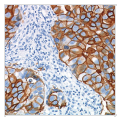

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DAB Staining of FFPE Slides

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1 Works for me dx.doi.org/10.17504/protocols.io.bdiei4be

Human BioMolecular Atlas Program (HuBMAP) Method Development Community

ABSTRACT

Standard IHC staining, such as this DAB protocol, is included in the validation of new antibodies at the University of Florida Tissue Mapping Center, TMC-Florida/Zurich. This procedure includes all of the reagents necessary to perform a DAB based immunohistochemistry reaction. DAB Substrate (3,3'-diaminobenzidine) produces a brown reaction product in the presence of peroxidase (HRP) enzyme.

ATTACHMENTS

New ELITE HRP Run Report.docx

GUIDELINES

Microwave retrieval conditions are specific to the microwave in use. Correct temperature is achieved when small bubble form along the plastic coplin, but a boil does not occur.
A water jacket (beaker of room temperature water at a level that is 1cm from the cap level of the retrieval solution plastic coplin jar) is used to equillibrate the temperature and avoid hot spots during the retrieval.

Do not allow slides to become dry during staining procedure.

MATERIALS

NAME	CATALOG #	VENDOR
Water		
Ethanol, 200 proof		
Hydrogen Peroxide, 30%	H325-500	Fisher Scientific
Methanol	A452-4	Fisher Scientific
Xylene	X3P-1GAL	Fisher Scientific
Coplin Staining Jar, Plastic Coplin staining jar, Holds ten slides back-to-back	107	Thermo Fisher
Shandon™ Shandon-Mount™	9990435	Thermo Fisher
Hematoxylin OPTIK DK	RS4576-A	
Bluing Reagent OPTIK	RS-4363-B	
Aqueous Clarifier	RS4361-B	
Vector DAB Staining Kit	SK-4100	Vector Laboratories
Vector Elite ABC Kit	PK-4000	
Immunohistochemistry Slide Humidity Chamber	68432A	Newcomer Supply

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NAME	CATALOG #	VENDOR
Avidin/Biotin Blocking Kit	SP-2001	Vector Laboratories
Normal Serum for Blocking (see guide below)		Vector Laboratories
Biotinylated anti-Primary Species Secondary (See selection guide below)		Vector Laboratories
Tween 20	P9416	Sigma Aldrich
TRIS-buffered saline (TBS 10X) pH 7.6	AAJ62662K3	Fisher Scientific
Citrate Buffer pH 6.0 10X	ab64214	Abcam

MATERIALS TEXT

SECONDARY ANTIBODY AND BLOCKING SERUM SELECTION INFORMATION:

Primary Antibody Species		Biotinylated Secondary Antibody		Blocking Serum
RABBIT		Goat anti-Rabbit (BA-1000)		Goat (S-1000)
MOUSE		Horse anti-Mouse (BA-2000)		Horse (S-2000)
RAT		Rabbit anti-Rat (BA-4000)		Rabbit (S-5000)
GOAT		Rabbit anti-Goat (BA-5000)		Rabbit (S-5000)
CHICKEN		Goat anti-Chicken (BA-9010)		Goat (S-1000)

All Catalog Numbrers Provided are for Vector Laboratories Products

It is easier to work with Tween-20 if a 10% solution is prepared in advance of performing this protocol.

SAFETY WARNINGS

Xylene is flammable and a neurotoxin. Use in a fumehood, keep containers tightly closed when not in use, and avoid open flames.

Hydrogen peroxide is ractive and can cause skin/eye damage. Handle with appropriate protective gear. Collect waste for chemical disposal

Methanol is flammable and an aquatic life toxin . Avoid flames, dispose of as chemical waste, collect first rinsate as waste.

Handle heated retrieval solutions with hot hands or heat resistant gloves.

DAB is a suspected carcinogen. Appropriate care should be exercised when using this reagent including gloves, eye protection, lab coats, and good laboratory procedures. Dispose in accordance with local regulations.

BEFORE STARTING

Replenish chemicals in slide deparaffinization/rehydration portion of staining line.

Prepare humidity chamber for slide incubations.

A bright field bench microscope is needed to monitor DAB development reaction.

Blocking serum and biotinylated secondary antibody must be compatible with primary antibody source species.

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Day 1 Prepare Slides for staining 1d

1 De-paraffinize and rehydrate slides.

Place slides in a xylene-compatible rack.

1.1 Immerse slide holder in first xylene station for 5 minutes. **00:05:00**

1.2 Immerse slide holder in second xylene station for 5 minutes. **00:05:00**

1.3 Immerse slide holder in first 100% Ethanol for 5 minutes. **00:05:00**

1.4 Immerse slide holder in second 100% Ethanol for 5 minutes. **00:05:00**

1.5 Transfer slide holder into 3% H_2O_2 in methanol for 10 minutes. **00:10:00**

Prepare the 3% H_2O_2 in methanol solution fresh each day (25ml of 30% H_2O_2 added to 225ml of methanol).

1.6 Immerse slide holder in 95% Ethanol for 5 minutes. **00:05:00**

1.7 Immerse slide holder in 75% Ethanol for 5 minutes. **00:05:00**

1.8 Immerse slide holder in 50% Ethanol for 5 minutes. **00:05:00**

1.9 Immerse slide holder in deionized water for 5 minutes. **00:05:00**

1.10 Immerse slide holder in deionized water for second wash for 5 minutes. **00:05:00**

Antigen Retrieval 1d

2 Perform heat mediated antigen retrieval using 1X citra buffer.

Alternative antigen retrieval methods can be used at this step based on individual antibody product sheet or laboratory experience/preferencve.

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2.1 Remove slides from slide holder and place into a plastic Coplin Jar filled with 10mM pH6.0 Na citrate buffer.
Slides may be placed back to back in Coplin jar.
Citrate buffer level must be over the top edge of the slides.

2.2 Place the Coplin Jar in a 400ml beaker filled with water leaving a centimeter of space between water level and cap bottom edge. Leave the cap loose, but held in place so that steam will not remove it.

2.3 Place beaker / Coplin jar assembly into microwave oven and heat for 7 minutes at 50% power.

2.4 Remove the beaker and jar from the microwave and incubate slides in the citrate buffer on the bench top for 18 more minutes.
During this incubation, make 1X TBST buffer by combing 90ml de-ionized water, 10ml 10X TBS, pH 7.6,
and 1ml 10% Tween-20.

3 Remove slides one at a time from the Coplin Jar and dip in de-ionized water to remove residual citra buffer.

3.1 Place slides in a humidity chamber and immediately cover secttions with 1X TBST buffer.
Equillibrate for 5 minutes

Blocking and Applying Primary 1d

4 Avidin block
To make the avidin solution: combine 1mL of 1X TBST buffer with 6 drops of Avidin from vector avidin biotin kit.

4.1 Take the slide, tap off buffer and cover section with Avidin Solution for 20 minutes. **00:20:00**

5 Biotin block
To make the biotin solution: combine 1mL of 1X TBST buffer plus 6 drops of Biotin from a vector avidin biotin kit.

5.1 Take the slide, tap off buffer and cover section with the Biotin solution for 20 minutes. **00:20:00**

5.2 Tap off biotin solution and cover sections in 1X TBST buffer .

6 Serum block
To make the serum block, combine 1mL of 1X TBST buffer and 20uL of Normal Serum .

The blocking solution is prepared with from the serum from the same animal species in which the biotinylated secondary antibody is made.

6.1 Tap off buffer and remove the excess moisture with gauze. Cover section with serum block and incubate for 60 minutes in the hummidity chamber

. **01:00:00**

6.2 Remove excess serum with gauze.

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7 Primary antibody incubation

Prepare desired dilutions of primary antibody in Antibody Diluent (Thermo Fisher)

7.1 Apply primary antibody solution directly over the section, cover with a plastic slip cut from a clean plastic bag surface and place in hummidity chamber. Incubate in a 4˚C refrigerator overnight. **Overnight**

Day 2 Detect Antibody Staining 1d

8 Remove chamber from the refrigerator, carefully lift off and dispose of plastic bag coverslips.
Wash slides with 1X TBST buffer for 5 minutes at room temperature . While washing, prepare ABC Reagent and secondary antibodies.

ABC Reagent needs to be made at least 30 minutes before it is used. To make the solution, use 20uL of reagent A and 1mL of buffer. Mix, then add 20uL of reagent B. Mix again.

To make secondary solution combine 1ml of 1X TBST buffer with 15ul of serum matching species secondary is raised in. Add 5ul of Vector biotinylated secondary antibody. Secondary antibody must be directed against species of origin for primary antibody.

9 Incubation with biotinylated secondary antibody(s).

9.1 Tip buffer of slides and remove excess moisture with gauze. Cover sections with the secondary antibody solution and incubate for 30 minutes at room temperature in a humidty chamber. **00:30:00**

10 Wash slide with 1X TBST buffer for 5 minutes. **00:05:00**
Tip buffer of of slides and remove excess moisture with gauze.

11 Cover the sections with ABC reagent (step 8) and incubate for 30 minutes at room temperature. **00:30:00**

12 Wash slides in 1X TBST buffer for 5 minutes. **00:05:00**
Slides can be held in buffer until ready for DAB staining.

13
DAB reaction
To make the DAB solution: add 1 drop of DAB to 1mL of Immpact Diluent.

13.1 Wipe excess buffer from one slide. Place the slide on to the stage of a bright field microscope, and identify an area for observing DAB development.

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13.2 Cover the section on the slide with DAB solution and incubate until desired intensity develops. Once desired intensity is reached, rinse and leave in water until ready to counterstain.

Collect DAB waste for hazardous chemical disposal.

Counterstain and mount 1d

14 Counterstain.
Prepare staining line in keeping with the steps below. Remove slides from water and place them into a xylene compatible slide holder.

14.1 Dip slide holder into hematoxylin for 10 seconds. **00:00:10**

14.2 Rinse with running tap water by immersing slide holder into water until it is clear.

14.3 Immerse slide holder into Clarifier for 20 seconds. **00:00:20**

14.4 Immerse slide holder into water for 1 minute.

14.5 Immerse slide holder into Bluing Reagent for 1 minute. **00:01:00**

14.6 Immerse slide holder into water for 1 minute.

14.7 Immerse slide holder into 50% Ethanol for 5 minutes. **00:05:00**

14.8 Immerse slide holder into 75% Ethanol for 5 minutes. **00:05:00**

14.9 Immerse slide holder into 95% Ethanol for 5 minutes. **00:05:00**

14.10 Immerse slide holder into 100% Ethanol for 5 minutes. Transfer slide holder into a second 100% Ethanol staining station for another 5 minutes. **00:10:00**

14.11 Immerse slide holder into first xylene for 3 minutes. Transfer slide holder into second xylene station for another 3 minutes. **00:06:00**

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15 Mount a coverslip on to the slide using a permanent mountant.

Working in the fume hood, remove one side at a time from the Xylene and mount coverslip with approximately 2 drops of Shandon Mount. Blot the edge of the coversliped slide on a paper towel to remove excess mountant and allow to dry in the fume hood.

16 When slides are dry, image using a brightfield microscopy sytem. The antibody staining will appear brown and nuclei will be counterstained light blue.

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