibody staining solution onto the Parafilm and invert the coverslip onto the staining solution so that the cells face downwards.

4.9 Incubate for 00:45:00 min in the dark. 45m

4.10 Wash the coverslips once in PBS, stain with DAPI (or other nuclear stain), and mount onto glass slides.

4.11 GPNMB can be observed predominantly at the trans-Golgi network but can also be seen at lysosomal compartments.