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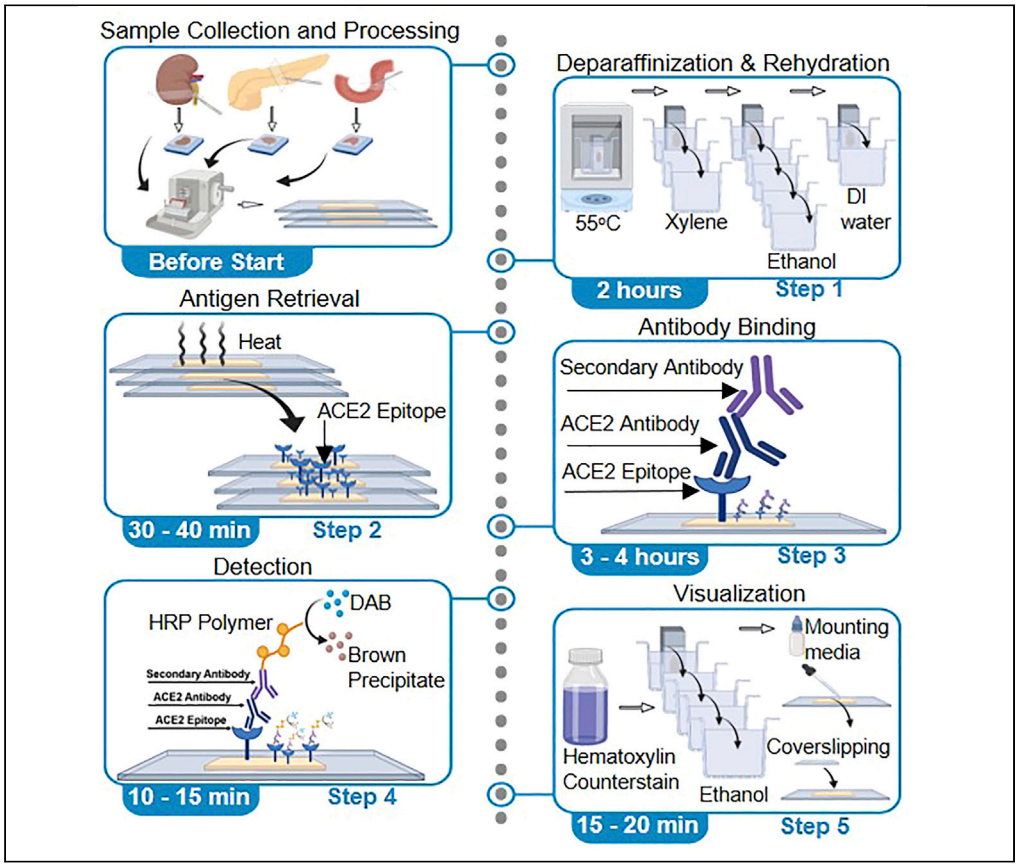
ACE2 chromogenic immunostaining protocol optimized for formalin-fixed paraffin-embedded human tissue sections



Angiotensin-converting enzyme 2 (ACE2) is a key cellular entry factor for severe acute respiratory syndrome coronavirus 2. Hence, identifying cell types that express ACE2 is important for understanding the pathophysiology of coronavirus disease 2019. We performed extensive testing of multiple primary antibodies across various human tissue types. Here, we describe an optimized protocol for immunostaining of ACE2 in formalin-fixed paraffin-embedded human pancreas, small intestine, and kidney tissue sections obtained from organ donors and autopsies.

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Highlights

We present an immunostaining protocol for rabbit monoclonal IgG anti-ACE2, clone EPR4435(2)

ACE2 staining has been optimized in human pancreas, small intestine, and kidney

Protocol has been optimized using tissue sections from human organ donors and autopsies

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ACE2 chromogenic immunostaining protocol optimized for formalin-fixed paraffin-embedded human tissue sections

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SUMMARY

Angiotensin-converting enzyme 2 (ACE2) is a key cellular entry factor for severe acute respiratory syndrome coronavirus 2. Hence, identifying cell types that express ACE2 is important for understanding the pathophysiology of coronavirus disease 2019. We performed extensive testing of multiple primary antibodies across various human tissue types. Here, we describe an optimized protocol for immunostaining of ACE2 in formalin-fixed paraffin-embedded human pancreas, small intestine, and kidney tissue sections obtained from organ donors and autopsies.

For complete details on the use and execution of this protocol, please refer to Kusmartseva et al. (2020).

BEFORE YOU BEGIN

The protocol below describes the specific steps for staining ACE2 in formalin-fixed paraffin-embedded (FFPE) human pancreas, small intestine, and kidney tissue obtained from organ donors and at autopsies. However, with appropriate optimization, this protocol could potentially be applied to many other human tissue types (Hamming et al., 2004).

Human tissue samples

1. Process human tissues to formalin fixed paraffin embedded (FFPE) blocks using standard methods (Sadeghipour and Babaheidarian, 2019).

   Note: Human tissue samples in the original study using this protocol (Kusmartseva et al., 2020) were collected by the Network for Pancreatic Organ donors with Diabetes (nPOD) (https://www.jdrfnpod.org/) program, as approved by the University of Florida Institutional Review Board, and at the University Medical Center New Orleans (New Orleans, LA), as approved by the Louisiana State University Institutional Review Board; both in accordance with federal guidelines involving informed consent from each individual's legal representative.

   Note: The nPOD Standard Operating Procedure (SOP) that outlines protocol for histological preparation of nPOD samples could be found here.

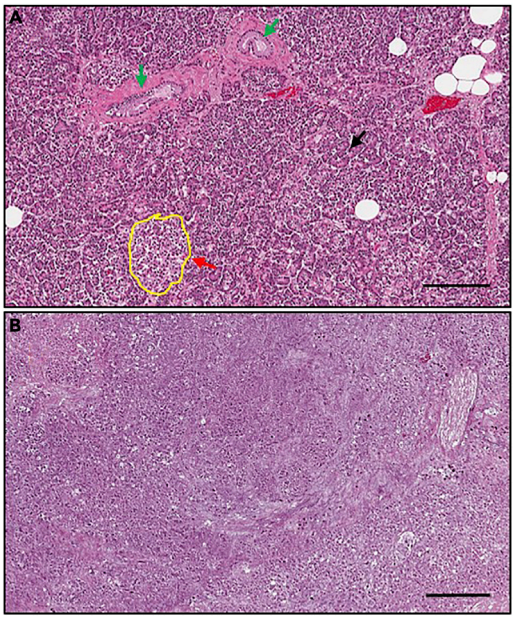

Figure 1. Tissue quality assessment in postmortem human pancreata by H&E staining

(A) Representative image of postmortem sample with sufficient tissue quality showing preserved pancreatic tissue architecture with clearly identifiable pancreatic islets (red arrow), acinar cells (black arrow) and ducts (green arrows); yellow line contours the islet border, scale bar: 200 μm.

(B) Representative image of postmortem pancreatic tissue sample with insufficient tissue quality showing severe autolysis with loss of cytoarchitectural features; scale bar: 200 μm.

Assessment of tissue quality in postmortem samples

2. Using a microtome, cut 4μm sections from FFPE tissue blocks.
3. Prior to selecting and staining tissue sections from an organ donor or autopsy case, stain a consecutive serial section with hematoxylin and eosin (H&E) according to manufacturer's instructions (Abcam).

   Note: ThermoScientific Micron HM 325 microtome was used in this protocol. Other commercially available microtomes are also suitable.

   Note: 4μm section thickness is recommended for the majority of tissue types.

   Optional: Other commercially available H&E kits are also suitable.

4. Visually assess tissue quality.
   a. If the tissue shows sufficient tissue quality (i.e., preserved tissue architecture with clearly identifiable tissue-specific structures; example of pancreatic tissue, Figure 1A), proceed with ACE2 staining.
   b. In contrast, if the tissue shows significant (>30%) signs of autolysis and advanced degradation (example of pancreatic tissue, Figure 1B) do not attempt ACE2 staining as false positive signaling may occur.

Staining preparation checklist

Timing: 1 h

5. Preheat a dry oven to 55°C.
6. Check that all reagents in the deparaffinization and rehydration containers are at a sufficient level to cover the entire surface of the slides.
7. Add tap water to a bench top staining tray just enough to create a humidified environment for performing the slide staining step.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal IgG anti-ACE2 (clone EPR4435(2)), dilution factor (1:150)	Abcam	Cat# ab108252; RRID: AB_10864415
Rabbit IgG Isotype Control	Novus	Cat# NB810-56910; RRID: AB_844243
Biotinylated goat anti-rabbit IgG (H+L)	Vector Laboratories	Cat# BA-1000; RRID: AB_2313606
Biological samples		
Human control organ donor duodenum FFPE blocks	nPOD repository https://www.jdrfnpod.org/	Network for Pancreatic Organ Donors with Diabetes, RRID:SCR_014641 Cat# nPOD_6493, RRID:SAMN15879546
Human control organ donor kidney FFPE blocks	nPOD repository https://www.jdrfnpod.org/	Network for Pancreatic Organ Donors with Diabetes, RRID:SCR_014641
Human control organ donor pancreas FFPE blocks	nPOD repository https://www.jdrfnpod.org/	Network for Pancreatic Organ Donors with Diabetes, RRID:SCR_014641 Cat# nPOD_6493, RRID:SAMN15879546
Human COVID-19 autopsy pancreas FFPE blocks	Department of Pathology, Louisiana State University	https://www.medschool.lsuhsc.edu/pathology/
Chemicals, peptides, and recombinant proteins		
Antibody diluent reagent	Life Technologies	Cat# 003118
Normal goat serum	Vector Laboratories	Cat# H-1200
Antigen Unmasking Solution, Citrate-Based	Vector Laboratories	Cat# H-3300-250
Optik Hematoxylin	Avantik	Cat# R54576-A
Optik Aqueous Clarifier	Avantik	Cat# R54361-B
Optik Bluing Reagent	Avantik	Cat# R54363
Xylene[1]	Fisher Scientific	Cat# X3P-1gal
Ethanol 200 proof[1]	Decon Labs	Cat# 2701G
Methanol[1]	Fisher Scientific	Cat# A433P-4
Hydrogen peroxide[2]	Fisher Scientific	Cat# H325-500
Citrate Buffer, pH 6.0 (10×)	Abcam	Cat# ab64214
Shandon-Mount Mounting Media	Thermo Scientific	Cat# 1900331
Critical commercial assays		
H&E Staining Kit	Abcam	Ab245880
Avidin/Biotin Blocking Kit	Vector Laboratories; www.vectorlabs.com	Cat# SP-2001
ABC Elite standard detection kit	Vector Laboratories; www.vectorlabs.com	Cat# PK-6100
ImmPACT DAB[3]	Vector Laboratories; www.vectorlabs.com	Cat# SK-4105
Software and algorithms		
Aperio eSlideManager	Leica Biosystems	Version 12.4.0.5043
Other		
Microtome	n/a	n/a
Benchtop dry oven	n/a	n/a
Microwave oven, 600 Watt	n/a	n/a
Fume hood	n/a	n/a
Brightfield microscope	n/a	n/a
Aperio CS2 Slide Scanner (optional)	Leica Biosystems	n/a

[1] ⚠ CRITICAL: Flammable, highly toxic. Use only in a functioning fume hood. Wear gloves and a lab coat. Dispose of spent chemicals as hazardous waste.

[2] Strong oxidizer and can react with skin. Use only in a functioning fume hood. Wear gloves and a lab coat. Dispose of spent chemicals as hazardous waste, and avoid introducing any reagent to sink or water waste.

[3] 3,3′-Diaminobenzidine (DAB) is a known carcinogen, gloves and lab coat should be worn during use.

MATERIALS AND EQUIPMENT

Wash Buffer		
Reagent	Final concentration	Volume
Tris Buffered Saline (10×)	1×	10 mL
Tween-20 (10%)	0.1%	1 mL
ddH_2O	n/a	89 mL
Total	**n/a**	**100 mL**

Store at 20°C–22°C. Dispose of after 1 week.

STEP-BY-STEP METHOD DETAILS

Deparaffinize and rehydrate sections

Timing: 2 h

Paraffin is removed followed by hydrogen peroxide quenching to avoid detection of endogenous peroxidase activity, and tissue is rehydrated through incubation in graded ethanol solutions.

Note: Fresh xylenes and graded ethanol solutions should be prepared daily for use in this protocol. For large numbers of stained slides, we recommend replacing these solutions after processing every 50 slides.

Note: Deparaffinization and rehydration steps should be carried on in a chemical hood to minimize exposure to harmful chemicals.

1. Remove paraffin from FFPE sections.
   a. Melt paraffin to drive off any residual water trapped behind sections.
      i. Place slides in a xylene compatible, non-metal slide rack.
      ii. Bake the rack of slides in a 55°C dry oven for one hour.
   b. Dissolve surrounding and infiltrating paraffin from the sections. Perform all steps at room temperature (20°C–22°C).
      i. Place rack of slides in xylene for 5 min.
      ii. Transfer rack of slides to fresh xylene, incubate for 5 min.
      iii. Transfer slides to fresh xylene, incubate for 5 min.
   c. Clear the xylene from slides. Perform all steps at room temperature (20°C–22°C).
      i. Place rack of slides in 100% ethanol for 5 min.
      ii. Transfer rack of slides to fresh 100% ethanol, and incubate for 5 min.

Note: It is recommended that two slides be prepared for each case and tissue so that rabbit IgG isotype control can be stained in parallel with the ACE2 stained slide.

Note: To confirm the antibody specificity, preincubation of the ACE2 antibody with the synthetic peptide to which the antibody was raised could be utilized as an additional staining control.

2. Block endogenous peroxidases.
   a. Place rack of slides in freshly prepared 3% hydrogen peroxide diluted in methanol, and incubate for 10 min at room temperature (20°C–22°C).

Optional: Peroxidase blocking can alternatively be performed after the antigen retrieval steps using a commercially available hydrogen peroxide blocking reagent following the manufacturer's instructions.

3. Rehydrate sections through graded ethanol solutions. Perform all steps at room temperature (20°C–22°C).
   a. Transfer slide rack to 95% ethanol and incubate 5 min.
   b. Transfer slide rack to 75% ethanol and incubate 5 min.
   c. Transfer slide rack to 50% ethanol and incubate 5 min.
   d. Transfer slide rack through 2 changes of deionized (DI) water for 2 min each.

Pause point: The assay could be paused at step 1 b (i) provided the incubation time does not exceed 72 hours.

Note: Used xylene and ethanol solutions must be disposed of according to federal, state and organization laws.

⚠ CRITICAL: From this point on, do not allow slides to become dry as background will occur in the staining.

Perform antigen retrieval

Timing: 30–40 min

Heat retrieval is used to reverse fixation crosslinking and enable antibody recognition of the antigen.

4. Prepare retrieval chamber.
   a. Prepare 1× citrate buffer by diluting 1 part of 10× citrate buffer stock solution with 9 parts deionized water.
   b. Transfer slides from rack to a plastic coplin jar filled with 1×X citrate buffer.

Note: Slides may be placed back-to-back in the coplin jar to increase capacity.

   c. Adjust the citrate solution level to cover the top of the slides.
   d. Place coplin jar into a 400 mL Pyrex beaker and fill beaker with tap water up to one cm from cap edge.

Note: Leave lid to coplin jar slightly loose to allow steam to escape.

5. Retrieve tissue sections.
   a. Program a microwave oven at 600 watts for 7 min.
   b. Place coplin/water jacket assembly in center of the microwave and press start.
   c. When microwave cycle ends, remove beaker from microwave and tighten lid.
   d. Allow coplin/water jacket assembly to cool on the bench top for 20 min.
   e. Incubate slides 1 min in DI water to rinse off buffer.
   f. Using PAP pen, make a square or circle hydrophobic barrier around the tissue section to keep staining reagent localized to the tissue and minimize reagent waste.
   g. Place slides in prepared humid staining chamber and cover section surface in wash buffer.

Optional: Use of humid staining chamber is strongly recommended. However, alternatively, use of PAP pen alone should create humid environment and keep tissue sections from drying provided that the incubation time with each staining reagent does not exceed the recommended duration.

Optional: Borg Decloaker RTU (Biocare Medical, LLC) can be used as an alternative antigen retrieval solution, following manufacturer's instructions.

Optional: Heat retrieval methods involving a different heating device, such as a water bath, have been successfully used in this assay. For the water bath epitope retrieval, we recommend the following setting: temperature 97°C; time 30 minutes. The antigen retrieval solution should be pre-warmed to 97°C before adding it to coplin jar with slides. The temperature in water bath should be closely monitored to ensure correct heat.

Optional: It is possible to adapt this method to use a preferred retrieval reagent provided titer is tested and adjusted.

Block tissue sections

Timing: 70 min–3 h

Endogenous tissue components are blocked to avoid background stain development.

6. Block biotin present in the tissue using Avidin/Biotin blocking kit (Vector Laboratories). Perform all steps at room temperature (20°C–22°C).
   a. Wipe buffer from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.
   b. Cover tissue section surface with biotin solution and incubate 15 min.
   c. Wash slides in wash buffer for 5 min.
   d. Wipe buffer from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.
   e. Cover tissue section surface with avidin solution and incubate for 15 min.
   f. Wash slides in buffer for 5 min.
   g. Wipe buffer from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.

Optional: Biotin blocking is not necessary if your tissue type does not contain endogenous biotin.

7. Perform normal goat serum blocking.
   a. Cover the tissue section surface with goat serum diluted to 2% in wash buffer and incubate 30 min at room temperature (20°C–22°C).

Note: Ensure that serum matches the species that the secondary antibody is produced in.

Pause point: The assay can be paused for up to 2 hours provided the goat serum solution level is adequate for the tissue to remain completely wet.

Apply primary antibody

Timing: 60 min - overnight (8–12 h)

Diluted primary antibody solution is placed on top of tissue sections to allow recognition of the ACE2 antigen

8. Perform primary antibody incubation.
   a. Dilute rabbit monoclonal ACE2 primary antibody at 1:150 in antibody diluent reagent.
   b. Wipe serum from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.
   c. Cover tissue section-containing area of slide with ACE2 antibody solution.
   d. Incubate for 1 h.

Optional: In a separate tube, dilute rabbit IgG isotype control at a final concentration to match that of the ACE2 antibody. Cover tissue section-containing area of an additional slide with isotype control antibody solution.

Note: Concentration of antibody used is dictated by retrieval method. Alterations to the retrieval conditions will necessitate adjustment of the primary antibody titer.

Pause point: The assay can be paused by placing the humidity chamber at 4°C and incubating overnight (8–12 hours).

Apply secondary and tertiary reagents

Timing: 80–85 min

Biotinylated secondary antibody and avidin-biotin-horseradish peroxidase reagent are sequentially applied to the sections to recognize and amplify the antibody/antigen complex.

Note: ABC reagent must be made according to manufacturer instructions prior to beginning the secondary antibody incubation so that the ABC completes a required 30-minute incubation step.

9. Perform secondary antibody incubation.
   a. Dilute biotinylated secondary antibody to 1:200 in wash buffer.
   b. Wash slides in wash buffer for 5 min.
   c. Wipe buffer from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.
   d. Cover tissue section with biotinylated secondary antibody solution, and incubate for 30 min.
10. Perform ABC Elite reagent incubation.
   a. Wash slides in wash buffer for 5 min.
   b. Wipe buffer from the surface of the slide with a folded Kimwipe, avoiding contact with the tissue.
   c. Cover tissue section in prepared ABC Elite reagent and incubate for 30 min.
   d. Wash slides in wash buffer for 5 min.

Pause point: The slides can be held up to 10 minutes in buffer prior to performing chromogenic development.

Detect the primary antibody signal

Timing: 30–40 min

Chromogenic detection of positive signal is achieved using DAB, and the section is counterstained with hematoxylin to enable visualization of unstained structures.

Note: DAB should be prepared immediately prior to use in order to avoid reagent precipitation.

11. Detect positive signal with ImmPACT DAB.
   a. Prepare working solution of DAB following kit instructions (combine one drop of DAB stock with one mL of DAB diluent).
   b. Wipe buffer from the surface of one slide with a folded Kimwipe, avoiding contact with the tissue.
   c. Cover surface of section in DAB solution and start a count-up timer.
   d. During DAB incubation, monitor brown color development under a brightfield microscope, typically 25–30 s.

e. When the signal reaches the desired intensity, immediately stop the reaction by submerging the slide in water and rinsing with mild agitation.
f. Place completed slide in a slide rack.
g. Repeat development (steps b–f) for all remaining slides.

Note: Develop Rabbit IgG isotype control slides for the same amount of time as ACE2 slide development.

12. Counterstain slides with hematoxylin.
    a. Place slide rack in tap water for 5 min to hydrate.
    b. Transfer slide rack to hematoxylin for 15 s.
    c. Wash in rapid changes of tap water until color ceases to drain from slides.
    d. Incubate rack of slides in Clarifier solution for 20 s.
    e. Wash 2 × 1 min in tap water.
    f. Incubate rack of slides 1 min in Bluing solution.
    g. Wash 2 × 1 min in tap water.
13. Dehydrate slides in graded ethanol solutions.
    a. Place rack of slides in 50% ethanol for 2 min.
    b. Transfer rack of slides to 75% ethanol for 2 min.
    c. Transfer rack of slides to 95% ethanol for 2 min.
    d. Transfer rack of slides to 100% ethanol for 2 min.
    e. Incubate rack of slides for 5 min in 100% ethanol.
14. Clear the slides by placing them twice in xylene for 5 min.
15. Mount glass coverslips to the slides with Shandon-Mount mounting media.
16. Let slides dry for at least 1 h.
17. Scan stained slides and view digitized images using slide scanner system such as Aperio CS2.

Note: Use fresh hematoxylin solution after every batch of 50 slides. Used hematoxylin solution must be disposed of according to federal, state and organization laws.

Optional: Alternatively, any xylene compatible permanent mounting media could be used to mount glass coverslips to the slides.

Optional: Alternatively, staining results can be observed using a brightfield microscope.

EXPECTED OUTCOMES

The described method consistently produces staining of specific sites in each organ tissue tested (Figure 2). The enterocytes of the duodenum display strong ACE2 staining restricted to the brush borders of these cells (Figures 2A and 2B). In the kidney (Figures 2C and 2D), intense, prominent ACE2 staining is observed in the apical brush borders of the proximal tubular cells. In contrast, the cytoplasm of these cells and endothelial cells of the vessels produce a weaker signal. Kidney glomeruli are negative for ACE2. In the pancreas, microvasculature endothelium in the islets and acinar tissue and some pancreatic ducts show distinct ACE2 positivity with tissue background devoid of signal (Figures 2E and 2G). The vast majority of larger pancreatic vessels are ACE2 negative. (Figures 2E and 2H). The ACE2 staining pattern in autopsy pancreatic tissue samples from patients with COVID-19 closely resembles the pattern of ACE2 expression observed in the pancreas of individuals without a history of SARS-CoV-2 infection (Figure 3).

LIMITATIONS

Staining results are heavily affected by the condition of the material tested. Adequate formalin fixation of non-degraded tissue is required.

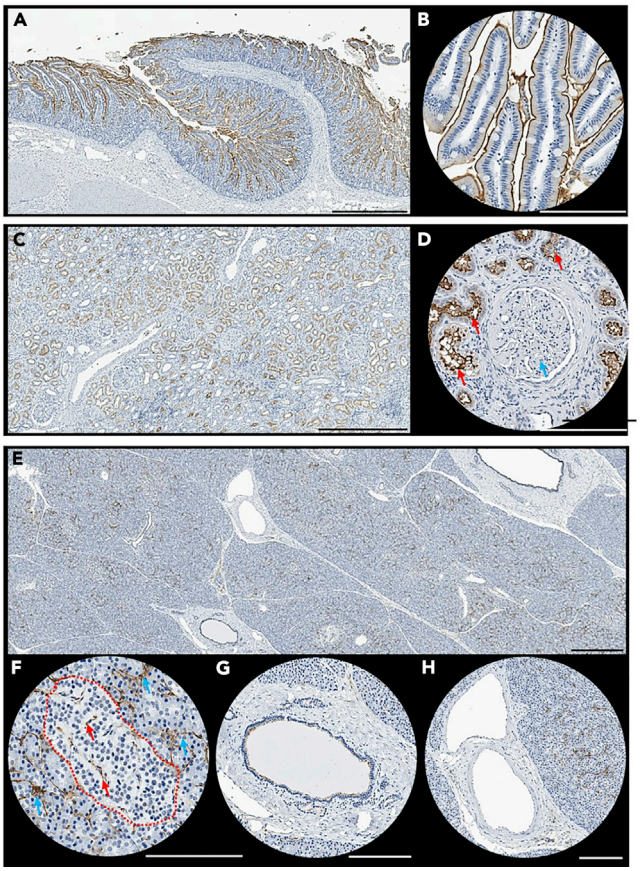

Figure 2. Organ-specific ACE2 staining pattern

(A) Representative image of human duodenum stained for ACE2; scale bar: 900 μm.

(B) Representative image of human duodenal microvilli showing ACE2 staining in the brush borders of the enterocytes; scale bar: 200 μm.

(C) Representative image of human kidney stained for ACE2; scale bar: 900 μm.

(D) Representative image of human renal cortex showing ACE2 staining in the brush borders of the proximal tubular cells (red arrows) and ACE2 negative glomerulus (blue arrow); scale bar: 200 μm.

(E) Representative image of human pancreas stained for ACE2; scale bar: 900 μm.

(F) Representative image showing ACE2 staining in the pancreatic microvasculature endothelium in islet (red arrow) and acinar (blue arrows) regions; red dash line contours the islet boarder, scale bar: 200 μm.

(G) Representative image showing ACE2 staining in the pancreatic duct; scale bar: 200 μm.

(H) Representative image showing absence of ACE2 in major vessels of the pancreas; scale bar: 200 μm.

TROUBLESHOOTING

Problem 1

Tissue lifts from slide.

Potential solution

Use fresh sections mounted on plus charged slides.

Problem 2

Staining of tissue fails in a cell type that is known to be positive.

Potential solution

ACE2 is highly expressed in microvasculature. If a positive control tissue fails, the assay should be repeated to confirm that all steps were included in stated order.

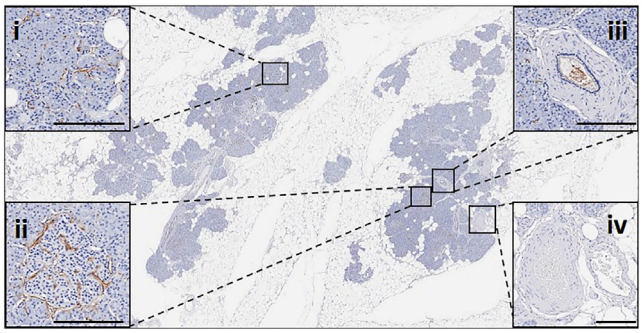

Figure 3. ACE2 localization in postmortem pancreas of a COVID-19 patient

Pancreatic tissue section from a patient with COVID-19 stained for ACE2. Insets highlight ACE2 staining in microvasculature within acinar (i) and islet (ii) regions, some ductal epithelium (iii), but not in major blood vessels (iv); scale bars: 2 mm; insets 200 μm.

Check DAB chromogen and kit component expiration dates.

Problem 3

Staining of tissue is weak.

Potential solution

Increase chromogen incubation time while observing reaction development with a brightfield microscope.

Problem 4

Poor signal to background discrimination.

Potential solution

Confirm the tissue is staining appropriately by performing a primary antibody titration experiment. Repeat the experiment staining one slide each with 1:75, 1:150 and 1:300 primary antibody dilutions. Results should be in keeping with the serial dilution showing 1:75>1:150>1:300.

Problem 5

Failure to find intense signal within autopsy tissue samples stained with the serially diluted primary antibody.

Potential solution

Confirm tissue quality by staining a consecutive tissue sections for H&E. If tissue shows less than 30% autolysis and degradation, repeat a primary antibody titration experiment by staining one slide each with wider range (1:20 to 1:600) of antibody dilutions. Do not attempt ACE2 staining if significant (>30%) autolysis and advanced degradation observed as false positive signaling may occur.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark Atkinson (atkinson@pathology.ufl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, M.J. and I.K.; investigation, M.J., P.J., and I.K.; writing – original draft, M.J.; writing – review & editing, I.K., P.J., A.L.P., R.S.V.H., and M.A.A.; funding acquisition, M.A.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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