

## IMMUNOPATHOLOGY

### 1 PURPOSE

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

### 2 SCOPE

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

### 3 RESPONSIBILITIES

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

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

### 4 EQUIPMENT and 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 (Zymed)  
Dewaxing reagents- xylene, 100%, 95%, and 70% ethanol (EtOH), water, reagent containers (Tissue Tek)  
Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Methanol (MeOH)  
DAKO Autostainer Plus, slide racks, reagent vials  
Pipettes and tips, serological pipettes  
Antigen retrieval: Citra (BioGenex), vegetable steamer (95°C)  
Tris buffered saline with Tween (TBST) - used for washes or rinses  
Sniper, normal serum or IgG from primary antibody host species (see Appendix 1)  
Alkaline phosphatase blocker (DEEB from Dako)  
Polymer systems for horseradish peroxidase (HRP) and alkaline phosphatase (AP) (MACH 2, Biocare)  
Biotinylated Goat anti-guinea pig, Avidin-Biotin-AP kit (Zymed)  
HRP (DAB) and AP (Liquid Fast Red) chromogen kits (Dako)  
Hematoxylin (Dako)  
Aqueous mounting media, coverslips  
Slide label printer

### 5 SAFETY

5.1 All reagents should be handled with the necessary personal protective equipment according to MSDS.

JDRF nPOD Standard Operating Procedure		
SOP Number and Version: 72.0	Supersedes: nPOD 3	IMMUNOPATHOLOGY
Original Effective Date: 07/01/07		
Version Effective Date: 05/04/11		Page 2 of 5

## 6 PROCEDURE

- 6.1 Prepare all solutions according to manufacturer's recommendations. Optimize antibody detection by antigen retrieval screening, titration, and validation according to clinical practice standards.
- 6.2 Obtain serial unstained paraffin slides from the histology lab (see SOP Histology).
- 6.3 Clear and rehydrate paraffin sections according to the schedule below:

Reagent	Time (minutes)
Xylene	5
Xylene	5
100% EtOH	2
100% EtOH	2
3% H <sub>2</sub> O <sub>2</sub> in MeOH	10
95% EtOH	1
95% EtOH	3
70% EtOH	1
Water	1

- 6.4 Perform antigen retrieval using citrate buffer in a steamer at 95°C for 30 minutes.
- 6.5 Wash slides in buffer for ≤ 5 minutes.
- 6.6 Load slides on Dako Autostainer (see Appendix 2).
- 6.7 Dako Autostainer settings:
- 6.7.1 Block with Sniper for 15 minutes. Rinse once.
  - 6.7.2 Incubate first primary antibody and negative control reagents (mouse or rabbit IgG) for 30 minutes. Rinse twice.
    - 6.7.2.1 Apply primary antibody to tissues at optimal concentrations as previously determined. Dilute primary antibody in antibody diluent.
  - 6.7.3 Incubate secondary antibody (Mach 2 HRP Polymer) for 30 minutes. Rinse twice.
  - 6.7.4 Develop first chromogen (DAB) for 4 minutes. Rinse twice.
  - 6.7.5 Block endogenous alkaline phosphatase (AP) with DEEB for 10 minutes. Rinse twice.
  - 6.7.6 Block with 10% normal goat serum (for insulin primary) or Sniper (for glucagon) for 10 minutes. Rinse twice.
  - 6.7.7 Incubate with second primary antibody or control reagent (guinea pig serum, mouse IgG) for 15 minutes. Rinse twice.
  - 6.7.8 Incubate with secondary reagent- biotinylated goat anti-guinea pig (insulin) or goat anti-mouse AP conjugate for 30 minutes. Rinse twice.
  - 6.7.9 Incubate with tertiary reagent- avidin-biotin- AP conjugate (insulin) or antibody diluents for 30 minutes. Rinse twice.
  - 6.7.10 Develop with alkaline phosphatase (AP) staining system (Liquid Fast Red) for 4 minutes. Rinse twice with water.
  - 6.7.11 Counterstain with Hematoxylin for 1 minute. Rinse twice with water.
- 6.8 Remove slides from rack and allow to air dry for ≤2 hours.
- 6.9 Coverslip using aqueous mounting media and label the slide (See Appendix 2 and SOP Case Processing).
- 6.10 Scan stained slides using an Aperio CS scanner and organize by donor and sample type using the Spectrum Plus information management system (Aperio, Vista, CA) (SOP Online Pathology).
- 6.11 Expected results and quality control measures:

JDRF nPOD Standard Operating Procedure		
SOP Number and Version: 72.0	Supersedes: nPOD 3	IMMUNOPATHOLOGY
Original Effective Date: 07/01/07		
Version Effective Date: 05/04/11		Page 3 of 5

- 6.11.1 Normal donors are expected to have intense (4+) islet cell staining for both insulin and glucagon cells without smearing or background on non-islet cells.
- 6.11.2 Spleen samples from all donors are expected to show 10-40% Ki67- or CD3-positive cells.
  - 6.11.2.1 Ki-67 will be expressed in nuclei only.
  - 6.11.2.2 CD3 will be expressed on cell membranes.
- 6.11.3 Positive and negative controls will be included in every run (see Appendix 2).
  - 6.11.3.1 Positive controls (PC) consist of two samples:
    - 6.11.3.1.1 One PC is a slide from a control donor.
    - 6.11.3.1.2 Spleen is used as a positive tissue control for Ki67 and CD3.
  - 6.11.3.2 Negative controls (NC) consist of two samples:
    - 6.11.3.2.1 One NC is a slide from pancreas of the test case that is incubated with antibodies from the host species used to generate the primary antibodies in the double stain.
    - 6.11.3.2.2 Spleen is used as a negative tissue control for endocrine cell markers.

## 7 REFERENCES

- 7.1 DAKO IHC Staining Methods – Educational Guide
- 7.2 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.
- 7.3 SOP 57 Case Processing
- 7.4 SOP 70 Histology
- 7.5 SOP 73 Online Pathology

## 8 REVISION HISTORY

Version	Date	Revision

Prepared by	Li Zhang		
Approved by	Martha Campbell-Thompson		
	Name	Signature	Date

<b>JDRF nPOD Standard Operating Procedure</b>		
SOP Number and Version: 72.0	Supersedes: nPOD 3	IMMUNOPATHOLOGY
Original Effective Date: 07/01/07		
Version Effective Date: 05/04/11		Page 4 of 5

## Appendix 1

### Primary Antibodies Used in nPOD Immunohistochemistry Protocols

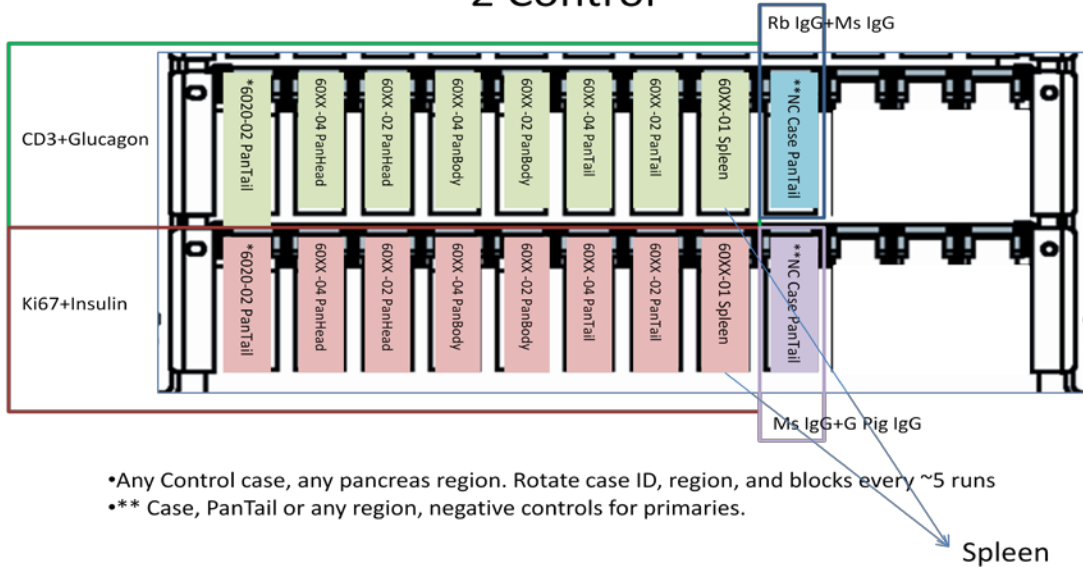
<b>Antigen</b>	<b>Host</b>	<b>Antibody Clone</b>	<b>Vendor</b>
<b>Insulin</b>	Guinea Pig	A0564	Dako
<b>Ki67</b>	Mouse	M1B-1	Dako
<b>CD3</b>	Rabbit	A0452	Dako
<b>Glucagon</b>	Mouse	ab10988	Abcam
<b>Somatostatin</b>	Rabbit	A0566	Dako
<b>Pancreatic Polypeptide</b>	Rabbit	A0619	Dako
<b>Synaptophysin</b>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

JDRF nPOD Standard Operating Procedure		
SOP Number and Version: 72.0	Supersedes: nPOD 3	IMMUNOPATHOLOGY
Original Effective Date: 07/01/07		
Version Effective Date: 05/04/11		Page 5 of 5

## Appendix 2

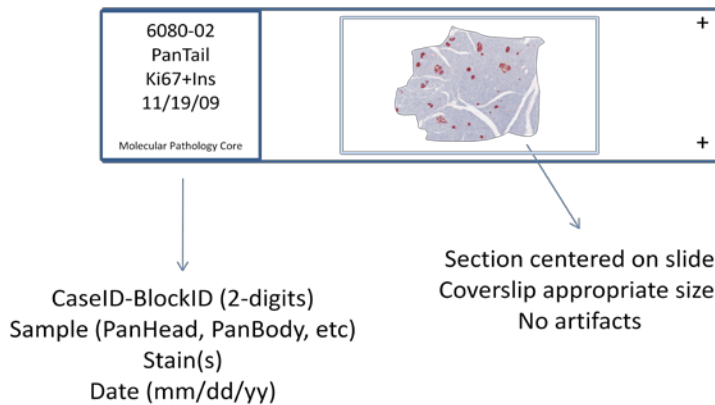
### Dako Autostainer Set-up and Slide Labeling

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



11/30/2010

### Elements of a nPOD IHC Slide



Scan into Spectrum, Sample=Specimen

11/30/2010