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## Indirect Proximity Ligation Assay (PLA) - Brightfield

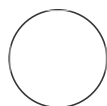
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### ABSTRACT

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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85848

**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

- 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)
- Wash 2x  00:05:00 with Tris-HCl 
- 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