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IHC staining

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ABSTRACT

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

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 titrating 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

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

Created: May 07, 2020

Last Modified: Apr 10, 2023

PROTOCOL integer ID:
36770

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	UFC30V V00
ImmEdge hydrophobic 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

A	B	C
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.

BEFORE START INSTRUCTIONS

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

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

Equipment

Thermo Scientific™ Lab Vision™ PT Module

NAME

Programmed Water bath

TYPE

ThermoFisher

BRAND

A80400012

SKU

<https://www.thermofisher.com/order/catalog/product/A80400012#/A80400012>

LINK

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

Note

IMPORTANT: Use fresh xylene for every deparaffination

Equipment

Leica ST4020 Small Linear stainer

NAME

Stainer

TYPE

Leica

BRAND

14050946425

SKU

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

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
- 16 Transfer the slides in the first 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm
- 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 hydrophobic 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 μ L (18 mm²) per sections

A	B	C	D	E	F
Estimated Surface area (mm)	10x10	15x15	18x18	20x20	20x45
Volume (μ L)	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	Impact 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 mm²) 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

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
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Volume (μL)	50	70	100	150	350
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- 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

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
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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

Note

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

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 International Catalog #48212-154**

ACRYTOL mounting medium **Electron Microscopy Sciences Catalog #13518**

Safety information

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

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 μ L 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