Apr 23, 2024

Western Blot in Mouse Brain Tissue for detecting pRab proteins

DOI
dx.doi.org/10.17504/protocols.io.261ge545jg47/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.261ge545jg47/v1

Protocol Citation: madalynn.erb Erb 2024. Western Blot in Mouse Brain Tissue for detecting pRab proteins. **protocols.io https://dx.doi.org/10.17504/protocols.io.261ge545jg47/v1**

Protocol status: Working
We use this protocol and it's working

Created: April 23, 2024

Last Modified: May 31, 2024

Protocol Integer ID: 98678

Keywords: ASAPCRN, prab proteins in mouse brain tissue, detecting prab protein, detecting prab proteins protocol, prab proteins protocol, western blot in mouse brain tissue, mouse brain tissue, western blot

Funders Acknowledgements:
ASAP
Grant ID: ASAP-000592

Disclaimer

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 : H_2O

TBST (20L)

400 mL : 1M Tris pH 7.5

600 mL : 5M NaCl

19 L : H_2O

20 mL : Tween 20

10X Transfer Buffer

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

Fill to 1000 mL with H_2O

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 H_2O

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

Troubleshooting

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 µL of chilled lysis buffer for every 100 mg of brain tissue

6.2 For organ tissue add 1000 µL of chilled lysis buffer for every 100 mg of brain tissue

6.3 For hemi brains or whole brains add 600 µL 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 25m

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

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 H_2O 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 H_2Or 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 H_2O 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 µL of solution A and 500 µL solution B for each membrane

31.2 Develop for at least 00:01:00 before imaging 1m