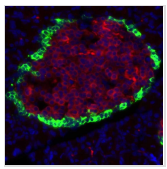

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RNA in situ hybridization on pancreatic sections using RNAscope® technology

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This protocol describes the steps for performing RNA in situ hybridization simultaneously for two targets using RNAscope Multiplex Fluorescent reagent Kit v2 assay on fixed-frozen pancreatic tissue sections. It is suitable for pancreatic tissue isolated from rats and mice at postnatal to adult stages. We routinely apply this protocol to assess gene expression qualitatively and semi-quantitatively at the cellular level. Briefly, pancreata are fixed in 4% paraformaldehyde solution and cryoprotected overnight in 30% sucrose. Tissue are then embedded, frozen, sectioned and mounted on slides. Tissue section pretreatments, RNAscope probe hybridization and fluorescence detection are performed essentially as described by the manufacturer with minor modifications.

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Croze ML, Flisher MF, Guillaume A, Tremblay C, Noguchi GM, Granziera S, Vivot K, Castillo VC, Campbell SA, Ghislain J, Huising MO, Poitout V. Free fatty acid receptor 4 inhibitory signaling in delta cells regulates islet hormone secretion in mice. Mol Metab. 2021 Mar;45:101166. doi: 10.1016/j.molmet.2021.101166. Epub 2021 Jan 20. PMID: 33484949

pancreas, islet, RNA in situ hybridization

protocol ,

Caroline Tremblay

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☒ Phosphate Buffered Saline 10x (solution) **Bio Basic Inc. Catalog #PD8117**

☒ Paraformaldehyde 10% buffered **Newcomer Supply Catalog #13301A**

☒ Sucrose Ultra Pure **Bioshop Catalog #SUC507.5**

☒ Superfrost Plus Microscope Slides **Fischer Scientific Catalog #12-550-15**

☒ OCT (Optimal Cutting Temperature compound) **Sakura Finetek Catalog #4583**

☒ MilliQ water **Contributed by users**

☒ 100% Ethanol **Contributed by users**

☒ ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #H-4000**

☒ ProLong™ Gold Antifade Mountant **Thermo Fisher Catalog #P36930**

☒ RNAscope Multiplex Fluorescent Detection Reagents V2 **Avanced Cell Diagnostics Catalog #323110**

☒ 1 Liter SSC [20X] (Sodium Chloride-Sodium Citrate) (0.3M sodium citrate, 3M NaCl, pH7.0) **G-Biosciences Catalog #R019**

☒ Opal 620 Reagent Pack **Perkin Elmer Catalog #FP1495001KT**

☒ Opal 520 Reagent Pack **Perkin Elmer Catalog #FP1487001KT**

☒ Opal 690 Reagent Pack **Perkin Elmer Catalog #FP1497001KT**

☒ Opal 570 Reagent Pack **Perkin Elmer Catalog #FP1488001KT**

☒ DAPI (2.5mg/mL) **Contributed by users**

HybEZ II system
hybridization oven

Advanced Cell Diagnostics	PN 321710/321720

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Surgipath® Clear Disposable Base Molds
Leica 75809-376

Well, 250ml. w/ lid, green, xylene resistant
TBS SS-WLG

When working with PFA, always work in a chemical hood.

Before performing the RNAscope assay, calculate the numbers of slides, you will need (samples + controls), verify that you have enough of each solutions (H_2O_2, Target retrieval, protease solution, probes, wash buffer, SSC buffer, Amp1-2-3, HRP#C1-C2-C3-C4, Opal dies and mounting media).

Preparation of cryosections 1d

1 Tissue fixation 19h

When working with PFA, always work in a chemical hood.

Harvest the pancreas and place it in a 50 ml Falcon tube containing **30 mL** of cold 4 % PFA (**12 mL** of 10 % PFA + **18 mL** of PBS).

Fix for **Overnight** at **4 °C** in the dark.

Then, working in a chemical hood, delicately remove the pancreas with forceps and place it on brown paper to absorb the fixative.

Place the organ in a new 50 ml Falcon tube containing **30 mL** of a 30 % sucrose solution (**9 g** of sucrose and **30 mL** of PBS).

Store **15:00:00** at **4 °C** in the dark.

The next day, delicately remove the pancreas with forceps and place it on brown paper to absorb the sucrose solution. Place it in a mold, cover with OCT and freeze at **-80 °C** until ready for sectioning.

2 Preparing cryosections 2h

Set the cryostat and the pedestal temperature at **-20 °C** .

Gather all of the needed material (OCT, slides, pencil, blades, paintbrushes, aluminium foil, slide box, tissues).

Cut cryosections at **8 µm** thickness and collect on Superfrost Plus microscopic slides.

Store the sections at **-80 °C** until ready for staining.

Pretreatments 2h

3 Preparation 35m

Turn on the HybEz oven and set to **40 °C** .

Place a wet Whatman paper on the Humidity Control Tray.

Insert the covered tray in the HybEz oven and humidify the chamber for at least **00:30:00** .

Wash the slides in a slide holder with PBS for at least **00:05:00** shaking from time to time.

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Prepare 1x Target Retrieval solution (1 bottle + **630 mL** milliQ water) in a **1 L** glass beaker.
Cover and heat the solution at maximum on a hot plate with constant stirring. Monitor the temperature with an electrical thermometer. When the temperature reaches **98 °C** reduce the heat setting to maintain a gentle boil.
Set to boil **700 mL** of milliQ water in a **1 L** glass beaker that will be used to prewarm the slides (step 5).

4 **Hydrogen Peroxide treatment** 10m

Collect the slides from the PBS wash, remove excess PBS and lay slides on a flat surface.
Add enough drops of Hydrogen Peroxide solution to cover the entire tissue section (3--4 drops).
Incubate at **Room temperature** for **00:10:00** .
Remove the solution and immediately wash with milliQ water using the slide holder. Repeat wash 3 times.

5 **Target Retrieval** 30m

Using forceps briefly transfer the slides in the slide holder into the boling milliQ water before transferring the slides into the boiling Target Retrieval solution. Cover with foil and incubate for **00:15:00** .

Do not let the slides cool down in the Target Retrieval solution.

Incubation time for the Target Retrieval step may vary. For mice pancreas incubate for maximum 8 minutes and 30 secondes.

Transfer the slides to a slide holder filled with milliQ water at **Room temperature** for **00:05:00** Wash the slides in milliQ water 3 more times for **00:05:00** each, shaking from time to time.
Wash the slides for **00:05:00** in **100 % (v/v)** ethyl alcohol and allow to completely dry at **Room temperature** .
Using the hydrophobic pen, create a barrier around the sections.

6 **Protease III treatment** 20m

Once the slides have completely dried, put them in the HybEz slide rack and add enough RNAscope Protease III drops to covert the tissue (3-4 drops).
Place the slide rack in the HybEz Humidity Control tray, close the lid and insert into the HybEz oven.
Incubate at **40 °C** for **00:15:00** .
During this time warm the 50x RNAscope Wash buffer in a **37 °C** water bath.
At the end of the incubation remove the HybEz Humidity Control tray from the oven.
Remove the slide rack and replace the tray back into the oven.
Flick each slides to remove the Protease III solution and wash 3 times in milliQ water at **Room temperature** for **00:05:00** in a slide holder.

RNAscope Assay 1d

7 **Probe hybridization (2 probe method)** 2h 14m

Determine the quantity of probes, transfer to 1.5 ml tubes and warm for **00:10:00** in a **37 °C** water bath.
Prepare the probe mixes as required (1:50 volume ratio C2:C1).
Remove the excess water by flicking the slides.
Place the slides in the slide rack and add **200 µL** of probe mix to cover each section.
Insert the slide rack into the HybEz oven and incubate for **02:00:00** at **40 °C** .

Do not mix probes of the same channel.

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During the incubation, prepare **3 L** of 1x Wash Buffer (to **2940 mL** of milliQ water add 1 bottle (**60 mL**) of prewarmed (**40 °C**) 50X RNAscope Wash Buffer) and 5X SSC (to **187.5 mL** milliQ water add **62.5 mL** 20X SSC).

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Then store slides **Overnight** in 5x SSC at **Room temperature** .

1h 21m

8 Amp hybridization steps

Amp1

Remove the excess liquid by flicking the slides and add enough Amp1 to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:30:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Amp2

Remove the excess liquid by flicking the slides and add enough Amp2 to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:30:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Amp3

Remove the excess liquid by flicking the slides and add enough Amp3 to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:15:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

1h 6m

9 Develop HRP-C1 signal

Remove the liquid by flicking the slides and add enough HRP-C1 to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:15:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Remove the liquid by flicking the slides and add enough Opal fluorophore (diluted 1:1500 in TCA buffer) to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:30:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Remove the liquid by flicking the slides and add enough HRP blocker to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:15:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

1h 6m

10 Develop HRP-C2 signal

Remove the liquid by flicking the slides and add enough HRP-C2 to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:15:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Remove the liquid by flicking the slides and add enough Opal fluorophore (diluted 1:1500 in TCA buffer) to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:30:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

Remove the liquid by flicking the slides and add enough HRP blocker to cover the tissue.

Insert the slide rack into the HybEz oven and incubate for **00:15:00** at **40 °C** .

Wash the slides in a slide holder 2 times for **00:02:00** in wash buffer at **Room temperature** .

30m 30s

11 Counterstain and mount

Remove the liquid by flicking the slides and add enough DAPI to cover the tissue.

Incubate for **00:00:30** at **Room temperature** .

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Remove the liquid by flicking the slides and add 1-2 drops Prolong Gold Antifade Montant to each slide and cover with a cover slip.

Allow to dry for at least **00:30:00** . Store slides in the dark at **4 °C** .

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