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Western Blot in Mouse Brain Tissue for detecting pRab proteins

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madalynn.erb Erb¹

¹Van Andel Research Institute

ASAP Collaborative Rese...



madalynn.erb Erb
Van Andel Research Institute

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We use this protocol and it's working

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Abstract

Protocol for detecting pRab proteins in mouse brain tissue



Materials

TBS (20L)

🧪 400 mL : 1M Tris pH 7.5

🧪 600 mL : 5M NaCl

🧪 19 L : H₂O

TBST (20L)

🧪 400 mL : 1M Tris pH 7.5

🧪 600 mL : 5M NaCl

🧪 19 L : H₂O

🧪 20 mL : Tween 20

10X Transfer Buffer

- 🧪 700 mL mH₂O
- 🧪 30.4 g Tris base
- 🧪 144 g Glycine

Fill to 🧪 1000 mL with H₂O

Store at 🌡 Room temperature

1X Transfer Buffer:

- 🧪 100 mL 10X Transfer Buffer
- 🧪 200 mL Methanol
- 🧪 700 mL mH₂O

Store at 4°C

10X Running Buffer :

Dissolve the following components in 500 mL H₂O:

- 🧪 30.0 g Tris base
- 🧪 144.0 g glycine
- 🧪 10.0 g SDS (Sodium dodecyl sulfate)

Fill to 🧪 1000 mL with H₂O



The pH of the buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.

5X Lammeli sample buffer

10% SDS

50% glycerol


25% 2-Mercaptoethanol

0.31M tris pH6.8

0.01% bromophenol blue




Lysate preparation

1 Freeze freshly dissected brain tissue on dry ice and store at  -80 °C











2 Make lysis buffer - keep on ice

A	B	C	D
Stock solutions	for 10mL	for 15mL	For 20mL
Tris HCl 1M pH7.5	500uL	0.75 mL	1.0 mL
EGTA 0.1M	100uL	0.15 mL	0.2 mL
Triton X 100	100uL	0.15 mL	0.2 mL
PIC I 100X	100uL	0.15 mL	0.2 mL
PIC III 20X	500uL	0.75 mL	1.0 mL
1.35M Sucrose (5X)	2.0mL	3.0 mL	4.0 mL
Complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich Cat #11836170001)	1 tablet	1 tablet	2 tablets
water		8.55	
Total			15.0

3 Remove tissue from  -80 °C and keep on dry ice before starting experiment

4 Weigh each tissue sample and record mass



- 5 Transfer each samples to clean 5mL test tube for homogenization
- 6 Place samples on ice and add appropriate amounts of lysis buffer based on sample mass
 - 6.1 For regional brain dissections add  1000 μ L of chilled lysis buffer for every  100 mg of brain tissue
 - 6.2 For organ tissue add  1000 μ L of chilled lysis buffer for every  100 mg of brain tissue
 - 6.3 For hemi brains or whole brains add  600 μ L of chilled lysis buffer for every  100 mg of brain tissue
- 7 Allow samples to thaw in chilled lysis buffer for about 5 minutes
 - 7.1 Check samples with a clean pipette tip. If tissue has thawed, move onto the next step.
- 8 Use a tissue homogenizer to lyse the tissue
 - 8.1 10 seconds homogenization at maximum speed
 - 8.2 tissue should be kept on ice during and after homogenization
- 9 Allow tissue to rest on ice for  00:10:00 -  00:15:00 after homogenization
- 9.1 Bubbles should disappear during this time
- 9.2 After  00:10:00 -  00:15:00 check lysate. If there are tissue chunks repeat homogenization step.

25m

25m



- 10 Transfer the samples to Eppendorf tubes and centrifuge at 4 °C during 00:30:00 at 21.000 rcf 30m
- 11 Remove samples from centrifuge and keep on ice
- 12 Transfer supernatant to new Eppendorf tube
- 12.1 Transfer 15 µL of supernatant to new Eppendorf tube for Bicinchoninic acid (BCA) assay to measure protein concentration
- 12.2 Store supernatant at -80 °C until ready to run western blot
- 12.3 Save the pellet and store at -80 °C - pellet contains triton X insoluble proteins
- 13 Perform BCA analysis to measure protein concentrations

Western Blot

3h 55m

- 14 Dilute tissue lysates 1:5 in 5X Lammeli sample buffer

10% SDS
50% glycerol
25% 2-Mercaptoethanol
0.31M tris pH6.8
0.01% bromophenol blue

For 50 mL :
 7.75 mL 2M Tris pH6.8
 25 mL glycerol
 5 g SDS
 12.5 mL 2-Mercaptoethanol
Fill with miliQ H₂O to 50 mL (approx. 4.75 mL).



Add bromophenol blue

First mix Tris with 4ml H₂O and add SDS. Let it mix for about an hour. Then add BME and glycerol and continue to mix for approximately one more hour (SDS will eventually go into solution). QS to 50ml with additional H₂O. Add bromophenol blue, aliquot and store at -20C.

- 15 Denature the proteins at 70 °C for 00:10:00 in lysis buffer / Lammeli sample buffer mix

10m

- 16 Load 80 µg of protein into each well of SDS page gel

- 16.1 For Rab proteins use 12.5% acrylamide gels
For LRRK2 use 7.5% acrylamide gels

- 17 Run gels at 120V

- 17.1 For Rab proteins run gels 01:00:00
For LRRK2 run gels 01:45:00

2h 45m

- 18 Transfer gels at 20V Overnight at Room temperature

1h

Blot membranes

2h 35m

- 19 Wash membranes in H₂O for 00:02:00 then 5 min in TBST

2m

- 20 Wash membranes in TBST for 00:05:00


5m

- 21 Incubate membrane in Ponceau S solution (Sigma P7170) for 00:05:00



5m

- 22 Rinse membrane in H₂O and image




23 Wash 2 times in TBST for  00:05:00 each wash



5m

24 Incubate membrane in 5% milk in TBST (BioRad 1706404XTU) for  01:00:00 at  Room temperature

1h

25 Wash 2 times in TBST for  00:02:00 each wash

2m

26 Incubate  Overnight at  4 °C with primary antibody diluted in 5% milk in TBST

1h

26.1

A	B	C	D
Target	Species	Manufacturer	Dilution
pRab10	rabbit	Abcam ; ab230261	1:500
Total Rab10	rabbit	Cell Signaling ; 8127S	1:1000
pRab8a	rabbit	Abcam ; ab230260	1:500
pRab12	rabbit	Abcam ; ab256487	1:500
Total Rab12	rabbit	Protein Tech ; 18843-1-AP	1:1000
GAPDH	mouse	Protein Tech ; 60004-1-Ig	1:5000
Actin	mouse	Milipore; MAB1501	1:2000
LRRK2	rabbit	Abcam ; ab133474	1:1000
p935 LRRK2	rabbit	Abcam ; ab133450	1:1000
p1292 LRRK2	rabbit	Abcam ; ab203181	1:1000

Primary antibodies for Rab proteins and LRRK2

27 Wash 3 times in TBST for  00:05:00

5m

28 Dilute HRP-conjugated secondary antibodies 1:10,000 in 5% milk in TBST

28.1




A	B	C
goat anti rat	112-035-175	Jackson Immunoresearch
goat anti mouse	115-035-174	Jackson Immunoresearch
mouse anti rabbit	211-032-171	Jackson Immunoresearch
goat anti guinea pig	ab97155	Abcam

Secondary Antibodies



29 Wash membrane 3 times in TBST for  00:05:00 and once in TBS

5m

30 Wash membrane in TBS for  00:05:00

5m

31 Image using ECL reagents (Amersham)

31.1 use  500 μ L of solution A and  500 μ L solution B for each membrane

31.2 Develop for at least  00:01:00 before imaging

1m