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

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Proteinase K digestion

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ABSTRACT

This protocol describes the steps for performing proteinase K digestion and subsequent visualization by immunofluorescence, which can be helpful in determining insolubility of alpha-synuclein aggregates.

ATTACHMENTS

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MATERIALS

-Pre-cut brain sections (30-50 µm thick)

-0.1 M PB

-Triton-X100 (detergent)

-Normal donkey serum (S30-100ML, Sigma-Aldrich)

-Proteinase K (e.g. Proteinase K, Invitrogen, #4333793)

-Primary antibodies (e.g. Anti-ChAT AB144P, Merck; anti-TH ab113, abcam; anti-p62, ab109012, abcam; anti-alpha synuclein(phosphoS129), ab51253, abcam)

-Secondary antibodies

-12-well-plates with netwell inserts (e.g. Corning Costar Netwell)

-Hard-drying mounting medium (recommended ProLong Diamond, ThermoFisher Scientific)

-Aluminum foil

-Microscopy slides

-Glass coverslips

-Designated container for storage of microscopy slides

-Water bath with integrated shaker, temperature adjustable (e.g. GFL-1083)

-Orbital shaker (e.g. Heidolph Duomax 1030)

-Platform shaker (e.g. Heidolph Unimax 1010)

-Optional: DAPI, Sigma-Aldrich, D9542-5MG, 1:10,000 of 5 mg/ml

SAFETY WARNINGS

Recommended PPE:

- Lab coat/disposable gown

- Safety goggles

- Examination gloves

BEFORE START INSTRUCTIONS

-Prepare 0.1 M PB (PB)

-Prepare PBT (0.1 M PB with 0.3% Triton X-100)

-Prepare NDS solution (10% normal donkey serum diluted in PBT)

-Prepare proteinase K solution (12 µg/ml in 0.1 M PB)

-Set water bath to 65°C

Proteinase K digestion - Procedure

18h 8m

1 -Place individual sections in 12-well-plate with netwell inserts in PB.

2 -Wash sections for 3x 5 min on platform shaker in PB, change solution between washing steps. 15m

3 -Place sections in 12-well-plate with netwell inserts in PK solution. Use 4 ml solution per well.

4 -Perform PK digestion by immediately putting the filled 12-well-plate in the water bath (65 °C) followe 10m
10 min shaking. 65 °C

5 -Take well-plate out of the water bath and wash sections 4x 5 min on platform shaker in 0.1 M PB at room temp., change solution between washing steps. 20m

Room temperature

6 -Block sections for 60 min at room temp. in NDS solution in 12-well-plates. We recommend 4 ml soluti 1h
per well for good results.

7 -For incubation with primary antibodies, transfer sections in a new 12-well-plate 16h
but without netwell inserts. This allows better shaking overnight. Solution for incubation with primary antibodies should contain NDS solution and the respective primary antibodies diluted according to manufacturer recommendation. Incubate sections on orbital shaker with gentle shaking at 4°C **overnight** (time may vary). We recommend at least 1 ml solution for each well. 4 °C

8 -On the next day, transfer sections into 12-well-plate with netwell inserts and wash 20m
sections 4x 5 min in PBT on platform shaker at room temperature. Change washing solution after each washing step.

9 -Next, transfer sections back in 12-well-plate without netwell inserts for incubation with secondary antibodies.

10 -Incubate sections with fluorophore-conjugated, species-specific secondary antibodies in 2h
NDS solution for 2 hours on orbital shaker at room temperature. For dilution of the secondary antibodies we generally use 1:500. We recommend at least 1 ml solution for each well. Cover the plate with aluminum foil during antibody incubation to avoid light exposure. Room temperature

11 -Thereafter, transfer sections back to 12-well-plate with netwell inserts and wash sections

for 5x 5min in PBT. For all washing steps, 12-well-plate should be covered with aluminum foil to avoid light exposure.

STEP CASE

Stain with DAPI

8 steps

-In case you want to additionally stain with DAPI, perform only 2 washing steps with PBT and add DAPI (1:10.000 of 5 mg/ml) to the third PBT washing step and let sections wash for 10 min. Perform a final washing step without DAPI in PBT solution. For all washing steps, 12-well-plate should be covered with aluminum foil to avoid light exposure.

12 -After washing, mount sections on microscopy slides using a fine brush.

13 -Let sections dry a few minutes. 3m

14 -Apply a small amount of hard-drying mounting medium sufficient to cover the sections. Carefully avoid the formation of air bubbles. Gently apply a coverslip over the sections and the mounting medium.

15 -Let cure overnight in the **dark** at room temperature. Room temperature

Proteinase K digestion - After the Procedure

16 -Dispose of waste and excess reagents/solution according to institutional guidelines.

17 -Clean tools/working station.

18 -Once the mounting medium is cured, slides are ready for observation.

19 -Fully mounted microscopy slides should be stored in a designated container at 4°C until time of observation. 4 °C