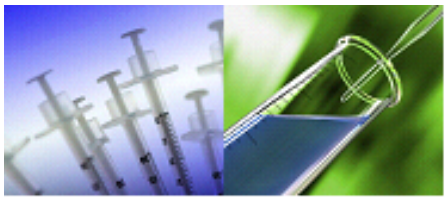

nPOD
Network for Pancreatic Organ Donors with Diabetes

STANDARD OPERATING PROCEDURE

Immunopathology

OPPC-SOP-72

Prepared by:	Myriam Padilla and Paul Joseph	**Original Effective Date:**	07/01/2007
Revised by:	Paul Joseph	**Version Effective Date:**	05/06/2021
Reviewed by:	Maria Beery	**Reviewed Date:**	04/17/2019
Approved by:	Irina Kusmartseva	**Approved Date:**	05/06/2021

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Network for Pancreatic Organ Donation with Diabetes (nPOD)
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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.

RESPOSIBILITIES: 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 (H_2O_2)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)

- 10% CAT Hematoxylin (BioCare)
- Bluing Reagent (Avantix)
- Liquimount mounting media, coverslips

PROCEDURE:

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 ddH_2O
- 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:

Reagent	Time (minutes)
Xylene	5
Xylene	5
100% EtOH	3
100% EtOH	3
100% EtOH	3
95% EtOH	3
95% EtOH	3
Water	

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 ddH_2O 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 ddH_2O 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 uL 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 uL of Warp Red to the slide for 2 minutes, while monitoring the color change thoroughly. Dip in ddH_2O waste jar and then place in separate ddH_2O 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 uL 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

1.2 Campbell-Thompson, M., et al. *Pancreatic adenocarcinoma patients with localized chronic severe pancreatitis show increased single beta cell numbers without alterations in fractional insulin area*. Diabetologia. 2009.

1.3 SOP 57 Case Processing

1.4 SOP 70 Histology

1.5 SOP 73 Online Pathology

REVISION HISTORY

Version	Date	Revision
1	05/04/11	Updated materials, reagents, quadruple stain IHC procedure

	Name	Signature	Date
Prepared by:	Myriam Padilla and Paul Joseph		04/17/2019
Approved by:	Irina Kusmartseva		05/06/2021

Appendix 1

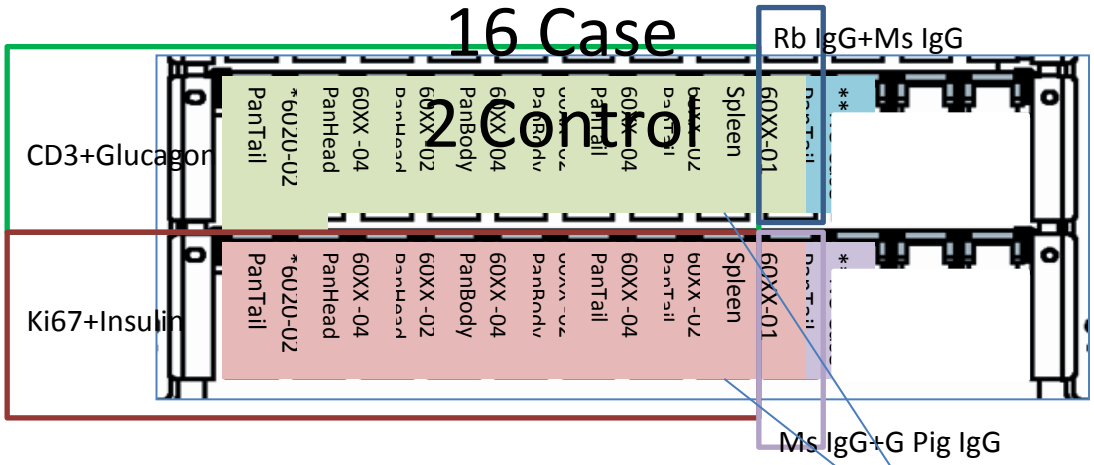
Primary Antibodies Used in nPOD Immunohistochemistry Protocols

Antigen	Host	Antibody Clone	Vendor
Insulin	Guinea Pig	A0564	Dako
Ki67	Mouse	M1B-1	Dako
CD3	Rabbit	A0452	Dako
Glucagon	Mouse	ab10988	Abcam
Somatostatin	Rabbit	A0566	Dako
Pancreatic Polypeptide	Rabbit	A0619	Dako
Synaptophysin	Mouse	SY38	Dako
CK19	Mouse	M0772	Dako
CD4	Mouse	4B12	Dako
CD8	Mouse	C8/114B	Dako
CD20	Mouse	L26	Dako
CD45	Mouse	2B11+PD7/26	Dako
CD68	Mouse	PG-M1	Dako

Appendix 2

Dako Autostainer Set-up and Slide Labeling

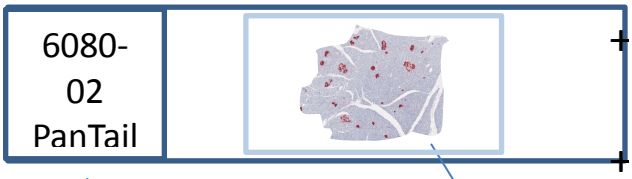
Dako Autostainer:
18 IHC Slides per Run
16 Case
2 Control



- Any Control case, any pancreas region. Rotate case ID, region, and blocks every ~5 runs
- ** Case, PanTail or any region, negative controls for primaries.

11/30/2010

Elements of a nPOD IHC Slide



CaseID-BlockID (2-digits)
Sample (PanHead, PanBody, etc)
Stain(s)
Date (mm/dd/yy)

Section centered on slide
Coverslip appropriate size
No artifacts

Scan into Spectrum, Sample=Specim

11/30/2010