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pS129 alpha-synuclein Western blot

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ABSTRACT

This protocol details the investigation of the amount of total alpha-synuclein and pS129 alpha-synuclein in cells.

ATTACHMENTS

dh36biqa7.pdf

DOI

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MATERIALS TEXT

MATERIALS AND REAGENTS:

- Western blot running tank
- iBlot transfer machine
- Clx LICOR scanner
- bench centrifuge
- LDS sample buffer
- MES running buffer

- 4-12 % Bis-Tris gel
- iBlot PVDF transfer stacks
- PBS
- TBS
- Paraformaldehyde
- Odyssey blocking buffer (PBS)
- Odyssey blocking buffer (TBS)
- HEPES
- NaCl
- Glycerol
- EGTA
- MgCl2
- Triton X-100
- Protease/phosphatase inhibitors
- Primary and secondary antibodies (see “additional information")

ADDITIONAL INFORMATION:

For primary antibody incubation, add both total asyn antibody and pS129 asyn antibody. The two antibodies have to be from different species.

Possible combinations:

Ms-αSynuclein BD 610787 1:1000 + Rb-pS129 αSynuclein clone MJFR13 ab168381 1:500 (suggested by Yumanity and used at BWH)
Rb-αSyn (14H2L1) Thermo 701085 - 1:3000-1:5000 + Ms-pS129 αSyn clone m81A (V. Lee) (Biolegend 11221) 1:500 (suggested by Yumanity, never tried at BWH

Use the following secondary antibodies:

IRDye 800CW – Dk anti-Ms 1:5000
IRDye 800CW – Dk anti-Rb 1:5000
IRDye 680RD – Dk anti-Ms 1:10,000
IRDye 680RD – Dk anti-Rb 1:10,000

Membrane can be cut before blocking in order to obtain a piece for asyn and pS129-asyn detection and another piece for a control protein (e.g. Actin or GAPDH). Process the membrane piece for control protein in parallel, following standard protocols (regular blocking and incubation solutions, tween concentrations, antibodies concentrations).

Extraction buffer preparation:

Prepare the extraction buffer (as described by J Mazzulli et al. 2006) containing:

[M]**20 Milimolar (mM)** HEPES

[M]**150 Milimolar (mM)** NaCl

10% Glycerol

[M]**1 Milimolar (mM)** EGTA

[M]**1.5 Milimolar (mM)** MgCl2

1% Triton X-100
+ protease and phosphatase inhibitors

The described buffer is aimed at extracting proteins from the triton-soluble fraction. If other fractions are needed (e.g. SDS-soluble fraction) use specific extraction buffers and procedures

DETERGENT-BASED CELL LYSIS 20m

1

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Add **100 µl** of lysis buffer to the cell pellet and mix thoroughly by pipetting up and down until the cell pellet is completely resolved into a homogeneous solution.

2 Adjust the lysis buffer volume to the cell pellet size, keep the sample at **Room temperature** as soon as the lysis buffer has been added, and work quickly to prevent proteolysis.

3 Starting with a frozen cell pellet, keep it on dry ice initially and thaw it on regular ice as soon as everything is set up.

4 If the cell pellet is collected right before conducting the Western blot, keep it **On ice** until the lysis buffer is added.

5  10m

Vortex and heat samples for **00:10:00** at **100 °C** .

6  10m

Spin down at maximal speed for **00:10:00** . Transfer the supernatant to a new Eppendorf tube and discard the cell pellet.

7 Store the supernatant at **-20 °C** in order to proceed with the protocol at a later point or as a positive control for later experiments.

8 Quantify protein concentration through a classical BCA assay (or other available techniques).

WESTERN BLOT 1h 35m

9 

For each sample, mix an appropriate amount of protein with NuPAGE® LDS Sample Buffer (4x), reducing agent (10x) and sterile H2O for protein linearization and monomerization.

10 Load no more than **40 µg** protein per well (e.g. **30 µg**).

11 Heat samples for **00:10:00** at **95 °C** . 10m

12 

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Prepare **500 mL** of running buffer 1X by mixing **475 mL** diH20 with **25 mL** NuPAGE® MES SDS Running Buffer (20x).

13 Place a 4-12 % Bis-Tris Gel in the gel electrophoresis chamber (after removing the tape and the comb and after rinsing) and fill the chamber with running buffer.

14 Use gel-loading tips to load the samples and **4 µl** of the protein ladder (Precision Plus Protein™ Dual Color Standards) onto the gel.

15 Run the gel at 150 V for approximately **00:30:00** – **00:55:00** until the protein ladder. 1h 25m

> The loading dye indicate a sufficient electrophoretic separation.

16 Use the iBlot Gel Transfer Device and the iBlot PVDF Transfer Stacks for dry blotting of proteins.

17 Boot the gel transfer device, select program "P0 7 min" (20v 1:00; 23v 4:00; 25v 2:00) and place the iBlot anode stack ("bottom"), with the PVDF transfer membrane on top, into the gel transfer device.

18 Separate the two plastic plates of the gel cassette using a gel knife. Cut off excessive parts of the gel. Transfer the gel onto the PVDF membrane. Gently remove any air bubbles using a wetted blotting roller.

19 Wet a Whatman paper in diH2O and place it on top of the gel. Gently remove any air bubbles using the wetted blotting roller.

20 Place the iBlot cathode stack ("top") on top and even the layers with the blotting roller. Place the iBlot sponge on the inner side of the iBlot lid in the right orientation.

21 Close the iBlot gel transfer device and start the program.

FIXATION, BLOCKING AND ANTIBODY INCUBATION 5h 5m

22 Fix membrane in 4% paraformaldehyde in PBS for **00:40:00** rocking at **Room temperature** . 40m

23 

Wash 3X with TBS (use and discard paraformaldehyde in compliance with the laboratory safety regulations).

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24 5m

Wash the PVDF membrane by gently rocking it in TBS for **00:05:00** .

25 When transferring the PVDF membrane or exchanging the solution in the blot box, make sure that the PVDF membrane never dries out.

26 1h

Remove the TBS and add a sufficient volume of one part Odyssey blocking buffer (TBS) mixed with one part of TBS (1:1). Incubate for **01:00:00** on a rocker.

27 Exchange the blocking solution with the primary antibody solution, i.e. one part Odyssey blocking buffer (TBS) mixed with one part TBS + the primary antibodies at the desired dilution.

28 1h

Incubate at **4 °C** on a rocker **Overnight** .

29 10m

Wash the PVDF membrane with TBS + 0.01% Tween20 on a rocker 3 times for **00:10:00** .

30

Exchange with the secondary antibody solution, i.e. one part Odyssey blocking buffer (TBS) mixed with one part TBS (1:1) + the secondary antibody at the desired dilution.

31 2h

Incubate on a rocker for **02:00:00** at **Room temperature** protected from light, i.e. wrap the blot box in aluminum foil.

32 10m

Wash the PVDF membrane with TBS + 0.01% Tween20 on a rocker 3 times for **00:10:00** protected from light.

33

Wash the PVDF membrane again with TBS until Tween-20 has been entirely removed and scan the blot using the Odyssey CLx Infrared Imager; for bands quantification use the Odyssey Software version 3.0.

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