ABSTRACT
Insulin and glucagon hormones were detected in paraffin sections (4 μm thickness) of formalin-fixed human pancreases. Insulin and glucagon were detected immunohistochemically using mouse anti-human insulin mAb (ascites; Sigma) and mouse anti-porcine glucagon mAb (ascites; Sigma), respectively, with biotinylated anti-mouse IgG and avidin-biotin-complex (ABC reagent; PK-2200, Vector Labs). Background staining was checked using the corresponding isotype control Ig instead of the primary antibody. 3-amino-9-ethylcarbazole (AEC) was used as the chromogen. Stained sections were imaged using a light microscope with attached camera (Olympus BX41).

EXTERNAL LINK
https://doi.org/10.1371/journal.pone.0191360

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION


DOI
dx.doi.org/10.17504/protocols.io.kv6cw9e

EXTERNAL LINK
https://doi.org/10.1371/journal.pone.0191360

PROTOCOL CITATION
Lora Starrs, Debra Brown, Sarah Popp, Charmaine Simeonovic 2018. Immunohistochemical staining of insulin and glucagon in islets of formalin-fixed human pancreas. protocols.io
https://dx.doi.org/10.17504/protocols.io.kv6cw9e

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

KEYWORDS
Insulin immunohistochemistry, glucagon immunohistochemistry, human pancreas, ihc

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Materials:

1. Prepare graded alcohols and xylene for deparaffinizing tissue sections: 2 x xylene (250 ml/slide container), 2 x absolute ethanol (250 ml/slide container), 1 x 90% ethanol (250 ml), 1 x 70% ethanol (250 ml).

1. Prepare acetate buffer components:
   (i) 0.1N acetic acid: 290 μl glacial acetic acid in 50 ml deionized water
   (ii) 0.1M sodium acetate: 410 mg anhydrous CH₃COONa in 50 ml deionized water.
   Prepare 0.1M acetate buffer (pH 5.2) by mixing 10.5 ml 0.1N acetic acid and 39.5 ml 0.1M sodium acetate.

1. Prepare 18% (w/v) sodium azide.

2. Prepare stock solution of 3-amino-9-ethylcarbazole (AEC; chromogen, 8 mg/ml: 40 mg AEC in 5 ml N-N-dimethyl formamide; protect from light and refrigerate at 4°C.

3. Prepare M.O.M. diluent: 200 μl M.O.M. protein concentrate stock solution (Immundetection kit, PK-2200, Vector) in 2.5 ml phosphate-buffered saline (PBS) for use either as a blocking step to minimize non-specific Ig binding or for diluting antibodies.

4. Mabs and pAbs:
   Mouse anti-human insulin mAb (ascites), Sigma-Aldrich #12018
   Mouse anti-porcine glucagon mAb (ascites), Sigma-Aldrich #G2654
   Biotinylated anti-mouse IgG, M.O.M immunodetection kit, Vector Labs # PK-2200
   Mouse IgG1κ, eBioscience # 14-4714-85

7. Other reagents:
   Hydrogen peroxide (30% w/w), Chem-Supply Pty Ltd (Australia) #HA154-500M
   Methanol, Merck #106009
   Sodium azide, Sigma-Aldrich #S2002
   3-Amino-9-ethylcarbazole (AEC), Sigma-Aldrich #A5754
   Animal free blocker, Vector Labs #SP-5030
   Stock protein concentrate, M.O.M immunodetection kit, Vector Labs # PK-2200
   Avidin-biotin complex (ABC), M.O.M immunodetection kit, Vector Labs # PK-2200
   N-N-dimethyl formamide, Sigma #D158550
   Glycergel mounting medium, Dako #C0563

1 See Guidelines, “Before starting”
Deparaffinize slides in each xylene for 1 min. rehydrate slides in graded alcohols beginning in absolute ethanol (10 dips)/ container of absolute ethanol), followed by 90% ethanol (10 dips) and 70% ethanol (10 dips). Wash well in running tap water for 5 min.

Wash 2 x 2 min in 250 ml phosphate-buffered saline (PBS).

Wipe around sections with a tissue, encircle the sections using a diamond pencil and place in clean 250 ml slide container of fresh PBS. Wipe away excess PBS around each section using a tissue.

Block endogenous peroxidase activity by incubating sections in 3% hydrogen peroxide in methanol (peroxidase block working solution) for 5 min. For peroxidase block working solution add 10 μl hydrogen peroxide to 10 ml methanol; take 5ml of 3% H₂O₂/methanol and add 28 μl 18% (w/v) sodium azide.

Wash off peroxidase block using PBS and transfer slides to a clean 250 ml slide container of fresh PBS. Wash slides 3x over 10 min.

Dilute primary antibodies in diluted stock protein concentrate (diluent prepared from M.O.M. Immunodetection kit).

Wipe around sections using tissue to remove excess PBS.

Apply:
(i) 1/500 dilution of anti-insulin mAb (stock ~ 20 mg/ml) or 40 μg/ml mouse IgG₁κ (as isotype control), diluted in M.O.M. diluent, 125-150 μl/section at room temperature for 30 min.
or
(ii) 1/500 dilution of anti-glucagon mAb (stock ~ 31 mg/ml) or 62.5 μg/ml mouse IgG₁κ (as isotype control), diluted in M.O.M. diluent, 125-150 μl/section at room temperature for 30 min.

Wash off primary antibody with PBS and transfer slides to slide container with 250 ml PBS. Wash 2 x 2min.

Wipe around sections using tissue and incubate with 1/250 diluted secondary biotinylated-anti-mouse IgG (M.O.M immunodetection kit), 150 μl/section, for 10 min at room temperature.

Wash off secondary antibody with PBS and transfer to slide container with 250 ml PBS. Wash slides 2 x 2min.

Wipe around sections using tissue and cover with Vectastain ABC reagent (M.O.M immunodetection kit), for 5 min at

Citation: Lora Starrs, Debra Brown, Sarah Popp, Charmaine Simeonovic (02/08/2018). Immunohistochemical staining of insulin and glucagon in islets of formalin-fixed human pancreas. https://dx.doi.org/10.17504/protocols.io.kv6cw9e

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room temperature.

14 Wash off ABC reagent with PBS and transfer to slide container with 250 ml PBS. Wash slides 3x in 10 min.

15 Prepare AEC working solution: 4.75 ml acetate buffer (see Guidelines), 0.25 ml AEC stock solution and 25 µl 3% H₂O₂. Filter using a disposable 0.2 µm filter. Use within 2 hours of preparation, refrigerate for short-term storage. Protect from light.

16 Wipe around sections using tissue and cover the sections with AEC solution for 30 min at room temperature.

17 Wash off AEC solution with deionized water and transfer slides to slide container with 250 ml deionized water. Wash 3x in 10 min.

18 Lightly counterstain with Gill’s hematoxylin, wash in deionized water (2 x) and briefly dip in ammonium water (100 µl ammonia in 250 ml deionized water), 2 x 2 sec. Wash in deionized water (2 x in 250 ml) and coverslip using glycergel mounting medium.

19 Photograph sections using a light microscope with camera attachment.