Mar 23, 2023 Version 1

Dual antibody immunohistochemistry staining V.1

DOI
dx.doi.org/10.17504/protocols.io.81wgbyoryvpk/v1

Angelina Spence[1], Mako Goldston[1], Kausalia Vijayaragavan[1], Marc Bosse[1], Sean Bendall[1], Mike Angelo[1]

[1]Stanford University

Human BioMolecular Atlas Program (HuBMAP) Method Development Community
Tech. support email: **Jeff.spraggins@vanderbilt.edu**

Marc Bosse
Stanford University

DOI: https://dx.doi.org/10.17504/protocols.io.81wgbyoryvpk/v1

Protocol Citation: Angelina Spence, Mako Goldston, Kausalia Vijayaragavan, Marc Bosse, Sean Bendall, Mike Angelo 2023. Dual antibody immunohistochemistry staining. **protocols.io https://dx.doi.org/10.17504/protocols.io.81wgbyoryvpk/v1**

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

Created: March 20, 2023

Last Modified: January 31, 2024

Protocol Integer ID: 79109

Keywords: chromogenic, double stain, immunohistochemistry, IHC, multiplex, dual antibody, dual antibody immunohistochemistry, immunohistochemistry, antibody, multiplex ion beam imaging time, multiplex ion beam imaging time of flight, standard ffpe tissue, ihc, staining procedure

Abstract

This protocol provides guidelines to perform dual antibody immunohistochemistry (IHC) on standard FFPE tissue in the Sean C. Bendall and Michael R. Angelo labs. The staining procedure is principally performed to validate the co-occurrence of two targets observed in Multiplex Ion Beam Imaging Time of Flight (MIBI_TOF) analysis.

Materials

	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	Agilent (Dako)	S2367
	UltraPure water	Invitrogen	10977-015
	Gelatin (cold water fish skin)	Sigma-Aldrich	G7765-250
	Xylene HISTOLOGICAL GRADE	Sigma-Aldrich	534056-500
	Bovine Albumin (BSA), heat shock treated	Fisher Scientific	BP1600-100
	Centrifugal filters (0.1µm)	Millipore	UFC30VV00
	ImmPRESS Duet (Anti-Mouse IgG HRP, Anti-Rabbit AP)	Vector Labs	MP-7724
	ImmPRESS Duet (Anti-Mouse IgG AP, Anti-Rabbit HRP)	Vector Labs	MP-7714
	ImmPACT Red alkaline phosphatase	Vector Labs	SK-5105
	ImmPACT NovaRed peroxidase	Vector labs	SK-4805
	Vector Blue	Vector labs	SK-5300
	ImmPACT DAB Peroxidase (HRP) Substrate	Vector Labs	SK-4105
	Bloxall	Vector Labs	SP-6000-100
	Horse serum	Vector Labs	S-2000
	VectaMount Permanent Mounting Medium	Vector Labs	H-5000
	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

	A	B	C
	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

Troubleshooting

Protocol IO references

1 This protocol refers to detailed procedures found in these respective Protocol IO:
- IHC staining **dx.doi.org/10.17504/protocols.io.bf6ajrae**
- Sequenza **dx.doi.org/10.17504/protocols.io.bmc6k2ze**
- MIBI and IHC solutions **dx.doi.org/10.17504/protocols.io.bmc6k2ze**

Slide preparation

2 Slides are baked, deparaffinized and processed for antigen retrieval and washed in PBS wash buffer as described in the IHC staining protocol.

3 The slides are then placed in an immunostain humid chamber.

4 To block endogenous peroxidase and alkaline phosphatase, the section of each slide is covered with 3-4 drops of Bloxall (Vector Laboratories, SP-6000-100), and incubated for 30 min at room temperature.

5 After blocking, the slides are briefly rinsed with PBS wash buffer and mounted on the Sequenza assembly (ref. Sequenza protocol).

6 Add 2 mL of PBS wash buffer for each slide and let the entire volume of buffer flow through.

7 Add 200 µL of blocking buffer (ref. MIBI IHC solutions) and incubate 1h.

Primary antibody staining titration

7.1

Note

The ImpressDuet (Vector Laboratories) requires the use of a mouse IgG antibody for the antigen A and a rabbit IgG antibody for the antigen B. A version of ImpressDuet kit (Vector Laboratories, MP-7714) uses a secondary antibody anti-mouse conjugated with alkaline phosphatase (AP) and an anti-rabbit conjugated with peroxidase (HRP). The ImpressDuet kit (Vector Laboratories, MP-7724) offers the reciprocal with an secondary antibody anti-mouse conjugated with HRP and an anti-rabbit conjugated with AP. The kits also includes a brown substrate for HRP and magenta substrate for AP. These kits are designed to obtain reliable results on tissue sections where the two target antigens do not over-lap (co-localize) in the same structure of the same cell, but rather are expressed in different cell compartments or different cell types. One may choose the substrate combination empirically that offers the best contrast between the two antigens of interest.

Perform an antibody titration for each antigen target. The recommended starting titers would be 1 µg/mL and 0.25 µg/mL with known antibody concentration or use the provider recommended dilution titer and 4x less (e.g. 1:400 and 1:1600). From the results of the first titration, iterate with a second titration to narrow it down. This initial titration can be done using the HRP and DAB detection. The DAB revelation time should be between 30s to 1 min, no less.

Note

DAB chromogenic reaction is revealed by a brown coloration in positive areas.
Rapid brown appearance (<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.
Revealing 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.

8 Once the initial titer is determined, perform a single color stain for each target with each substrate. Choose the color substrate that gives the best sensitivity for a putative target. In contrast to DAB, some substrates have a wide range of development time which gives more flexibility to increase sensitivity. For example, the alkaline phosphatase substrate Vector blue (SK-5300) has an optimal revelation time of 20-30 min but can be extended from 2 to 4 hours.

9 Once the secondary-enzyme conjugate, the color substrate, and the development time for each antigen target have been determined, proceed to perform the double staining.

Double staining

10 Dilute the antibody with the lowest titer first (Antibody 1) in antibody diluent buffer. Then dilute the second antibody (Antibody 2) using the solution of Antibody 1 with the lower titer.

11 After blocking, add 200 µL antibody diluent and let the entire volume of buffer flow through.

12 Add 100 µL of the diluted antibodies per slide and incubate at 4°C overnight.

13 The next day, add 2 mL of PBS wash buffer and let the entire volume of buffer flow through.

14 Bring all the reagents for double IHC staining to room temperature.

15 For each slide, add 3 drops of the secondary antibody-enzyme mix corresponding the desired color for each target (ImpressDuet, MP-7714 or MP-7724, Vector Laboratories).

16 Incubate for 10 min at room temperature.

17 Add 2 mL of PBS wash buffer and let the entire volume of buffer flow through.

Development

18 Start revealing with the alkaline phosphatase substrate first. Prepare the alkaline phosphatase substrate working solution by adding the components in the recommended buffer as instructed by the manufacturer.

Note

As an example, Vector Blue alkaline phosphatase substrate recommended buffer is 100mM Tris-HCl pH 8.2-8.5 , 0.1% Tween for antigen retrieved tissue section.

19 Dismount the Sequenza assembly and put the slides on a immunostain humid chamber. Add immediately an excess of substrate working solution to cover the tissue section.
.

Note

Some substrates may develop better in the dark. Put the immunostain humid chamber cover during development.

20 Stop the development by removing the excess and incubate for 5 min in recommended buffer (100 mM Tris-HCl pH 8.5, Tween 0.1% or PBS).

21 Rinse in water prior to the peroxidase development.

22 Prepare the peroxidase substrate working solution by adding the components in the recommended buffer as instructed by the manufacturer.

23 Put the slides back into the immunostain humid chamber. Immediately add an excess of the peroxidase substrate working solution to cover the tissue section and develop for 40s.

24 Stop the development by removing the excess and transfer in water.

25

Note

It is not recommended to counterstain with hematoxylin. Some substrates are partially soluble in xylene. Do use xylene-based mounting medium.

26 Dehydrate the tissue in 1x ethanol 70%, 1x ethanol 80%, 2x ethanol 95%, 2x ethanol 100%.

27 Coverslip in a non-aqueous mounting media such as VectaMount (Vector Laboratories, H-5000).

Note

Slides can be placed in a 70°C oven for 1h to accelerate drying.

28 Let the slides dry completely before visualization.

Protocol references

This protocol refers to detailed procedures found in these respective Protocol IO:

- IHC staining **dx.doi.org/10.17504/protocols.io.bf6ajrae**
- Sequenza **dx.doi.org/10.17504/protocols.io.bmc6k2ze**
- MIBI and IHC solutions **dx.doi.org/10.17504/protocols.io.bmc6k2ze**