May 21, 2024

Multiplex Labeling with Tyramide Fluorophores for Detecting CIAP-Resistant PSER129 and Proteinase K-Resistant aSyn in situ (Killinger 2024)

DOI
dx.doi.org/10.17504/protocols.io.x54v92dq4l3e/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.x54v92dq4l3e/v1

Protocol Citation: Solji Choi, Bryan Killinger 2024. Multiplex Labeling with Tyramide Fluorophores for Detecting CIAP-Resistant PSER129 and Proteinase K-Resistant aSyn in situ (Killinger 2024). **protocols.io** https://dx.doi.org/10.17504/protocols.io.x54v92dq4l3e/v1

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

Created: May 15, 2024

Last Modified: May 21, 2024

Protocol Integer ID: 100194

Funders Acknowledgement:
Michael J. Fox Foundation
Grant ID: ASAP-021030
NIH-NINDS
Grant ID: 1R01NS128467

Abstract

This protocol aims to examine the association of calf-intestinal alkaline phosphatase (CIAP)-resistant alpha-synuclein phosphorylated at serine 129 (PSER129) and proteinase K (PK)-resistant alpha-synuclein (aSyn) in the mouse brain, particularly in M83 transgenic mice treated with preformed fibrils. M83 lines exhibit a notably higher abundance of endogenous PSER129 compared to wild-type mice.

Materials

- **Dilution media:**

	A	B
	Tris-HCl, pH 7.4	50 mM
	NaCl	150 mM
	Triton- X100	0.5%

Alkaline Phosphatase, Calf Intestinal (20 u/µl) **Promega Catalog #**M2825

- **CIAP buffer:**

	A	B
	NaCl	100 mM
	Tris-HCl	50 mM
	MgCl2, pH 7.9	10 mM
	Autoclave and store RT	

- **Blocking buffer:**

	A	B
	Dilution media	100 mL
	Normal serum	3 mL
	BSA	2 g
	Triton X100	0.4 mL
	Mix well so the Triton is completely dissolved	

- **Borate buffer:**

	A	B
	Borate buffer, pH 8.5	0.05 M
	DI H2O	300 mL
	Sodium tetraborate decahydrate	5.72 g
	Mix well to dissolve completely. Adjust to pH 8.5	

- **Components:**

	A	B
	Borate buffer	10 mL

	A	B
	H2O2	1 uL
	TF	5 uL

- **Sodium Citrate Buffer, pH 6.0 (1L):**

	A	B
	Sodium citrate-Trisodium salt (Dihydrate) in 1000 mL DI water	2.94 g
	Tween-20	0.5 mL
	Mix well	

- **PBS:**

	A	B
	Tris-HCl, pH 7.2	50 mM
	NaCl	158 mM

Proteinase K **Thermo Fisher Scientific Catalog #**EO0491

Protocol materials

Proteinase K **Thermo Fisher Scientific Catalog #**EO0491 Materials, Step 25

Alkaline Phosphatase, Calf Intestinal (20 u/µl) **Promega Catalog #**M2825 Materials, Step 5

Day 1

1d 1h 10m

1 Wash free-floating tissue (3 x 10 minutes) in dilution media (DM).

Dilution media:

	A	B
	Tris-HCl, pH 7.4	50 mM
	NaCl	150 mM
	Triton- X100	0.5%

1.1 Wash free-floating tissue for 00:10:00 in dilution media (DM). (1/3) 10m

1.2 Wash free-floating tissue for 00:10:00 in dilution media (DM). (2/3) 10m

1.3 Wash free-floating tissue for 00:10:00 in dilution media (DM). (3/3) 10m

2 Incubate the samples with 1% Triton X-100 in DM for 00:10:00 . 10m

3 Wash in DM for 00:10:00 . 10m

4 Wash the tissues in CIAP buffer (2x10 minutes).

CIAP buffer:

	A	B
	NaCl	100 mM
	Tris-HCl	50 mM
	MgCl2, pH 7.9	10 mM
	Autoclave and store RT	

4.1 Wash the tissues in CIAP buffer for 00:10:00 . (1/2) 10m

4.2 Wash the tissues in CIAP buffer for 00:10:00 . (2/2) 10m

5 Incubate the tissues with CIAP at a dilution of 1:333 for 24:00:00 at 37 °C on a shaker. 1d

- Alkaline Phosphatase, Calf Intestinal (20 u/µl) **Promega Catalog #**M2825 .
- In 500uL CIAP buffer, add 1.5 µl CIAP (30 units).

Day 2 8h 30m

6 Wash in DM (3 x 10 minutes).

6.1 Wash in DM for 00:10:00 . (1/3) 10m

6.2 Wash in DM for 00:10:00 . (2/3) 10m

6.3 Wash in DM for 00:10:00 . (3/3) 10m

7 Endogenous peroxidase inhibition and serum blocking step (1-hour incubation):

- 0.3% H_2O_2+0.1% Sodium Azide in 50 mL blocking buffer.
- **Blocking buffer:**

	A	B
	Dilution media	100 mL
	Normal serum	3 mL
	BSA	2 g
	Triton X100	0.4 mL

	A	B
	Mix well so the Triton is completely dissolved	

8 Dilute primary antibody in blocking buffer. Incubate Overnight at 4 °C . 8h

- Recombinant Anti-Alpha-synuclein (phospho S129) antibody (EP1536Y, ab51253), dilution factor: 1:50K
- In 30 mL blocking buffer, add 0.6 µL PSER129 antibody.

Day 3 17h 20m

9 Wash in DM (3 x 10 minutes).

9.1 Wash in DM for 00:10:00 . (1/3) 10m

9.2 Wash in DM for 00:10:00 . (2/3) 10m

9.3 Wash in DM for 00:10:00 . (3/3) 10m

10 HRP-Secondary antibody incubation 1:1000 dilution (01:00:00). 1h

- Solvent is 100 mL DM + 1 mL normal serum + 1 g BSA

11 Wash in DM (2 x 10 minutes).

11.1 Wash in DM for 00:10:00 . (1/2) 10m

11.2 Wash in DM for 00:10:00 . (2/2) 10m

12 Wash in borate buffer for 00:10:00 . 10m

- **Borate buffer:**

	A	B
	Borate buffer, pH 8.5	0.05 M
	DI H2O	300 mL
	Sodium tetraborate decahydrate	5.72 g
	Mix well to dissolve completely. Adjust to pH 8.5	

13 Incubate with tyramide fluorophore (TF) for 00:30:00 while blocking light. After this step, always protect the tissues from light. 30m

- **Components:**

	A	B
	Borate buffer	10 mL
	H2O2	1 uL
	TF	5 uL

14 Wash in DM (2 x 10 minutes).

14.1 Wash in DM 00:10:00 . (1/2) 10m

14.2 Wash in DM 00:10:00 . (2/2) 10m

15 View under the microscope to confirm successful staining.

16 Heat water bath to 80 °C - 85 °C for 01:30:00 before the primary antibody elution step. 1h 30m

17 Place the dish containing sodium citrate buffer in the water bath and heat it for 00:10:00 . 10m

- **Sodium Citrate Buffer, pH 6.0 (1L):**

	A	B
	Sodium citrate-Trisodium salt (Dihydrate) in 1000 mL DI water	2.94 g
	Tween-20	0.5 mL
	Mix well	

18 Wash the tissues in sodium citrate buffer for 00:10:00 — 10m

19 Incubate the tissues in the heated sodium citrate buffer for 00:30:00 . — 30m

20 Cool the dish containing tissues to Room temperature (at least 00:20:00). — 20m

21 Wash in DM for 10 min x 2 times.

21.1 Wash in DM for 00:10:00 . (1/2) — 10m

21.2 Wash in DM for 00:10:00 . (2/2) — 10m

22 Mount the tissues on Superfrost Plus Microscope Slides (Fisherbrand), and completely dry it for at least 02:00:00 . — 2h

23 Heat water bath to 37 °C for 00:30:00 before the PK digestion step. — 30m

24 Place the dish containing PBS in the water bath and heat it for 00:10:00 . — 10m

- **PBS:**

	A	B
	Tris-HCl, pH 7.2	50 mM

	A	B
	NaCl	158 mM

25 Add the PK to the PBS at a dilution of 1:666 and mix well.

- Proteinase K **Thermo Fisher Scientific Catalog #**EO0491
- 30 mL PBS, add 45 µL PK.

26 Incubate the mounted tissues in the PK containing PBS for 00:30:00 . 30m

27 Wash the slide in PBS (2 x 5 minutes).

27.1 Wash the slide in PBS for 00:05:00 . (1/2) 5m

27.2 Wash the slide in PBS for 00:05:00 . (2/2) 5m

28 Incubate the slide in 4% PFA for 00:30:00 at Room temperature on a shaker. 30m

29 Wash in DM (2 x 5 minutes).

29.1 Wash in DM for 00:05:00 . (1/2) 5m

29.2 Wash in DM for 00:05:00 . (2/2) 5m

30 Block the tissues on slide using Bloxall endogenous blocking solution (Vector Laboratories) for 00:10:00 at Room temperature on a shaker. 10m

31 Dilute primary antibody in blocking buffer. Incubate Overnight at 4 °C . 8h

- Recombinant Anti-Alpha-synuclein antibody (EPR20535, ab212184), dilution factor: 1:20K
- In 30 mL blocking buffer, add 1.5 µL aSyn antibody.

Day 4 3h 50m

32 Wash in DM (3 x 10 minutes).

32.1 Wash in DM for 00:10:00 . (1/3) 10m

32.2 Wash in DM for 00:10:00 . (2/3) 10m

32.3 Wash in DM for 00:10:00 . (3/3) 10m

33 HRP-Secondary antibody incubation 1:1000 dilution (01:00:00). 1h

- Solvent is 100 mL DM + 1 mL normal serum + 1 g BSA

34 Wash in DM (2 x 10 minutes).

34.1 Wash in DM for 00:10:00 . (1/2) 10m

34.2 Wash in DM for 00:10:00 . (2/2) 10m

35 Wash in borate buffer for 00:10:00 . 10m

- **Borate Buffer:**

	A	B
	Borate buffer, pH 8.5	0.05 M
	DI H2O	300 mL
	Sodium tetraborate decahydrate	5.72 g
	Mix well to dissolve completely. Adjust to pH 8.5	

35.1 Incubate with tyramide fluorophore (TF) for 00:30:00 while blocking light. 30m

35.2 Wash in DM (2 x 10 minutes). 20m

- Wash in DM 00:10:00 . (1/2)
- Wash in DM 00:10:00 . (2/2)

35.3 **Components:**

	A	B
	Borate buffer	10 mL
	H2O2	1 uL
	TF	5 uL

36 View under the microscope to confirm successful staining.

37 Wash in PBS (2 x 10 minutes).

37.1 Wash in PBS for 00:10:00 . (1/2) 10m

37.2 Wash in PBS for 00:10:00 . (2/2) 10m

38 Counterstain with DAPI for 00:20:00 at Room temperature . 20m

- 1:2000 dilution in ddH_2O or PBS.

39 Wash the tissues in PBS (2 x 10 minutes).

39.1 Wash the tissues in PBS for 00:10:00 . (1/2) 10m

39.2 Wash the tissues in PBS for 00:10:00 . (2/2) 10m

40 Cover the slide with Fluoroshield, and coverslip. Seal with nail polish on all sides of the coverslip. Always protect the slides from light. Slides can be stored at 4 °C .