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# Indirect Proximity Ligation Assay (PLA) - Brightfield

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ABSTRACT

Indirect Proximity Ligation Assay (PLA) is a powerful molecular technique used to detect and visualize protein-protein interactions, protein modifications, and protein complex formations within cells or tissues. This method is based on the principles of proximity-dependent ligation and utilizes specific antibodies to detect the nitration of proteins on free-floating brain sections. Here we describe the PLA protocol that we routinely use in our laboratory to detect nitrated alpha-synuclein and nitration of mitochondrial enzymes such as SOD2 and the mitochondrial complex 1 subunit NDUFB8.

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

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**Keywords:** post-translational modification, alpha-synuclein, oxidative stress

MATERIALS

Duolink InSitu PLA probe anti-rabbit PLUS kit: DUO92002-100RXN

Duolink InSitu PLA probe anti-mouse MINUS kit: DUO92004-100RXN

Duolink InSitu Detection Reagents Brightfield kit: DUO92012-100RXN

Duolink wash buffer A: DUO82047-20L

Histomount mounting medium: Life Technologies 008030

**Antibodies:**
mouse anti-3-NT: 1:250; ab61392, Abcam
rabbit anti-human alpha-synuclein (clone MJFR1): 1:4000, ab138501, Abcam
rabbit anti-SOD1: 1:1000; ADI-SOD-110, Enzo Life Sciences
rabbit anti-NDUFB8: 1:300; 14794, Proteintech

## Day 1

1

Pick 35um cut brain sections and transfer them to 1.5 mL Eppendorf tubes

Note: all incubation and wash steps are performed by shaking Eppendorf tubes at 250rpm (e.g., thermomixer)

2 Wash 2x 00:05:00 with Tris-HCl 5m

3 **Peroxidase quenching** with BloxAll solution

use 300 µL and incubate for 30min

4 Wash 3x 00:05:00 with wash buffer A (see materials) 5m

5 **Antigen-retrieval** with citrate buffer (+tween, pH = 6) at 95°C for 00:04:00 4m
Note: Heat the solution to 95°C before adding on the samples

6 Wash 3x 00:05:00 with wash buffer A 5m

7 **Blocking:** Incubate in Duolink Blocking Solution ( 300 µL )at 37°C for 01:00:00 1h

8 **Primary Antibody Incubation**: Dilute antibodies in Duolink Antibody Diluent ( 200 µL µl/tube) 5m
and incubate Overnight room temperature.
for nitrated alpha-synuclein: mouse anti-3-NT and rabbit anti-h-alpha-synuclein
for nitrated SOD2: mouse anti-3-NT and rabbit anti-SOD2
for nitrated NDUFB8: mouse anti-3-NT and rabbit anti-NDUFB8
**see materials for dilutions and catalog numbers**

## Day 2 10m

9 Wash 3x 00:10:00 with wash buffer A 10m

10 **PLA probe incubation**: 1h 30m
Prepare anti-mouse MINUS and anti-rabbit PLUS probes following manufacturer's instructions, add solution on sections and incubate at 37°C for 01:30:00

11 Wash 3x 00:10:00 with wash buffer A 10m

12 **Ligation:** 1h 15m
Dilute the Duolink Ligation Buffer 1:5 in high-purity water
Add the ligase (diluted 1:40) just before incubation (keep cold on freezer block!)
add the ligation solution on sections and incubate at 37°C for 01:15:00

13 Wash 3x 00:05:00 with wash buffer A 5m

14 **Amplification**: 2h 15m
Dilute the Duolink Amplification Buffer 1:5 in high-purity water
Add the polymerase (diluted 1:80) just before incubation (keep cold on freezer block!)
Add the amplification solution to the sections and incubate at 37°C for 02:15:00

15 **Detection:** 1h 10m
Dilute the Duolink Detection Brightfield Stock 1:5 in high-purity water
add solution to the sections and incubate at RT for 01:10:00

16 Wash 2x 00:05:00 with wash buffer A 10m
Wash 1x 00:05:00 with Tris-buffered saline (no detergent)

17 **Developing:**
Dilute the Duolink Brightfield Substrate solutions in high-purity water:
§ Substrate A (1:70)
§ Substrate B (1:100)
§ Substrate C (1:100)
§ Substrate D (1:50)

Incubate sections at RT for 1-5min (depending on desired intensity outcome)

18 Wash 3x 00:05:00 with wash Tris-buffered saline 5m

19 Mount the samples on slides and let them air-dry

20 **Nuclear staining** (optional): Incubate for 00:01:00 11m
Wash the staining off with running tap water for 00:10:00

21 Dehydrate of samples in 1min each: 70% ethanol, 95% ethanol, 2x 100% ethanol 2x xylene

22 Coverslip samples with Histomount mounting medium