Oct 11, 2018

Working

Immunohistochemistry

PLOS One

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dx.doi.org/10.17504/protocols.io.s8dehs6

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ABSTRACT

Immunohistochemistry using paraffin sections.
There are several options for some steps and all the options are listed on Steps section.
The suitable options for each antibody is noted on Guidelines&warnings section.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0205494

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Fujisawa M, Moh-Moh-Aung A, Zeng Z, Yoshimura T, Wani Y, Matsukawa A (2018) Ovarian stromal cells as a source of cancer-associated fibroblasts in human epithelial ovarian cancer: A histopathological study. PLoS ONE 13(10): e0205494. doi: 10.1371/journal.pone.0205494

PROTOCOL STATUS

Working

GUIDELINES

Individual procedure for each antibody:

FOXL2
1 > 2 > 3.1 > 4 > 5.1 > 6.1 > 7 > 8.1

ASMA/FOXL2 double staining
1 > 2 > 4(ASMA) > 5.3 > 6.2 > 3.1 > 4(FOXL2) > 5.1 > 6.1 > 7 > 8.2

FAP/FOXL2 double staining
1 > 2 > 3.1 > 4(FOXL2) > 5.1 > 6.1 > 3.1 > 4(FAP) > 5.3 > 6.2 > 7 > 8.2

CD163
1 > 2 > 3.2 > 4 > 5.2 > 6.1 > 7 > 8.1

CD138
1 > 2 > 3.2 > 4 > 5.2 > 6.1 > 7 > 8.1

CD31/FOXL2 double staining
1 > 2 > 3.1 > 4(FOXL2) > 5.1 > 6.1 > 3.2 > 4(CD31) > 5.3 > 6.2 > 7 > 8.2

Type4 Collagen/FOXL2 double staining
1 > 2 > 3.1 > 4(FOXL2) > 5.1 > 6.1
>3.1 > 3.3 > 4(Type4 collagen) > 5.3 > 6.2 > 7 > 8.2

CD45/FOXL2 double staining
1 > 2 > 3.1 > 4(FOXL2) > 5.1 > 6.1 > 3.1 > 4(CD45) > 5.3 > 6.2 > 7 > 8.2

CD163/FOXL2 double staining

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1 > 2 > 3.1 > 4(FOXL2) > 5.1 > 6.1 > 3.2 > 4(CD163) > 5.3 > 6.2 > 7 > 8.2

Antibody	Species	Clone or Code No.	Manufacturer	Dilution	Antigen Retrieval	Detection System	Chromogen (color)
FOXL2	goat, polyclonal	NB100-1277	Novus Biologicals, Littlecon, CO	1:400	Citrate pH6	Polink-2 Plus HRP[a]	DAB(brown)
ASMA	mouse, monoclonal	1A4	Dako, Carpinteria, CA	1:100	None	ImmPRESS-AP[b]	Vector Red (red)[b]
FAP	rabbit, polyclonal	NB100-58755	Novus Biologicals, Littlecon, CO	1:100	Citrate pH6	ImmPRESS-AP[b]	Vector Red (red)[b]
CD138	mouse, monoclonal	B-A38	Cell Marque, Rocklin, CA	1:100	EDTA pH8	Simple Stain MAX PO[c]	DAB(brown)

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CD163	mouse, monoclonal	10D6	Novocastla, Newcastle, UK	1:200	EDTA pH8	Simple Stain MAX PO[cd]	DAB(brown)[d]
CD45	mouse, monoclonal	2B11+PD7/26	Dako, Carpinteria, CA	1:100	Citrate pH6	ImmPRESS-AP[b]	Vector Red (red)[b]
CD31	mouse, monoclonal	JC70A	Dako, Carpinteria, CA	1:40	EDTA pH8	ImmPRESS-AP[b]	Vector Red (red)[b]
Collagen Type4	mouse, monoclonal	CIV22	Cell Marque, Rocklin, CA	1:500	Citrate pH6[e]	ImmPRESS-AP[b]	Vector Red (red)[b]

[a]GBI labs, Bothell, WA.

[b]Vector laboratories, Burlingame, CA.

[c]Nichirei Biosciences, Tokyo, Japan.

[d]For the double staining with FOXL2, CD163 was visualized with ImmPRESS-AP and Vector Red (red).

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[e]This microwave treatment was followed by Proteinase K treatment (0.002% Proteinase K, 3minutes, in room temperature).

Deparaffinization and rehydration

1
- Incubate slides in xylene for 5 min.
- Incubate slides in another jar of xylene for 5 min.
- Incubate slides in 100% ethanol for 5 min.
- Incubate slides in another jar of 100% ethanol for 5 min.
- Incubate slides in 90% ethanol for 5 min.
- Incubate slides in 80% ethanol for 5 min.

Blockage of endogenous peroxide

2
- Make 3% H_2O_2 in methanol just before use
- Incubate slides for 10 min.
- Rinse slides in tap water for 5 min.
- Rinse slides in DW for 5 min.

Antigen retrieval

3 3.1 Citrate buffer (pH 6) Microwave
- Enclose slides with citrate buffer (600ml) in a pressure cooker
- Microwave continuously for 20 min (500W).
- Open the lid and cool down naturally at room temperature (15-20 min.)
- Rinse slides in DW for 2 min. (three times)
- Wash slides in TBST for 5 min.

3.2 EDTA solution (pH8) Microwave
- Enclose slides with EDTA solution (600ml) in a pressure cooker
- Microwave continuously for 20 min (500W).
- Open the lid and cool down naturally at room temperature (15-20min.)
- Rinse slides in DW for 2 min. (three times)
- Wash slides in TBST for 5 min.

3.3 Proteinase K treatment
- Wipe excess liquid around the tissue
- Apply 80µl of Proteinase K solution on the tissue
- Incubate for 3 min. at room temperature
- Wash slides in TBST for 2 min. (four times)

NOTE

Citrate buffer stock solution (x10):

Citric acid monohydrate	0.9556g
Trisodium citrate dihydrate	13.23g

Dissolve in DW

Final volume 500ml

Dilute this ten times before use

NOTE

EDTA stock solution (0.5M):

EDTA 2Na	186.12g

dissolve in DW and adjust pH with 1M NaOH

Final volume and pH 1000ml pH8.0

Dilute this 500 times before use

NOTE

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Proteinase K: 20mg/ml (Roche, Cat No. 03 115 887 001)

Proteinase K dilution buffer:

TrisHCl 1M pH7.6	2ml
DW	98ml
$CaCl_2$	0.294g

Dilute Proteinase K 1000 times with dilution buffer just before use

Primary antibody

4
- Wipe excess liquid around the tissue
- Apply 40-50µl of primary antibody solution
- Incubate for 1.5 hours at room temperature
- Wash slides in TBST for 3 min. (three times)

NOTE

Dilute antibody with Antibody diluent (DAKO S8090) in advance
Ventana antibody diluent is not suitable for Polink-2 system!!

Secondary antibody

5 5.1 Polink-2 Plus HRP
- Wipe excess liquid around the tissue
- Apply 1 drop of Reagent1 (enhancer)
- Incubate for 15 min. at room temperature
- Wash slides in TBST for 2 min. (three times)
- Wipe excess liquid around the tissue
- Apply 1 drop of Reagent2 (Polymer HRP)
- Incubate for 25 min. at room temperature
- Wash slides in TBST for 2 min. (three times)

5.2 Simple stain MAX PO
- Wipe excess liquid around the tissue
- Apply 1 drop
- Incubate for 45 min. at room temperature
- Wash slides in TBST for 2 min. (three times)

5.3 ImmPRESS-AP
- Wipe excess liquid around the tissue
- Apply 1 drop
- Incubate for 45 min. at room temperature
- Wash slides in TBST for 2 min. (three times)

Histochemistry

6 6.1 Peroxidase-DAB
- Wipe excess liquid around the tissue
- Apply 60-100µl of reagent
- Incubate until positive signal becomes strong enough, checking with microscope (usually 3-8 min.)
- Rinse in tap water for 10 min.
- or Rinse in DW for 3 min., three times, if next immunostaining follows this

6.2 Alkaline phosphatase-Vector Red
- Wipe excess liquid around the tissue
- Apply 60-100µl of reagent
- Incubate until positive signal becomes strong enough, checking with microscope (usually 3-15 min.)
- Rinse in tap water for 10 min.
- or Rinse in DW for 3 min., three times, if next immunostaining follows this

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Counterstaining

7
- Shake excess water off the slides
- Incubate slides in Hematoxyline for 15 sec. at room temperature
- Wash slides in tap water for 10 min.

Dehydration and Mount

8 8.1 Ethanol dehydration
- Shake excess water off the slides
- Incubate slides in 100% ethanol for 5 min.
- Incubate slides in another jar of 100% ethanol for 5 min.
- Incubate slides in another jar of 100% ethanol for 5 min.
- Incubate slides in another jar of 100% ethanol for 5 min.
- Incubate slides in Hemo-De (xylene alternative) for 5 min.
- Incubate slides in another jar of Hemo-De for 5 min.
- Incubate slides in another jar of Hemo-De for 5 min.
- Mound with solvent-based medium

8.2 Heat dehydration (for slides stained with Vector Red)
- Rinse slides in DW for 3 min.
- Wipe excess water
- Put slides on a hot plate at 50 degree for 30-60 min.
- Incubate slides in Hemo-De (xylene alternative) for 5min.
- Incubate slides in another jar of Hemo-De for 5 min.
- Mount with solvent-based medium

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