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## mIHC staining (OHSU, Coussens' lab) SOP (TMA\_TNP)

Forked from a private protocol

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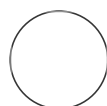
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**Protocol status:** Working  
We use this protocol and it's working

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ABSTRACT

This protocol describes multiplex immunohistochemical staining of human FFPE tissues. A staining "cycle" is defined as being one heat-mediated antigen retrieval step followed by either 1 or 2 or 3 staining "rounds". A "round" is defined as protein block, primary antibody incubation, secondary antibody incubation, chromogen development, whole slide scanning (visualization), and chromogen removal (with alcohol). If 2+ antibodies are used within a cycle a HRP inactivation step is used between rounds in order to block any chromogen development from the first round from contributing to signal in the second round. The multiple rounds in a single cycle must use antibodies produced in different species, i.e. rabbit and mouse, in order to ensure single target chromogen amplification.

ATTACHMENTS

[Discovery\\_panel\\_23\\_CBB\\_06.18.2020.xlsx](#)

## Bake and Deparaffinize slides

- 1 Incubate slides at 58-60 for 30 min. Cool for 5 minutes, then bake for another 30 mins.
- 2 Deparaffinize slides: Xylene 2 x 5 min, 100% ETOH 2 x 2 min, 95% ETOH 2 x 2 min, 70% ETOH 2 x 2 min, 50% ETOH 1 x 2 min, diH2O 2 x 2 min
- 3 Counterstain in Hematoxylin (Dako S3301) for 1min, diH2O 4-5 dips, then put in TBST.
- 4 Cover section (Cover Glass Signature Series, Thermo Scientific) with TBST (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl plus 0.05% Tween-20).
- 5 Scan the slides @ 20x magnification with the Leica Aperio slide scanner. Scan according to slide scanning protocol. Of note- first time slides are scanned requires setting-up slide settings (aka presets, same scan area and focus points) that will be used to scan that slide for the entire rest of the multiplex protocol.
- 6 Agitate the slides 3-5 minutes in TBST, then gently remove coverslip.
- 7 Wash slides in diH2O (4-5 dips).

## Perform Antigen Retrieval

- 8 Make antigen retrieval solution: Dilute 10x citrate #HK087-5K BioGenex (diluted from 10x with milliQ H2O) to make a 1x stock. You will need 250 mls total.
- 9 Perform antigen retrieval (AR): Cover plastic jar and microwave at full power for 90-120 seconds until liquid is boiling. Place slides into boiling citrate and place into the steamer for 20 minutes (make sure steamer has water in the basin).
- 10 Allow slides to cool down at room temperature (20-30 min).
- 11 Wash slides in diH2O (4-5 dips) and TBST (1 x 1 min).

## Block endogenous peroxidase activity

- 12 Incubate slides with Dako dual endogenous enzyme block (Dako catalog number S2003) for 10 minutes @ RT.
- 13 Wash slides in diH2O (4-5 dips) and TBST (1x 1 min).

## Protein blocking

Block section in Blocking buffer (5% normal goat serum, 2.5% BSA, 1X PBS) for 10 min @ RT

- 14 (use about 100-200µl /section).
- 15 Aspirate blocking buffer (or drain off). No washing necessary.

### Primary Antibody Incubation

- 16 Apply Primary antibody (100-200 µl/section) to section (dilute in 0.5X block buffer). Incubate in humidified chamber at 30 min @RT or overnight @4°C.
- 17 TBST wash, 3 x 2 min with agitation.

### Secondary Antibody (HRP conjugated) Incubation

- 18 Incubate slides in primary antibody-targeting secondary antibodies conjugated with HRP-polymer (e.g. HistoFine(M/R/G) Simple Stain MAX PO, Nichirei Bioscience Inc, 1-2 drops/section) @RT for 30 mins.
- 19 TBST wash, 3 x 2 min with agitation.
- 20 Wash slides in diH<sub>2</sub>O briefly. Do not mix TBST and AEC solution.

### Development of chromogen (AEC), i.e. visualization

- 21 Prepare AEC solution according to manufacturer's recommendation (Vector Lab, SK-4200).
- 22 Pipette AEC solution onto tissue, being sure to completely cover tissue.
- 23 Incubate 5-40 min (usually 20 min, depends on the marker) @RT.
- 24 Wash in diH<sub>2</sub>O (1 min, to de-activated AEC enzymatic reaction), then place slides in TBST.

### Perform whole slide scanning

- 25 Cover section (Cover Glass Signature Series, Thermo Scientific) with TBST. Drain excess fluid, and dry bottom of slide to remove streaks.
- 26 Scan the slides @ 20x magnification with the Leica Aperio slide scanner. See slide scanning protocol for details.
- 27 Agitate slides in TBST for 3-5 minutes, then gently remove coverslip.

### Remove chromogen with alcohol

- 28 Put slides into diH<sub>2</sub>O briefly, 70% ETOH briefly, 100% ETOH with agitation until signal-clearance (usually 2-3 min). BE SURE to check under the microscope that all color has been removed.
- 29 Rehydrate slides: 70% ETOH 1 x 1 min, 30% ETOH 1 x 1 min, dH<sub>2</sub>O wash 4-5 x briefly (make sure for complete elimination of EtOH), TBST 1 x 1 min
- 30 At this stage- go to step 8 if need to start next cycle. Go to step 31 if need to start a new round

(within a cycle).

## Inactivation of HRP on secondary antibodies

- 31** Incubate slides with Dako dual endogenous enzyme block (Dako catalog number S2003) for 10 minutes @ RT.
- 32** Wash slides in diH<sub>2</sub>O (4-5 dips) and TBST (1x 1 min).
- 33** Go to step 16 in order to add next primary antibody (to complete next round).