Immunohistochemistry (IHC) – Novolink Polymer Detection Systems

Purpose

The purpose of this SOP is to outline the correct procedures for performing Immunohistochemistry (IHC) using the Novolink Polymer Detection System kit. It is adapted from the Novolink Polymer Detection Systems instruction manual and is intended for the use by all staff, students, and users of the Gregory Laboratory.

Safety First

Before starting, you must read all relevant Health & Safety documentation, and fully read this SOP. Please speak to any of the Gregory Lab technical staff if there is anything you are unsure about before embarking on your experiment, we are always happy to help.

- ✓ COSHH and Risk assessments
- ✓ SOP

Special note

See appendix A for methods for making up solutions.
See appendix B for a list of commonly used antibody dilutions, pre-treatments and ordering information.

1. Prepare treatment materials

- ✓ Get Novolink kit from the fridge, bring to room temperature
- ✓ Make up 1:10 TBS (tris:saline)
- ✓ If using, make up citric acid buffer (see step 3)
- ✓ Get sequenza racks and coverplates

2. Deparaffinise FFPE sections and remove formalin pigment

Put your slides in a staining rack and take them through a series of xylene and alcohol baths (see below). Briefly agitate the rack when placing it in each bath and drain well before moving onto the next one.

Xylene	**3 min**
Xylene	**3 min**
Alcohol	**2 min**
Alcohol	**2 min**
Saturated Alcoholic Picric acid	**15 min**
Wash in warm, running tap water	**15 min**

3. Antigen retrieval

It is always a good idea to refer to the datasheet provided with the antibody for up to date information regarding pre-treatments and dilutions, different antibodies require different conditions. Appendix B provides a quick reference guide to commonly used antibodies.
The pre-treatment most often used in The Gregory Lab is heat induced epitope retrieval with citric acid buffer (pH6), you will need to make up a working solution (see appendix A for methods for making solutions).

- ☞ For 10mM (working) solution dilute 1:10.
  E.g.,50ml of 100mM citric acid buffer and 450ml of dH_2O.

Using the pressure cooker

The settings, times and temperature on your pressure cooker may be different to the one used in the Gregory Lab. The instructions below are optimised for a 3L Drew and Cole 'Pressure King Pro' domestic pressure cooker.

Put **500ml dH_2O** in the basin of the pressure cooker.
Place slides in a slide rack, pop the rack in a suitable pressure cooker pot and pour in antigen retrieval buffer (e.g., citric acid or tris/EDTA). Make sure the slides are fully covered.
Put the lid on and lock it by turning it anticlockwise until you hear a click.
Turn the pressure release valve to the closed position.

Press the 'browning/meat' button and use the + and – buttons to adjust the time to **5 min** (if it starts before you're ready, press cancel and try again). After a short delay, the pressure cooker will start automatically. The full cycle takes around **20 min.**
When the cycle has finished there will be a short beep sound and the display will read 'end', press the cancel button. You can wait for the pressure to release naturally or you can move the valve to the open position to release the pressure more quickly.
Beware! The slides and pressure cooker will be very hot and should be handled with extreme caution. Remove the lid of the pressure cooker, lift the basin out and place it in the sink.
Cool the slides down in running tap water.

4. Coverplating

Use **dH_2O** to fit coverplates to your slides. Make sure there are no bubbles present and press securely into the sequenza rack.
Fill the wells to the top with **dH_2O**, leave for **5 min.** If the water flows through too quickly, or not at all, remove the coverplate from the slide and try again.

5. Using Novolink Polymer Detection kit

Apply the following Novolink Polymer Detection kit reagents. See below for how to make working primary antibody and DAB solutions.

Peroxidase block (3 drops)	**30 min**
TBS	**5 min**
Protein block (3 drops)	**15 min**
TBS	**5 min**
Primary antibody	**30 min**
TBS	**5 min**
Post primary block (2 drops)	**30 min**
TBS	**5 min**
Novolink Polymer (2 drops)	**30 min**
TBS	**5 min**
DAB	**5 min**
dH_2O	**5 min**

Making up primary antibody

Every antibody is different. Check the data sheet that came with your antibody for the concentration and recommended working dilution. Each slide requires 100µl of antibody solution and is diluted with wash buffer, in the Gregory Lab we generally use TBS.

When making up antibody we need to know
- What is the volume of solution required?
- What is the dilution?
- How much antibody will be needed?

DAB

The ratio of DAB chromogen to DAB substrate buffer is 50µl:1000µl. This is plenty of solution for 10 slides.
Pipette **100µl** of DAB solution into the well of each coverplate, leave for **5 min.**

Fill up wells with **dH_2O**, leave for **5 min.**

6. Remove coverplates and Counterstain Slides

Carefully remove each slide with their attached cover plate from the sequenza, gently lift the slide off the coverplate (remembering to lift rather than slide them off).
Put the slides in a slide rack and wash well in running tap water for **5 min**.

Counterstaining
Counterstain your slides in the below solutions, and then dehydrate and clear. As with dewaxing, briefly agitate the rack at each stage and drain well before moving into the next reagent.

Haematoxylin	**2 min**
Wash in running tap water	**1 min**
Lithium carbonate	**30 sec**
Wash in running tap water	**1 min**

7. Dehydrate, Clear and Mount

Drain off as much water as possible from the rack, then fully dehydrate the slides by taking them through a series of alcohols.

70% Alcohol
100% Alcohol
100% Alcohol

In each alcohol bath, agitate the slides for 30-60 seconds or until they are 'clear'. You will see no water steaks and the glass will appear solid. Alternatively, agitate the rack briefly and leave for two minutes. Drain the rack very well before moving onto the next alcohol.

Using the same technique as for the alcohols, clear the slides in at least two changes of xylene.
When the slides are 'clear' of alcohol there will be no streaks and they will 'disappear' when submerged in xylene.
Your slides are now ready to be coverslipped.

Appendix A

Solution	Use	Method		Storage
Citric acid buffer (check which citric acid first)	Antigen retrieval 100mM citric acid buffer	dH_2O Citric acid anhydrous Adjust to pH6	1L 19.21g	4^0C (cold room) Dilute 1:10 for working solution
	Antigen retrieval 100mM citric acid buffer	dH_2O Citric acid monohydrate Adjust to pH6	1L 21.01g	4^0C (cold room) Dilute 1:10 for working solution
Tris/EDTA buffer	Antigen retrieval	dH_2O Tris EDTA Adjust to pH9	1L 1.21g 0.37	4^0C (cold room)
5N Hydrochloric acid	pH buffer	dH_2O HCL	115ml 85ml	Acids cupboard
5N Sodium hydroxide	pH buffer	dH_2O NaOH	500ml 135g	Cupboard
TBS (Tris Buffered Saline)	Wash	Tris buffer Saline Solution	100ml 900ml	Make fresh
Tris buffer	x10 stock solution	Saline solution Tris Adjust pH to 7.6. Then make up to 1.5L with dH_2O. Check pH, adjust if necessary.	500ml 1.21g	4^0C (cold room)
Saline solution	For Tris and TBS	dH_2O NaCl	5L 42.5g	Cupboard
Saturated alcoholic picric acid	Removing formalin pigment	Add picric acid to 100% IMS until no more powder can be dissolved. Filter before use.		

Appendix B

ANTIBODY	DILUTION	PRE-TREATMENTS	Control	Storage ˚C
BA4	1:100	Formic	BA4	4
TAU	1:2000	None	TAU	-20
α SYN	1:200	Citric & Formic	α SYN	-20
TDP43	1:4000	Citric	TDP43	-20
P62	1:1000	Citric	P62	-20
UBIQUITIN	1:500	Citric	UBIQUITIN	4
FUS	1:500	Citric	FUS	4
NF	1:1000	Citric	NF	4
GFAP	1:800	None	GFAP	4
BAPP (HUMAN)	see forensics	Citric	BAPP (HUMAN)	4
CD68	1:100	Citric	CD68	4
OLIG 2	1:100	Citric	OLIG 2 WM	-20
NOGO A	1:1500	Citric	NOGO A WM	
MBP	1:500	Formic	White Matter	4
NF Heavy	1:500	Citric & Formic	White Matter	-20
CD163	1:1000	Citric	Spleen	-20
Claudin	1:250	Citric	Blood Vessel	-20
Collagen-I	1:1000	Citric	Blood Vessel	-20
PDGFR-β	1:100	Citric	Blood Vessel	-20
SMA	1:1000	Citric	Blood Vessel	4
PEDM	1:5000	Citric & Formic	Huntingtons	-20
CD20	1:500	None	CD20	
CD45	1:100	Citric	CD45	4
CR343 (HLA-DP, DQ, DR)	1:500	Citric	CR343	4
c21orF2	1:100	Citric		
VAPB	1:200	Citric		
Alsin	1:100	Citric		
PolyQ	1:7500	Citric & Formic	Huntingtons	-20
MGR2	1:250	Citric	Cerebellum	-20
RIP3	1:500	Citric & Formic	Lung Cancer	
MLKL	1:100	Citric & Formic	Skin	
CD34	1:100	Citric	Tonsil	
Fibrinogen	1:1000	Formic	Fibrinogen Control	-20
Fibrinogen	1:4000	Citric	Fibrinogen Control	4
TMEM119	1:50	Citric	LN control	-20
p22phox	1:250	Citric	Lung Control	-20
Iba1	1:3000	Citric	Cerebral Cortex	-20
HERV	1:250	Citric	Kidney	
Collagen-IV	1:750	Citric	Blood Vessel	-20
CD31	1:200	Citric	Blood Vessel	-20
CD44	1:2500	Citric	norm brain	4
NRROS	1:50	Citric	norm brain/lung	-20
GLP-1				-20
α-Internexin	1:1500	Citric	NF control	-20
Nesprin	1:250	Citric	Kidney	-20
Yap-1	1:100	Citric	Breast Cancer	-20
Lamin	1:500	Citric	brain	-20