Immunohistochemistry

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ABSTRACT

This protocol outlines the steps used to perform immunohistochemistry on murine and human tumor samples.

In brief: Study animals underwent full necropsies, including gross examination of all organs for macroscopic disease. Harvested tissues were fixed in 10% neutral buffered formalin (10:1 formalin:tissue volume ratio) for at least 48 hours, embedded in paraffin, sectioned (4mm) and used for various immunohistochemical and histochemical assays. Sections were stained with hematoxylin and eosin (H&E) or primary antibodies for markers of interest. Slides were baked for 30 minutes at 60°C and deparaffinized on the Leica Bond-Max Automated Immunostainer. Following antigen retrieval (see table below), slides were blocked for 5 minutes at room temperature with Leica Bond Peroxide Block, then for 20 minutes (25°C) with 10% normal goat serum (Jackson ImmunoResearch, cat: 005-000-121) in TBS or TCT buffer (Casein based TBS with Tween20). After primary antibody staining for 30 minutes or 60 minutes at 25°C, Leica Bond Polymer was applied for 8 minutes (25°C). Leica Bond Mixed Refine (DAB, cat: D59800) detection was performed at 2x for 10 minutes (25°C), and a Leica hematoxylin counterstain (Biocare, cat: NM-HEM-M) was added for 3-4 minutes. Slides were cleared with xylene and mounted with synthetic resin mounting medium and a 1.5 cover slip.
Slides were scanned in brightfield with 20x objective using a Nanozoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, New Jersey) or the Aperio ScanScope AT (Leica Biosystems). The digital images were imported into Visiopharm software (Hoersholm, Denmark) or Aperio eSlide Manager (Leica Biosystems) and analyzed after identifying regions of interest and excluding normal tissue.

Equipment needed:
Sakura Tissue-Tek VIP6 AI Vacuum Infiltration Processor
Sakura DRS2000 H&E Stainer (and staining racks/arms)
Leica Bond Rx Autostainer

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MATERIALS TEXT

MATERIALS

Bond Wash 10x **Leica**

Biosystems Catalog #AR9590

Bond Polymer Refine Detection Kit **Leica**

Biosystems Catalog #DS9800

PowerVision Polymer anti-Mouse IgG HRP **Leica**

Biosystems Catalog #DPVM-110HRP

Primary Antibody Diluent BOND **Leica**

Biosystems Catalog #AR9352

Grossing and specimen processing

1 Gross one specimen at a time at the Mopec grossing station.
Carefully verify the cassette information to the specimen ID on the specimen container.
All contaminated biohazard bags must be disposed of in the approved biohazard containers.
Load the cassette holding rack into Sakura Tissue-Tek VIP6 AI.
Process samples on Program 8 (see program table below).

Grossing and Specemin Processing

2 **Program number: 8**
Full Name: 8 Hour Processing

Initial	Step	Reagent	Temp (0C)	Time
	3	75% Ethanol	--	40:00 min
	4	95% Ethanol	--	40:00 min
	5	95% Ethanol	--	40:00 min
	6	100% Ethanol	--	40:00 min
	7	100% Ethanol	--	40:00 min
	8	Clear-Rite	--	40:00 min
	9	Clear-Rite	--	40:00 min
	10	Clear-Rite	--	40:00 min
	11	Paraffin	60C	40:00 min
	12	Paraffin	60C	40:00 min
	13	Paraffin	60C	40:00 min
	14	Paraffin	60C	40:00 min

Grossing and specimen processing

3 1. Ensure the Tissue-Tek TEC embedding station paraffin reservoir is full of paraffin before the processor has completed and the cassettes need to be removed from the processor. If the reservoir needs to be filled, transfer molten paraffin from the TBS Liquid Paraffin Dispenser.
2. Remove samples from the Sakura Tissue-Tek VIP6 AI and transfer them to the warming reservoir in the Tissue-Tek TEC embedding station.
3. Samples must be embedded immediately, or RNA degradation will occur.
4. Remove one cassette from the cassette holding reservoir at a time for embedding.
5. Using a square base mold, fill with molten paraffin from the dispenser, remove the cassette lid, carefully remove the specimen with forceps and orient the tissue on edge making sure it is flush and secure on the bottom of the mold.
6. Place the cassette on the top of the mold and top off with molten paraffin; move on to the cooling plate.
7.Trim FFPE blocks using the Wax Trimmer.

Cutting FFPE slides

4 1. Turn the water bath on and fill with distilled water; heating element should be set at 37°C.
2. Bring Histocool block out from freezer, fill up with distilled water. Wait 10-15 minutes before pre-cooling FFPE blocks.
3. Prior to cutting: Match each FFPE block with the corresponding RFS form and accompanying slides to verify the identifiers match.

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4. Place the blocks (tissue side down) on the HistoCool block; pre-cooling paraffin blocks is necessary to obtain quality sections.
5. Allow the blocks to soak on the ice for approximately 20 minutes (depending on fixation).
6. Face each block to obtain a full tissue section.
7. Replace microtome blades as necessary, in order to avoid cutting artifact.
8. Place ribbons onto water bath, picking up sections on corresponding slides.
9. Remove the FFPE blocks from the HistoCool block and pat dry all excess water from the blocks before placing in the plastic bag to return to investigator.
10. In any of the cassettes do not have tissue, or if there were possible problems during embedding that prevent sectioning, annotate the specimen ID on the RFS requisition form.
11. Place slides in slide-drying rack, allow slides to air-dry overnight.
12. Dry paraffin sections at 60°C 1 hour.
13. Remove the sections from the oven and allow cooling at room temperature.

H&E staining

5 1. Load slides into the black H&E slide staining rack(s) and attach to a plastic staining arm.
2. Open the Sakura DRS2000 by gently pushing on the bottom door and place the staining arm (with the attached rack(s)) in the S1/S2 loading station; close the door.
3. Press the F1 [START] button on the console, the Method at the top of the console should read F FPE H&E.
4. If the correct staining method is not displayed, Press the F2 [METHOD] and the up and down buttons until the correct staining protocol is highlighted.
5. Press the F1 [START] button to start the staining process.
6. When staining program finishes (indicated by stainer beeping), press the F1 [REMOVE] button, open the door, carefully remove the E1/E2 Xylene bucket (Program End), and carry to the coverslipping area.
7. Transfer the slide racks to one of the Xylene buckets at the coverslipping area
8. Place the Xylene bucket back into the Program End station, close the stainer door, and press the F1 [CONFIRM] button.

6 **Program Name: FFPE H&E**

Step	Reagent	Time
1	Xylene	3:00 min
2	Xylene	3:00 min
3	Xylene	3:00 min
4	100% ETOH	2:00 min
5	100% ETOH	2:00 min
6	95% ETOH	2:00 min
7	95% ETOH	2:00 min
8	80% Ethanol	2:00 min
9	Water (Rinse)	1:00 min
10	Hematoxylin	10:00 min
11	Water (Rinse)	1:00 min
12	Clarifier	0:10 min
13	Water (Rinse)	2:00 min
14	Bluing Solution	0:30 min
15	Water (Rinse)	4:00 min
16	95% Ethanol	1:00 min
17	Eosin Y Phloxine	1:30 min
18	95% Ethanol	0:30 min
19	95% Ethanol	1:00 min
20	95% Ethanol	1:00 min
21	100% Ethanol	2:00 min
22	100% Ethanol	2:00 min
23	100% Ethanol	2:00 min
24	Xylene	3:00 min
25	Xylene	3:00 min
26	Xylene	Program End

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7 1. Coverslip the slides using Cytoseal XYL and a VWR 24x40 coverglass.
2. Wipe the back of the slide and drain the sides thoroughly to remove excess mounting media/xylene, then place on a cardboard palette to dry overnight.
3. Fill out the H&E QC sheet and place the slides and the sheet in the QC bin for the Director/Ops Manager's QC approval.

IHC STAINING PROTOCOL – Mouse Polymer DAB

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Step	Reagent	Dispense Amount	Time
1	*(Leica) Peroxide Block	150uL	5min 0sec
2	*(Leica) Bond Wash	150 uL	0sec
	*(Leica) Bond Wash	150 uL	0sec
3	*(Leica) Bond Wash	150 uL	0sec
4	TCT Buffer	150 uL	10min0sec
5	*(Leica) Bond Wash	150 uL	0sec
	*(Leica) Bond Wash	150 uL	0sec
5	**Primary/Isotype**	150 uL	0sec
	Pimary/Isotype	150uL	60min0sec
6	*(Leica) Bond Wash	150 uL	0sec
7	*(Leica) Bond Wash	150 uL	0sec
8	*(Leica) Bond Wash	Open	0sec
9	*(Leica) Bond Wash	150 uL	0sec
	*(Leica) Bond Wash	150 uL	20min0sec
	*(Leica) Bond Wash	150 uL	2min 0sec
	*(Leica) Bond Wash	150 uL	2min 0sec
	*(Leica) Bond Wash	150 uL	2min 0sec
10	Leica PowerVision anti-Mouse IgG HRP	150 uL	0sec
11	Leica PowerVision anti-Mouse IgG HRP	150 uL	12min 0sec
12	*(Leica) Bond Wash	150 uL	2min 0sec
13	*(Leica) Bond Wash	Open	5min 0sec
14	*(Leica) Bond Wash	Open	0sec

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15	*(Leica) Bond Wash	Open	2min 0sec
16	*(Leica) Bond Wash	150 uL	0sec
17	Deionized Water	150 uL	0sec
18	*(Leica) Mixed DAB Refine	150 uL	1min 0sec
19	*(Leica) Mixed DAB Refine	150 uL	9min 0sec
20	Deionized Water	150 uL	0sec
21	Deionized Water	Open	0sec
22	Deionized Water	150 uL	0sec
23	*(Leica) Hematoxylin	150 uL	0sec
24	*(Leica) Hematoxylin	150 uL	4min 0sec
25	Deionized Water	150 uL	0sec
26	*(Leica) Bond Wash	Open	0sec
27	Deionized Water	150 uL	0sec

9 Dehydrate and clear the slides through the IHC dehydration line:
3 changes of 95% Ethanol; 1 minute each
3 changes of 100% Ethanol; 1 minute each
5 changes of Xylene; 1 minute each

Coverslip the slides using Cytoseal XYL and a VWR 24x40 (or x50) coverglass.
Wipe the back of the slide and drain the sides thoroughly to remove excess mounting media/xylene, then place on a cardboard palette to dry overnight.

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