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Immunohistochemistry - uPAR in mouse lung tissue

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Sojin Kim[1]

[1]Icahn School of Medicine at Mount Sinai

Sojin Kim
Icahn School of Medicine at Mount Sinai

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

Immunohistochemistry (IHC) is a method to detect specific target antigens (proteins) in tissue sections using antibodies. Immunocytochemistry (ICC) uses similar techniques to localize cellular proteins in cell preparations. Both IHC and ICC are powerful tools that provide insights into gene expression, spatial
relationships, and biomarker identification in various applications. These applications include basic research, assessment of normal and disease states within human and animal tissues, and assessment of plant pathology. The target antigen, bound by the detection antibody, is visualized using chromogenic or fluorescence detection. In chromogenic detection, the detection antibody is conjugated to an enzyme. The enzyme, usually horseradish peroxidase or alkaline phosphatase, catalyzes the conversion of its respective chromogen to a colored precipitate at the site of the antigen. This precipitate can be visualized by using brightfield microscopy. Certain chromogens can also be visualized by using electron, darkfield or fluorescence microscopy. In fluorescence detection, the detection antibody is conjugated to a fluorophore, which can be visualized using fluorescence microscopy.

Materials

Tissue preparation

- ImmEdge Hydrophobic Barrier PAP Pen (Vector Laboratories, Cat.# H-4000, **link**)
- ImmPrint Histology Pen (Vector Laboratories, Cat.# H-6100, **link**)
- Xylene
- Ethanol
- PBS (pH 7.5)

Antigen Retrieval

- Graduated cylinder (for measuring 1.6 L)
- Serologic pipette (for measuring 15 mL)
- Timer
- Antigen Unmasking Solution, Citrate-Based (Vector Laboratories, Cat.# H-3300-250, **link**)
- PBS (pH 7.5)

For the pressure cooker method:

- Pressure cooker
- Metal staining rack

For the microwave method:

- Microwave
- Plastic Coplin jar and staining rack

Quench/Block

- BLOXALL Endogenous Blocking Solution, Peroxidase and Alkaline Phosphatase (Vector Laboratories, Cat.# SP-6000-100, **link**)

Primary antibody

- Mouse uPAR Antibody (Biotechne, Cat.# AF534, **link**)
- Bovine Serum Albumin (BSA), Immunohistochemical Grade (Vector Laboratories, Cat.# SP-5050-500, **link**)

Secondary antibody

- Horse Anti-Goat IgG Antibody (H+L), Biotinylated (Vector Laboratories, Cat.# BP-9500-50, **link**)

Substrate/Chromogen

- ImmPACT DAB Substrate Kit, Peroxidase (HRP) (Vector Laboratories, Cat.# SK-4105, **link**)

Counterstain

- Hematoxylin QS (Vector Laboratories, Cat.# H3404, **link**)

Coverslip/Mount

- Cover Slip, Square, #1.5 Corning 0211 Glass, 22 x 22mm (Thomas Scientific, Cat.# 1139W03, link)
- VectaMount Express Mounting Medium (Vector Laboratories, Cat.# H-5700-60, **link**)
- 2-Propanol, histology grade (Thermo Fisher, Cat.# 447080025, **link**)

Tissue Preparation

1 Cut and mount sections on slides (The thickness of sectioning: 5 µm))

Deparaffinize and Rehydrate

2 Place the slide in a 56 °C to 60 °C oven for 00:15:00 15m

3 Incubate as below: 52m 30s

1. Xylene, 00:05:00
2. Xylene, 00:05:00 . Shake off excess liquid
3. 100% EtOH, 00:03:00
4. 100% EtOH, 00:03:00 . Shake off excess liquid
5. 90% EtOH, 00:03:00 . Shake off excess liquid
6. 80% EtOH, 00:03:00
7. Rinse the slides in gently running tap water for 00:00:30 (avoid a direct jet which may wash off or loosen the section)
8. Place in PBS wash bath for further rehydration (00:30:00 , Room temperature)

4 Mark the tissue area with an ImmPrint Histology Pen.

Antigen Retrieval

5 **Tris-based vs. Citrated-based antigen retrieval reagents**

- Antibody/Epitope Sensitivity: Depending on the sensitivity of the specific antigen or antibody being targeted, one might be preferred over the other. Some epitopes might only be retrievable in acidic or alkaline conditions.
- Tissue Type: Different tissues may respond better to different pH conditions for optimal antigen retrieval.

6 **Option 1. Pressure Cooker**

6.1 Position slides into metal staining racks. Do not place slides close together; uneven staining may occur.

6.2 Pour 1600 mL of distilled water into a stainless steel pressure cooker.
Shake well before use, and then add 15 mL of the concentrated stock Antigen Unmasking Solution (Vector Laboratories, Cat.# H-3300-250)

6.3 Cover, but do not lock the lid of a pressure cooker, and bring a solution to a boil.
Place slides in a pressure cooker, ensuring slides are well immersed in diluted Antigen Unmasking Solution. Lock lid.

6.4 Start timing once the cooker has pressurized start timing. After 00:01:00, remove the pressure cooker from a heat source and run it under cold water. 1m
DO NOT OPEN LID UNTIL THE INTERNAL PRESSURE HAS BEEN COMPLETELY REDUCED.

6.5 Open the lid, remove slides, and place immediately into a tap water bath.
DO NOT LET SECTIONS DRY OUT.

6.6 Wash sections in PBS buffer (pH 7.5) for 00:05:00 5m

7 **Option 2. Microwave**

7.1 Position slides in a plastic rack (do not use standard glass histology rack and vessel)

8 Add the appropriate antigen retrieval buffer to the rack. Do not tighten the lid.

9 Set it to full power and wait until the solution comes to a boil. Boil for 00:20:00 from this point. Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out. 20m

10 Remove the vessel and run cold tap water into it for 10 min. Use care with a hot solution.

Quench/Block

11 **How to choose a blocking reagents**
Blocking agents minimize background signals from endogenous enzyme activity, biotin, and non-specific binding of tissue elements (charged particles, macromolecules, Fc receptors) with detection reagents.

12 Apply Blocking solution (Vector Laboratories, Cat.# SP-6000) to tissue sections and incubate for 00:10:00 Room temperature 10m
Appropriate care should be exercised when using this reagent to avoid contact with eyes and skin. Dispose by local regulations

13 Wash sections with PBS for 00:05:00 5m

Primary Antibody

14 **How to choose a primary antibody**
1. Specific for antigen of interest
2. Consider tissue species and preparation (fixation)
3. Consider antigen retrieval requirements

15 Allow the slides to drain, shake off excess fluid with a brisk motion, and carefully wipe each slide around the sections.

16 Dilute the primary antibody or negative control reagent to its optimal dilution in diluent (100 uL/tissue sections). The diluent alone may be used as a negative control.
- For uPAR: Prepare 1:50 and 1:100 antibody dilution in BSA (Vector Laboratories, Cat.# SP-5050-500)

17 Apply 100 µL primary antibody solution to the appropriate slides, covering the tissue sections. Tilt each slide in two different directions so the liquid is spread evenly over the slide.

18 Incubate for at least 01:00:00 . at 37 °C in humidified chamber. Longer incubations are advised for low-density antigens. 1h

19 Rinse gently with PBS from a wash bottle. Place the slide in a PBS wash bath for 00:05:00 . 5m

Secondary Antibody

20 **How to choose a secondary/Tertiary antibody**
1. Choose HRP or AP enzyme system
2. Consider sensitivity requirements
3. Consider species of primary antibody

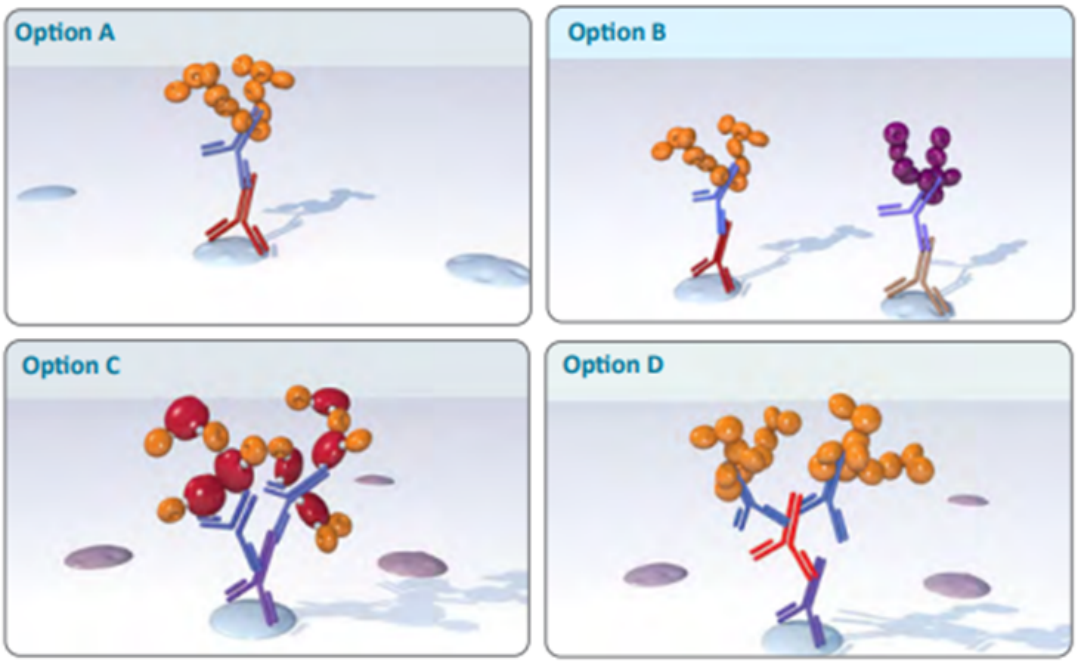
4. Consider tissue species

Consider species cross-reactivity

When choosing the optimal detection system for your application, it is important to consider not only the species of the primary antibody but also the species of the tissue under examination. If the species of the primary antibody and the species of the tissue are closely related (for example, rat and mouse), the biotinylated secondary antibody may cross-react with endogenous IgG in the tissue section. This can lead to background staining. The following options may minimize background staining in these instances:

1. Directly label the primary antibody with biotin
2. Use a biotinylated secondary antibody specifically adsorbed to remove cross-reacting antibodies of closely-related species
3. Use the M.O.M. (Mouse on Mouse) Immunodetection System for applications of mouse primary antibodies on mouse tissue

4 Options



1. Option A: Convenient. Consistent. Ready-to-use. Non-biotin based
2. Option B: Dual label (two antigen detection). Convenient. Consistent. Ready-to-use. Non-biotin based
3. Option C: Economical. Biotin-based
4. Option D: Highest sensitivity. Non-biotin-based

21 Apply a secondary antibody matched to the primary antibody.

- For uPAR: Apply Horse Anti-Goat IgG Antibody (H+L), Biotinylated (Vector Laboratories, Cat.# BP-9500-50) to the tissue section.

22 Incubate for 00:30:00 at Room temperature in a humidity chamber 30m

23 Rinse gently with PBS from a wash bottle. Place the slide in a PBS wash bath for 00:05:00 5m

Substrate/Chromogen

24 **How to choose enzyme substrate**

1. Color
2. Compatibility with other system reagents (counterstains, mounting media, and other substrates for multiplexing)

First Substrate \ Second Substrate	Alkaline Phosphatase			Peroxidase					
	ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100	Vector Blue (blue) SK-5300	BCIP/NBT (indigo) SK-5400	ImmPACT VIP & Vector VIP (purple) SK-4605 & SK-4600	ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105, SK-4103, SK-4100	DAB-Ni (gray-black) SK-4100	ImmPACT NovaRED & Vector NovaRED (red) SK-4805 & SK-4800	ImmPACT SG & SG (blue-gray) SK-4705 & SK-4700	ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205, SK-4285, SK-4200
Alkaline Phosphatase: ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100		−	−	−	+	+	−	+	−
Alkaline Phosphatase: Vector Blue (blue) SK-5300	+		−	+	+	+	+	+	+
Alkaline Phosphatase: BCIP/NBT (indigo) SK-5400	+	−		+	+	+	+	+	+
Peroxidase: ImmPACT VIP & Vector VIP (purple) SK-4605, SK-4600	−	+	−		+	+	−	+	−
Peroxidase: ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105, SK-4103, SK-4100	+	+	+	+		−	−	+	+
Peroxidase: DAB-Ni (gray-black) SK-4100	+	−	−	+	+		+	−	−
Peroxidase: ImmPACT NovaRED & Vector NovaRED (red) SK-4805, SK-4800	−	+	+	−	+	+		+	−
Peroxidase: ImmPACT SG & SG (blue-gray) SK-4705, SK-4700	+	−	−	+	+	−	−		+
Peroxidase: ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205, SK-4285, SK-4200	−	−	−	−	+	−	−	+	

+ Indicates good contrast − Indicates incompatibility of substrates for various reasons

How to choose nuclear counterstain

- A counterstain introduces color to specific cellular structures to provide contrast to the colored enzyme substrate. Counterstaining aids in visualization and target localization, facilitating interpretation of morphology and cell structure within the tissue section. Our nuclear counterstains are packaged as convenient, ready-to-use solutions for use on individual slides or in staining dishes.
- Blue, green, or red compatibility with substrate, mounting media

Substrate	Catalog Number	Vector Hematoxylin and Hematoxylin QS H-3401 and H-3404	Vector Methyl Green H-3402	Vector Nuclear Fast Red H-3403
ImmPACT DAB (brown)	SK-4105	Excellent Contrast	Excellent Contrast	Fair Contrast
ImmPACT DAB EqV	SK-4103	Excellent Contrast	Excellent Contrast	Fair Contrast
DAB (brown)	SK-4100	Excellent Contrast	Excellent Contrast	Fair Contrast
DAB-Ni (gray-black)	SK-4100	Excellent Contrast	Fair Contrast *	Good Contrast
ImmPACT AEC (red)	SK-4205	Excellent Contrast	Counterstain Incompatibility **	Color Incompatibility
ImmPACT AMEC Red (red)	SK-4285	Excellent Contrast	Counterstain Incompatibility **	Color Incompatibility
AEC (red)	SK-4200	Excellent Contrast	Counterstain Incompatibility **	Color Incompatibility
TMB (blue)	SK-4400	Color Incompatibility	Counterstain Incompatibility	Excellent Contrast
ImmPACT VIP (purple)	SK-4605	Fair Contrast	Excellent Contrast	Poor Contrast
Vector VIP (purple)	SK-4600	Fair Contrast	Excellent Contrast	Poor Contrast
ImmPACT SG (blue-gray)	SK-4705	Poor Contrast	Good Contrast	Excellent Contrast
SG (blue-gray)	SK-4700	Poor Contrast	Good Contrast	Excellent Contrast
ImmPACT NovaRED (red)	SK-4805	Excellent Contrast	Excellent Contrast ***	Color Incompatibility
Vector NovaRED (red)	SK-4800	Excellent Contrast	Excellent Contrast ***	Color Incompatibility
ImmPACT Vector Red (magenta)	SK-5105	Excellent Contrast	Excellent Contrast	Color Incompatibility
Vector Red (magenta)	SK-5100	Excellent Contrast	Excellent Contrast	Color Incompatibility
Vector Black (black)	SK-5200	Excellent Contrast	Excellent Contrast *	Excellent Contrast
Vector Blue (blue)	SK-5300	Color Incompatibility	Good Contrast	Excellent Contrast
BCIP/NBT (indigo)	SK-5400	Color Incompatibility	Excellent Contrast *	Excellent Contrast

* This substrate shows a slight decrease in sensitivity following the methyl green protocol. This decrease can be minimized by reducing the heat incubation and acetone rinse times in the methyl green protocol.
** Substrate dissolves in acetone wash.
*** A slight color change in ImmPACT NovaRED and Vector NovaRED reaction product may be seen using methyl green.

HRP vs AP

- HRP (peroxidase-based) detection system is a preferred choice for many IHC applications that
  require sharp, distinct localization of the target antigen.
- AP (Alkaline phosphatase-based) detection kit is a good alternative to using peroxidase-based
  reagents in specimens that exhibit problematic levels of endogenous peroxidase activity.

25 It is recommended to prepare the substrate mixture during the final wash step.
Please check the table above and choose the appropriate one.

- For uPAR: Add 1 drop of ImmPACT DAB Reagent 1 to 1 mL ImmPACT DAB Diluent. Mix well before use.

IMPORTANT: DAB is a suspected carcinogen. Appropriate care should be exercised when using this reagent including gloves, eye protection, lab coats, and good laboratory procedures. Dispose in accordance with local regulations.

26 Allow each slides to drain. Shake off excess fluid and carefully wipe the slide as before.

27 Incubate with the substrate working solution at Room temperature for 00:02:00 to 00:10:00 . Optimal development times should be determined by the investigator. 12m

28 Terminate the reaction before background staining appears in the negative controls by rinsing gently with distilled water from a wash bottle.

29 Wash for 00:05:00 in water. 5m

30 **Notes**

1. An unused working solution is stable for up to 14 days if stored at 2–8 °C or up to 5 days if stored at room temperature (approximately 23 °C). The working solution may change color during storage, but this will have no effect on the quality or intensity of the staining. No sample-obscuring precipitate will form.
2. It is not necessary or recommended to add detergent to ImmPACT DAB working solution to reduce surface tension (e.g., for use in an automated stainer).
3. The DAB reaction product can be intensified using a DAB Enhancing Solution (H-2200) after development.

Counterstain

31 Choose counterstain based on this information (**link**)

32 Rinse the slide in tap water.

33 Incubate sections with Hematoxylin QS for 00:00:05 to 00:00:45 50s

34 Rinse sections with running tap water until rinse water is colorless or dip for approximately 00:00:10 in water. 10s

Coverslip/Mount

1m

35 **How to choose mounting media**

1. Aqueous vs non-aqueous
2. Compatibility with substrate(s) and counterstain

Mounting Media/Substrate Compatibility

Substrate	VectaMount Express Mounting Medium	VectaMount Permanent Mounting Medium	VectaMount AQ Aqueous Mounting Medium
Peroxidase			
DAB	•	•	•
DAB-Ni	•	•	
ImmPACT DAB	•	•	•
ImmPACT DAB EqV	•	•	•
Vector VIP	•	•	
ImmPACT VIP	•	•	
Vector NovaRED	•	•	
ImmPACT NovaRED	•	•	
Vector SG	•	•	•
ImmPACT SG	•	•	•
AEC			•
ImmPACT AEC			•
ImmPACT AMEC Red			•
TMB		•	
ALkaline Phosphatase			
Vector Red	•	•	•
ImmPACT Vector Red	•	•	•
Vector Blue	•	•	•
Vector Black		•	
BCIP/NBT	•	•	•

For permanent, non-aqueous mounting: Dehydrate, clear and coverslip using a non-aqueous mounting media, such as VectaMount® Mounting Medium (H-5000) or VectaMount® Express Mounting Medium
(H-5700).
For aqueous mounting: Coverslip using an aqueous mounting media such as VectaMount® AQ Mounting Medium (H-5501).

36 Perform rapid dehydration with two washes in 99% histological grade isopropanol (also called 2-propanol or isopropyl alcohol) for at least 00:01:00 each. Histological-grade alcohol can also be used. However, isopropanol is recommended. Processing through a standard graded series of alcohol can also be substituted if desired. 1m

37 Remove a slide from the isopropanol, blot excess, and immediately apply mounting medium to the tissue section(s)*. Two drops of mounting medium are recommended for a standard-sized tissue section (less than 22 mm^2).

38 Slowly lower coverslip onto mounting medium avoiding air bubbles.

39 Allow slides to dry flat at ambient temperature.

40 Slides can be viewed immediately. However, 00:10:00 to 00:20:00 are required for coverslip to set. 30m

41 Store coverslipped slides at ambient temperature. We do not recommend storage at elevated temperature (oven).

Protocol references

Vector Laboratories - Immunohistochemistry Resource Guide
Millipore Sigma - Immunohistochemistry Procedure for Paraffin-Embedded Tissues