Apr 15, 2021

# IHC staining

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1 Works for me dx.doi.org/10.17504/protocols.io.bf6ajrae

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ABSTRACT

This is standard immunohistochemistry (IHC) procedure recommended in the Bendall and Angelo lab.

DOI

dx.doi.org/10.17504/protocols.io.bf6ajrae

PROTOCOL CITATION

Marc MB Bosse, Sean Bendall, Mike Angelo 2021. IHC staining. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bf6ajrae



CREATED

May 07, 2020

LAST MODIFIED

Apr 15, 2021

PROTOCOL INTEGER ID

36770

GUIDELINES

Staining tissue sections is fairly straightforward but there are a few things to be cognizant of when preparing samples:

- Always try to limit mechanical damage to the sample surface. This can occur when moving the samples with forceps, particularly when the mounting substrates are small.
- Once the samples have been rehydrated, they cannot dry out until the end of the protocol.
- Be careful at all times to not touch the tissue, in order to not leave any residue.
- Once the samples have been stained, fixed, and dehydrated, they have an indefinite shelf life and can be imaged at any time.
- The ImmPRESS Reagent is ready-to-use. No mixing or tittering of the ImmPRESS reagent is necessary to obtain immunohistochemical staining.
- For optimal performance, the ImmPRESS reagent should be equilibrated to room temperature before use.
- Unused working solution is stable up to 14 days if stored at 2-8°C. Working solution may change color during storage but this will have no effect on the quality or intensity of the staining.
- **Caution:** It is important that you do not use normal serum from the primary antibody source, as serum proteins from this source will be recognized by your secondary antibody – making your background staining worse, not better.

MATERIALS TEXT

| A | B | C |
|---|---|---|
| **Products** | **Provider** | **Catalogue No.** |
| Alcohol ethyl ETHANOL 200 PROOF | Gold Shield | 412811 |
| Alcohol ethyl ETHANOL 190 PROOF | Gold Shield | 412602 |
| TBS IHC Wash Buffer with Tween 20 | Cell Marque | 935B-09 |
| PBS IHC Wash Buffer with Tween 20 | Cell Marque | 934B-09 |
| Target Retrieval Solution, pH 9, (3:1) | Agilent (Dako) | S2375 |
| UltraPure water | Invitrogen | 10977-015 |
| Avidin/Biotin Blocking Kit | Biolegend | 927301 |
| Hydrogen peroxide | Sigma-Aldrich | 216763-100ML |
| Gelatin (cold water fish skin) | Sigma-Aldrich | G7765-250 |
| Xylene HISTOLOGICAL GRADE | Sigma-Aldrich | 534056-500 |
| Glutaraldehyde 8% Aqueous Solution EM Grade | EMS | 16020 |
| Normal Donkey serum | Sigma-Aldrich | D9663-10ML |
| Bovine Albumin (BSA), heat shock treated | Fisher Scientific | BP1600-100 |
| Centrifugal filters (0.1µm) | Millipore | UFC30VV00 |
| ImmEdge hybrophobic barrier pen | Vector labs | H-4000 |
| ImmPRESS UNIVERSAL (Anti-Mouse/Anti-Rabbit) IgG (HRP) | Vector Labs | MP-7500-50 |
| ImmPRESS Anti-Goat IgG KIT (HRP) | Vector Labs | MP-7405-15 |
| ImmPRESS Anti-Mouse IgG KIT (AP) | Vector Labs | MP-5402-15 |
| ImmPRESS Anti-Rabbit IgG KIT (AP) | Vector labs | MP-5401-15 |
| Levamisole | Vector Labs | SP-5000 |
| Horse serum | Vector Labs | S-2000 |
| VectaMount Permanent Mounting Medium | Vector Labs | H-5000 |
| ImmPACT DAB Peroxidase (HRP) Substrate | Vector Labs | SK-4105 |
| Transfer Pipettes, 4.6mL | Thermo Fisher | 251-1SPK |
| Thermo Scientific Gold Seal Cover Slips, #1.5 | Fisher Scientific | 12-518-108A |
| Harris Hematoxylin solution | Sigma-Aldrich | HHS16-500ML |
| Scott's tap water | Sigma-Aldrich | S5134-100ML |
| Cytoseal 60 | Thermo Fisher Scientific | 8310-4 |
| ACRYTOL mounting medium | EMS | 13518 |
| **Equipments** | **Provider** | **Cat No.** |
| Thermo Scientific™ Lab Vision™ PT Module | Thermo Fisher Scientific | A80400012 |
| Leica ST4020 Small Linear Stainer | Leica | 14050946425 |
| Digital incubators, INCU-Line®, IL 10 and IL 23 | VWR | 390-0384 |
| Bel-Art™ SP Scienceware™ Lab Companion Cabinet Style Vacuum Desiccators, Clear | Fisher Scientific | 08-648-109 |
| Oribital shaker | Boekel | 270200 |
| Immunostain Moisture Chamber | Ted Pella | 21051 |

SAFETY WARNINGS

All organic solvents should be manipulated under a chemical hood.



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BEFORE STARTING

Verify the stocks of all reagents and place an order or prepare solutions, if some reagents are running low.

## Slide baking and PT module preparation

1 ⚠

Bake the sections at **70 °C** for **00:20:00** in an dry incubator

**Optional** : **01:00:00** ; **Overnight**

last 10 min place the slide vertical with the label side up to allow the paraffin to drip down

**Note: Some tissues or section size may need longer baking time. Recommended to bake at least 1 hour for brain tissue or TMA. This can be extended to 16 h (overnight).**

2 **Prepare Target retrieval solution**

**2.5 mL** of target retrieval solution 10x (3-in-1)

in **22.5 mL** of Ultrapure TYPE 1 water

| A | B | C |
|---|---|---|
| Total volume (mL) | Volume (mL) Target retrieval | Volume (mL) H2O |
| 25 | 2.5 | 22.5 |
| 50 | 5 | 45 |
| 100 | 10 | 90 |

3 Put in the containers with the diluted target retrieval solution in the PT Module



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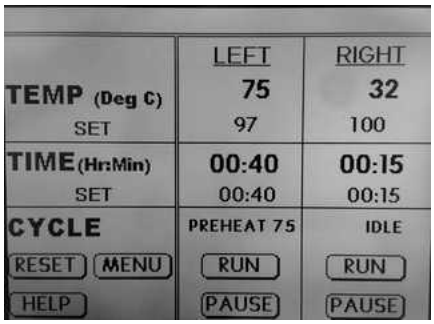
Thermo ScientificTM Lab VisionTM PT Module
Programmed Water bath

Thermofisher A80400012

PNG

4 **PT Module Preheat**
Press RUN on digital screen and check for PREHEAT 75 on display




## Slide deparafination

5 **Linear Stainer**
Pour out reagent containers and fill with fresh reagents:

Xylene 3x, 100% Ethanol 2x, 95% Ethanol 2x, 80% Ethanol 1x, 70% Ethanol 1x, ddH2O 2x, exit stainless steel tank = ddH2O

**IMPORTANT**: **Use fresh xylene for every deparaffination**



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Leica ST4020 Small Linear stainer
Stainer
**Leica** **14050946425**

PNG

6 Insert slides into slide carriers

Place the slide carrier into first xylene container

7 Press on **Menu**

Check for Processing time = 30 sec, Lift bar = 976, Number of dips = 3
Set Start position corresponding to the first slide carrier position
Exemple: If the first slide carrier is at position 4, use Plus (**+**) or Minus (**-**) button to increase or decrease to get **Start at: 04**

7.1 Then press **enter**

7.2 **Synchronize** when the PT module temperature has reached **75˚C** then Press **Run on the Linear Stainer**

7.3 Allow the rehydration process and wait until the slides reached the stainless steel tank and stop

8 Bring the stainless steel tank with the slides close to the PT module



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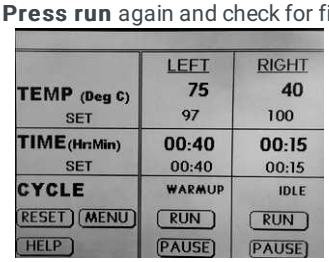
## Antigen Retrieval

9 Open the PT Module and insert the slides in the **Target retrieval solution** container

10 **Press run** again and check for first **WARMUP** then **HEAT** on display





11 Verify stock of 1x PBS wash buffer and prepare accordingly if running low

| A | B |
|---|---|
| **Reagents** | **Qty for 1000 mL** |
| PBS IHC Wash Buffer with Tween 20 (mL) | 50 |
| Bovine Albumin (BSA), heat shock treated (g) | 1 |
| Ultrapure (type 1) water (mL) | 949 |

12 Allow to run for 40 min at **97 °C** and then cool down for approximately 50 min and reach **65 °C**

13 When the alarm sounds Stop the PT module

14 Take out the slides and let cool down at room temperature for at least **00:05:00**

15 Prepare two Coplin jars filled with 1x PBS wash buffer

Transfer the slides in the first 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm



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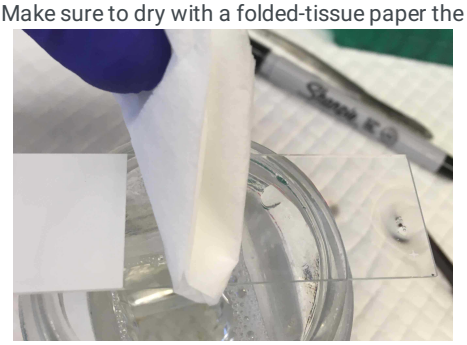
16

17 Transfer the slides to the second 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm

## Pap pen

18 ⚠

Make sure to dry with a folded-tissue paper the slide, leaving a square of wet surface surrounding of the tissue section



**Note: Do not let dry the tissue section**

19 Draw a square following the outside edges of the wet square with an hydrophibic ink pen (ImmEdge pen, Vector labs)

## Endogeneous enzyme Quenching

20 **Quenching Horseradish Peroxidase (HRP)**

20.1 Make a 1:10 dilution of 30% Hydrogen peroxide $H_2O_2$

| A | B | C |
|---|---|---|
| Total volume | Ultrapure TYPE 1 water (mL) | Hydrogen peroxide (mL) |
| 1 mL | 0.9 | 0.1 |
| 5 mL | 4.5 | 0.500 |
| 50 mL | 45 | 5 mL |

20.2 Incubate for 30 min by adding diluted $H_2O_2$ >100 µL (18 $mm^2$) per sections



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| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Estimated Surface area (mm) | 10x10 | 15x15 | 18x18 | 20x20 | 20x45 |
| Volume (µL) | 50 | 70 | 100 | 150 | 350 |

20.3 Prepare a Coplin jar filled with 1x PBS wash buffer

20.4 Transfer quenched tissue/ slides to the 1x PBS wash buffer and use orbital skaker set for 5 min, 70 rpm

20.5 **Proceed to step 22**

## 21 Quenching Alkaline Phosphatase

21.1 Make a 1:100 dilution of levamisole

| A | B | C |
|---|---|---|
| Total volume | Immpact diluent (mL) | Levamisol (mL) |
| 1 mL | 0.990 | 0.01 |
| 5 mL | 4.950 | 0.50 |

21.2 Incubate for 30 min by adding diluted Levamisole >100 µL (18 $mm^2$) per sections

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Estimated Surface area (mm) | 10x10 | 15x15 | 18x18 | 20x20 | 20x45 |
| Volume (µL) | 50 | 70 | 100 | 150 | 350 |

21.3 Prepare a Coplin jar filled with 1x PBS wash buffer

21.4 Transfer quenched tissue/ slides to the 1x PBS wash buffer and use orbital skaker set for 5 min, 70 rpm

**Blocking**

22 Add 100 µL of Blocking Buffer for 18 $mm^2$



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| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Estimated Surface area (mm) | 10x10 | 15x15 | 18x18 | 20x20 | 20x45 |
| Volume (µL) | 50 | 70 | 100 | 150 | 350 |

23 Place the slides in a moist chamber at **Room temperature** and incubate **00:20:00** to **01:00:00**

24 Dilute the primary antibody in antibody diluent buffer

## Primary antibody

25 Remove the blocking solution by tapping the slide on a side and immediately add the primary antibody
Refer to the chart for the volume of antibody to apply

| Estimated Surface area (mm) | 10x10 | 15x15 | 18x18 | 20x20 | 20x45 |
|---|---|---|---|---|---|
| Volume (µL) | 50 | 70 | 100 | 150 | 350 |

26 Verify stock of **Antibody Diluent (3% NHS)** and prepare accordingly if running low (Refer **MIBI and IHC solutions protocol)**

27 Place the moist chamber at 4˚C **Overnight** , preferably in a place with low disturbance (e.g. a designated area in a cold room)

## Wash buffer

28 Prepare two Coplin jars filled with 1x PBS wash buffer

28.1 Transfer the slides into the first Coplin jar and use orbital skaker set for 5 min, 70 rpm

28.2 Transfer the slides into the second Coplin jar and use orbital skaker set for 5 min, 70 rpm



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## Secondary antibody

29 Add adequate volume of universal anti-mouse/ anti-rabbit (Vector labs, MP-7500-50)
Note: 1 drop is ~40 µL

| Estimated Surface area (mm) | 10x10 | 15x15 | 18x18 | 20x20 | 20x45 |
|---|---|---|---|---|---|
| Volume (µL) | 50 | 70 | 100 | 150 | 350 |
| Estimated # of drops | 1 | 2 | 3 | 4 | 8-9 |

30 Place sample in a humidity chamber, transfer to **Room temperature** , and incubate **00:30:00**

30.1 Equilibrate at **Room temperature** the chromogenic substrate and revealing buffer

## Wash buffer

31 **go to step #28** than go to step 32

## Chromogenic development

32 Add 1 drop of chromogenic solution (DAB) in **1 mL** dilution buffer (ImmPACT DAB Peroxidase (HRP) Substrate, SK-4105, Vector lab)

32.1 Take the slide from 1x PBS wash buffer

32.2 Place the slide on a absorbant meterial (preferably white)

32.3 Start a count up timer and take immediatly 1mL DAB diluted solution with 1 mL pipette

32.4 At 10 s count up, add rapidly the solution in excess over tissue section; starting with the lowest titer

32.5 ⚠

Stop at 50 s count up by tapping the side of the slide to remove DAB and putting the slide back in the wash buffer



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DAB chromogenic reaction is revealed by a brown coloration in positive areas.

Optimal revelation time ranges between 30s to 1 min.

Rapid brown apperance (<15s) after adding the DAB indicates an over titration of the primary antibody.
Light brown or no coloration >1 min reveals is undertitered primary antibody or negative result.

Revealling time should be set constant at 40s but can be extented up to 1 min max.

Optimal titer at constant development time ensure consistency when large number of slides are processed.

33 **Optional**
Chromogenic development can be performed directly under a microscope.

This should be done at low magnification using a 5 x or a 10x objective. This way, it helps to cover a large field of view where positive areas can be observed.

At higher magnification (*e.g.* 40x), it is likely that the DAB solution will contaminate the objective and cause a permanent stain if not cleaned immediately with ROR (Residual Oil Remover, EMS, 74319-35) and lens paper.

This procedure can be used for single preliminary test but is not recommended for large scale.

**Importantly, make sure to clean the objectives and stage after use. Otherwise, DAB residues will stain permanently the surface in contact and increase deterioration of the microscope parts in the long term.**

## Counter stain

34 Prepare a 1L beaker filled with tap water in a sink

34.1 Set staining area by protecting counter top with an absorbant

34.2 Start a count up timer

34.3 ⚠

Add Hematoxylin to cover all tissue sections (Hematoxylin Solution, Harris Modified, Sigma-Aldrich, HHS16-500ML)

After 10s stop by dipping and rinsing in the tap water beaker



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Reveal one slide at the time

34.4 Add 1x Scott's tap water for 10s, until blue color is revealed

34.5 Put in 70% ethanol

## Deshydration and Clear Medium (toluene-acrylic based)

### 35 Prepare the linear stainer

Place the reagents in the following order:

70% Ethanol 1x, 80% Ethanol 1x, 95% Ethanol 2x, 100% Ethanol 2x, Xylene 3x, exit stainless steel tank = empty

35.1 Let air dry for at least **00:02:00** 2m

35.2 Dip 3x the slide quickly in xylene prior mounting

35.3 

Add 2 drops of mounting media on tissue area by squeezing down the bottle gently for Cytoseal 60 or ~70 µL of ACRYTOL from a small aliquot put in a 50 mL tube

Cytoseal 60 **VWR**

**Avantor Catalog #48212-154**

ACRYTOL mounting medium **Electron Microscopy**

**Sciences Catalog #13518**

Work under a fume hood. The mounting media contains toluene as solvent.

35.4 Place a glass coverslip no. 1.5 (Thermo Scientific, Gold Seal, coverslips, #1.5, Fisher Scientific, 12-518-108A)

35.5 Excess of mounting media can be removed by dipping back the slide in xylene and wiping excess along the slide edges

35.6 Let dry **00:15:00** under fume prior long term storage 15m



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## Deshydration and VectaMount Permanent Mounting Medium 1h 2m

### 36 Prepare the linear stainer

Place the reagents in the following order:

70% Ethanol 1x, 80% Ethanol 1x, 95% Ethanol 2x, 100% Ethanol 2x, exit stainless steel tank = empty

36.1 Let air dry for at least **00:02:00** 2m

36.2 ⚠

Add 2 drops of mounting media on tissue area using a transfer pipette or a pointed cotton swab

VectaMount permanent **Vector Laboratories Catalog #H-5000**

**NOTE: Please DO NOT USE micropipette (200 µL or 1 mL). Mounting media residues may soil the pipette tip and making it less accurate unless it is cleaned properly.**

36.3 Place a glass coverslip no. 1.5 (Thermo Scientific, Gold Seal, coverslips, #1.5, Fisher Scientific, 12-518-108A)

36.4 Bake at **70 °C** for **01:00:00** prior long term storage 1h



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