STANDARD OPERATING PROCEDURE
Immunopathology
OPPC-SOP-72

Prepared by: Myriam Padilla and Paul Joseph
Revised by: Paul Joseph
Reviewed by: Maria Beery
Approved by: Irina Kusmartseva

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Network for Pancreatic Organ Donation with Diabetes (nPOD)
BMSB Room 586
P.O. BOX 100275
Gainesville, FL 32610
IMMUNOPATHOLOGY

POLICY: Use universal safety precautions when handling human samples and personal protective equipment (e.g., face mask with shield, gloves, lab coat or apron). Use chemical and physical safety precautions when working with paraformaldehyde and sharps, respectively.

PURPOSE: The purpose of this Standard Operating Procedure (SOP) is to outline procedures for immunopathology preparation and analysis of nPOD samples.

SCOPE: This SOP will be applied to nPOD paraffin samples stained by immunohistochemistry.

RESPONSIBILITIES: Managers and supervisors - are responsible for making sure that technicians are properly trained and equipment and facility are maintained in good working order.

Laboratory personnel - are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs.

EQUIPMENT & MATERIALS: The materials, equipment and forms listed in the following list are recommendations only and alternative products as suitable may be substituted for the site-specific task or procedure.

- Primary and secondary antibodies (see Appendix 1), antibody diluent (Renaissance, Davinci, Dako)
- Dewaxing reagents- xylene, 100% and 95% ethanol (EtOH), water, reagent containers (Tissue Tek)
- 3% Hydrogen peroxide (H₂O₂) Pipettes and tips, serological pipettes
- Antigen retrieval: Borg’s Decloaker RTU (BioCare), water bath (98°C)
- Tris buffered saline with Tween (TBST) - used for washes or rinses
- ImmEdge pen
- Background Sniper (BioCare)
- Avidin-Biotin Kit (BioCare)
- MACH 2 Double Stain 1, MACH 2 Double Stain 2 (BioCare)Biotinylated Goat anti-guinea pig, Avidin-Biotin-AP kit (Zymed)
- Betazoid DAB Chromogen Kit (HRP; BioCare)
- Ferangi Blue Chromogen Kit (AP; BioCare)
- Warp Red Chromogen Kit (AP; BioCare)
- Deep Space Black Chromogen Kit (HRP; BioCare)
1.0 Preparation for Immunopathology

1.1 Prepare all solutions according to manufacturer’s recommendations. Optimize antibody detection by antigen retrieval screening, titration, and validation according to clinical practice standards. Use Renaissance diluent for Ki67 and Somatostatin, Davinci diluent for CD3 and CD45, or Dako for CD45.

1.2 Rotate the xylene and alcohol containers. Dispose the first containers of xylene, 100% EtOH, and 95% EtOH. Move all other containers up one position. Move fresh xylene and EtOH to the last positions.

1.3 Prepare antigen retrieval by placing container with 200 mL of Borg’s Decloaker RTU to water bath set to 98°C. Allow sufficient time to heat up before adding slides.

1.4 Prepare sufficient Tris Buffer Saline:
   1.4.1 900 mL ddH2O
   1.4.2 100 mL UltraPure 1M Tris-HCl pH 7.5
   1.4.3 500 uL Tween 20
   1.4.4 8.8 g NaCl

1.5 Place serial unstained paraffin slides in the slide dryer, set to 65°C, for 1 hour.

1.6 Transfer the dried slides to xylene overnight.

1.7 Clear and rehydrate paraffin sections according to the schedule below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time (minutes)</th>
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<tbody>
<tr>
<td>Xylene</td>
<td>5</td>
</tr>
<tr>
<td>Xylene</td>
<td>5</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>3</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>3</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>3</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>3</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td></td>
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</tbody>
</table>

1.7.1 Note: if you do not allow the slides to soak in xylene overnight, you can begin the rehydration process. However, you must add an additional soak in xylene and increase the time in xylene to 10 minutes, each.
2.0 Immunohistochemistry Staining

2.1 Antigen retrieval 1:

2.1.1 Incubate the slides in Borg’s Decloaker RTU, within the water bath, for 20 minutes. Position the slides so that the sides with the tissue are facing away from the thermometer. Place lid on container with slides.

2.1.2 After 20 minutes, record the temperature. Continue incubating for 20 minutes more.

2.1.3 Remove container with Borg’s and slides from the water bath. Record the temperature. Move the lid so that it is partially covering the container. Allow to cool at room temperature for 10 minutes.

2.2 Slowly add ddH2O to the container with the slides and Borg’s until the decloaker is gone and the slides are cooled down. This must be done slowly so that the water does not strip the slides of the tissues.

2.3 Transfer the slides to 3% H2O2 for 10 minutes.

2.3.1 **NOTE: During this time, replace Borg’s Decloaker RTU and place container back in the water bath.**

2.4 Transfer the slides to ddH2O.

2.5 Wash the slides in four containers of TBS (ten dunks each) and let them sit in a TBS bath for 5 minutes.

2.6 Wipe off residual TBS around the tissue and apply ImmEdge pen around tissue. Place in tray and apply TBS on tissue using dropper.

2.7 Tap off TBS and apply 4-5 drops of Background Sniper to tissue. Incubate for 2 minutes.

2.8 Tap off Background Sniper, wash slides in TBS containers and let them sit in the TBS bath for 5 minutes.

2.9 Apply 250-300 uL of primary antibody solution to the slides. Incubate for 20 minutes.

2.10 Tap off primary antibody solution, wash slides in TBS containers, and let them sit in the TBS bath for 5 minutes.

2.11 Apply 4-5 drops of MACH 2 Double Stain 1 to the tissues and incubate for 20 minutes.

2.12 Tap off the Double Stain 1 solution, wash slides in TBS containers and let them sit in the TBS bath for 5 minutes.

2.13 Prepare sufficient amount of Betazoid DAB Chromogen solution (1 drop of chromogen per 1 mL buffer).

2.14 Place slides in TBS container (without spinner).

2.15 Apply 200-250 uL of DAB to slide for appropriate amount of time, depending on the primary antibody, while monitoring color change thoroughly. Dip in ddH2O waste jar and then place in separate ddH2O container.

2.16 Transfer back to TBS container (without spinner).

2.17 Prepare sufficient amount of Ferangi Blue solution (1 drop of chromogen per 2.5 mL buffer).

2.18 Apply 200-250 uL of Ferangi Blue to slide for 2 minutes and 30 seconds. Dip in ddH2O waste jar and then place in separate ddH2O container.

2.19 Antigen Retrieval 2:
2.19.1 Incubate the slides in Borg’s Decloaker RTU, within the water bath, for 10 minutes. Position the slides so that the sides with the tissue are facing away from the thermometer. Place lid on container with slides.

2.19.2 After 10 minutes, record the temperature. Continue incubating for 10 minutes more.

2.19.3 Remove container with Borg’s and slides from the water bath. Record the temperature. Move the lid so that it is partially covering the container. Allow to cool at room temperature for 15 minutes.

2.20 Slowly add ddH₂O to the container with the slides and Borg’s until the decloaker is gone and the slides are cooled down. This must be done slowly so that the water does not strip the slides of the tissues.

2.21 Transfer the slides to 3% H₂O₂ for 10 minutes.

2.22 Transfer the slides to ddH₂O.

2.23 Wash the slides in four containers of TBS (ten dunks each) and let them sit in a TBS bath for 5 minutes.

2.24 Wipe off residual TBS around the tissue and apply ImmEdge pen around tissue. Place in tray and apply TBS on tissue using dropper.

2.25 Add 4-5 drops of Avidin to the tissue and incubate for 3 minutes.

2.26 Wash the slides in TBS and let them sit in TBS bath for 5 minutes.

2.27 Add 4-5 drops of Biotin to the tissue and incubate for 3 minutes.

2.28 Wash the slides in TBS and let them sit in TBS bath for 5 minutes.

2.29 Add 4-5 drops of Background Sniper to the tissue and incubate for 3 minutes.

2.30 Wash the slides in TBS and let them sit in TBS bath for 5 minutes.

2.31 Add 250-300 µL of Insulin (or Insulin + PP) primary antibody solution and incubate for 30 minutes.

2.32 Wash the slides in TBS and let them sit in TBS bath for 5 minutes.

2.33 Add 4-5 drops of MACH 2 Double Stain 2 to the tissue and incubate for 30 minutes.

2.34 Wash the slides in TBS and let them sit in TBS bath for 5 minutes.

2.35 Prepare sufficient amount of Warp Red Chromogen solution (1 drop of chromogen per 2.5 mL buffer).

2.35.1 **Note: Warp Red is only effective for approximately 15 minutes, so do not make large volumes.**

2.36 Place slides in TBS container (without spinner).

2.37 Apply 200-250 µL of Warp Red to the slide for 2 minutes, while monitoring the color change thoroughly. Dip in ddH₂O waste jar and then place in separate ddH₂O container.

2.38 Transfer slides back to TBS container (without spinner).

2.39 Prepare sufficient amount of Deep Space Black Chromogen solution (1 drop of chromogen per 1 mL buffer).

2.40 Apply 200-250 µL of Deep Space Black to slide for 10 seconds. Dip in ddH₂O waste jar and then place in separate ddH₂O container.

2.41 Rinse in diH₂O and then place in diH₂O bath (with spinner) for 5 minutes.

2.42 Place slides in 10% CAT Hematoxylin for 30 seconds.

2.43 Transfer slides to diH₂O, rinse, and place in diH₂O bath (with spinner) for 5 minutes.

2.44 Place slides in Bluing solution for 30 seconds.

2.45 Transfer slides to diH₂O, rinse, and place in diH₂O bath (with spinner) for 5 minutes.

2.46 Allow slides to dry overnight. Apply coverslips after the slides have fully dried.
REFERENCES:

1.0 Related Documents and Procedure

1.1 DAKO IHC Staining Methods – Educational Guide
1.3 SOP 57 Case Processing
1.4 SOP 70 Histology
1.5 SOP 73 Online Pathology

REVISION HISTORY

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<thead>
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<th>Version</th>
<th>Date</th>
<th>Revision</th>
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<tbody>
<tr>
<td>1</td>
<td>05/04/11</td>
<td>Updated materials, reagents, quadruple stain IHC procedure</td>
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### Appendix 1

Primary Antibodies Used in nPOD Immunohistochemistry Protocols

<table>
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<tr>
<th>Antigen</th>
<th>Host</th>
<th>Antibody Clone</th>
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