SEP 20, 2023

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Protocol Citation: Michael Henderson 2023. Dual In Situ Hybridization/Immunofluorescence. **protocols.io** https://protocols.io/view/dual-in-situ-hybridization-immunofluorescence-b3g4qjyw

MANUSCRIPT CITATION: Adapted from ACD Standard Protocol/Cheadle/Otero-Garcia Protocols

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

Created: Jan 05, 2022

Last Modified: Sep 20, 2023

Dual In Situ Hybridization/Immunofluorescence

In 1 collection

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ABSTRACT

This protocol details about the Dual In Situ Hybridization/Immunofluorescence in tissue.

ATTACHMENTS

338-741.pdf

GUIDELINES

ACD protocol notes that tissues should be fixed in 10% NBF for 16-32 hours, and embedded in paraffin. Then, sectioned and dried overnight at RT. They suggest sectioned tissue be used in less than a year (4°C) or less than 3 months at room temperature.

PROTOCOL integer ID: 56572

Keywords: Dual In Situ Hybridization, Immunofluorescence, RNAscope Multiplex Fluorescent v2 Assay, ASAPCRN

MATERIALS

Solutions

	A	B	C
	Needed (mL)	Stock Solution	Final Concentration
	5 L	dH2O	
	485 g	Tris base	0.5 M
	240 mL	Concentrated HCl	
	pH to 7.6		
	To 8L	dH2O	

0.5 M Tris (8 L)

Reagents

	A	B	C	D	E
	Vendor	Catalog #	Qty	Unit Price	Description
	RNAscope® Multiplex Fluorescent Reagent Kit V2	323100		1330	Contains H2O2, protease reagents, target retrieval reagent, wash buffer, HRP reagents
	RNAscope® 3-plex	320861		100	Polr2a (C1 channel) and PPIB (C2 channel), UBC (C3 channel)
	Positive Control Human Sigma	199664-25G	1	66.6	Sudan Black B
	Vector Laboratories	H-4000	1	120	ImmEdge Hydrophobic Barrier Pen
	Southern Biotech	0100-01	1	45.14	DAPI Fluoromount-G

Sudan black B **Sigma Aldrich Catalog #199664**

ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #H-4000**

Fluoromount-G **Southern Biotech Catalog #0100-01**

Preparing Tissue (Day 1): Prepare Tissue

1h 19m

1 Bake slides in a dry oven for 01:00:00 at 60 °C. Use slides within a week. 1h

2 De-paraffinize slides in fresh xylenes, then in 100% ethanol.

2.1 De-paraffinize slides for 00:05:00 in fresh xylenes. (1/4) 5m

2.2 De-paraffinize slides for 00:05:00 in fresh xylenes. (2/4) 5m

2.3 De-paraffinize slides for 00:02:00 in 100% ethanol. (3/4) 2m

2.4 De-paraffinize slides for 00:02:00 in 100% ethanol. (4/4) 2m

3 Place slides on absorbent paper and dry in the oven from 00:05:00 at 60 °C or until dry. 5m

Preparing Tissue (Day 1): Hydrogen Peroxide Treatment 1h 19m

4 Place slide horizontally in an incubation tray. Add ~5-8 drops of RNAscope Hydrogen Peroxide to cover each section. Incubate for 00:10:00 at Room temperature. 10m

5 Dab solution off and move to a rack in distilled water. Move up and down 5 times. Repeat with a fresh boat of distilled water.

Preparing Tissue (Day 1): Target Retrieval

1h 19m

6 Dilute Target Retrieval Regents (RNAscope) 1:10 in dH_2O (25 mL / 225 mL dH2O/boat). Mix well.

7 Place in microwave for 00:15:00 at 95 °C. 15m

8 Transfer slides to a slide boat with 200 mL distilled water for 00:00:15. 15s

9 Transfer the slides to 100% ethanol for 00:03:00. 3m

10 Dry the slides in a 60 °C incubator (or Room temperature) for 00:05:00. 5m

11 Draw a hydrophobic barrier onto slides with ImmEdge pen. Do NOT due for fluorescent slides. Let the barrier dry for 00:05:00. OPTIONAL PAUSE POINT Overnight at Room temperature. 10m

RNAscope Multiplex Fluorescent v2 Assay (Day 2): Protease T...

1h 15m

12 Place a wet Humidifying Paper in an incubation tray and warm for 00:30:00 at 40 °C (TC incubator). Keep the tray in the incubator when not in use. Insert the slides into the 30m

incubation tray.

13 Add ~5 drops RNAScope Protease Plus (Protease III-Cheadle) to cover each section and place tray into the incubator at 40 °C for 00:30:00 (standard) (00:15:00 -Otero-Garcia). 45m

> **Note**
>
> Prepare RNAscope assay reagents during this step.

14 Wash slides with 200 mL+ distilled water and slight agitation.

14.1 Wash slides with 200 mL + distilled water and slight agitation. (1/2)

14.2 Wash slides with 200 mL + distilled water and slight agitation. (2/2)

RNAscope Multiplex Fluorescent v2 Assay (Day 2): Preparation 1h 15m

15 **Wash Buffer**: Warm 50x Buffer to 40 °C for 00:10:00 to 00:20:00 . Add 980 mL distilled water to 20 mL of RNAscope Wash Buffer in a 1 L bottle. May need 1 L to 2 L per run. Mix well. Can be stored for up to one month. 30m

16 **Probes**: Prepare only those probes needed. 10m

> **Note**
>
> If you are only using C2 and C3, dilute in probe diluent instead of C1.

Warm probes for 00:10:00 at 40 °C , then let cool to Room temperature . Add 1 volume C2 and volume C3 probes to 50 volumes C1 probe in a tube (e.g. 200 µL C1 +

4 µL C2). Invert to mix. Store at 4 °C for up to 6 months.

17 **Reagents**: Warm AMP1-3, HRP-C1-3 and HRP blocks at Room temperature.

18 (Optional) **Saline Sodium Citrate**: 175.3 g NaCl + 88.2 g sodium citrate in 800 mL istilled water. Adjust to pH 7.0 with [M] 1 Molarity (M) HCl. Add water to a final volume of 1 L. Sterilize by autoclaving and store at Room temperature for up to 2 months.

RNAscope Multiplex Fluorescent v2 Assay (Day 2): Hybridize P...

1h 15m

19 Remove liquid from slides. Add 4-6 drops (6 drops= 180 µL) of the probe mix to slides. Incubate in incubator for 02:00:00 at 40 °C. 2h

20 Wash slides with Wash Buffer.

20.1 Wash slides with Wash Buffer 00:02:00 at Room temperature. (1/2) 2m

20.2 Wash slides with Wash Buffer 00:02:00 at Room temperature. (2/2) 2m

21 **OPTIONAL PAUSE POINT**: Store slides in 5x SSC Overnight at Room temperature. 2m

RNAscope Multiplex Fluorescent v2 Assay (Day 2): Hybridize A..

1h 15m

22 Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 Amp 1 to each slide. Incubate in incubator for 00:30:00 at 40 °C. 30m

23 Wash slides with Wash Buffer.

23.1 Wash slides with Wash Buffer 00:02:00 at Room temperature. (1/2) 2m

23.2 Wash slides with Wash Buffer 00:02:00 at Room temperature. (2/2) 2m

24 Repeat steps 22 and 23 for Amp 2 and Amp 3. Amp 3 only requires 00:15:00 at 40 °C. 15m

25 During this incubation, dilute necessary Opal Dye fluorophores in TSA Buffer (1:1500 standard).

RNAscope Multiplex Fluorescent v2 Assay (Day 2): Develop HR..

1h 15m

26 Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 HRP-C1 to each slide. Incubate in incubator for 00:15:00 at 40 °C. 15m

27 Wash slides with Wash Buffer.

27.1 Wash slides with Wash Buffer 00:02:00 at Room temperature. (1/2) 2m

27.2 Wash slides with Wash Buffer 00:02:00 at Room temperature. (2/2) 2m

28 Remove liquid from slides. Add 200 µL Opal 520 to each slide. Incubate in HybEZ Oven for 00:30:00 at 40 °C. 30m

29 Wash slides with Wash Buffer.

29.1 Wash slides with Wash Buffer 00:02:00 at Room temperature. (1/2) 2m

29.2 Wash slides with Wash Buffer 00:02:00 at Room temperature. (2/2) 2m

30 Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 HRP Blocker to each slide. Incubate in incubator for 00:15:00 at 40 °C. 15m

31 Wash slides with Wash Buffer.

31.1 Wash slides with Wash Buffer 00:02:00 at Room temperature. (1/2) 2m

31.2 Wash slides with Wash Buffer 00:02:00 at Room temperature. (2/2) 2m

32 STOP HERE IF USING JUST C1 PROBE. Continue to **Immunofluorescence**.

33 Repeat steps 26-32 with HRP-C2 and Opal 570, and again with HRP-C3 and Opal 690.

Note

*Note that after additional of fluorophores, slides should be kept out of the light as much as possible.

33.1 4% PFA fix for 00:15:00 at 4 °C. 15m

33.2 Then, wash with PBS-Otero-Garcia for 00:04:00. (1/2) 4m

33.3 Wash with PBS-Otero-Garcia for 00:04:00. (2/2) 4m

Day 2: Immunofluorescence 1h 15m

34 Wash in [M] 0.1 Molarity (M) Tris buffer, pH 7.6 00:05:00. Discard all Tris washes. 5m

35 Block in [M] 0.1 Molarity (M) Tris/2% FBS (Tris/FBS) 00:30:00+. Keep blocking solution for up to 2 weeks @ 4 °C. 30m

36 Dilute primary antibodies in Tris/FBS), and prepare humidified chamber(s) by soaking towel in the middle of the slide chamber(s).

37 Wipe excess fluid off back of slides and from around tissue and apply 200 µL of primary antibody to slides.

38 Incubate at 4 °C in humidified chamber 00:45:00 to 02:00:00 at Room temperature or Overnight at 4 °C. 4h 45m

Day 3 1h 15m

39 Rinse off antibody from tissue using Tris.

> **Note**
>
> Carefully direct spray from wash bottle around tissue, NOT directly on it.

40 Wash in Tris 00:05:00. 5m

41 Block in Tris/FBS 00:05:00. 5m

42 Dilute fluorophore-conjugated secondary antibody 1:500 in Tris/FBS and apply 200 µL to 4h

wiped slides. Incubate at Room temperature for 02:00:00 or Overnight at 4 °C.

43 Rinse off slides with Tris.

44 Wash in running tap H_2O for 00:05:00. 5m

45 Wash in Tris for 00:05:00 in green boats. 5m

46 Coverslip using non-photobleaching reagent (Prolong Gold with DAPI or FluorMount with DAPI). Allow to dry completely before imaging on scanner.