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RNAscope® Reference Guide

Guidelines and protocols for obtaining optimal RNA *in situ* hybridization results with any tissue type from any species

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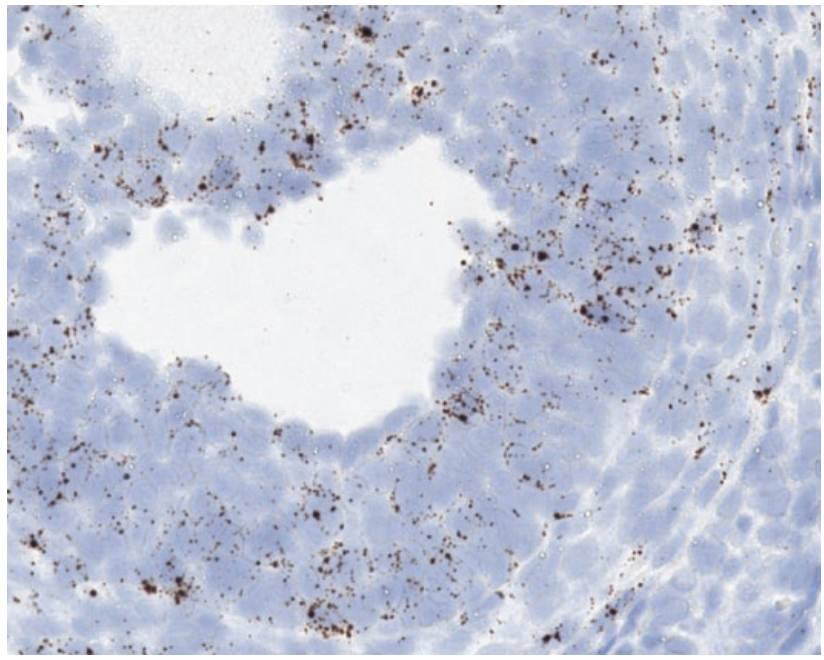
Introduction to RNAscope® ISH technology in any species, any tissue

There is a great need for robust and straightforward assays to evaluate target gene expression in tissues, particularly for preclinical studies. Most *in situ* RNA detection techniques, however, lack the robustness and sensitivity to reliably detect expression of many target genes in various species and tissue types.

Although popular, immunohistochemistry (IHC) techniques rely on the availability of antibodies which can be expensive and time-consuming to develop. Furthermore, in some cases, it may not be possible to develop appropriate antibodies, particularly in species other than human, rat and mouse. While microarrays and PCR both provide useful molecular profiles of diseases, important clinically relevant cell and tissue context information is lost along with the spatial variation of gene expression patterns.

To solve these problems, Advanced Cell Diagnostics (ACD) has developed the RNAscope® technology — an advanced platform for *in situ* RNA detection. Probes can be developed for almost any target in any species in just two weeks — and come with a performance guarantee. Thanks to a proprietary double Z probe design, RNAscope® ISH enables the detection of almost any RNA biomarker with single-molecule sensitivity and high specificity in tissues. It provides a universal solution to characterize tissue distribution of drug targets and biomarkers in a highly specific and sensitive manner, without the need for time-consuming antibody development and validation.

FIGURE 1. Expression of *Ki-67* in rat ovary tissue



Addressing the requirements of investigators using a wide range of species and tissue types, section 2 of this book presents data from a recent study of 24 tissue types from three commonly used preclinical animal models (rat, dog, and cynomolgus monkey) to provide a reference guide to optimizing the RNAscope® assay.

The data details which pretreatment is best for different tissue types and provides recommendations on which control probes to be applied for sample qualification.

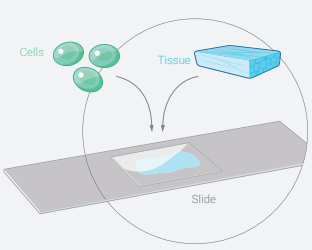
The data demonstrates that the fully automated RNAscope® 2.5 LS Assay is capable of detecting a broad range of RNA targets in multiple tissue types and is ideal for the histopathological evaluation of drug targets and biomarkers in various animal models.

The RNAscope® workflow

The RNAscope® assay can be performed manually or on fully automated staining systems, including the Discovery Ultra and Discovery XT Automated IHC/ISH slide staining systems from Roche Tissue Diagnostics and Leica Biosystems' BOND RX Research Advanced Staining System (Table 1). The RNAscope® procedure can be completed within a single day and consists of the following steps outlined below.

01

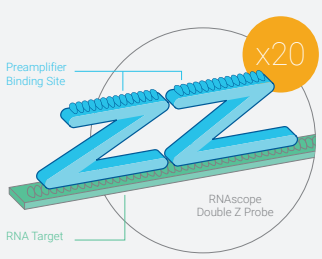
Permeabilize
cell or tissue



Fixed tissue sections or cells on slides are pretreated with a Pretreatment Kit to block endogenous peroxidase activity and optimally permeabilize samples to allow probe access to target RNA.

02

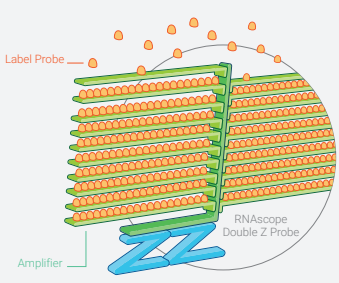
Hybridize
to target DNA



With ~20 specific double Z probe pairs per target, probes hybridize to target RNA molecules.

03

Amplify
signal

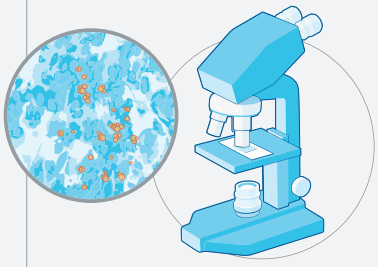


Detection reagents amplify the hybridization signals via sequential hybridization of amplifiers and labeled probes.

ACD also offers Pharma Assay Services to make this technology available for customers preferring to outsource tissue-based projects. The assay provides visualization of each individual RNA molecule as a punctate dot under a standard bright-field microscope or fluorescent microscope. These dots can be quantified by counting the number of signal dots in individual cells, either manually or by using image analysis tools, such as HALO™ software (Indica Labs), SpotStudio™ (Definiens) software and Aperio RNA ISH Algorithm (Leica Biosystems).

04

Visualize
with morphology



Each punctate dot signal represents a single target RNA molecule and can be visualized with a microscope.

05

Quantify
single-cell expression

Single-molecule signals can be quantified on a cell-by-cell basis by manual counting or automated image analysis.

RNAscope® Manual and Automated Chromogenic Assay Workflow

Steps	Manual assay	Leica automated staining systems	Ventana automated staining systems
Step 1. **Pretreatment**	**a.** Deparaffinization	**a.** Deparaffinization	**a.** Deparaffinization
	b. H_2O_2 block	**b.** Epitope retrieval	**b.** Epitope retrieval
	c. Epitope retrieval	**c.** Protease	**c.** Protease
	d. Protease	**d.** H_2O_2 block	**d.** H_2O_2 block
Step 2. **Hybridize**	**a.** Target probe hybridization	**a.** Target probe hybridization	**a.** Target probe hybridization
Step 3. **Amplify**	**a.** AMP1	**a.** AMP1	**a.** AMP1
	b. AMP2	**b.** AMP2	**b.** AMP2
	c. AMP3	**c.** AMP3	**c.** AMP3
	d. AMP4	**d.** AMP4	**d.** AMP4
	e. AMP5	**e.** AMP5	**e.** AMP5
	f. AMP6	**f.** AMP6	**f.** AMP6
			g. AMP7
Step 4. **Stain and detect**	**a.** DAB reaction	**a.** DAB reaction	**a.** DAB reaction
	b. Hematoxylin stain	**b.** Hematoxylin stain	**b.** Hematoxylin stain
	c. Image detection under standard microscope/scanner	**c.** Image detection under standard microscope/scanner	**c.** Image detection under standard microscope/scanner

TABLE 1. Detailed RNAscope® assay workflow

The importance of controls

Success with any assay begins with good and consistent quality control practices. ACD recommends two levels of quality control: a technical workflow check and a sample/RNA quality check.

Technical workflow quality control

A technical quality control check ensures that the assay is working correctly. This is easily performed using a control sample tested on two separate slides – one slide with a housekeeping gene positive control probe and another slide with a nonspecific bacterial gene negative control probe (i.e., dapB). When the assay is run successfully, the positive control slide will display strong staining and the negative control slide will display no staining. Including technical quality controls with every assay is recommended.

Sample/RNA quality control

Although the RNAscope® assay has universal conditions that work on most samples, tissue RNA quality and fixation conditions can vary. As a result, it is occasionally necessary to adjust the pretreatment conditions for optimal results. Section 1.3.1 of this book provides guidance on the optimization of pretreatment conditions, and section 2 details the optimal pretreatment conditions for 24 tissue types in rat, dog and cynomolgus monkey. However, checking tissue sample quality with positive and negative controls before running experiments is also recommended. This should result in a high positive control signal and no negative control background. If signal is low or background is detected, this is most often improved with adjustment to the pretreatment conditions as discussed in section 1.3.1.

Choosing appropriate control probes

Negative controls

ACD's universal negative control probe ensures that there is no background staining related to the assay and that the tissue specimen is appropriately prepared. The probe targets the dapB gene (GenBank accession #EF191515) from *Bacillus subtilis* strain SMY, a soil bacterium, and is suitable for all samples. Alternatively, probes from an unrelated species, for instance, a zebrafish probe on human tissue, can be used.

Positive controls

Positive control probes should be selected depending on the level of expression of the target gene as detailed in Tables 2 and 3.

	Positive Control Probe Gene	Expression Level (copies per cell)	Recommendations
Use with high-expression targets	UBC (ubiquitin C)	Medium/High (>20)	UBC is expressed at a medium to high level and should be paired with high-expressing targets. Applying UBC along side with a low-expressing target is not recommended as it can lead to false negative results. Because the RNAscope® assay is very sensitive, it may be possible to detect UBC RNA even with substantial degradation and under nonoptimal assay conditions.
More flexible option	PPIB (Cyclophilin B)	Medium (10–30)	PPIB is our recommendation for use as a positive control for most tissues. It is expressed at a sufficiently low level so as to provide a rigorous control for sample quality and technical performance. In the vast majority of studies, if PPIB is positive, then any target probe will detect your target RNA. PPIB is also often used as a reference in RT-PCR.
Use with low-expression targets	POLR2A (DNA-directed RNA polymerase II subunit RPB(1)	Low (3–15)	POLR2A is an additional low-copy, rigorous positive control, used for very low-expressing targets. It can be an alternative to PPIB for proliferating tissues, like tumors, and also for some non-tumor tissues (i.e., retinal tissue and lymphoid tissues).

TABLE 2. General recommendations for positive control probe selection for species other than rat, dog and cynomolgus monkey

Tissue	Rat	Dog	Cynomolgus Monkey
Adrenal gland	N.A.	Cl-POLR2A	Hs-PPIB
Colon	Rn-Ppib	Cl-POLR2A/Cl-UBC	Hs-PPIB
Duodenum	Rn-Ppib/Rn-Polr2a	Cl-POLR2A	Hs-PPIB
Epididymis	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Esophagus	Rn-Ppib	Cl-POLR2A	N.A.
Heart	Rn-Ubc	Cl-UBC	Hs-UBC
Jejunum	Rn-Ppib	N.A.	Hs-PPIB
Kidney	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Liver	Rn-Ppib	Cl-UBC/Cl-POLR2A	Hs-PPIB
Lung	Rn-Ppib/Rn-Ubc*	Cl-UBC	Hs-PPIB/Hs-UBC*
Lymph node	Rn-Ppib/Rn-Polr2a	Cl-POLR2A****	Hs-PPIB/Hs-POLR2A
Ovary	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Pancreas	Rn-Ppib/Rn-Ubc*	N.A.	Hs-PPIB
Prostate gland	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Retina	Rn-Polr2a***	Cl-POLR2A	Hs-POLR2A
Skeletal muscle	Rn-Ubc	Cl-UBC	Hs-UBC
Skin**	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Spinal cord	Rn-Ppib	Cl-POLR2A****	Hs-PPIB
Spleen	Rn-Ppib/Rn-Polr2a	Cl-POLR2A ****	Hs-PPIB****
Stomach	Rn-Ppib	Cl-POLR2A	Hs-PPIB
Testis**	Rn-Polr2a	Cl-POLR2A	Hs-POLR2A/Hs-PPIB
Thymus	Rn-Polr2a/Rn-Ppib	Cl-POLR2A****	Hs-PPIB
Tonsil	N.A.	N.A.	Hs-PPIB/Hs-POLR2A
Urinary bladder**	Rn-Ppib	Cl-POLR2A	Hs-PPIB

TABLE 3. Recommended positive control probe by tissue for rat, dog and cynomolgus as determined by ACD in the study described in the following pages

*May use UBC control probe if tissues have low RNA quality, in which case detection of low- to medium-expressing targets is challenging

**Due to limited sample quantity, only the standard pre-treatment condition was tested and provided optimal results

***Due to limited sample quantity, optimal control probe and pre-treatment conditions were determined using the RNAscope 2.0 LS reagent kit. Data not shown

****Due to limited sample quantity, only one control probe was tested and provided optimal results

Materials and methods

This section details the materials and methods used to obtain the data presented in section 2.

FFPE tissues

Multiple tissues from three commonly used animals for preclinical studies (rat, dog, and cynomolgus monkey) were harvested using a standard protocol at the drug safety research and development laboratory of Pfizer Global Research and Development (Groton, USA) (Table 4). Tissues were cut into 3 mm thickness, then fixed in 10% neutral-buffered formalin (NBF) for 24-48 hours. Fixed tissues were dehydrated in a graded series of ethanol and xylene, followed by infiltration of melted paraffin at 56°C in an automated processor. Tissue microarrays (TMAs) were constructed, sectioned at a thickness of 5 µm and mounted on the SuperFrost® Plus slides (Fisherbrand Cat #12-550-15).

Animal Models: Rat, Dog, and Cynomolgus Monkey	
Cardiovascular system	Heart
Endocrine glands/ exocrine glands	Liver, Pancreas, Adrenal gland
GI tract	Esophagus, Stomach, Duodenum, Jejunum, Colon
Hematopoietic system	Thymus, Lymph node, Spleen, Tonsil
Nervous system	Spinal cord, Retina
Reproductive system	Epididymis, Prostate, Testis, Ovary
Respiratory system	Lung, Bronchus
Skin/soft tissues	Skin, Skeletal muscle
Urinary tract	Kidney, Urinary bladder

TABLE 4. Tissue types from three animal models used in this study

Automated RNAscope® 2.5 LS assay

Ready-to-use reagents from RNAscope® 2.5 LS Reagent Kit-BROWN were loaded onto the Leica Biosystems' BOND RX Research Advanced Staining System according to the user manual (Doc. No. 322100-USM). FFPE tissue sections were baked and deparaffinized on the instrument, followed by epitope retrieval (using Leica Epitope Retrieval Buffer 2 at 95°C or at 88°C for 15 min) and protease treatment (15 min at 40°C). Probe hybridization, signal amplification, colorimetric detection, and counterstaining were subsequently performed. A schematic of the RNAscope® 2.5 LS Assay workflow on Leica Biosystems' BOND RX Research Advanced Staining System is presented in Figure 2.

RNAscope® probes

Control probes for low-, medium-, and high-expressing housekeeping genes (POLR2A, PPIB, and UBC, respectively) were tested for tissues from each species. Because the sequences of the human probes for housekeeping genes are over 95% homologous to the respective target mRNA sequences of cynomolgus monkey, human probes were used to test samples of cynomolgus monkey. The bacterial probe dapB was used as a negative control. Probes for the cell type biomarkers, proliferation markers, and apoptosis-related molecules used in this study were designed for each species. As summarized in Table 3, species-specific target probes were tested for all RNA targets except two genes, CD68 and KI67, for which human probes were used to detect cynomolgus monkey genes, due to 90-95% homology between the probe sequence and target mRNA sequence.

Image acquisition and data analysis

Images were acquired using a Leica Biosystems Aperio AT2 Digital Pathology Scanner, and RNA markers were analyzed based on the average RNA dot number per cell. RNA quantity was scored based on manual counting described as follows. Staining results were categorized into five grades according to the number of dots visualized under a bright-field microscope. 0: No staining or less than 1 dot to every 10 cells (40X magnification); 1+: 1-3 dots/cell (visible at 20-40X magnification); 2+: 4-10 dots/cell, very few dot clusters (visible at 20-40X magnification); 3+: >10 dots/cell, and less than 10% positive cells have dot clusters (visible at 20X magnification); and 4+: >10 dots/cell, and more than 10% positive cells have dot clusters (visible at 20X magnification). Figure 3 shows example of scoring for a variety of tissues, and Figure 6 (page 147) shows examples of gradual scoring.

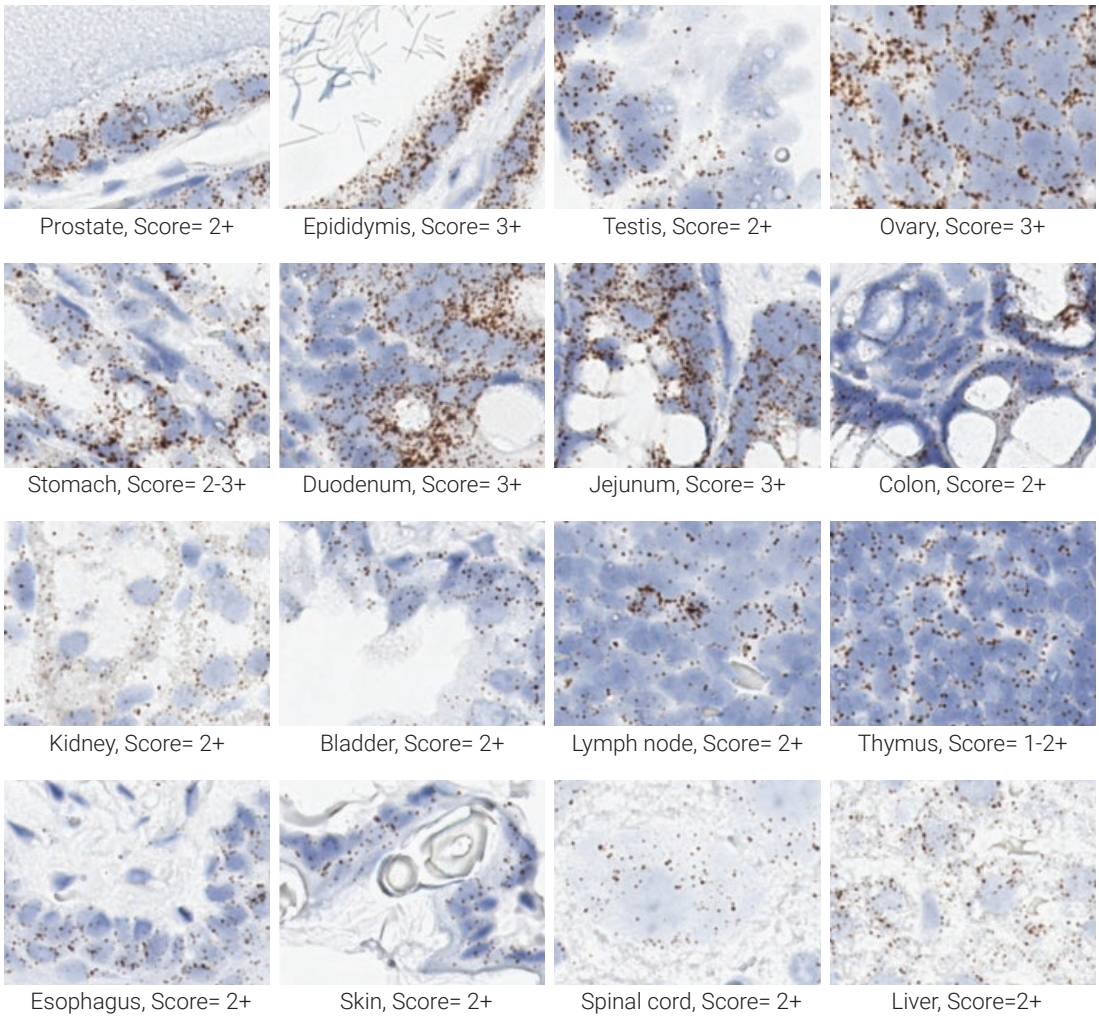


FIGURE 3. Representative images for tissue qualification using the control probe Rn-Ppib in multiple rat tissues

Optimal pretreatment conditions for different tissues in different species

The standard protocol of RNAscope® 2.5 LS Reagent Kit (on the Leica Biosystems' BOND RX Research Advanced Staining System) is designed to work for the majority of FFPE tissues.

In the study, to achieve optimal detection of RNA molecules in each tissue type, two different pretreatment conditions were compared — standard and mild — with a modification to the epitope retrieval step (Figure 2).

Standard pretreatment
BOND Epitope Retrieval Buffer 2 (ER2) at 95°C for 15 min, followed by protease digestion at 40°C for 15 min.

Mild pretreatment
ER2 at 88°C for 15 min, followed by protease digestion at 40°C for 15 min.

The optimal pretreatment condition for each tissue type was determined based on RNA signal level and the integrity of morphology. Table 4 describes optimal conditions determined in this study for tissues from rat, dog and cynomolgus monkey. Complete data with tissue images and recommended conditions for all samples are shown in section 2. The background signal, evaluated by the bacterial dapB probe, was very low or absent in all test samples with both pretreatment conditions. To optimize conditions for species other than rat, dog, and cynomolgus monkey, it is generally recommended to start with the default pretreatment setting, which is mild for lymphoid tissues and retina and standard for other tissues.

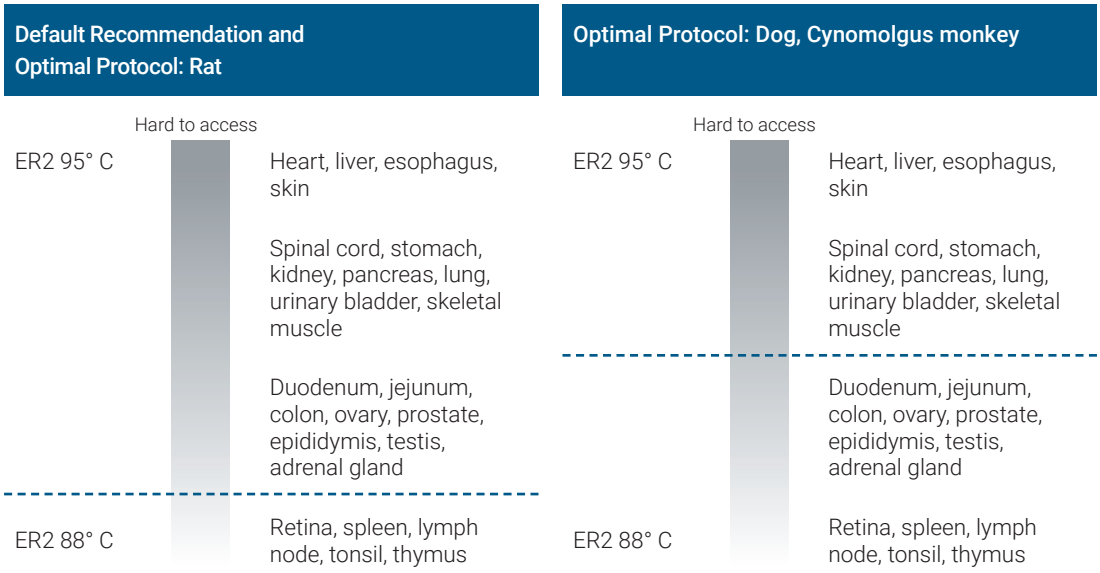


FIGURE 4. (A) Default pretreatment recommendation for different tissue types and the optimal pretreatment conditions for tissues from rat. (B) Optimal pretreatment conditions for tissues from dog and cynomolgus monkey. Tissues sharing the same pretreatment protocol may be grouped together for tissue microarray.

Optimal pretreatment is based on FFPE tissue prepared by Pfizer-Groton and the RNAscope® LS Reagent Kit for the Leica Biosystems' BOND RX Research Advanced Staining System. Further optimization may be required for samples prepared and processed differently – pretreatment conditions for manual RNAscope® assay and RNAscope® assays on the Discovery Ultra and Discovery XT Automated IHC/ISH slide staining systems from Roche are adjusted by time, NOT temperature.

Optimization recommendation for manual assay are:

- Target retrieval: 15 to 30 min
- Protease: 15 to 30 min

Optimization recommendation for Discovery Ultra and Discovery XT Automated IHC/ISH slide staining systems are:

- Target retrieval: 16 to 24 min
- Protease: 16 to 24 min

See respective user manuals for further guidance.

RNAscope® optimization results by species and tissues

This section presents data from 24 tissue types in rat, dog and cynomolgus monkey. The results shown here are intended to be used as a reference guide for similar studies.

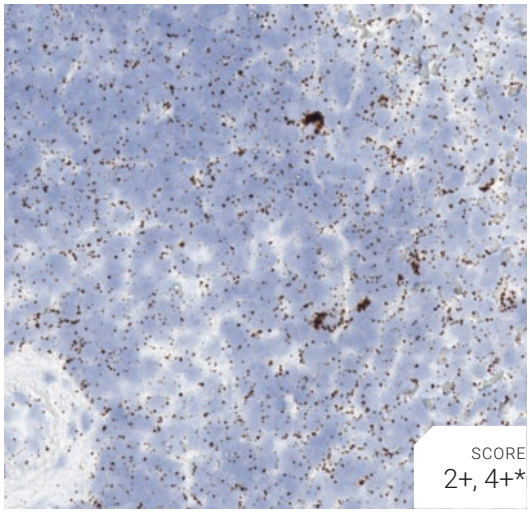
Rat

Rattus norvegicus

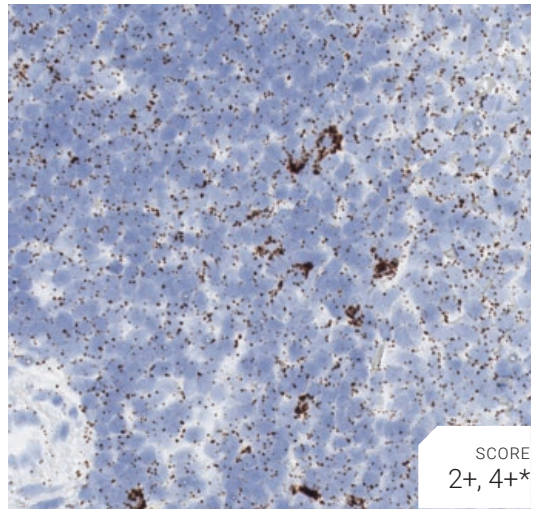
The following pages show positive control probe images with standard and mild pretreatment conditions. Optimal pretreatment condition is indicated for each tissue type.

Positive control probe: Rn-Ppib
Tissue: Spleen

Pretreatment: **Standard**



Pretreatment: **Mild**

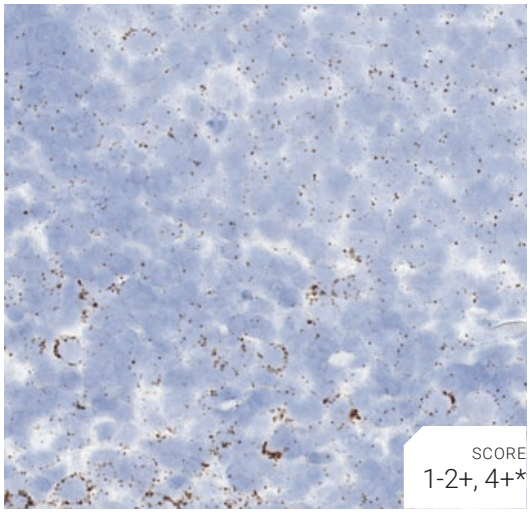


Observation: Loss of cell boundary using standard pretreatment.

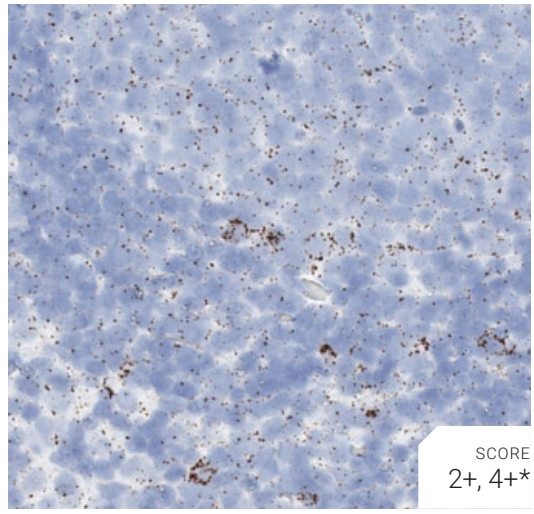
Optimal Pretreatment: Mild

Positive control probe: Rn-Ppib
Tissue: Lymph node

Pretreatment: **Standard**



Pretreatment: **Mild**



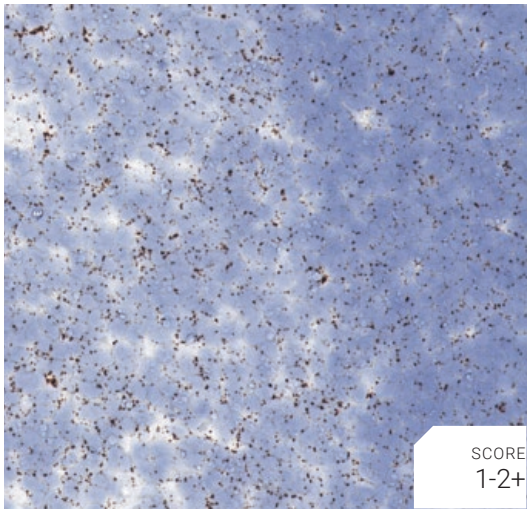
Observation: Loss of cell boundary using standard pretreatment.

Optimal Pretreatment: Mild

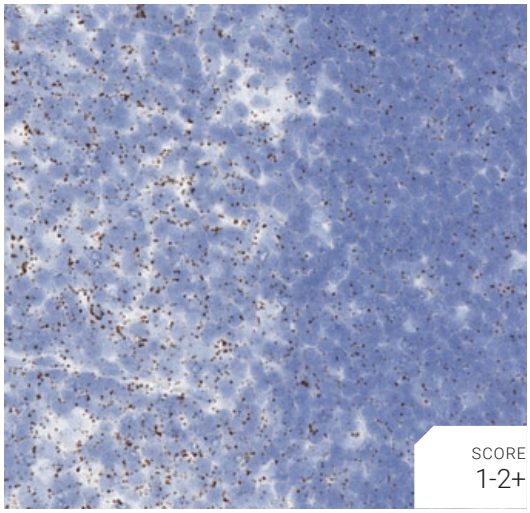
*A few cells have higher *Ppib* signal

Positive control probe: Rn-Ppib
Tissue: Thymus

Pretreatment: **Standard**



Pretreatment: **Mild**

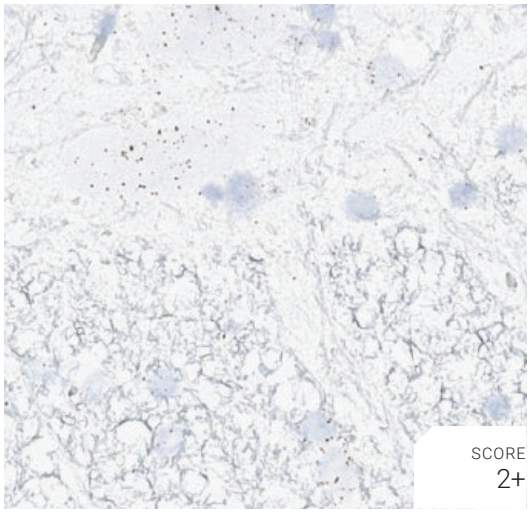


Observation: Loss of cell boundary using standard pretreatment.

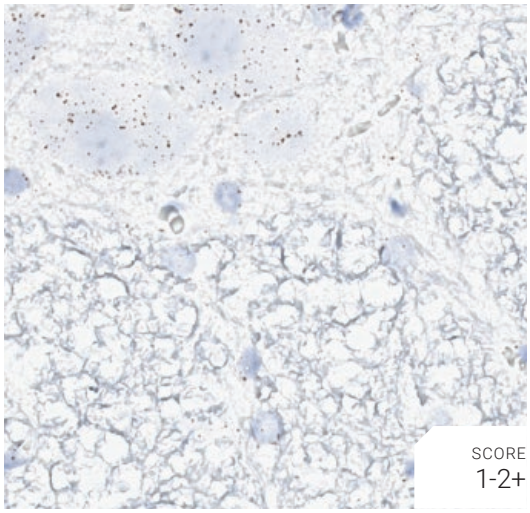
Optimal Pretreatment: Mild

Positive control probe: Rn-Ppib
Tissue: Spinal cord

Pretreatment: **Standard**



Pretreatment: **Mild**

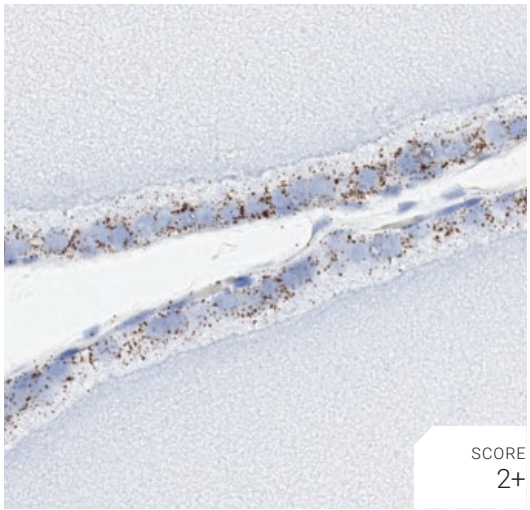


Observation: Standard pretreatment increases staining in white matter.

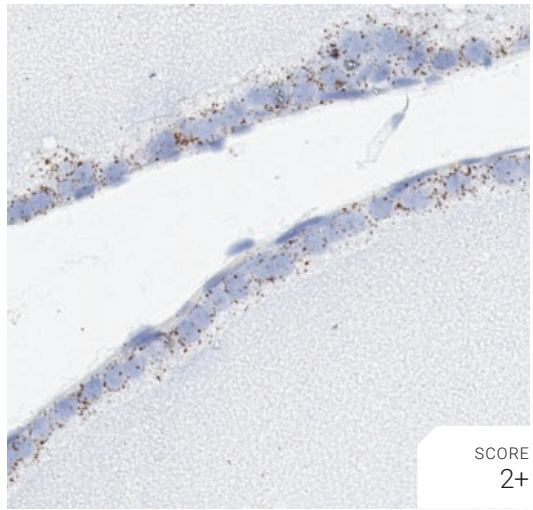
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Prostate gland

Pretreatment: **Standard**



Pretreatment: **Mild**

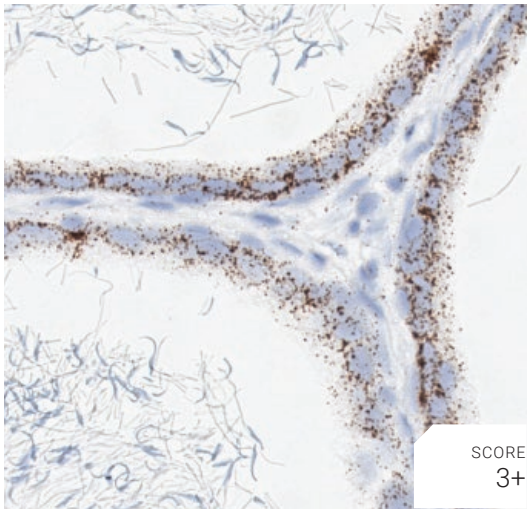


Signal: Standard ≥ Mild

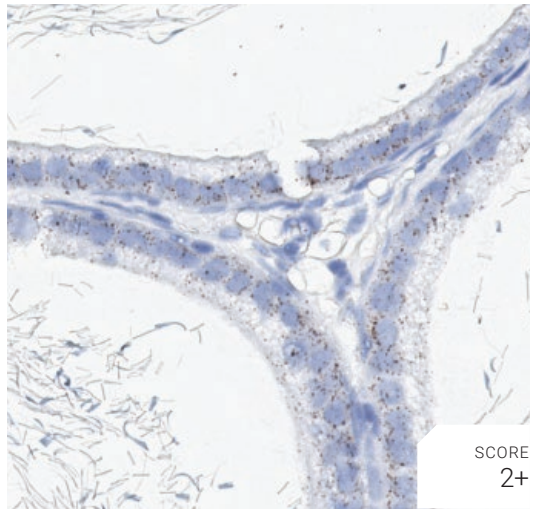
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Epididymis

Pretreatment: **Standard**



Pretreatment: **Mild**

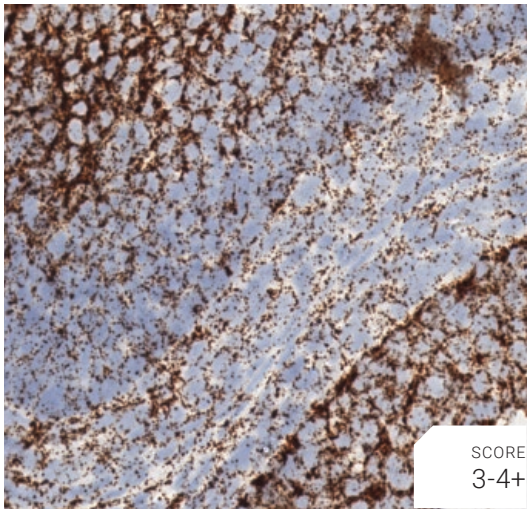


Signal: Standard ≥ Mild

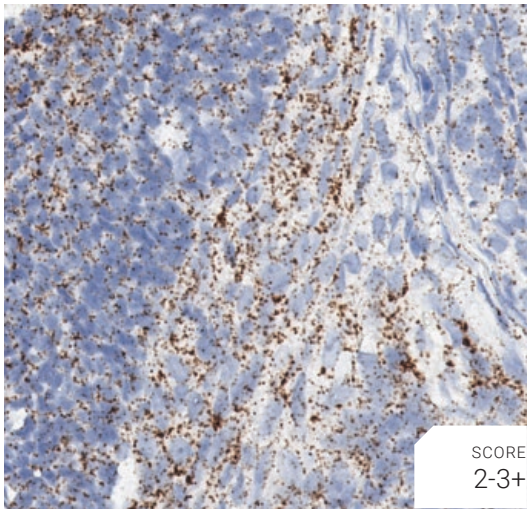
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Ovary

Pretreatment: **Standard**



Pretreatment: **Mild**

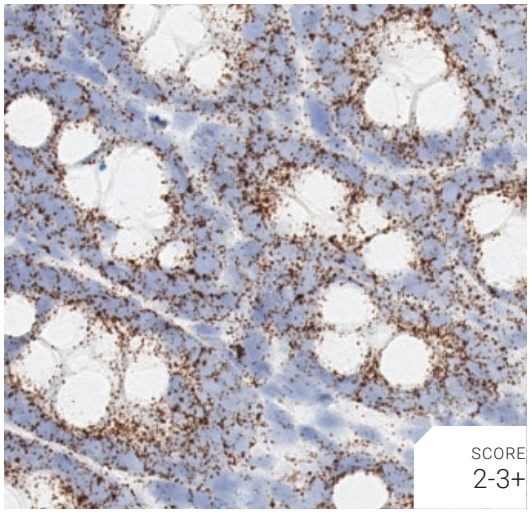


Signal: Standard ≥ Mild

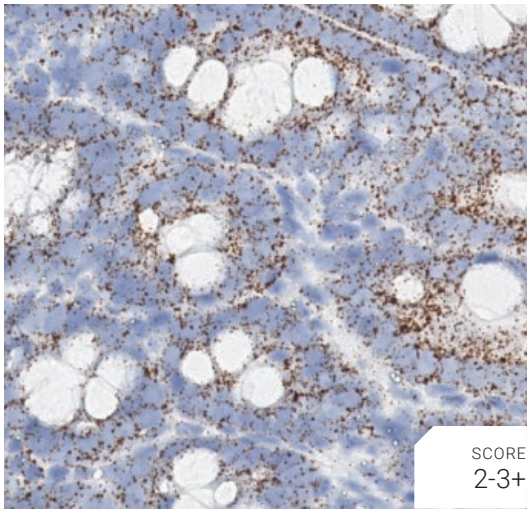
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Duodenum

Pretreatment: **Standard**



Pretreatment: **Mild**

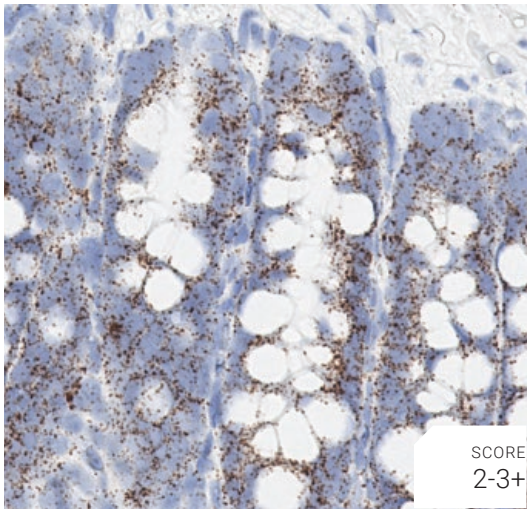


Signal: Standard ≥ Mild

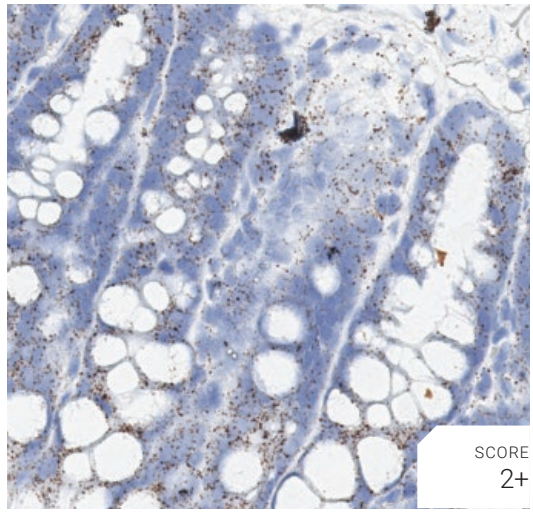
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Jejunum

Pretreatment: **Standard**



Pretreatment: **Mild**

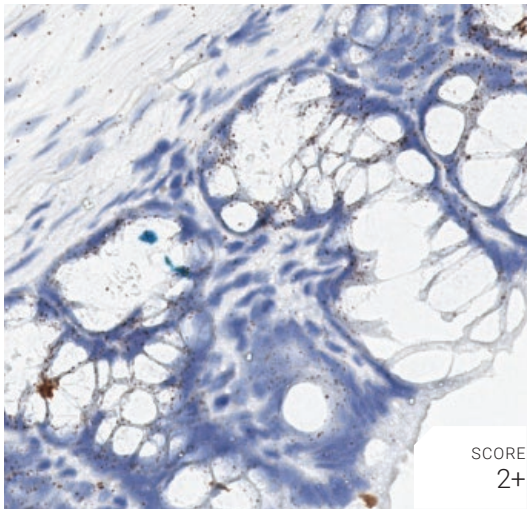


Signal: Standard ≥ Mild

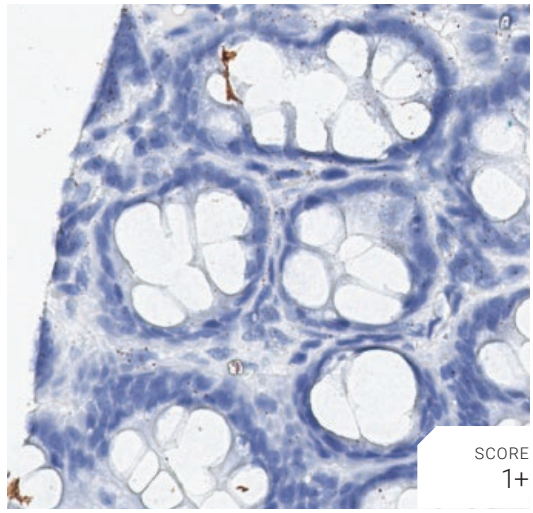
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Colon

Pretreatment: **Standard**



Pretreatment: **Mild**

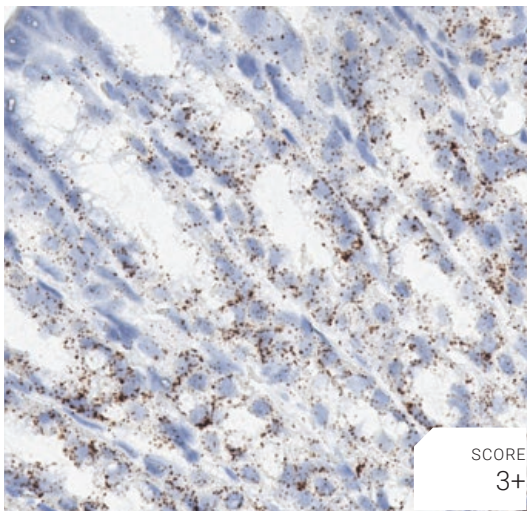


Signal: Standard ≥ Mild

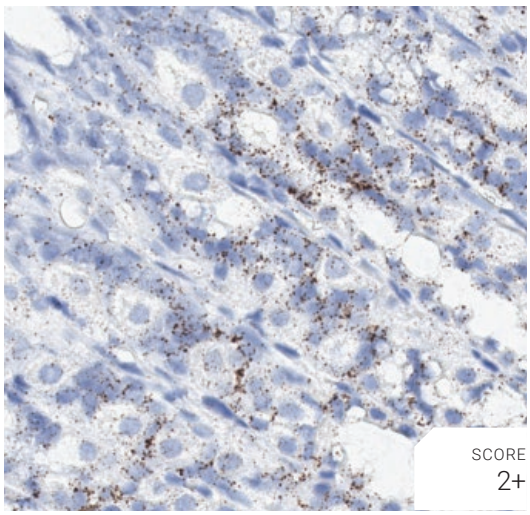
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Stomach

Pretreatment: **Standard**



Pretreatment: **Mild**

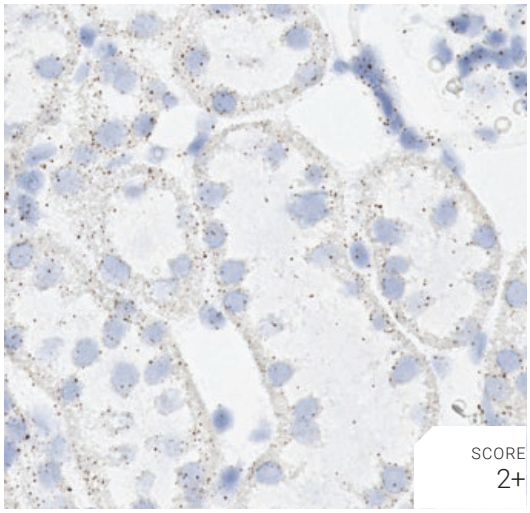


Signal: Standard ≥ Mild

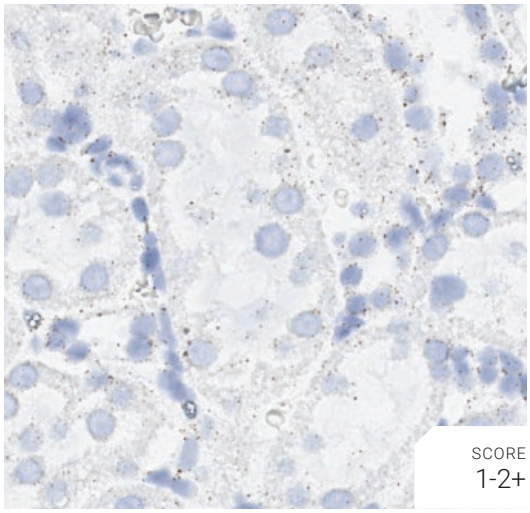
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Kidney

Pretreatment: **Standard**



Pretreatment: **Mild**

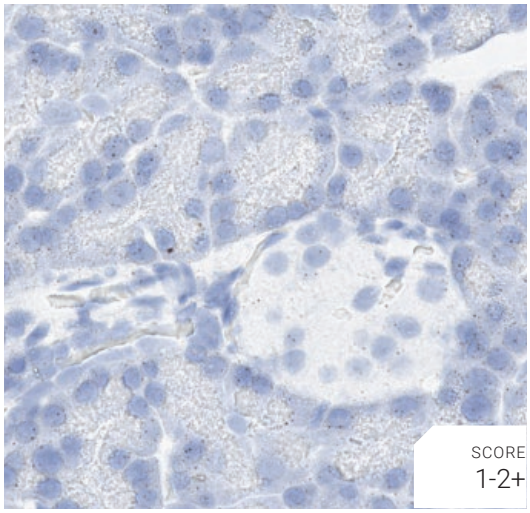


Signal: Standard ≥ Mild

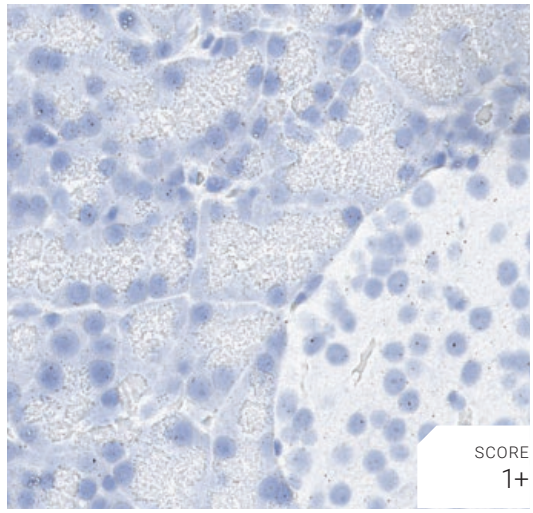
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Pancreas

Pretreatment: **Standard**



Pretreatment: **Mild**

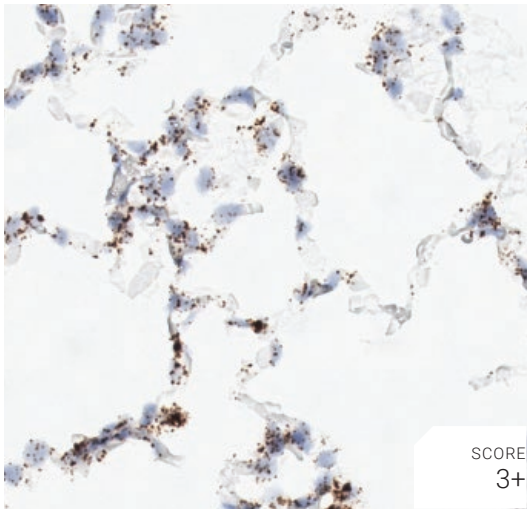


Signal: Standard ≥ Mild

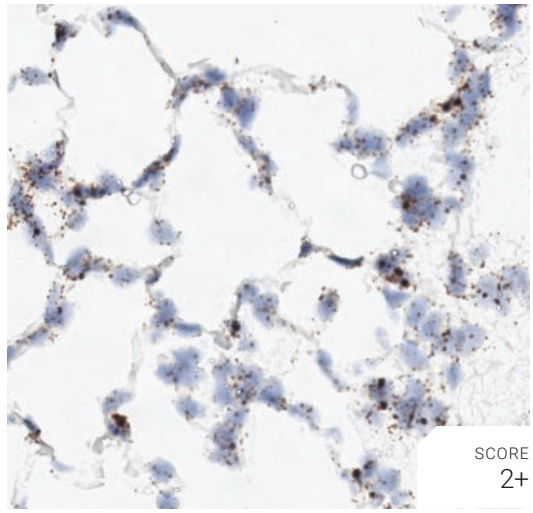
Optimal Pretreatment: Standard

Positive control probe: Rn-Ubc*
Tissue: Lung

Pretreatment: **Standard**



Pretreatment: **Mild**



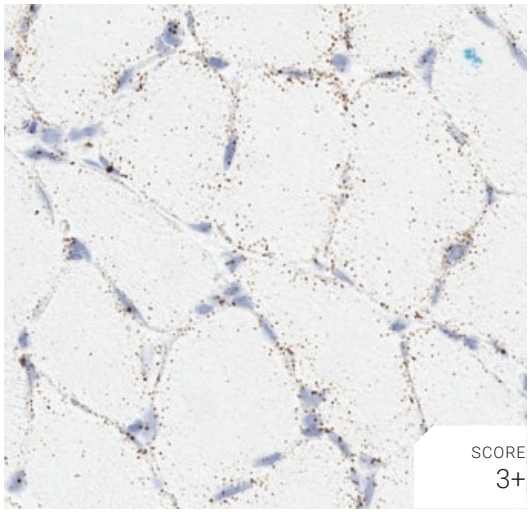
Signal: Standard ≥ Mild

Optimal Pretreatment: Standard

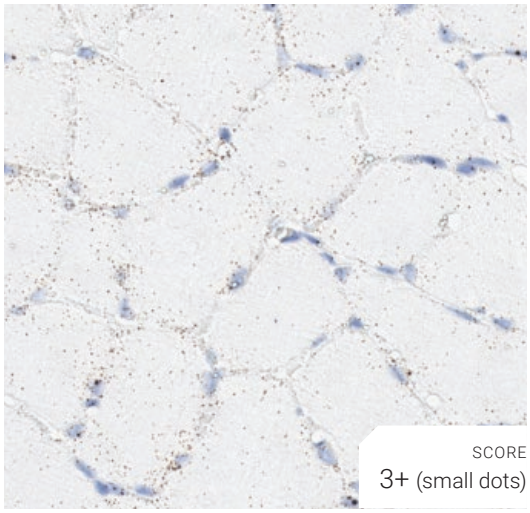
*Low Ppib detection in alveolar cells

Positive control probe: Rn-Ubc
Tissue: Skeletal muscle

Pretreatment: **Standard**



Pretreatment: **Mild**

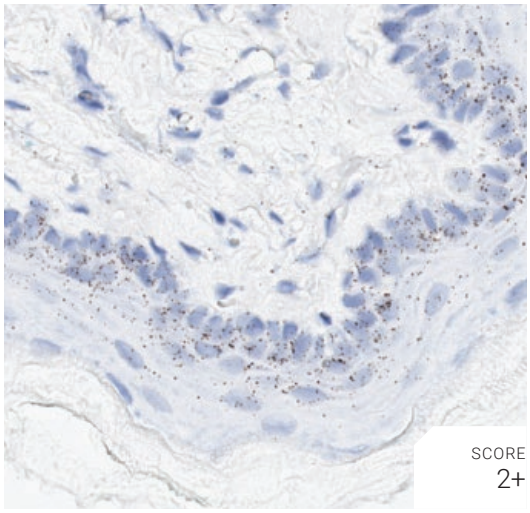


Signal: Standard ≥ Mild

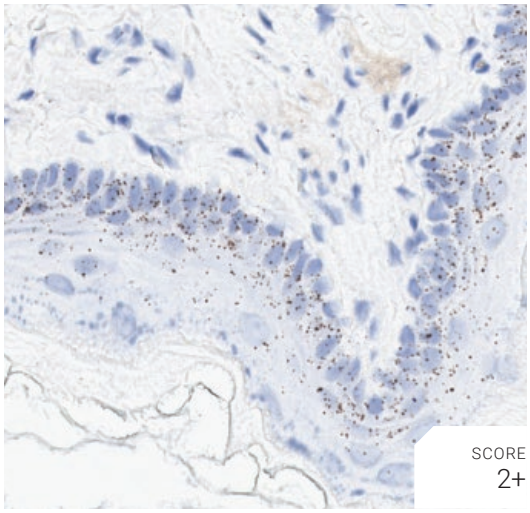
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Esophagus

Pretreatment: **Standard**



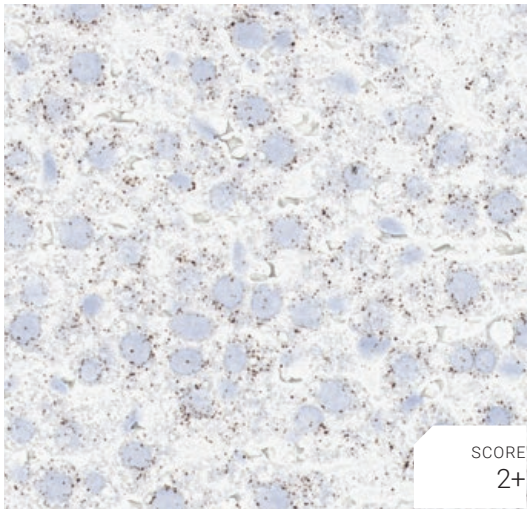
Pretreatment: **Mild**



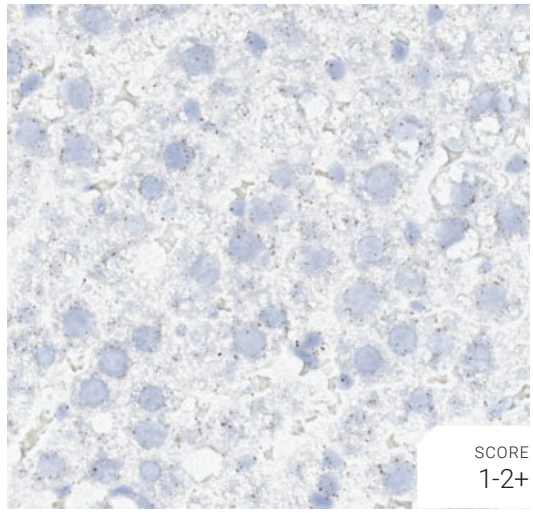
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Liver

Pretreatment: **Standard**



Pretreatment: **Mild**

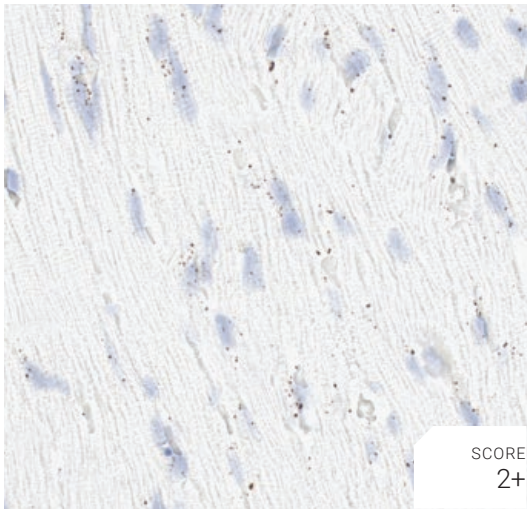


Signal: Standard ≥ Mild

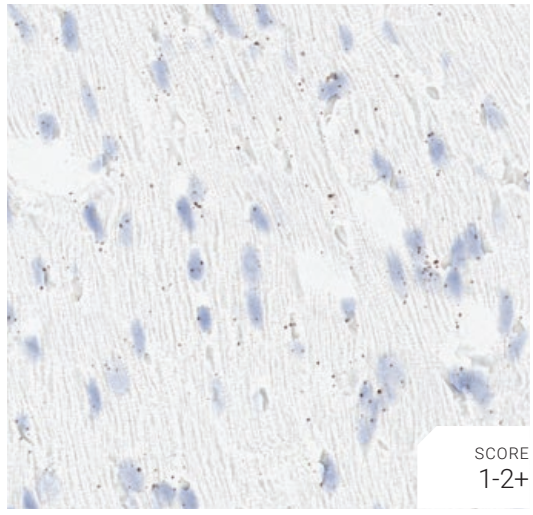
Optimal Pretreatment: Standard

Positive control probe: Rn-Ppib
Tissue: Heart

Pretreatment: **Standard**



Pretreatment: **Mild**



Signal: Standard ≥ Mild

Optimal Pretreatment: Standard

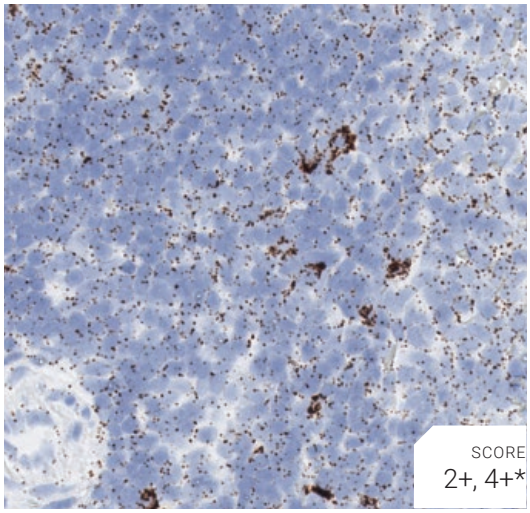
Rat

Rattus norvegicus

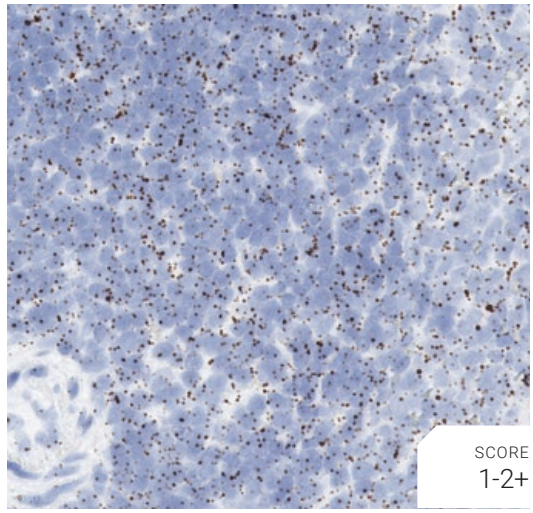
The following pages show the three positive controls (Ppib, Polr2a, Ubc) and the negative control images using optimal pretreatment condition. Recommended positive control probe is indicated for each tissue type.

Tissue: Spleen
Pretreatment: Mild

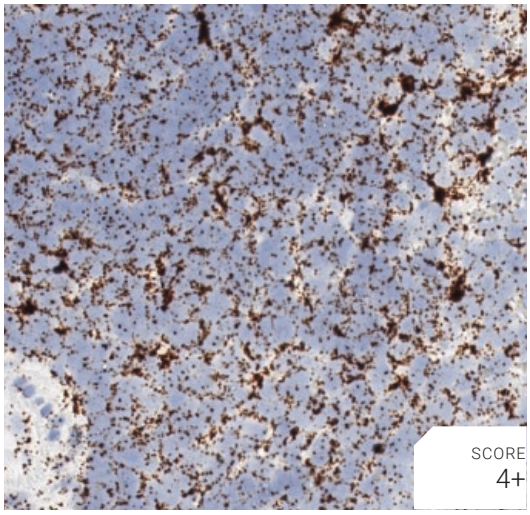
Control probe: **Rn-Ppib**



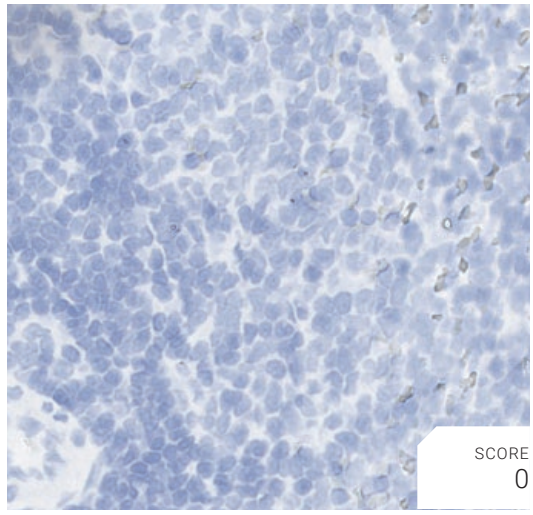
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**



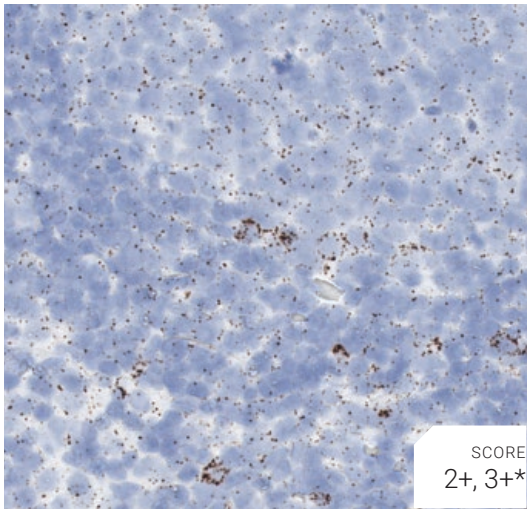
Recommended Positive Control Probe: Rn-Ppib/Rn-Polr2a**

*A few cells have higher *Ppib* signal

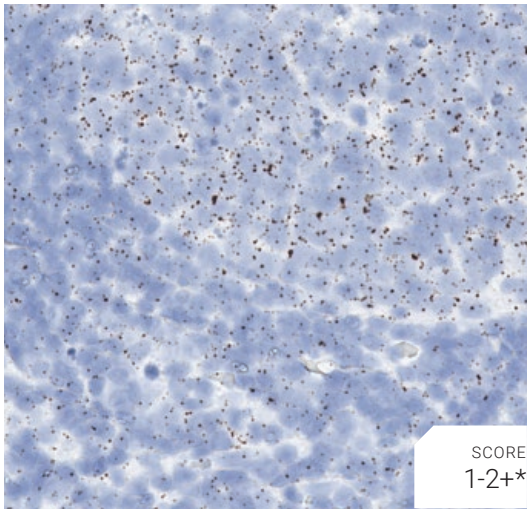
**Rn-Polr2a staining is more evenly distributed

Tissue: Lymph node
Pretreatment: Mild

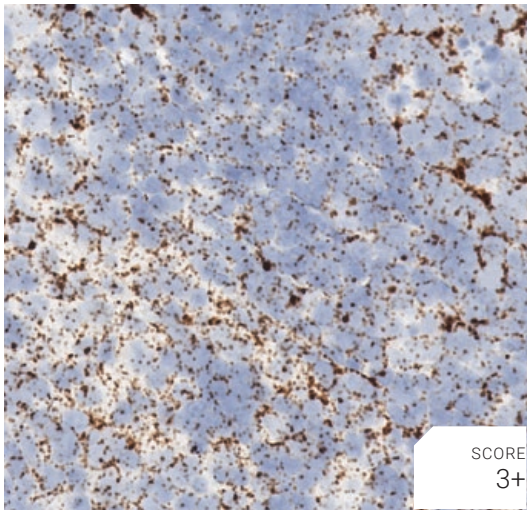
Control probe: **Rn-Ppib**



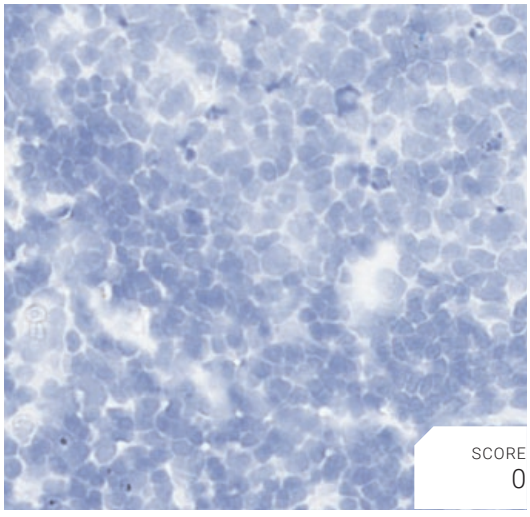
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**

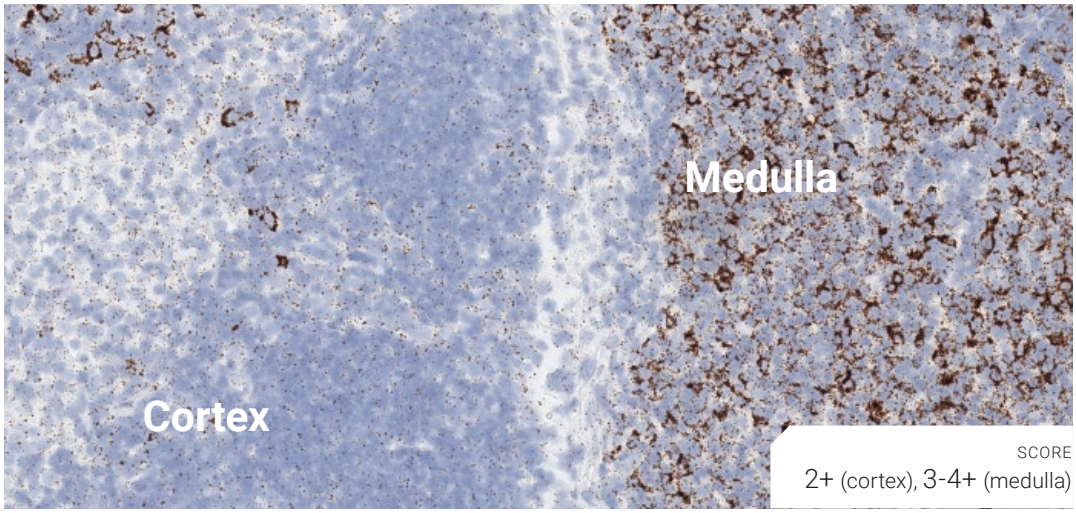


Recommended Positive Control Probe: Rn-Ppib/Rn-Polr2a*

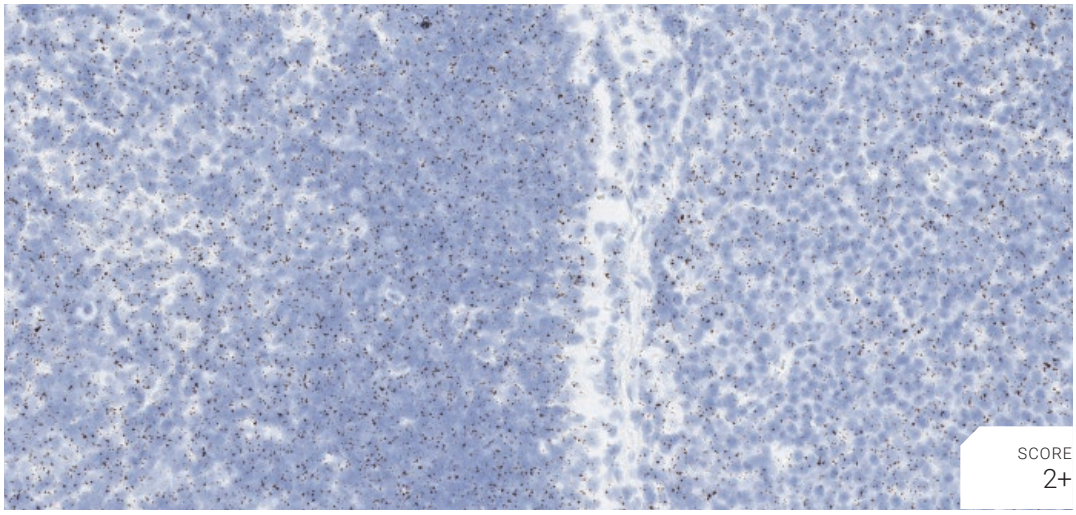
*Rn-Polr2a staining is more evenly distributed (next page)

Tissue: Lymph node
Pretreatment: Mild

Control probe: **Rn-Ppib**



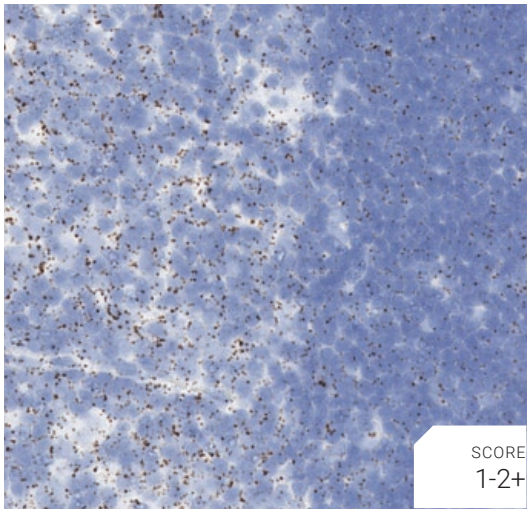
Control probe: **Rn-Polr2a**



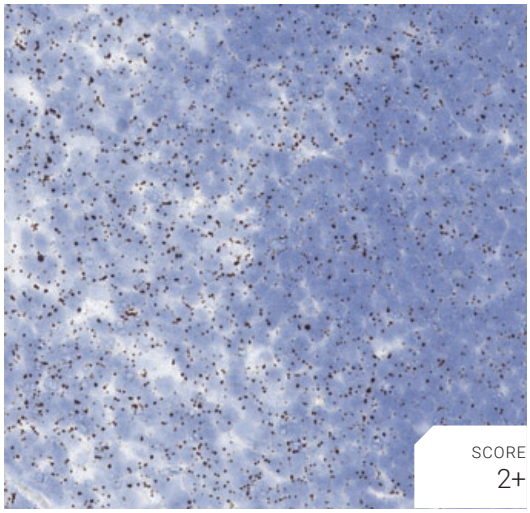
Recommended Positive Control Probe: Rn-Polr2a staining is more evenly distributed

Tissue: Thymus
Pretreatment: Mild

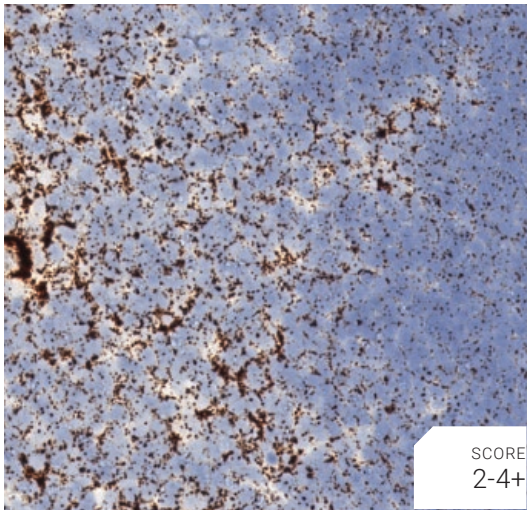
Control probe: **Rn-Ppib**



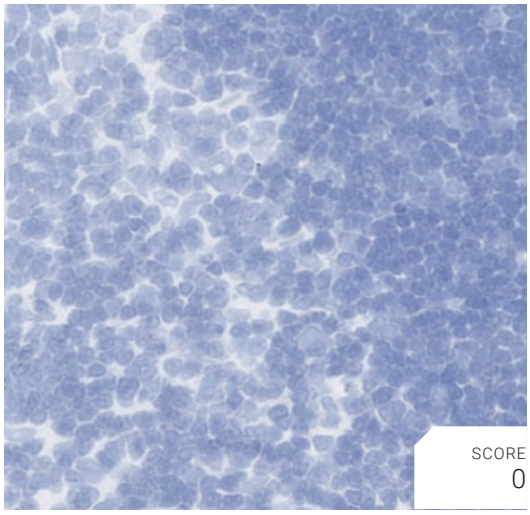
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**

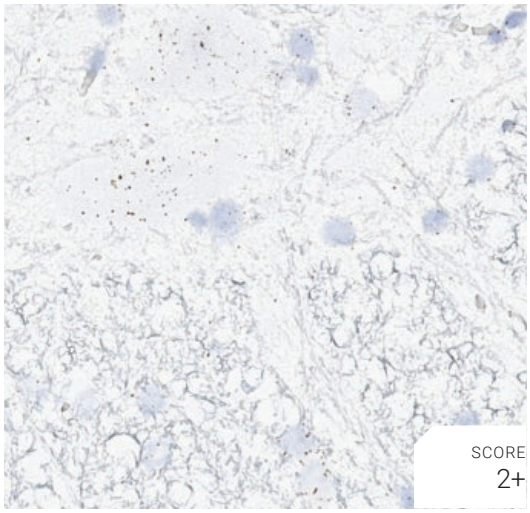


Recommended Positive Control Probe: Rn-Polr2a*/Rn-Ppib

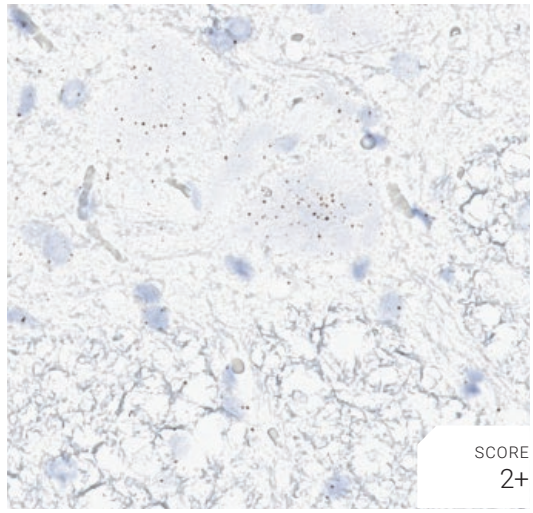
*Rn-Polr2a staining is more evenly distributed

Tissue: Spinal cord
Pretreatment: Standard

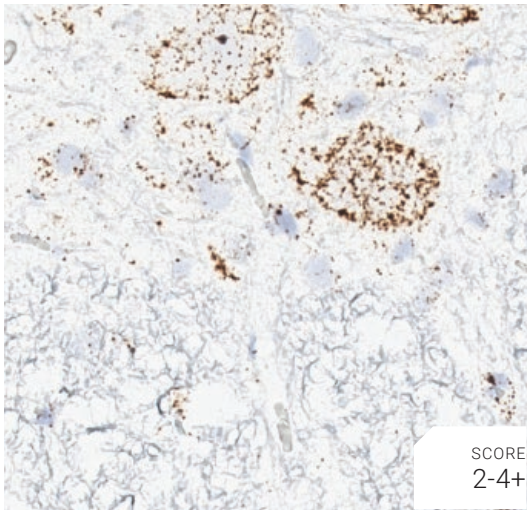
Control probe: **Rn-Ppib**



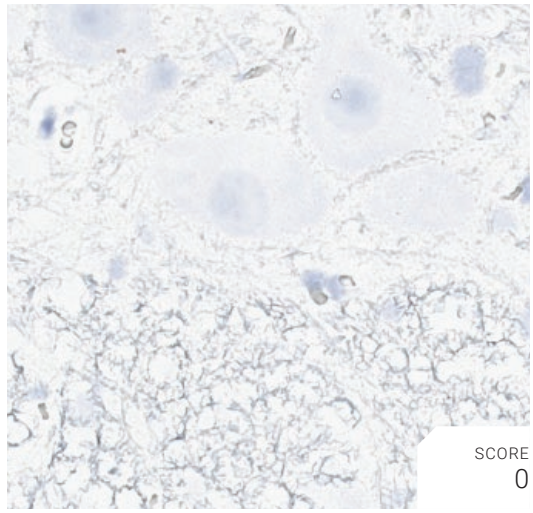
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



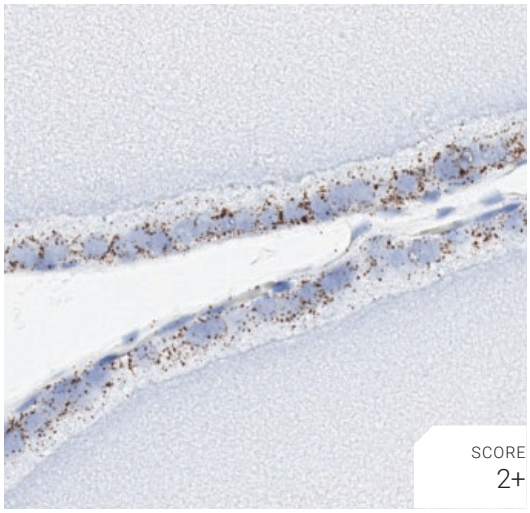
Control probe: **DapB**



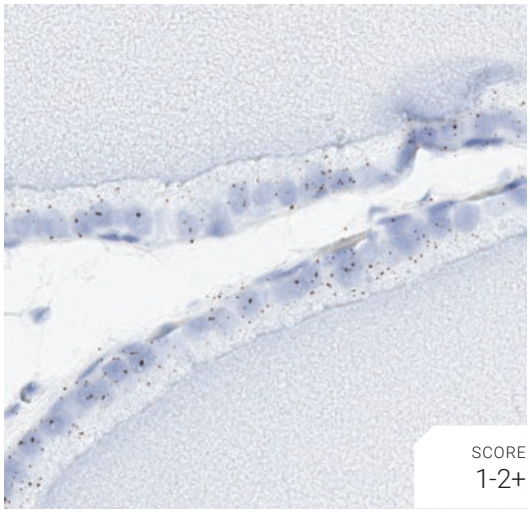
Recommended Positive Control Probe: Rn-Ppib

Tissue: Prostate gland
Pretreatment: Standard

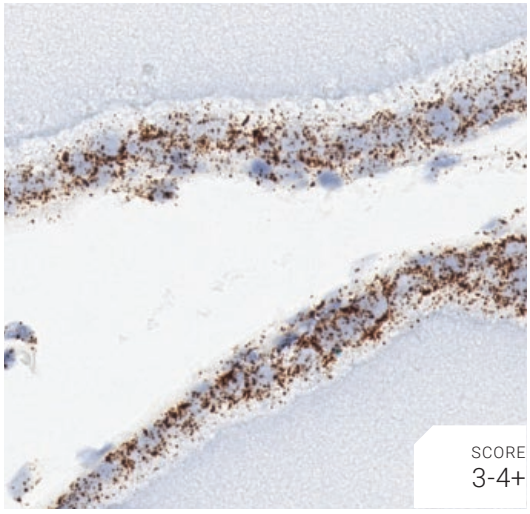
Control probe: **Rn-Ppib**



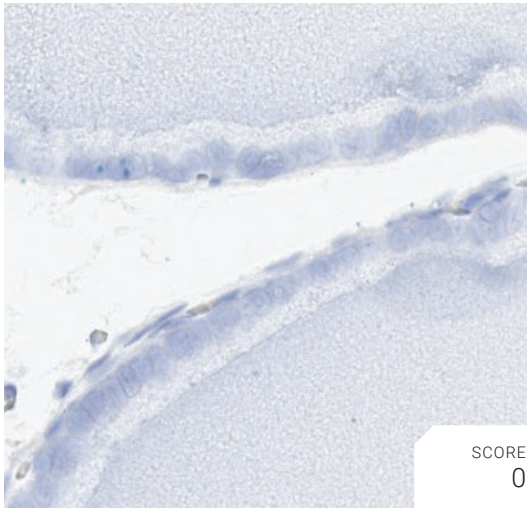
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



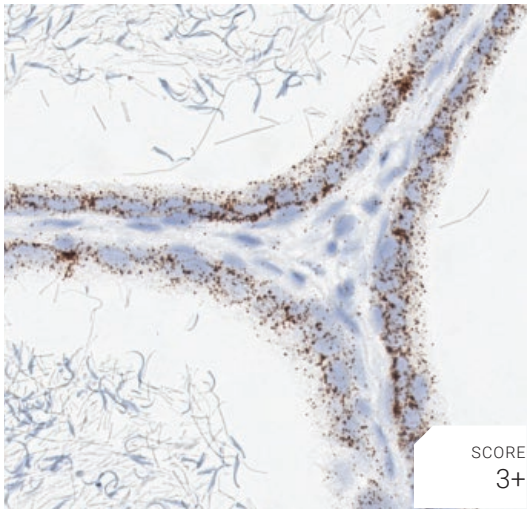
Control probe: **DapB**



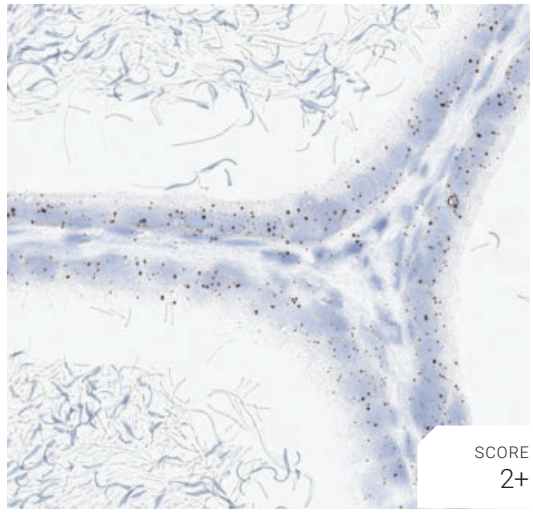
Recommended Positive Control Probe: Rn-Ppib

Tissue: Epididymis
Pretreatment: Standard

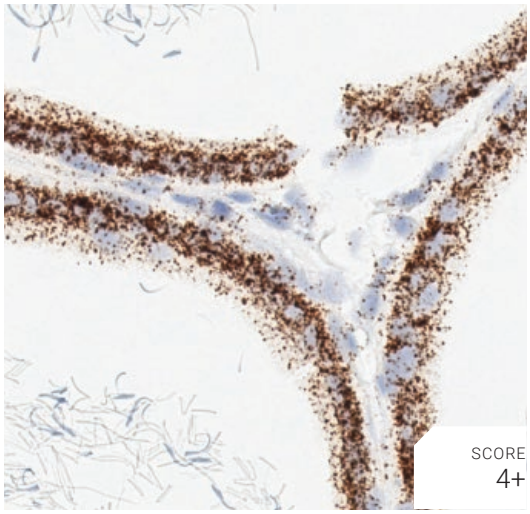
Control probe: **Rn-Ppib**



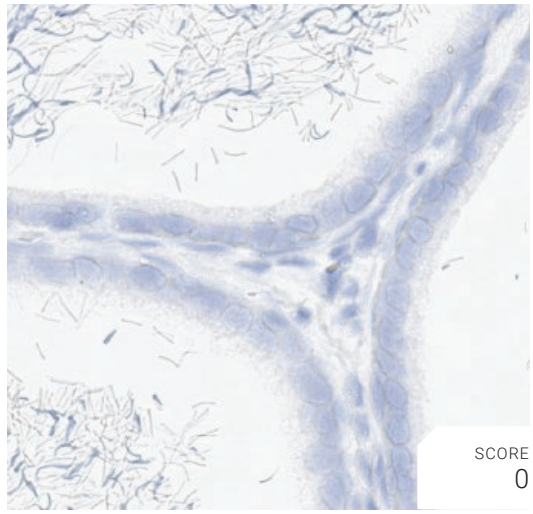
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



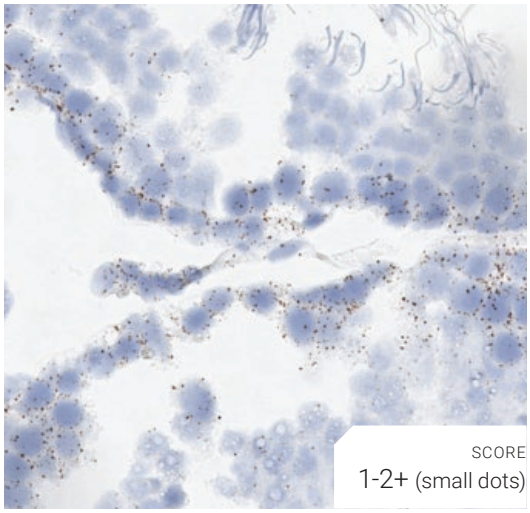
Control probe: **DapB**



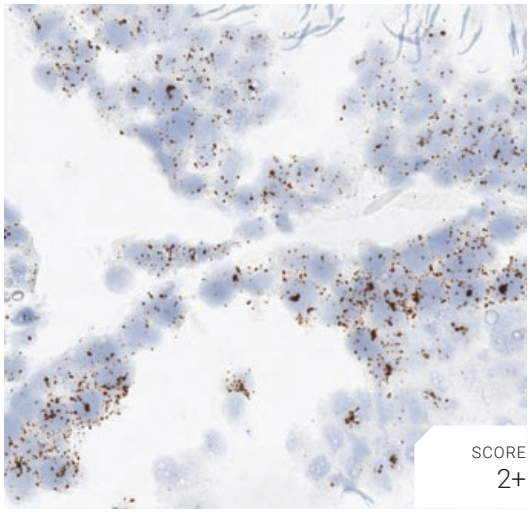
Recommended Positive Control Probe: Rn-Ppib

Tissue: Testis
Pretreatment: Standard

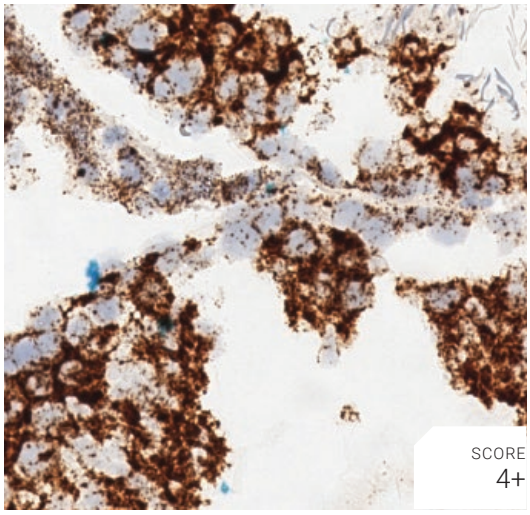
Control probe: **Rn-Ppib**



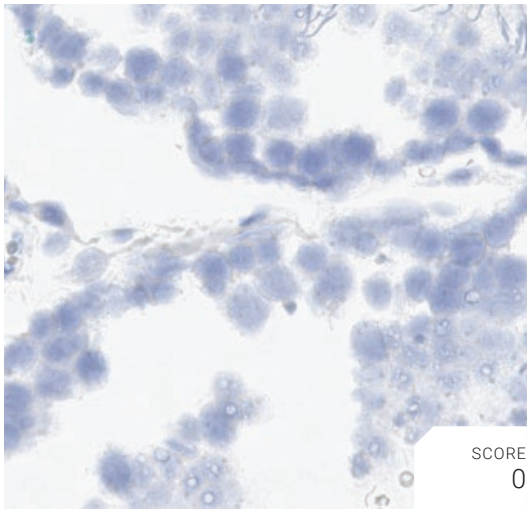
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



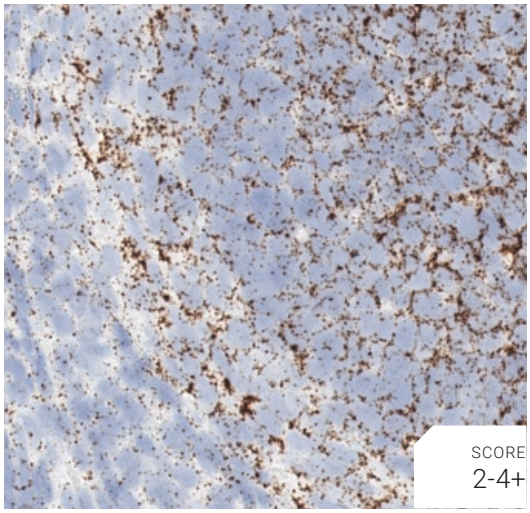
Control probe: **DapB**



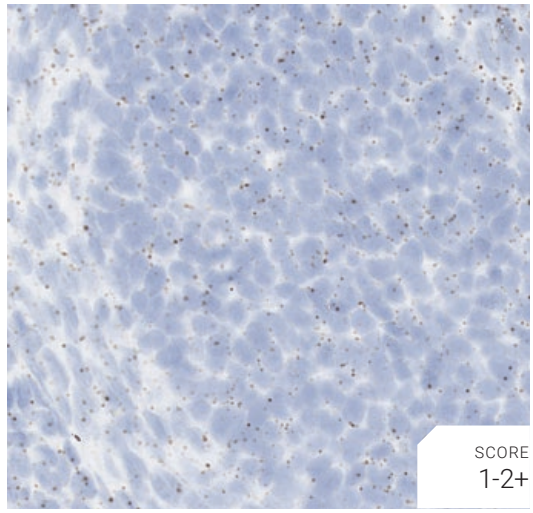
Recommended Positive Control Probe: Rn-Polr2a

Tissue: Ovary
Pretreatment: Standard

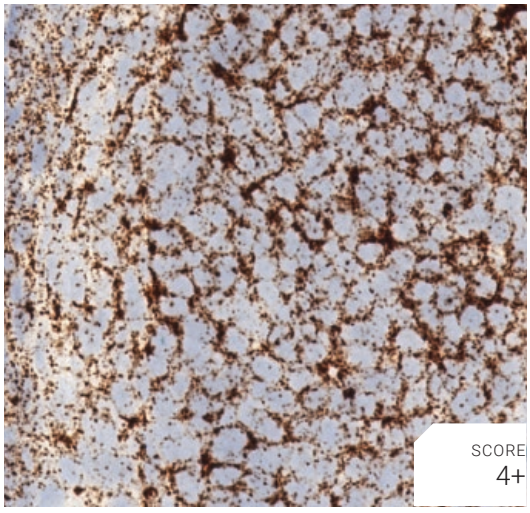
Control probe: **Rn-Ppib**



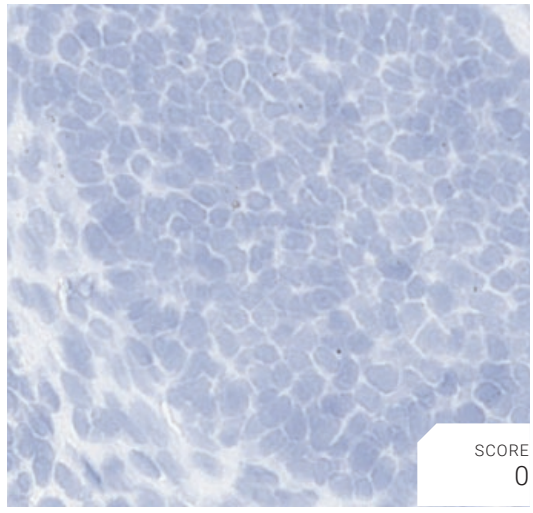
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



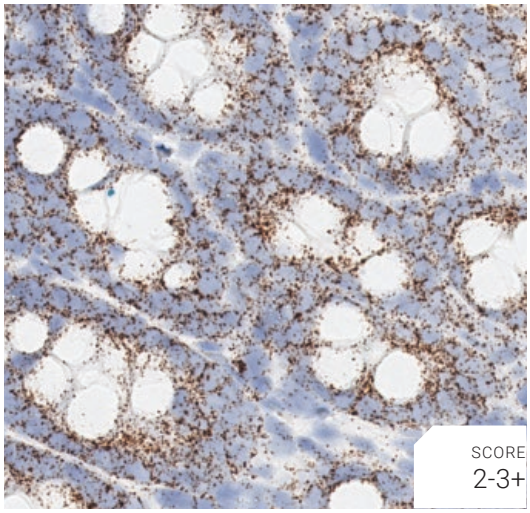
Control probe: **DapB**



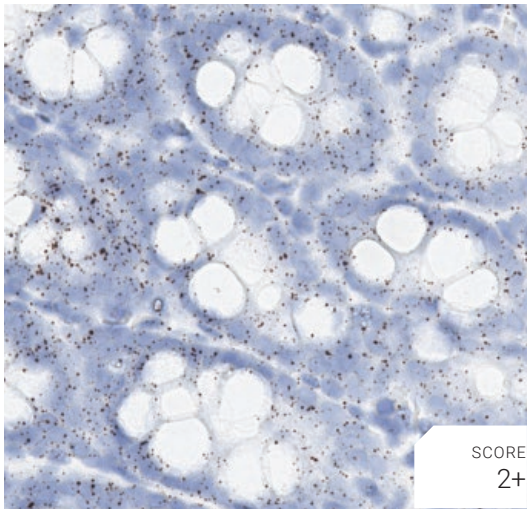
Recommended Positive Control Probe: Rn-Ppib

Tissue: Duodenum
Pretreatment: Standard

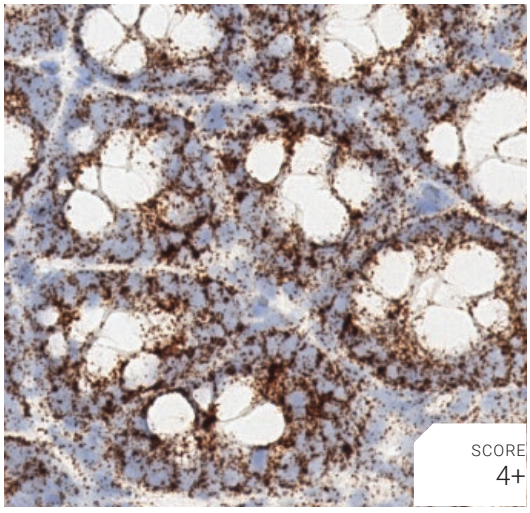
Control probe: **Rn-Ppib**



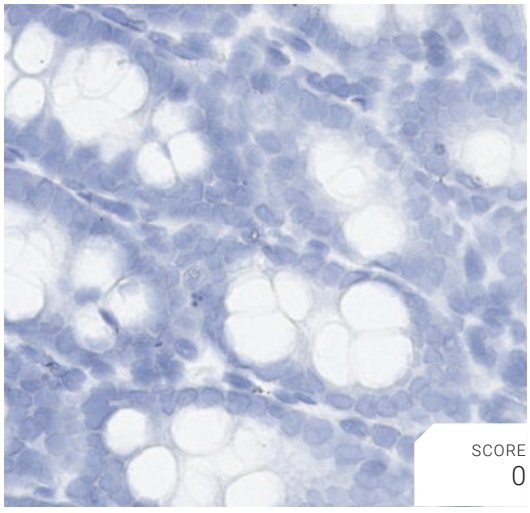
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**

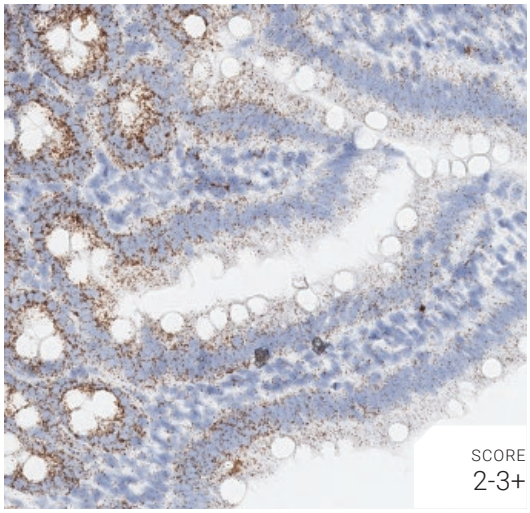


Recommended Positive Control Probe: Rn-Ppib /Rn-Polr2a*

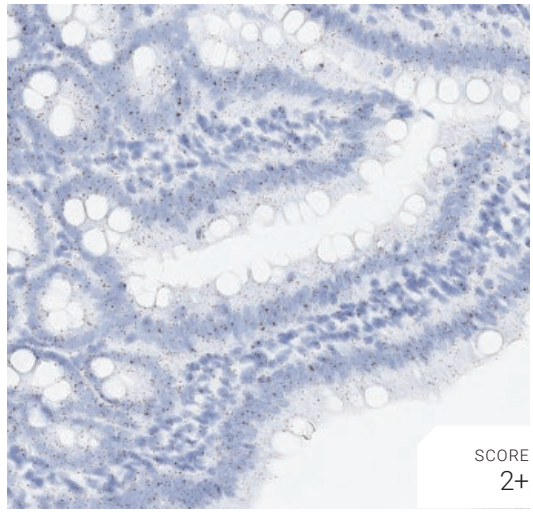
*Rn-Polr2a staining is more evenly distributed (next page)

Tissue: Duodenum (20X)
Pretreatment: Standard

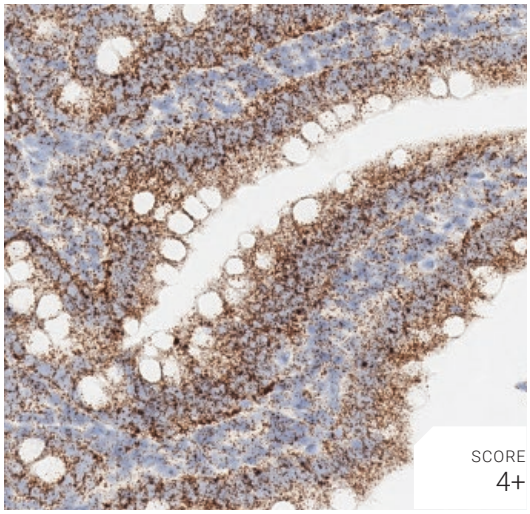
Control probe: **Rn-Ppib**



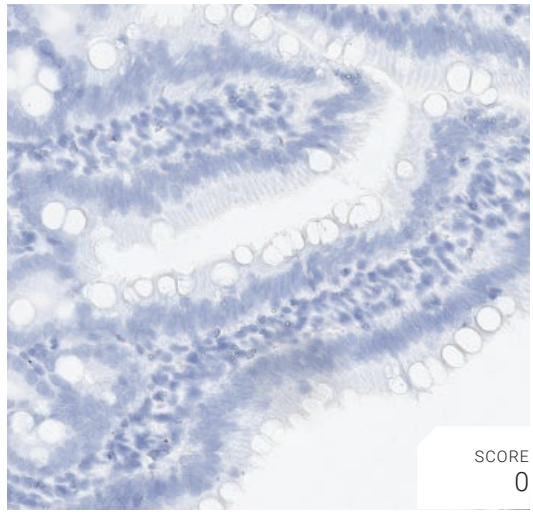
Control probe: **Rn-Polr2a***



Control probe: **Rn-Ubc**



Control probe: **DapB**

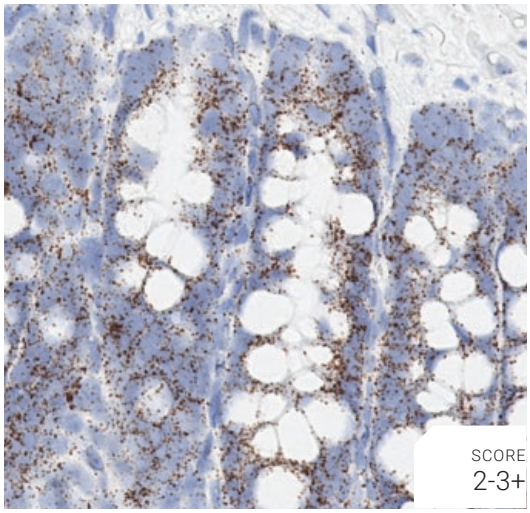


Recommended Positive Control Probe: Rn-Ppib

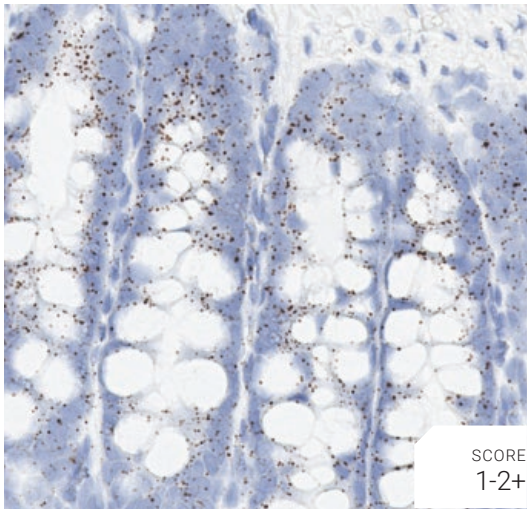
*Rn-Polr2a staining is more evenly distributed

Tissue: Jejunum
Pretreatment: Standard

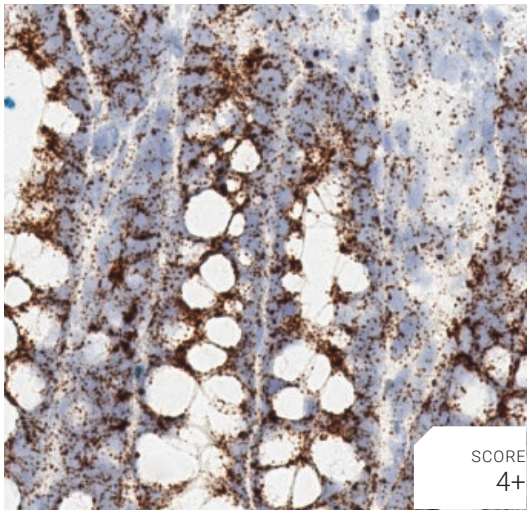
Control probe: **Rn-Ppib**



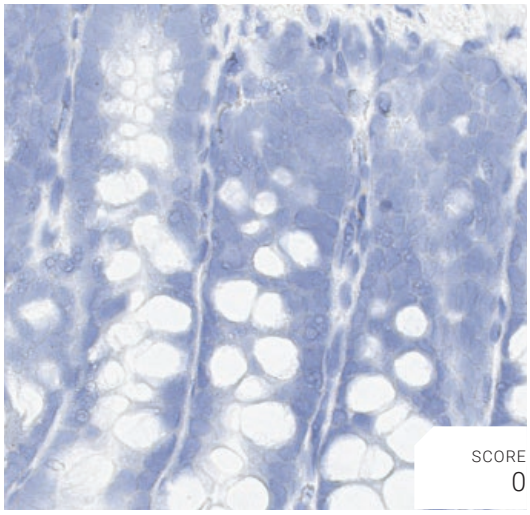
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



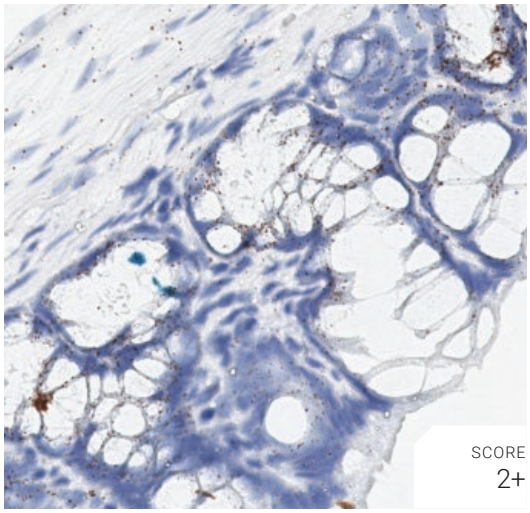
Control probe: **DapB**



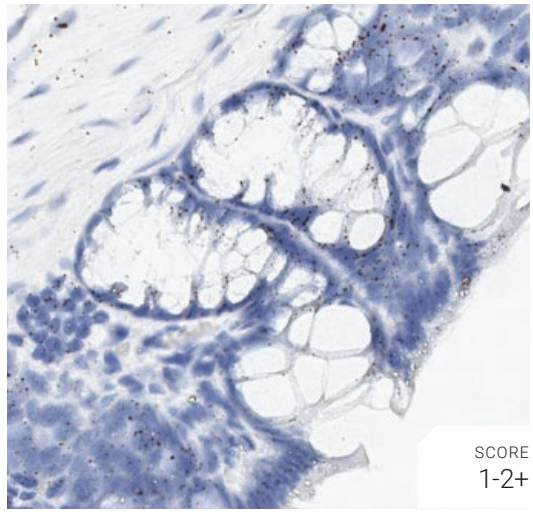
Recommended Positive Control Probe: Rn-Ppib

Tissue: Colon
Pretreatment: Standard

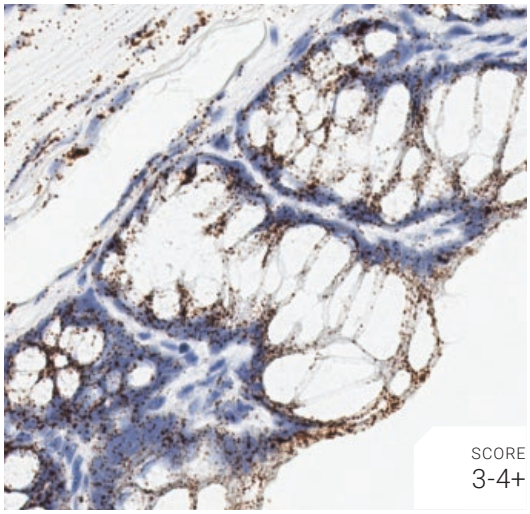
Control probe: **Rn-Ppib**



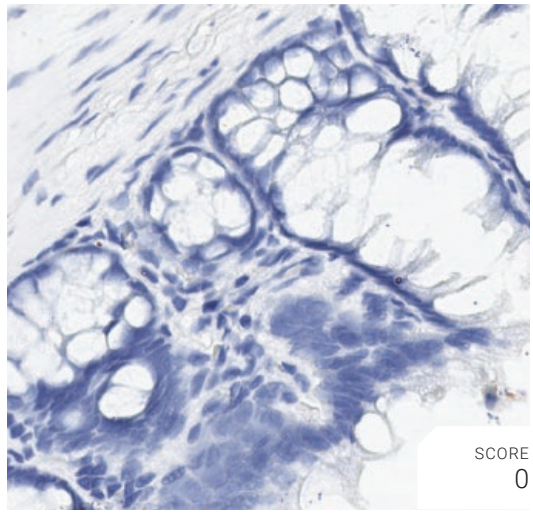
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



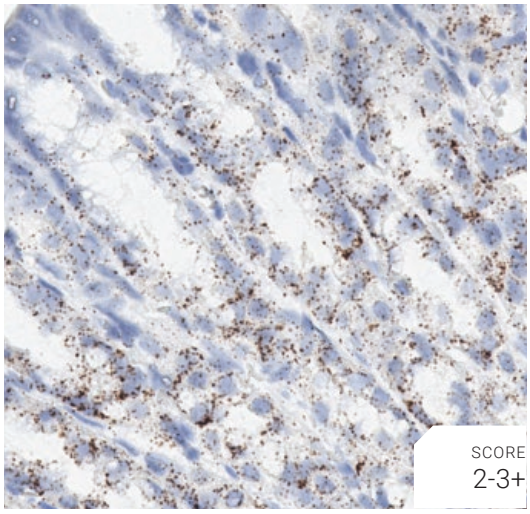
Control probe: **DapB**



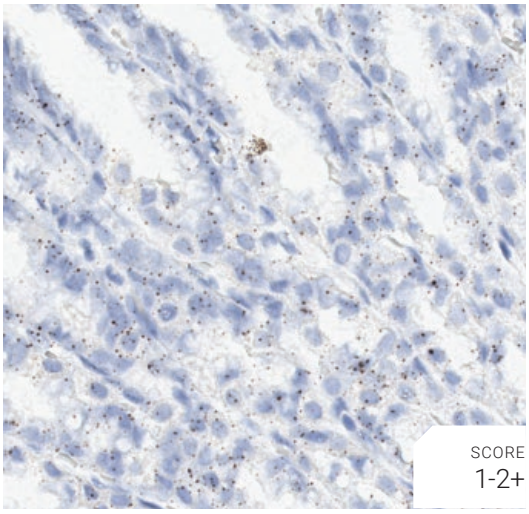
Recommended Positive Control Probe: Rn-Ppib

Tissue: Stomach
Pretreatment: Standard

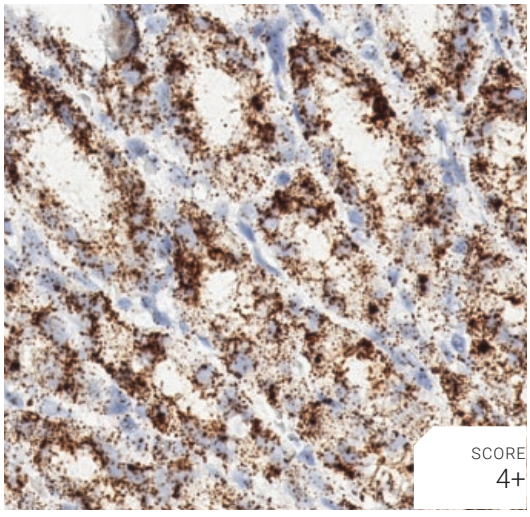
Control probe: **Rn-Ppib**



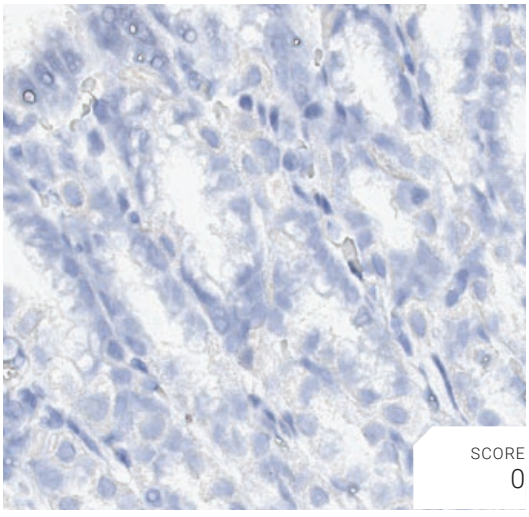
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



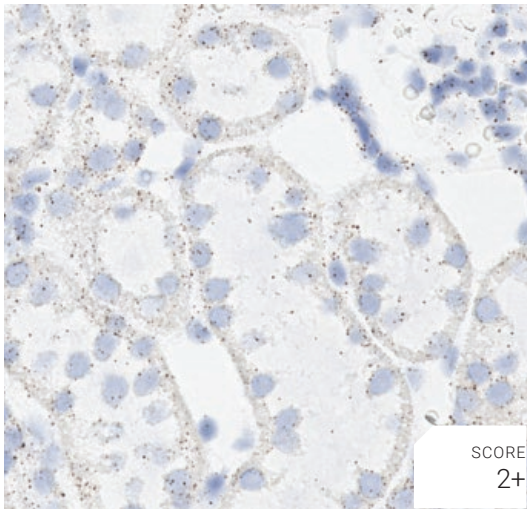
Control probe: **DapB**



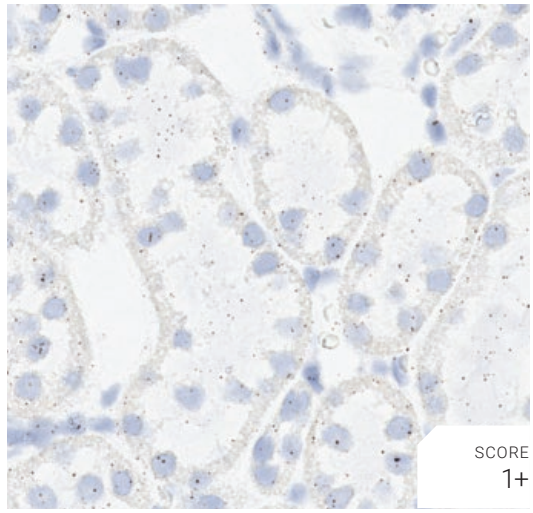
Recommended Positive Control Probe: Rn-Ppib

Tissue: Kidney
Pretreatment: Standard

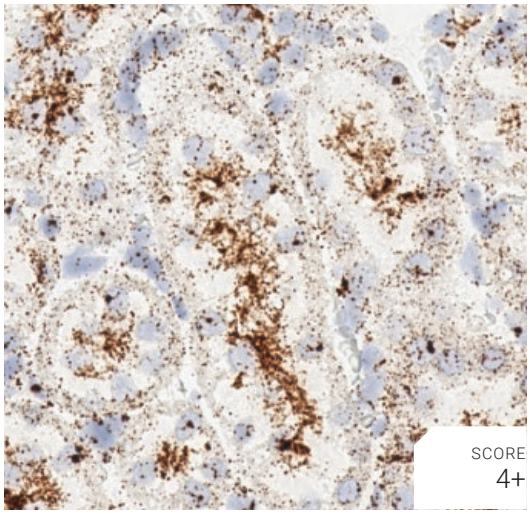
Control probe: **Rn-Ppib**



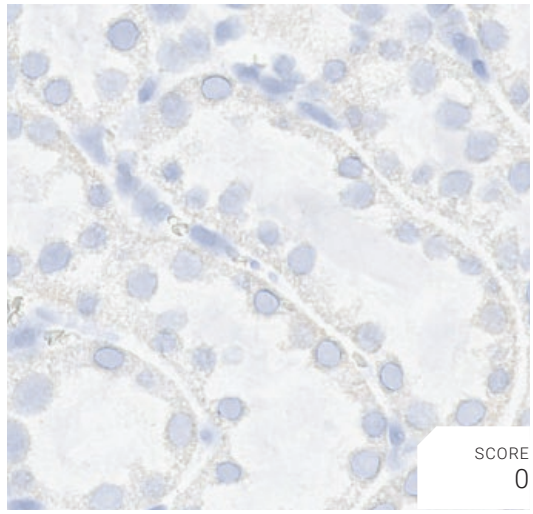
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



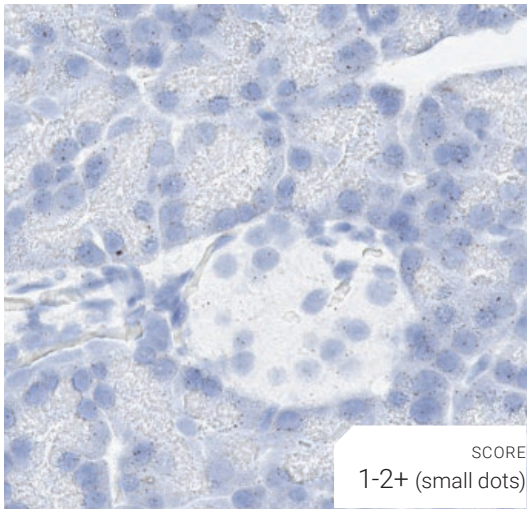
Control probe: **DapB**



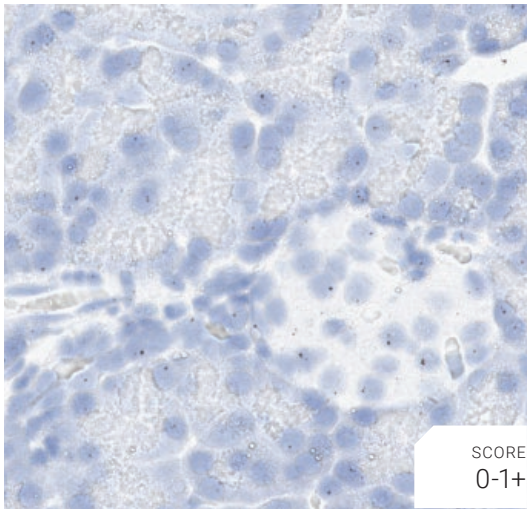
Recommended Positive Control Probe: Rn-Ppib

Tissue: Pancreas
Pretreatment: Standard

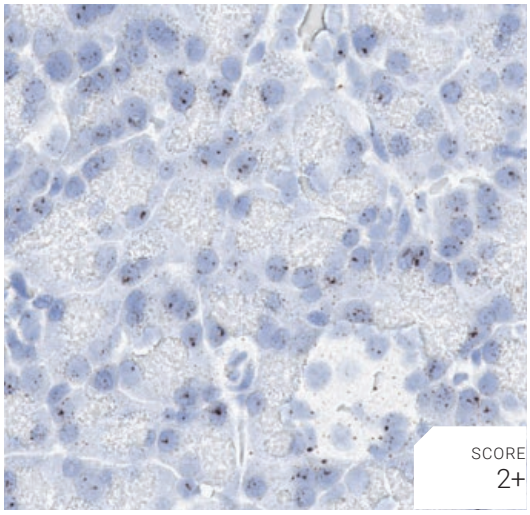
Control probe: **Rn-Ppib**



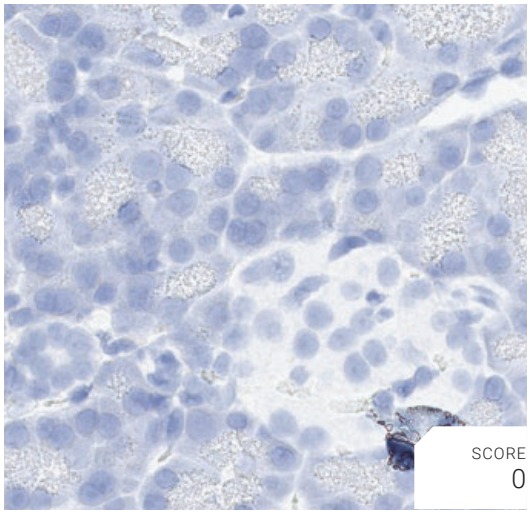
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



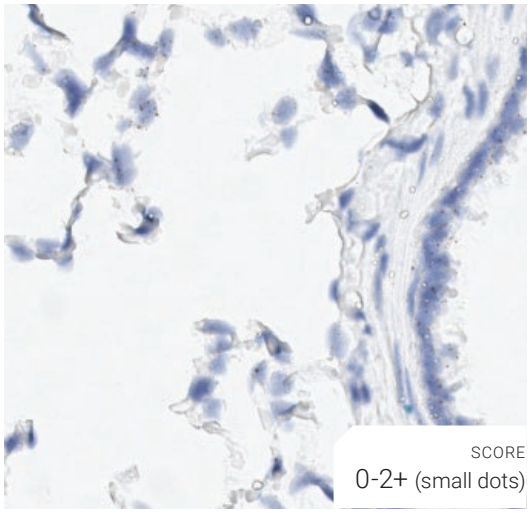
Control probe: **DapB**



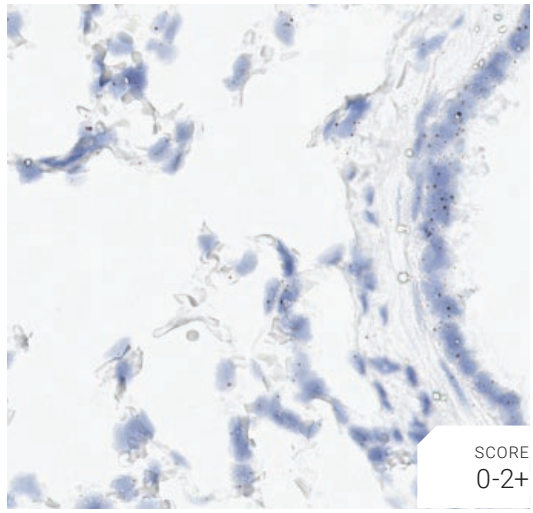
Recommended Positive Control Probe: Rn-Ubc/Rn-Ppib

Tissue: Lung
Pretreatment: Standard

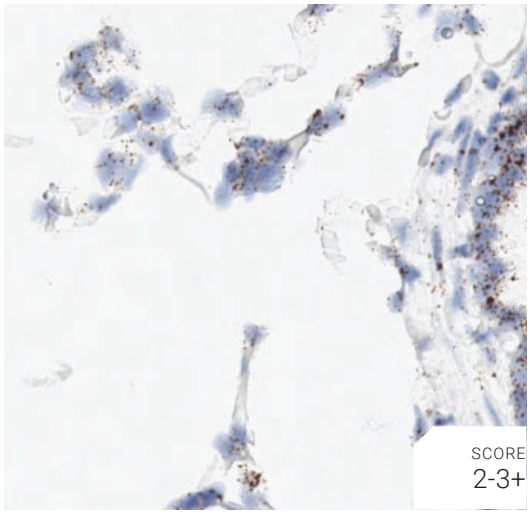
Control probe: **Rn-Ppib**



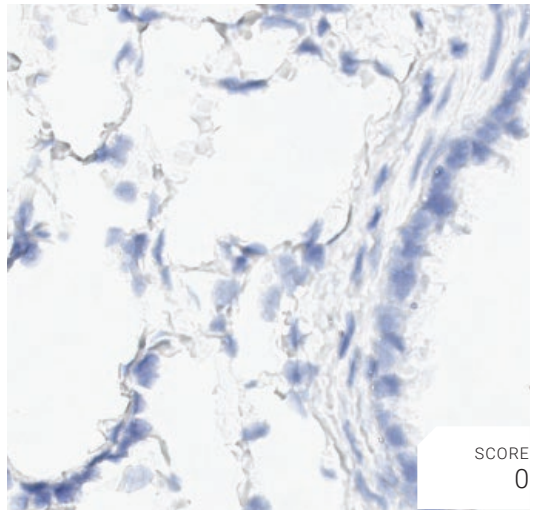
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**

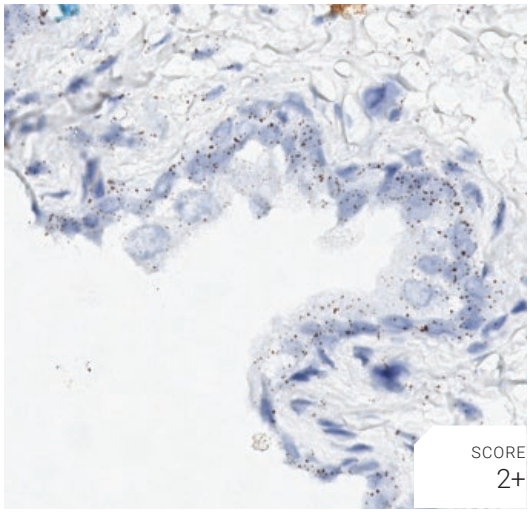


Recommended Positive Control Probe: Rn-Ubc/Rn-Ppib

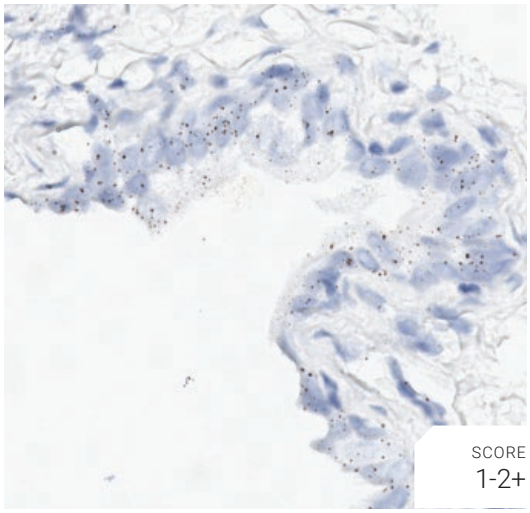
Staining: Bronchus > alveolar cells; Recommend Rn-Ubc to control alveolar cells

Tissue: Urinary bladder
Pretreatment: Standard

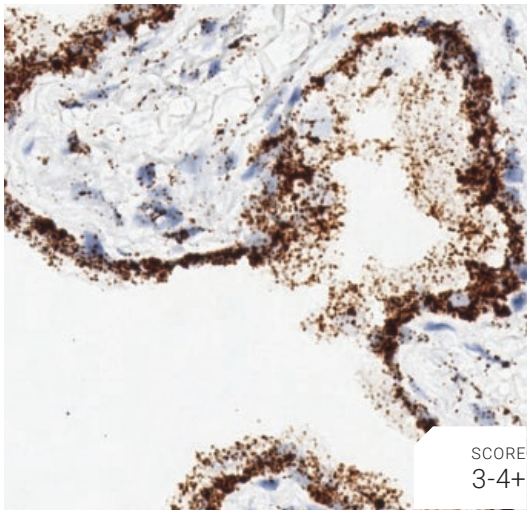
Control probe: **Rn-Ppib**



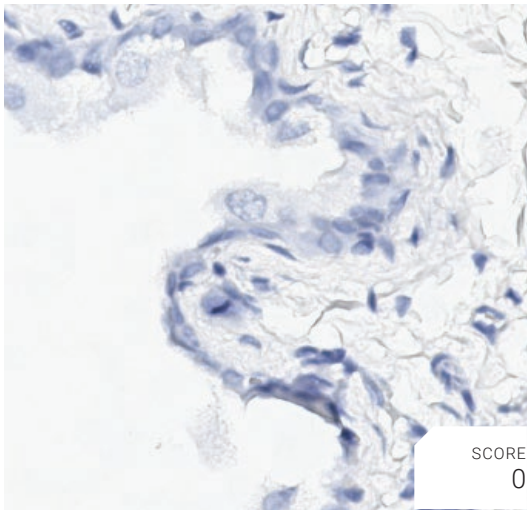
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



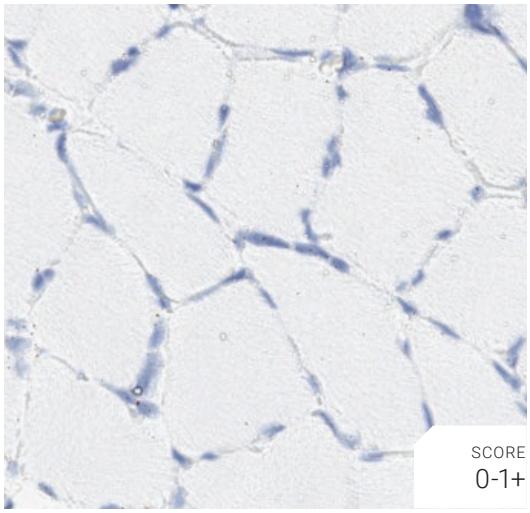
Control probe: **DapB**



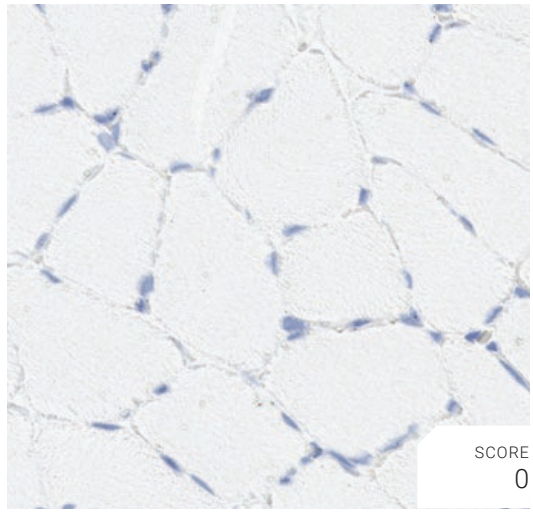
Recommended Positive Control Probe: Rn-Ppib

Tissue: Skeletal muscle
Pretreatment: Standard

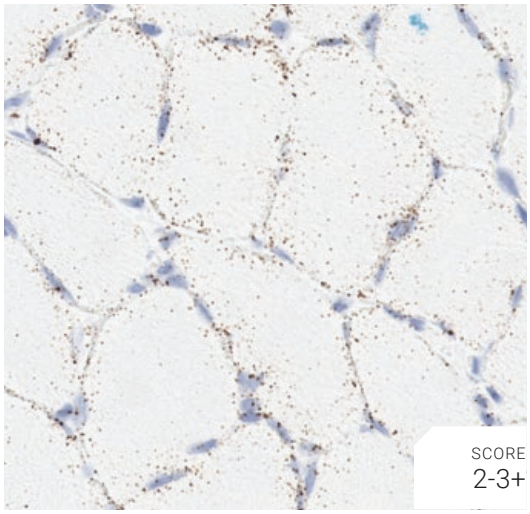
Control probe: **Rn-Ppib**



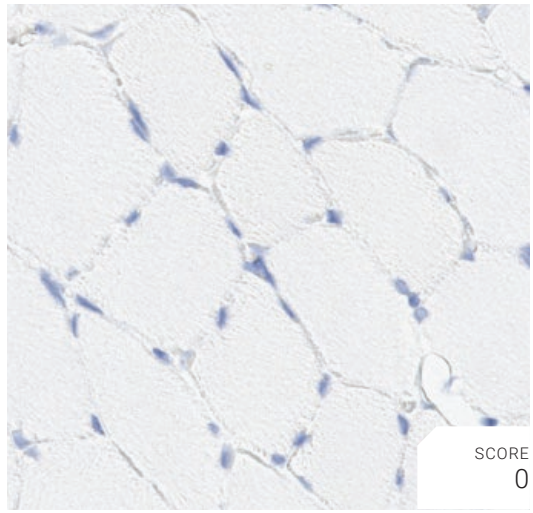
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



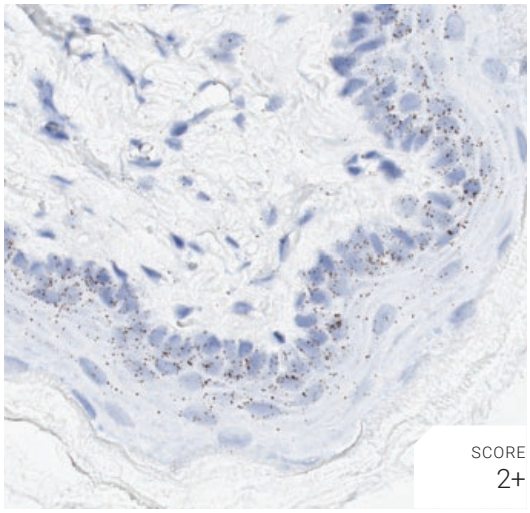
Control probe: **DapB**



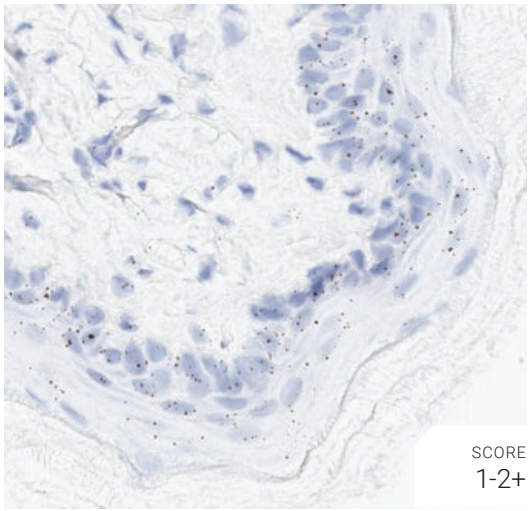
Recommended Positive Control Probe: Rn-Ubc

Tissue: Esophagus
Pretreatment: Standard

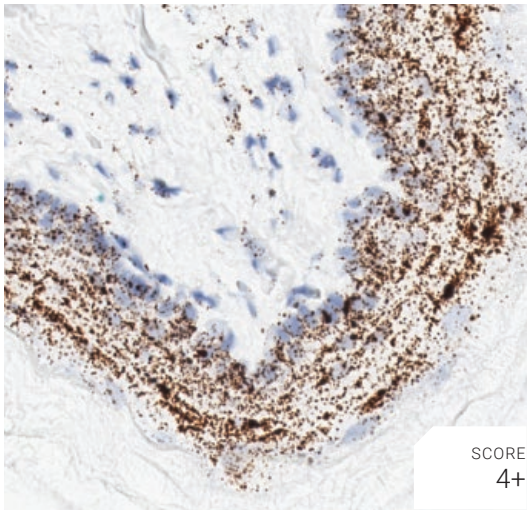
Control probe: **Rn-Ppib**



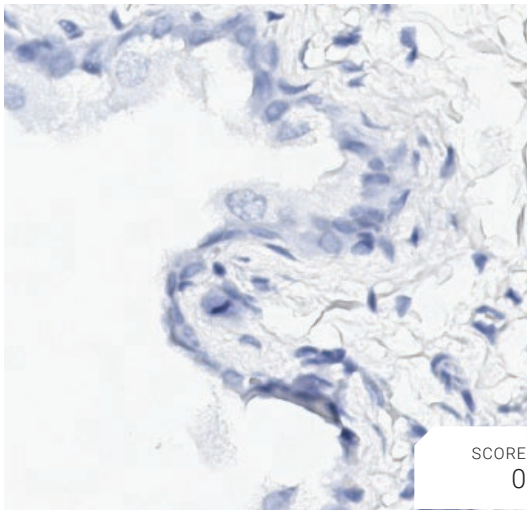
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



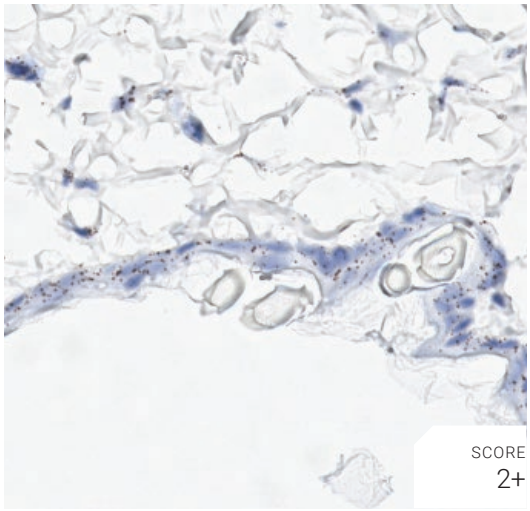
Control probe: **DapB**



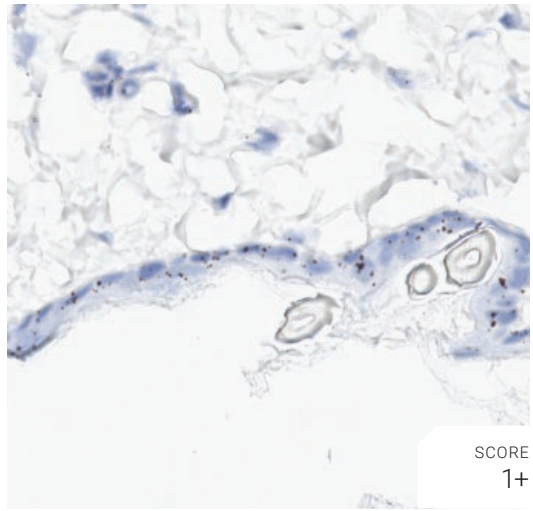
Recommended Positive Control Probe: Rn-Ppib

Tissue: Skin
Pretreatment: Standard

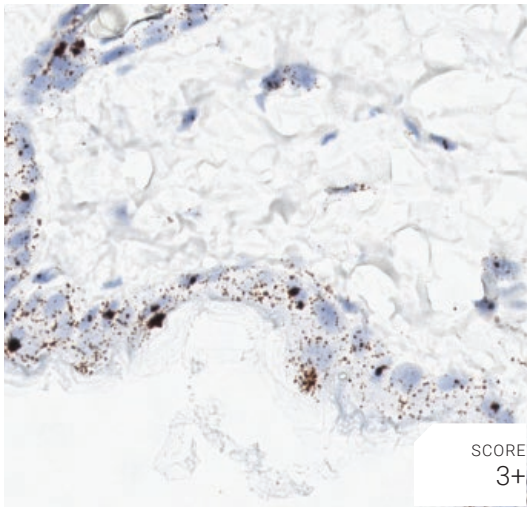
Control probe: **Rn-Ppib**



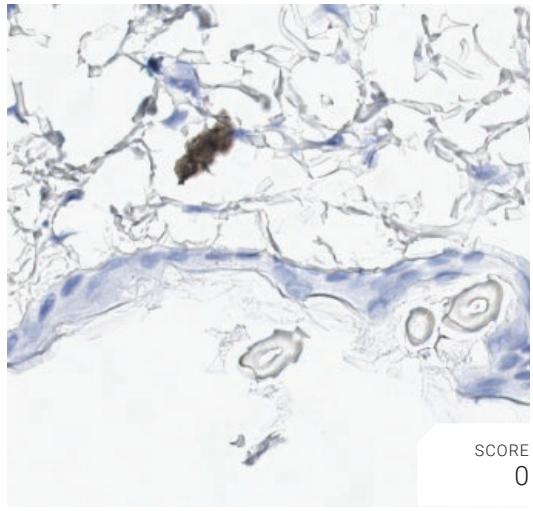
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



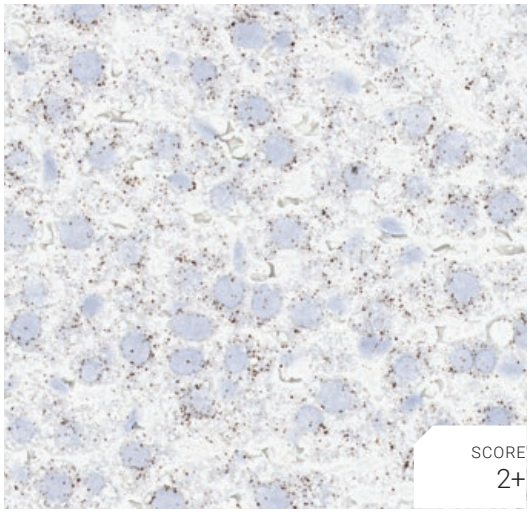
Control probe: **DapB**



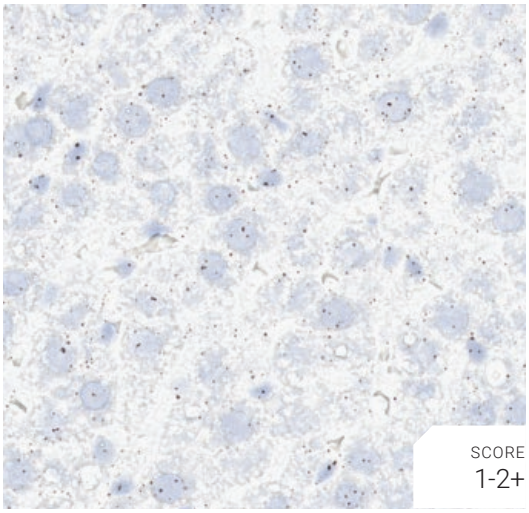
Recommended Positive Control Probe: Rn-Ppib

Tissue: Liver
Pretreatment: Standard

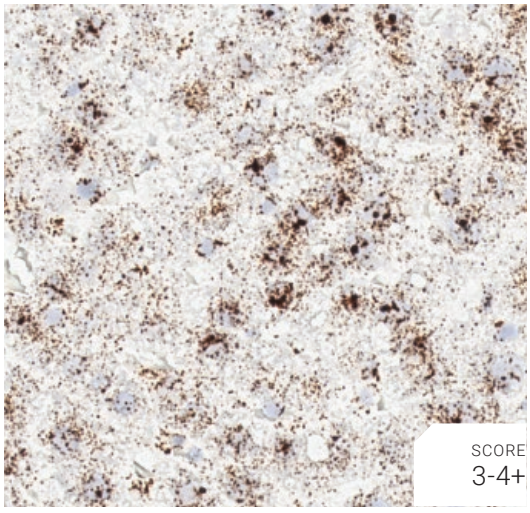
Control probe: **Rn-Ppib**



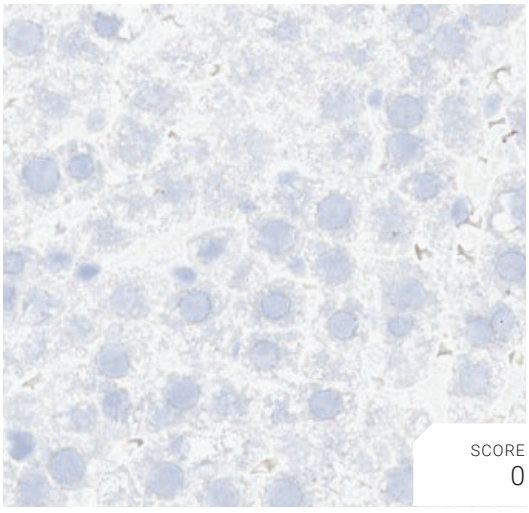
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



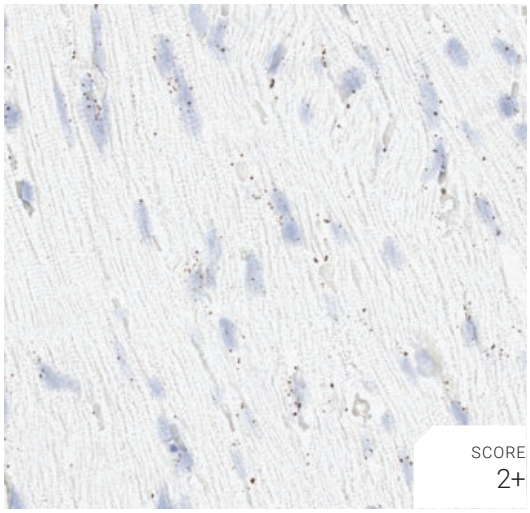
Control probe: **DapB**



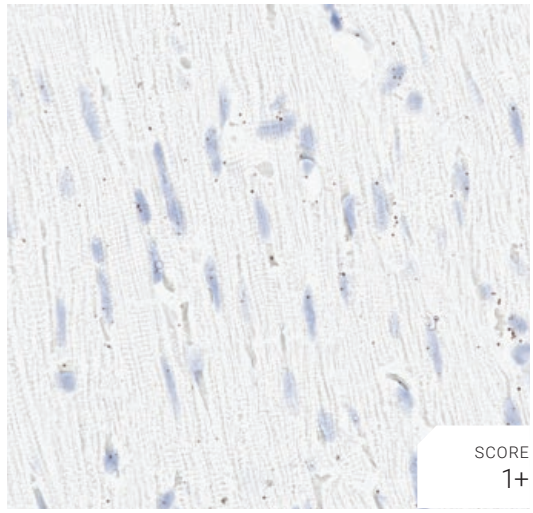
Recommended Positive Control Probe: Rn-Ppib

Tissue: Heart
Pretreatment: Standard

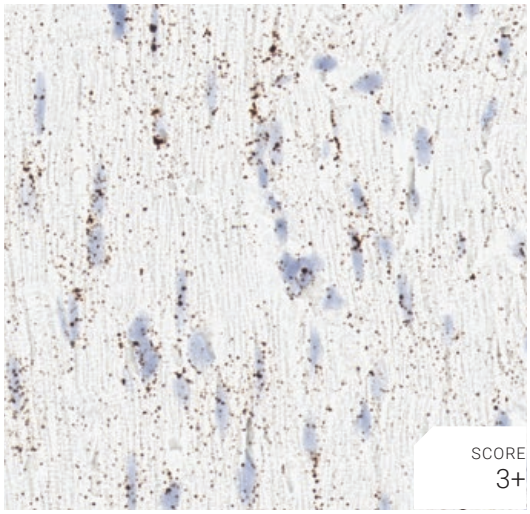
Control probe: **Rn-Ppib**



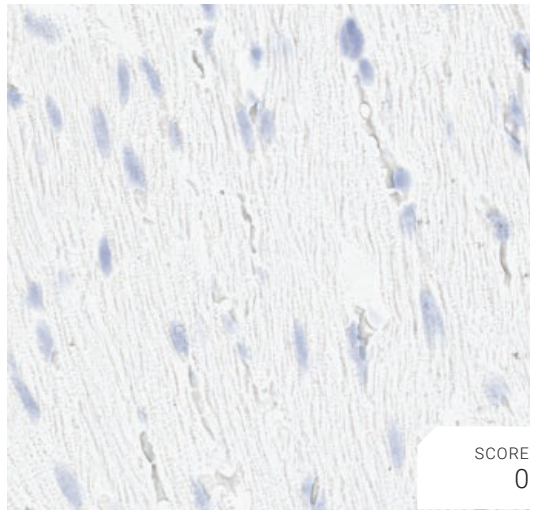
Control probe: **Rn-Polr2a**



Control probe: **Rn-Ubc**



Control probe: **DapB**



Recommended Positive Control Probe: Rn-Ubc

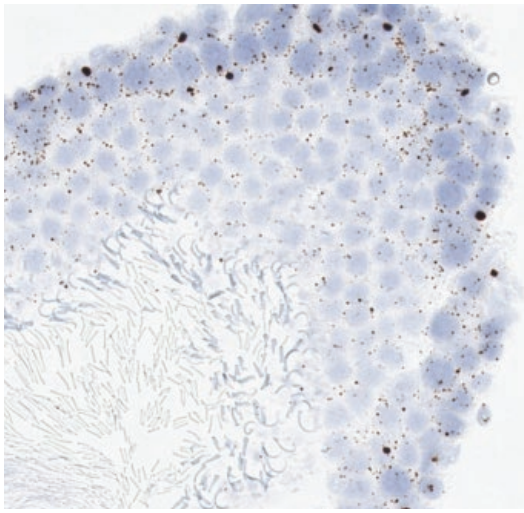
Rat

Rattus norvegicus

The following pages show the proliferation and apoptosis marker images using the optimal pretreatment condition for each tissue.

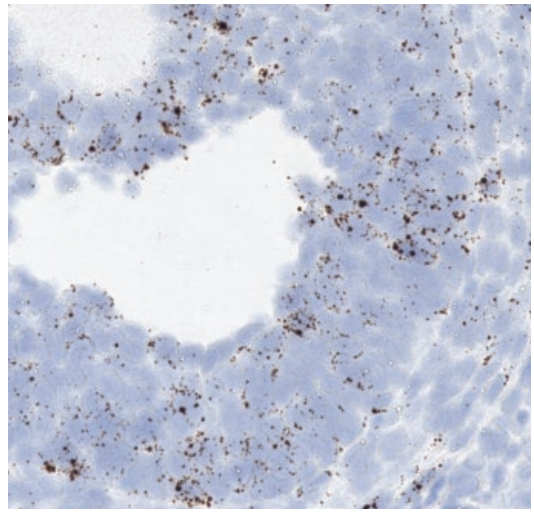
Tissue: Testis

Target probe: **Rn-Mki67 (Ki-67)**



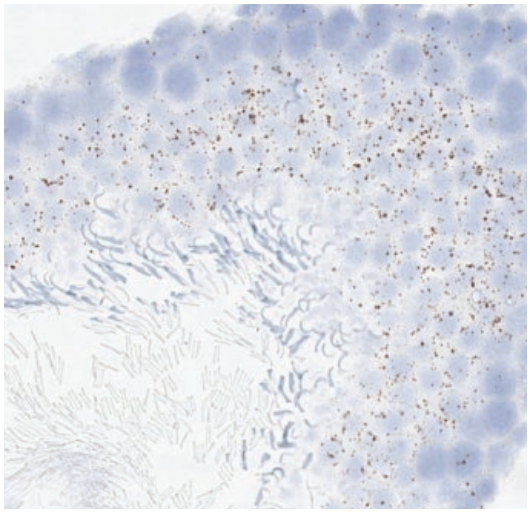
Tissue: Ovary

Target probe: **Rn-Mki67 (Ki-67)**



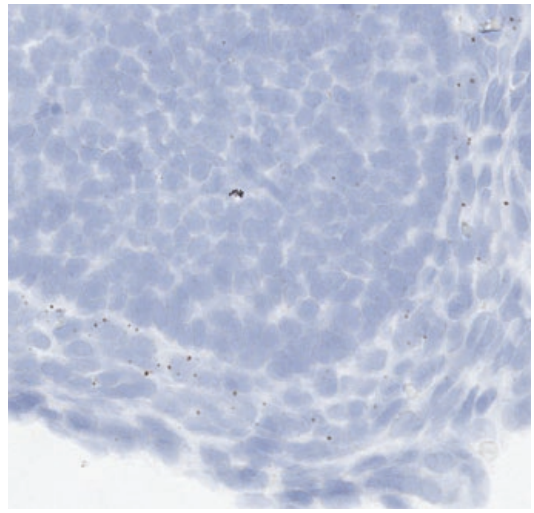
Tissue: Testis

Target probe: **Rn-Ccne1 (Cyclin E1)**



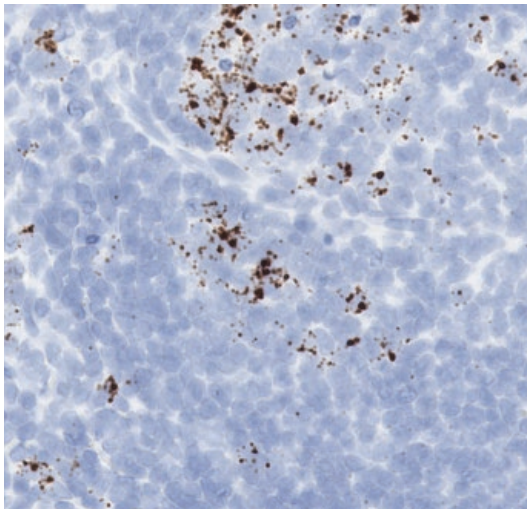
Tissue: Ovary

Target probe: **Rn-Ccne1 (Cyclin E1)**



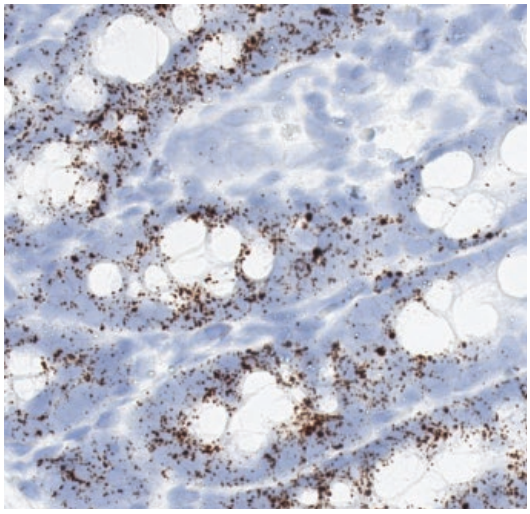
Tissue: Lymph node

Target probe: **Rn-Mki67 (Ki-67)**



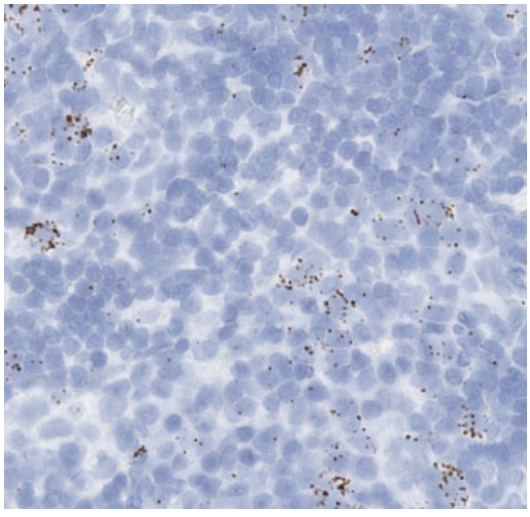
Tissue: Duodenum

Target probe: **Rn-Mki67 (Ki-67)**



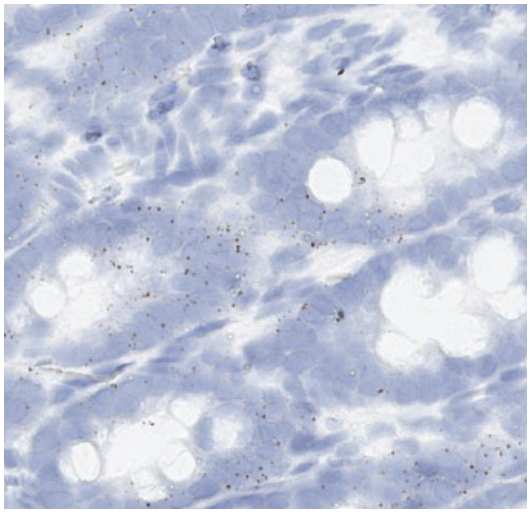
Tissue: Lymph node

Target probe: **Rn-Ccne1 (Cyclin E1)**



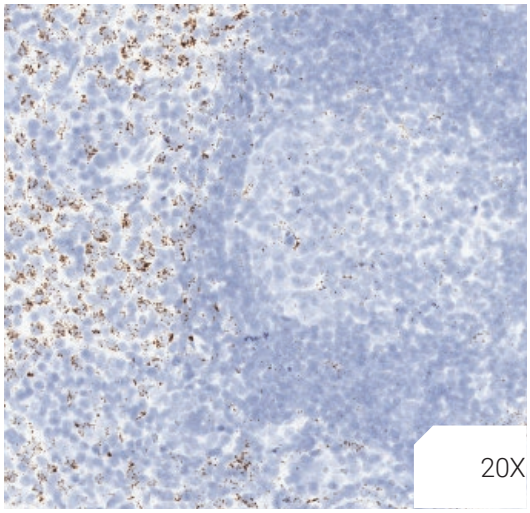
Tissue: Duodenum

Target probe: **Rn-Ccne1 (Cyclin E1)**



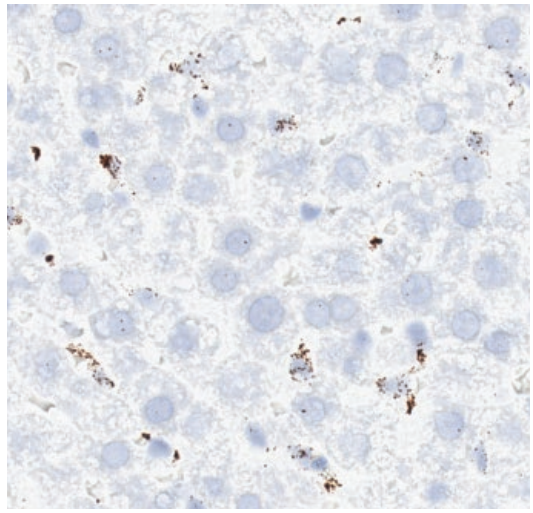
Tissue: Lymph node

Target probe: **Rn-Cd68**



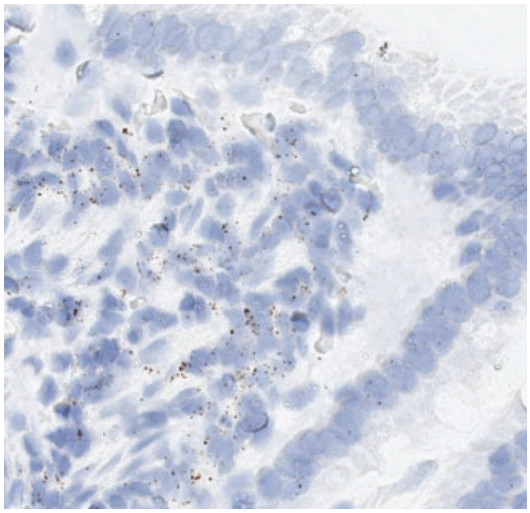
Tissue: Liver

Target probe: **Rn-Cd68**



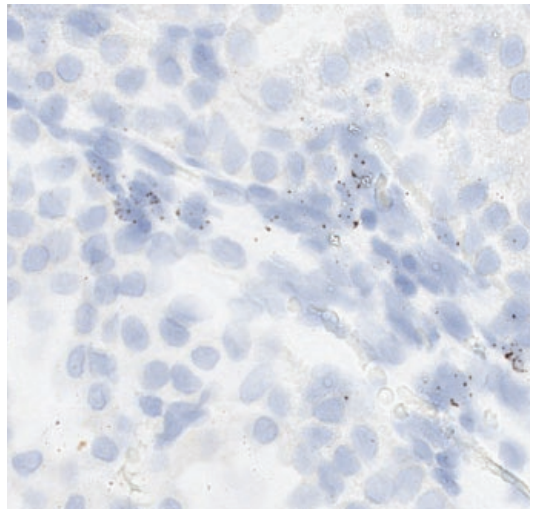
Tissue: Duodenum

Target probe: **Rn-Cd68**



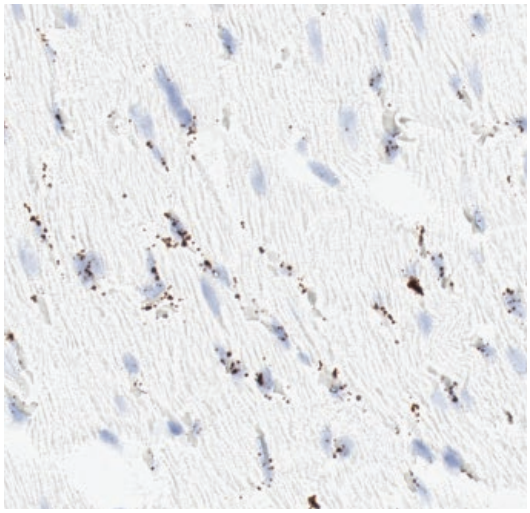
Tissue: Kidney

Target probe: **Rn-Cd68**



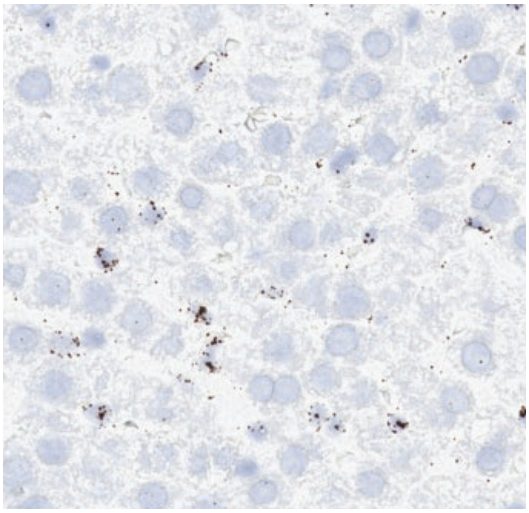
Tissue: Heart

Target probe: **Rn-Pecam1**



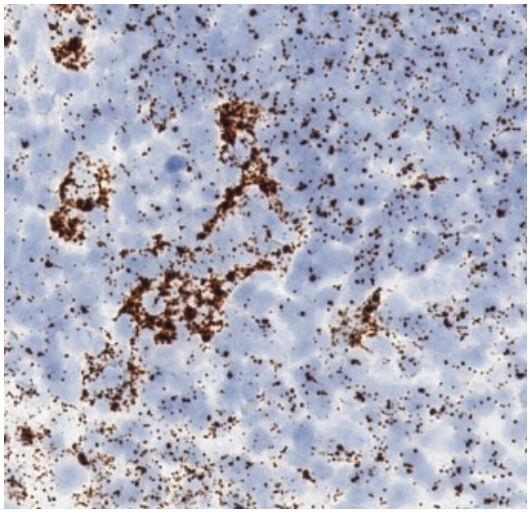
Tissue: Liver

Target probe: **Rn-Pecam1**



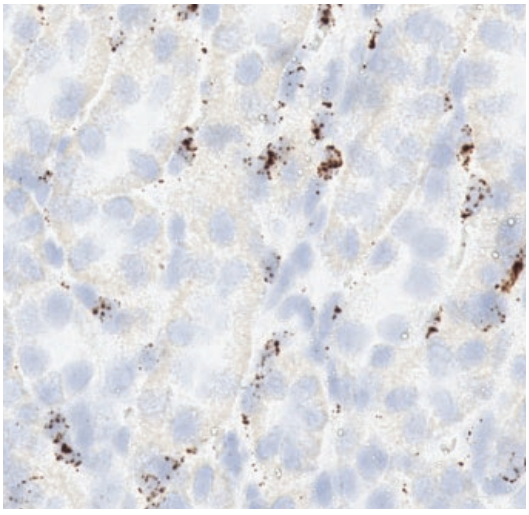
Tissue: Lymph node

Target probe: **Rn-Pecam1**



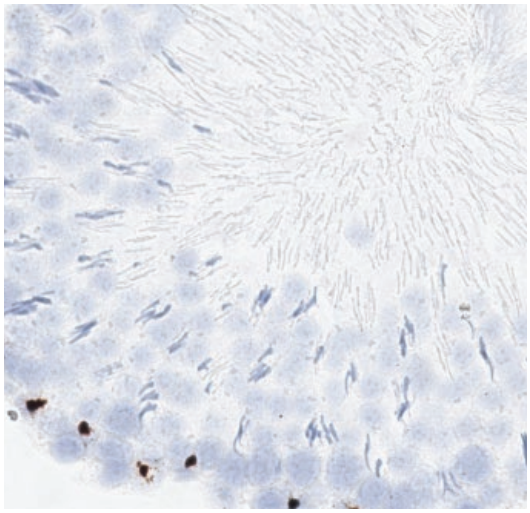
Tissue: Kidney

Target probe: **Rn-Pecam1**



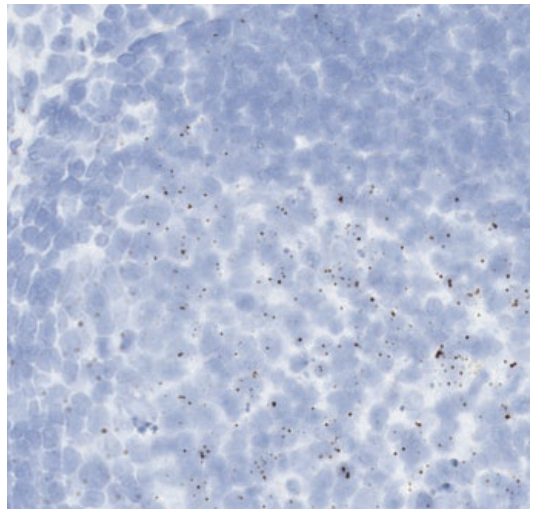
Tissue: Testis

Target probe: **Rn-Fas (CD95)**



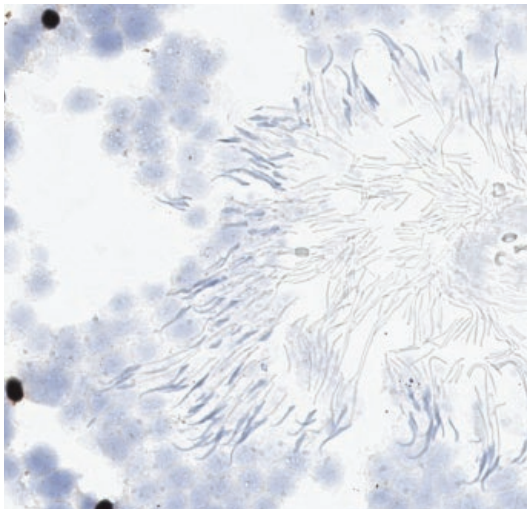
Tissue: Lymph node

Target probe: **Rn-Fas (CD95)**



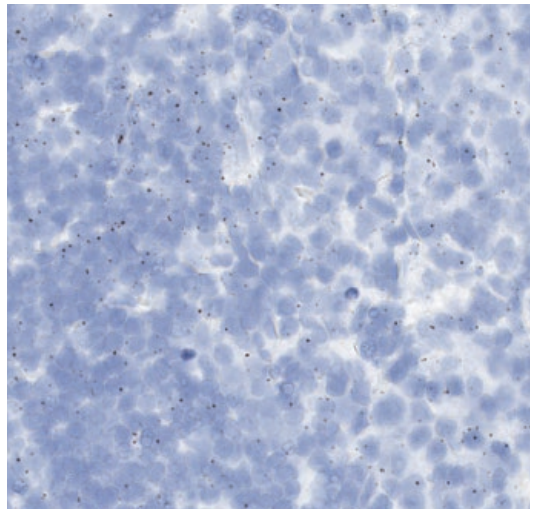
Tissue: Testis

Target probe: **Rn-Bbc3 (Puma)**



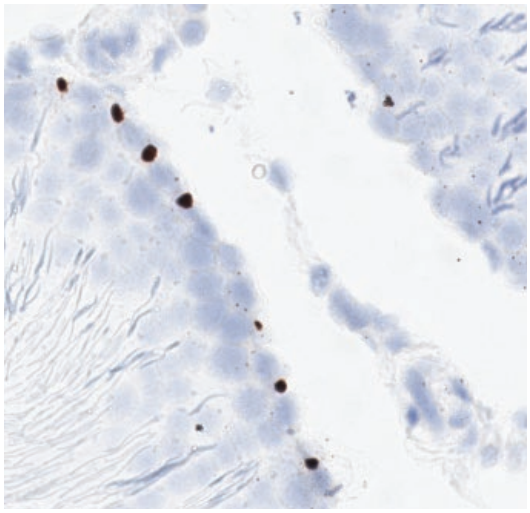
Tissue: Lymph node

Target probe: **Rn-Bbc3 (Puma)**



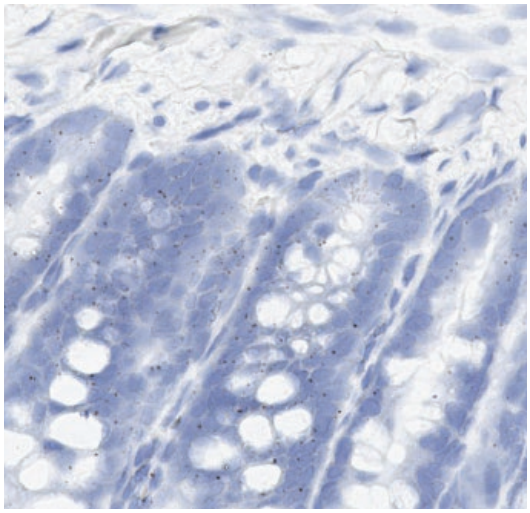
Tissue: Testis

Target probe: **Rn-Tnfrsf10b (DR5)**



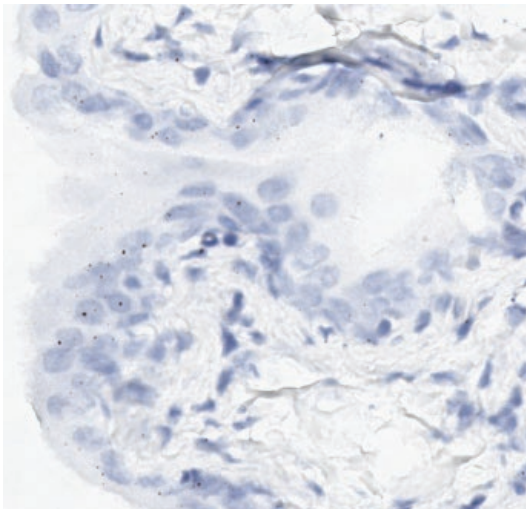
Tissue: Jejunum

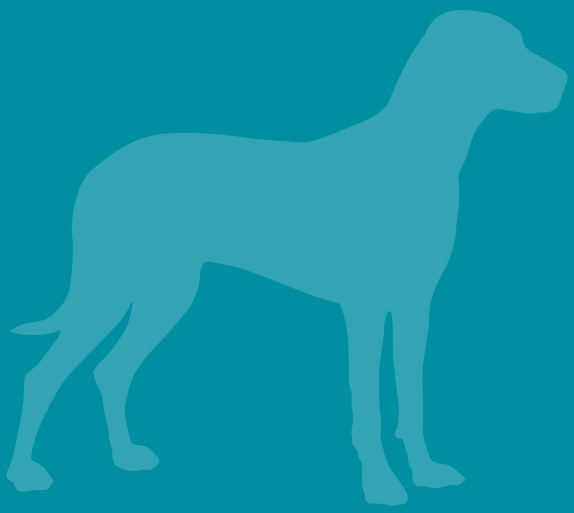
Target probe: **Rn-Tnfrsf10b (DR5)**



Tissue: Bladder

Target probe: **Rn-Tnfrsf10b (DR5)**





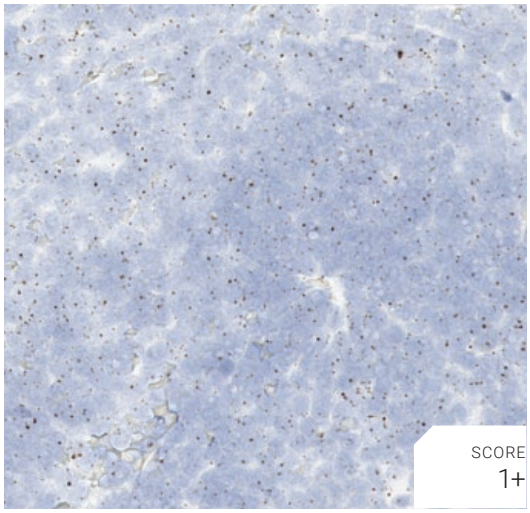
Dog

Canis lupus

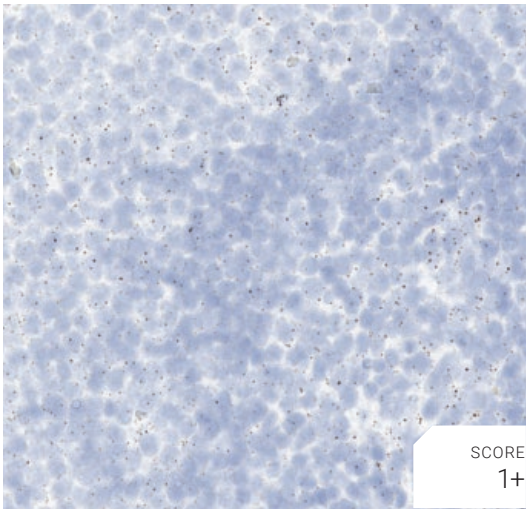
The following pages show positive control probe images with standard and mild pretreatment conditions. Optimal pretreatment condition is indicated for each tissue type.

Positive control probe: Cl-POLR2A
Tissue: Spleen

Pretreatment: **Standard**



Pretreatment: **Mild**

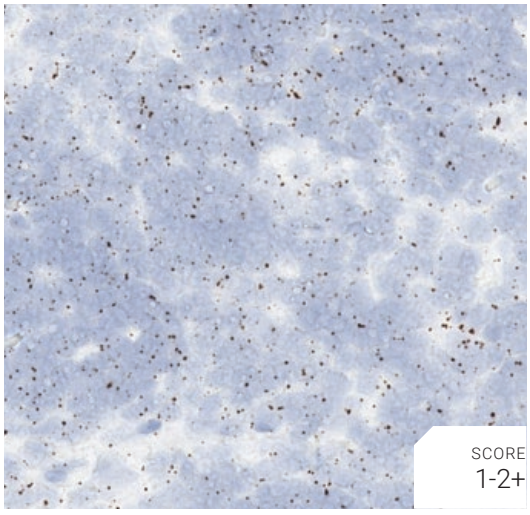


Observation: Loss of cell boundary using standard pretreatment.

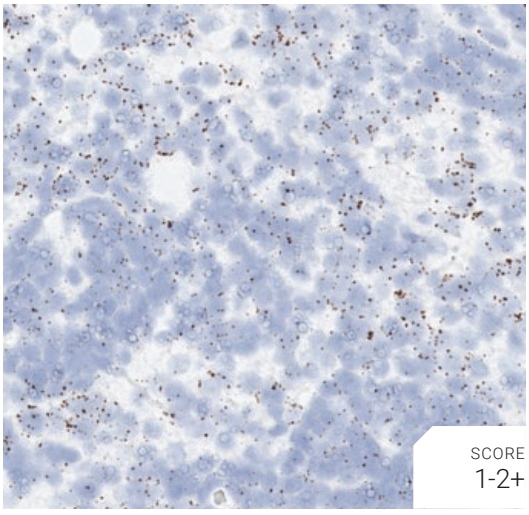
Optimal Pretreatment: Mild

Positive control probe: Cl-POLR2A
Tissue: Lymph node

Pretreatment: **Standard**



Pretreatment: **Mild**

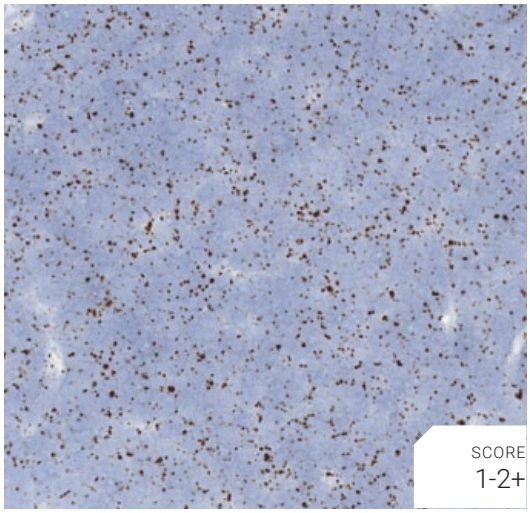


Observation: Loss of cell boundary using standard pretreatment.

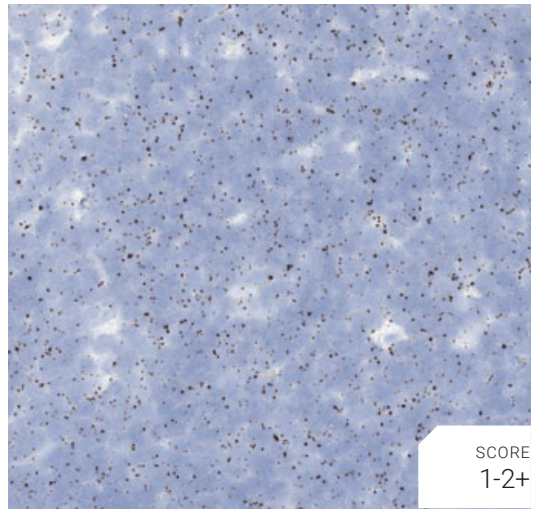
Optimal Pretreatment: Mild

Positive control probe: Cl-POLR2A
Tissue: Thymus

Pretreatment: **Standard**



Pretreatment: **Mild**

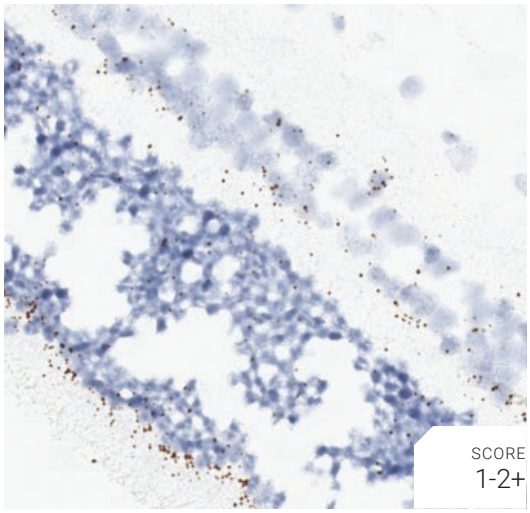


Observation: Loss of cell boundary using standard pretreatment.

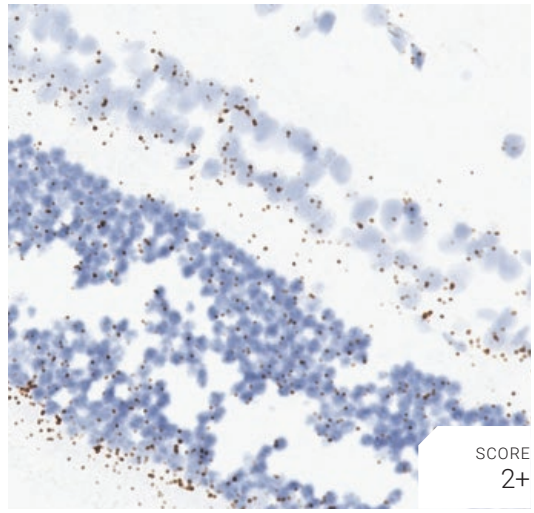
Optimal Pretreatment: Mild

Positive control probe: Cl-POLR2A
Tissue: Retina (eye)

Pretreatment: **Standard**



Pretreatment: **Mild**

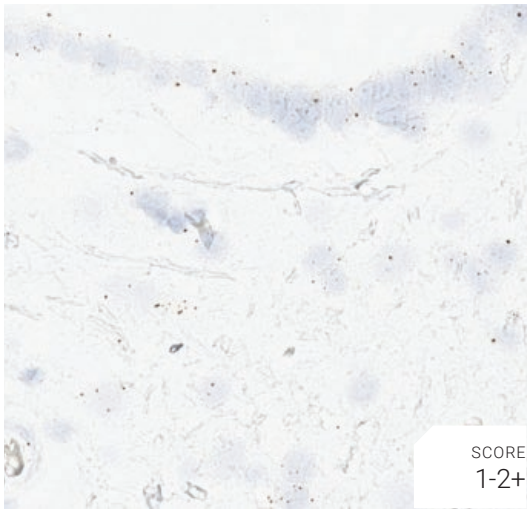


Observation: Standard pretreatment causes more cell detachment and reduced signals.

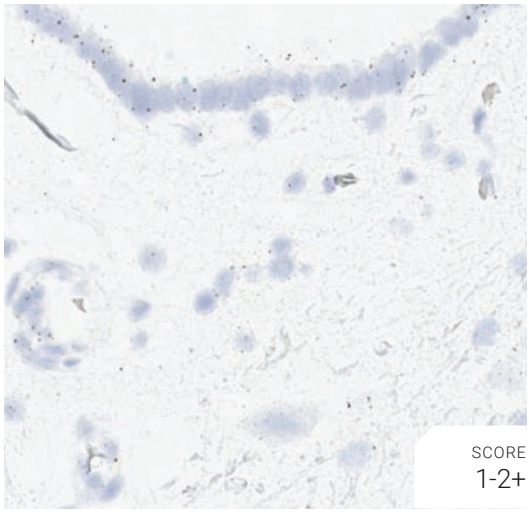
Optimal Pretreatment: Mild

Positive control probe: Cl-POLR2A
Tissue: Spinal cord

Pretreatment: **Standard**



Pretreatment: **Mild**

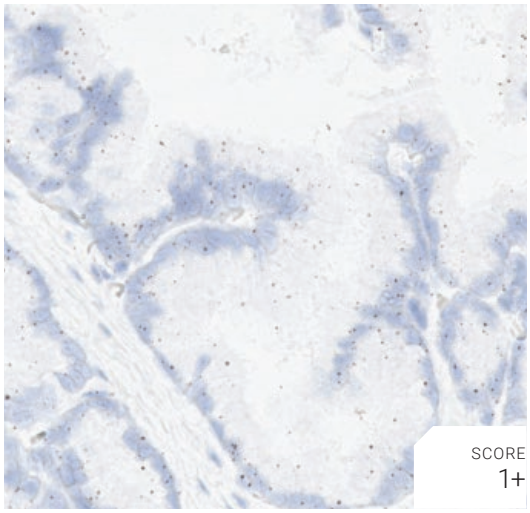


Observation: Standard pretreatment increases staining in white matter.

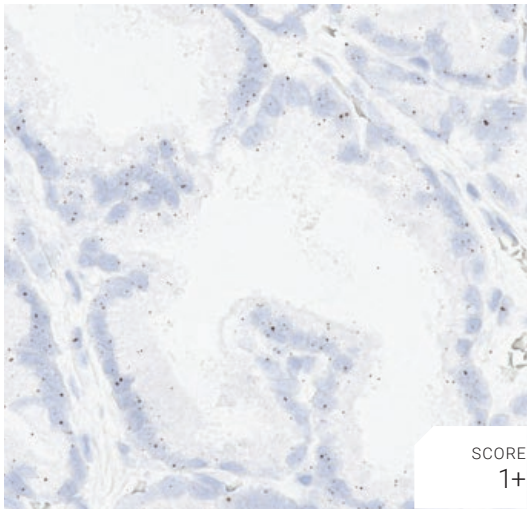
Optimal Pretreatment: Standard

Positive control probe: Cl-POLR2A
Tissue: Prostate gland

Pretreatment: **Standard**



Pretreatment: **Mild**



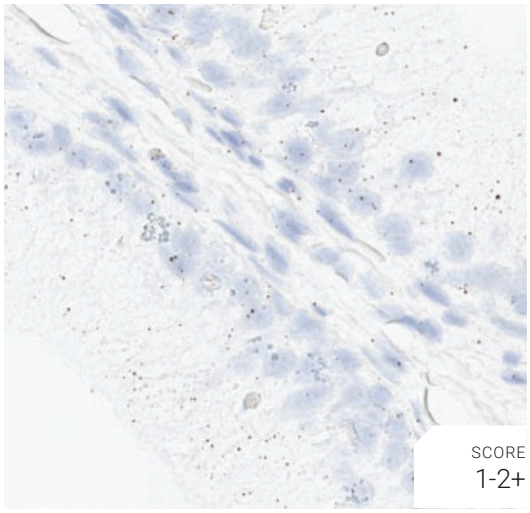
Observation: Loss of cell boundary using standard pretreatment.

Optimal Pretreatment: Mild*

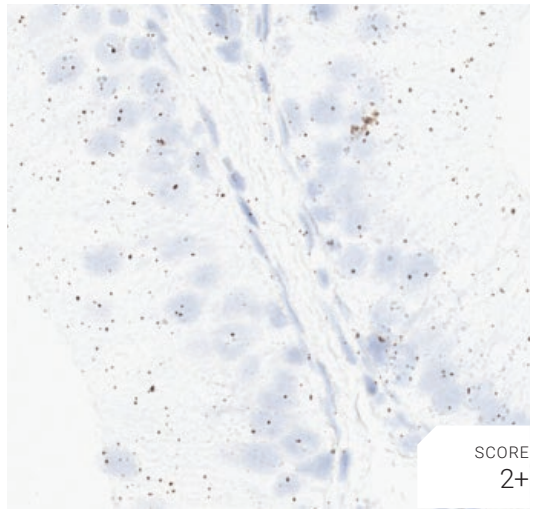
*Standard pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Epididymis

Pretreatment: **Standard**



Pretreatment: **Mild**

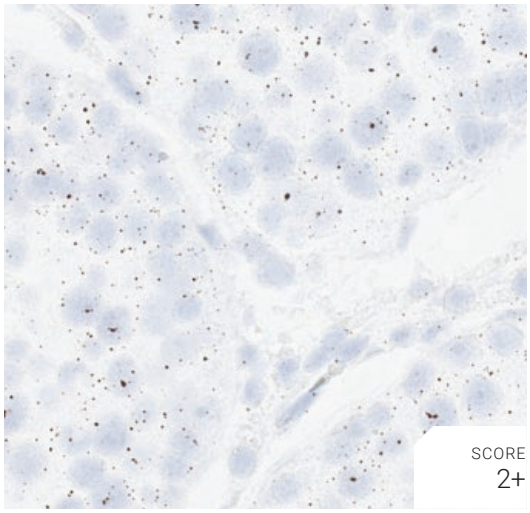


Observation: Loss of cell boundary using standard pretreatment.

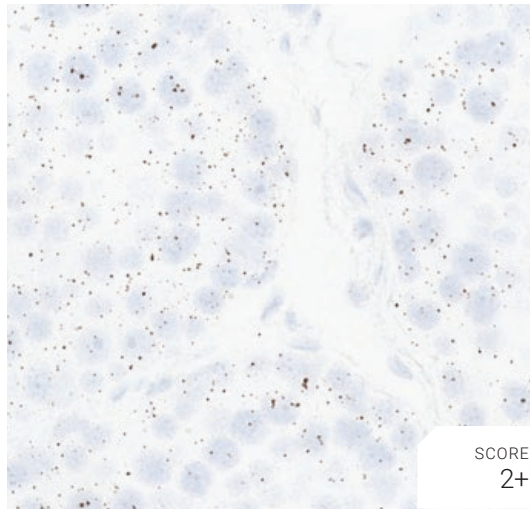
Optimal Pretreatment: Mild*

Positive control probe: Cl-POLR2A
Tissue: Testis

Pretreatment: **Standard**



Pretreatment: **Mild**



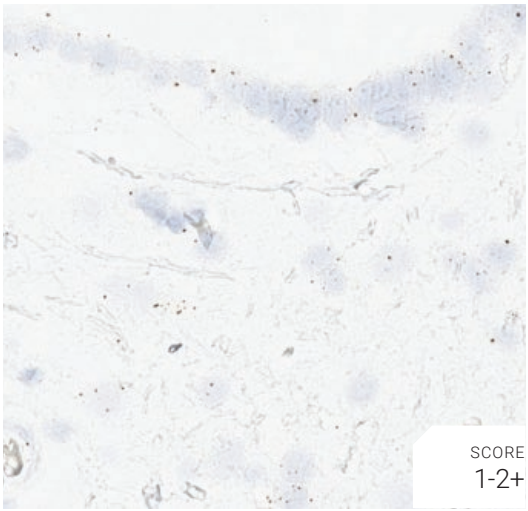
Observation: Standard pretreatment causes more cell detachment and reduced signals.

Optimal Pretreatment: Mild*

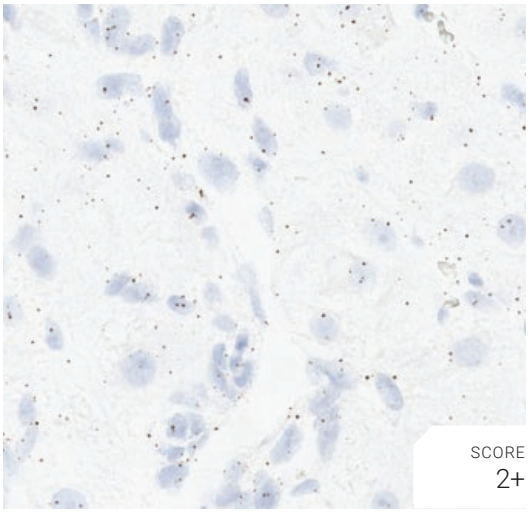
*Standard pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Ovary

Pretreatment: **Standard**



Pretreatment: **Mild**

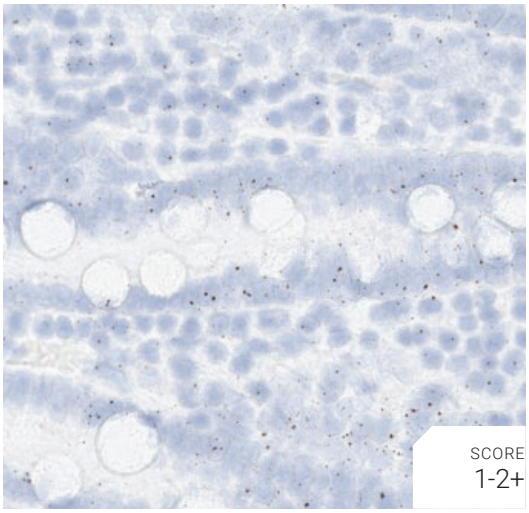


Observation: Standard pretreatment increases staining in white matter.

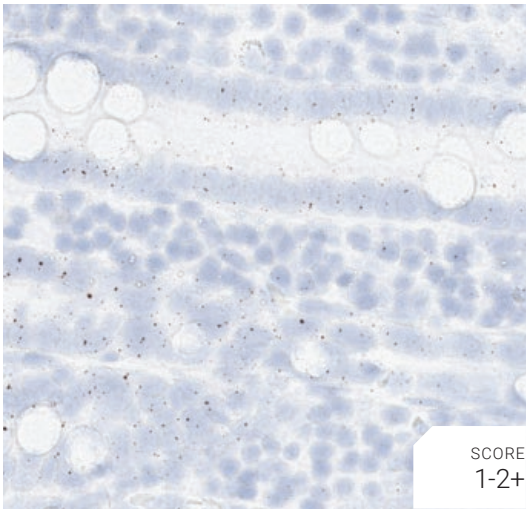
Optimal Pretreatment: Standard**

Positive control probe: Cl-POLR2A
Tissue: Duodenum

Pretreatment: **Standard**



Pretreatment: **Mild**



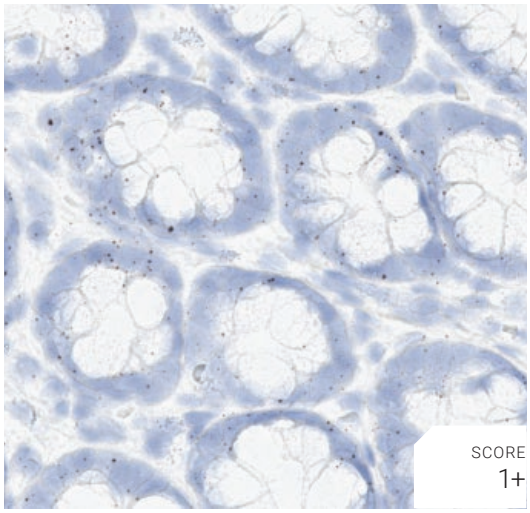
Observation: Loss of cell boundary using standard pretreatment.

Optimal Pretreatment: Mild*

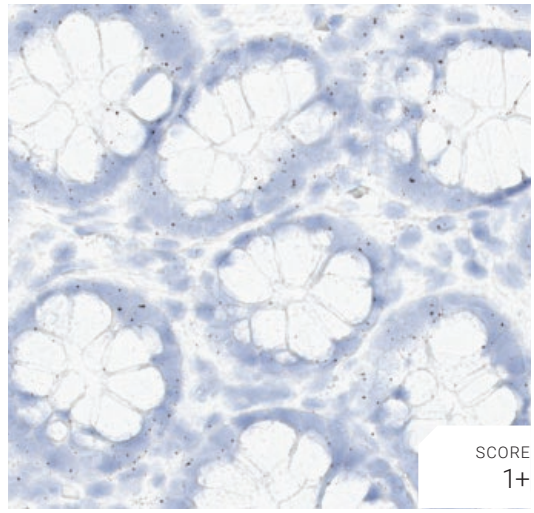
*Standard pretreatment is also suitable **Mild pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Colon

Pretreatment: **Standard**



Pretreatment: **Mild**

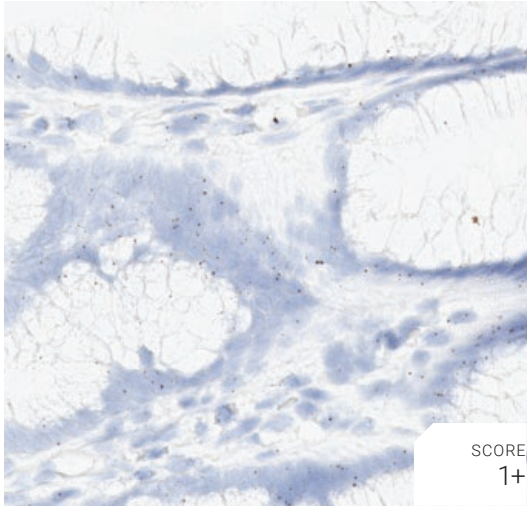


Observation: Loss of cell boundary using standard pretreatment.

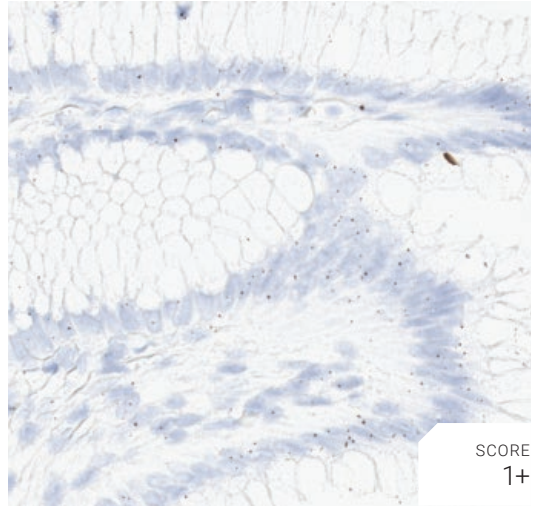
Optimal Pretreatment: Mild*

Positive control probe: Cl-POLR2A
Tissue: Stomach

Pretreatment: **Standard**



Pretreatment: **Mild**

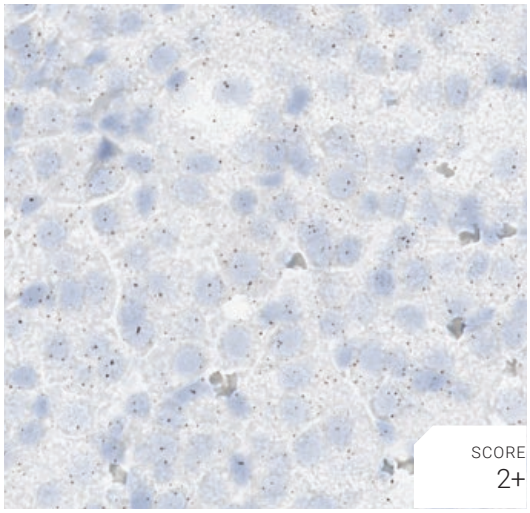


Optimal Pretreatment: Standard*

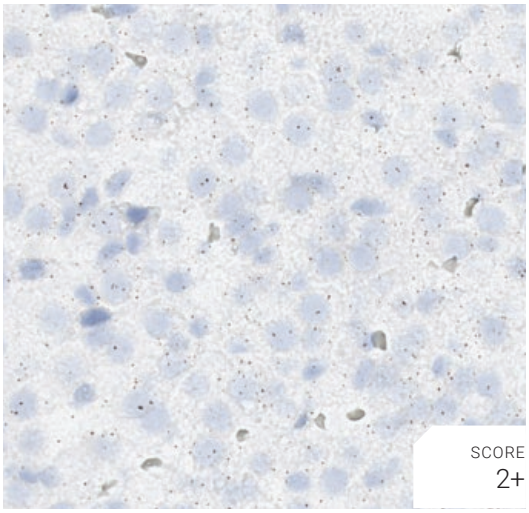
*Standard pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Adrenal gland–cortex

Pretreatment: **Standard**



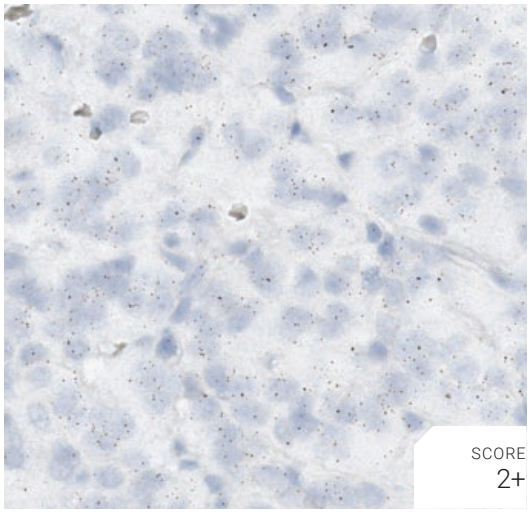
Pretreatment: **Mild**



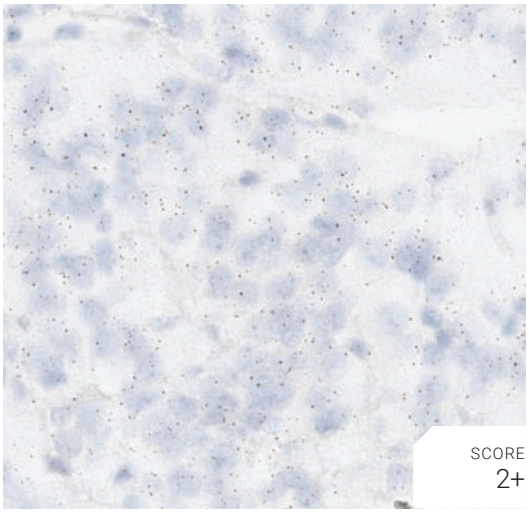
Optimal Pretreatment: Standard*

Positive control probe: Cl-POLR2A
Tissue: Adrenal gland–medulla

Pretreatment: **Standard**



Pretreatment: **Mild**



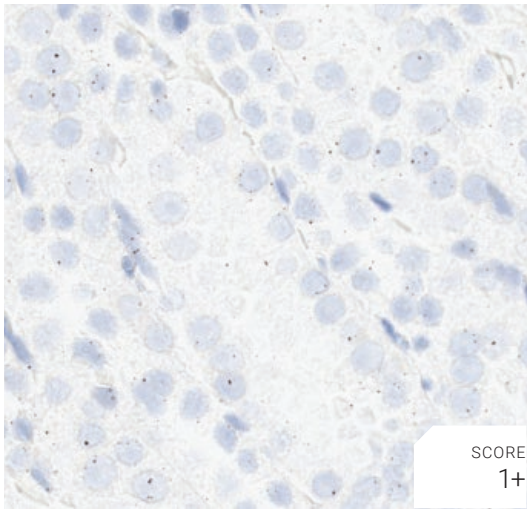
Observation: Loss of cell boundary using mild pretreatment.

Optimal Pretreatment: Standard*

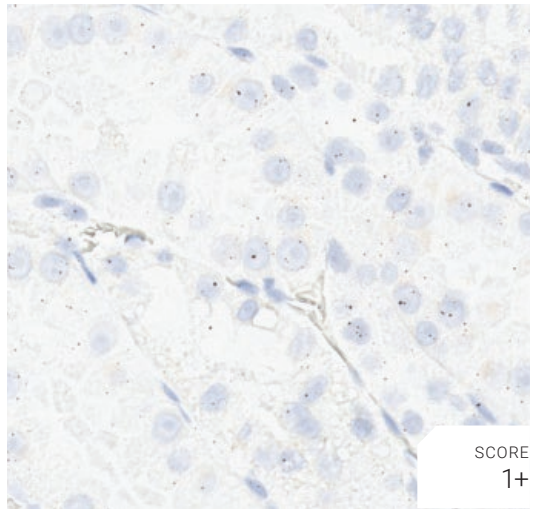
*Mild pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Kidney

Pretreatment: **Standard**



Pretreatment: **Mild**

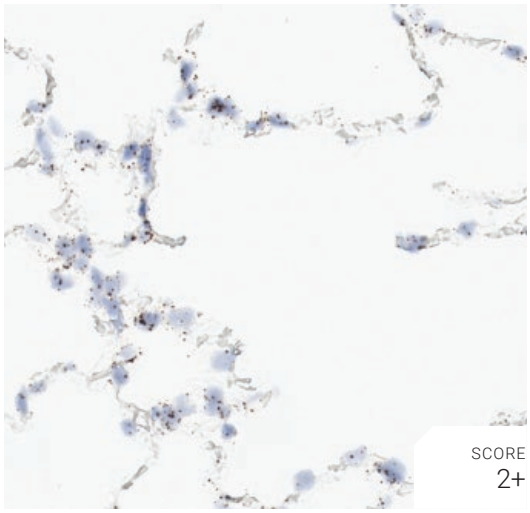


Observation: Loss of cell boundary using standard pretreatment.

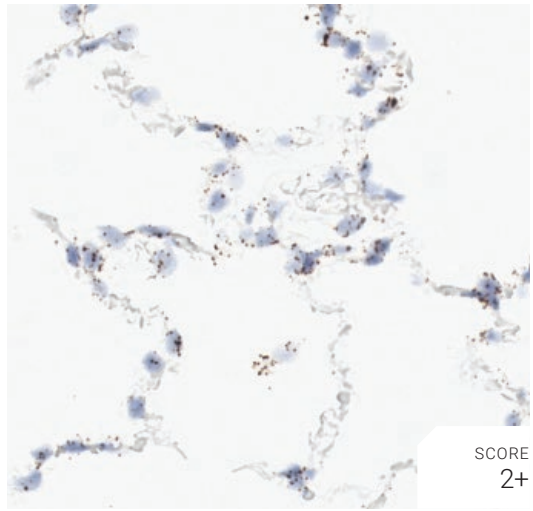
Optimal Pretreatment: Standard**

Positive control probe: Cl-UBC*
Tissue: Lung

Pretreatment: **Standard**



Pretreatment: **Mild**

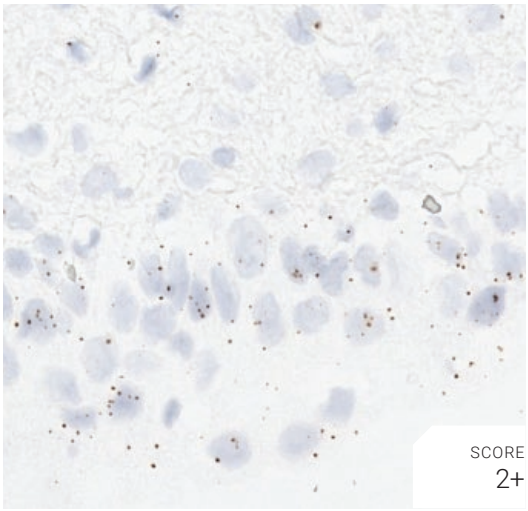


Optimal Pretreatment: Standard**

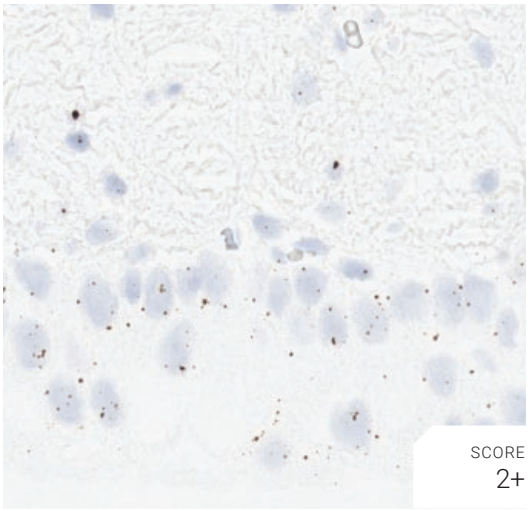
*Low PPIB/POLR2A detection in alveolar cells **Mild pretreatment is also suitable

Positive control probe: Cl-POLR2A
Tissue: Urinary bladder

Pretreatment: **Standard**



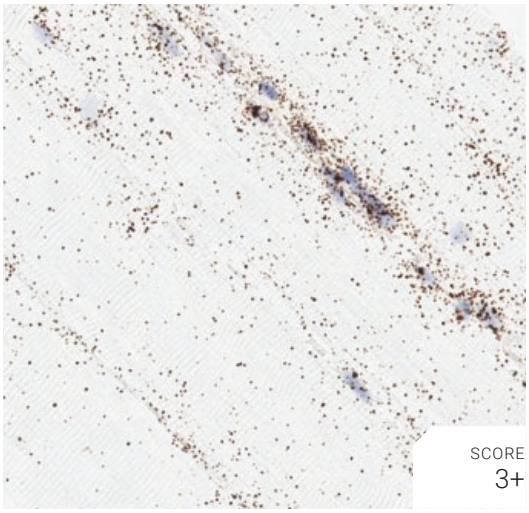
Pretreatment: **Mild**



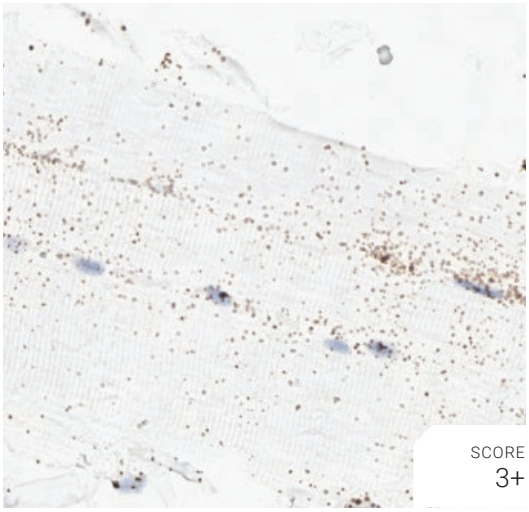
Optimal Pretreatment: Standard

Positive control probe: Cl-UBC*
Tissue: Skeletal muscle

Pretreatment: **Standard**



Pretreatment: **Mild**

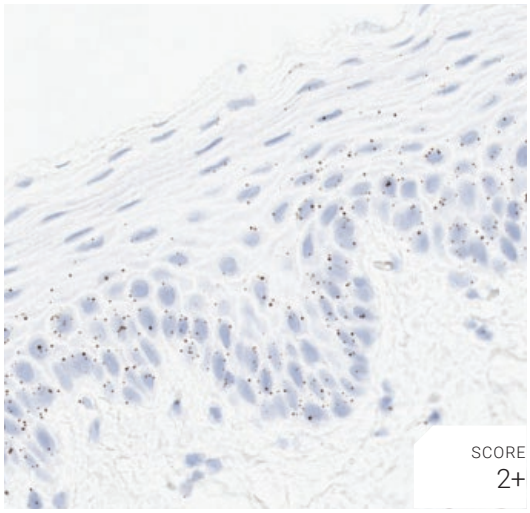


Optimal Pretreatment: Standard

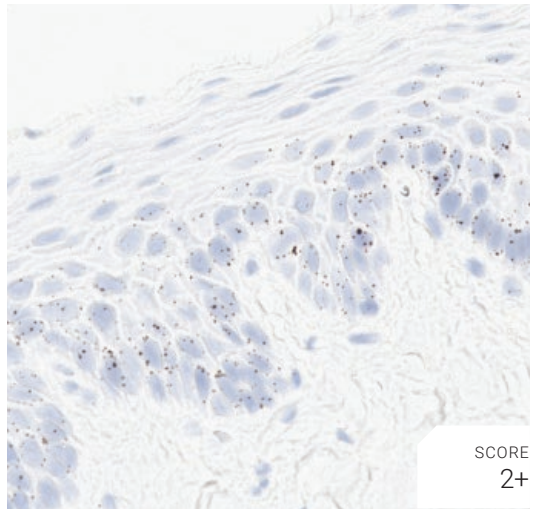
*Low PPIB/POLR2A detection in skeletal muscle cells

Positive control probe: Cl-POLR2A
Tissue: Esophagus

Pretreatment: **Standard**



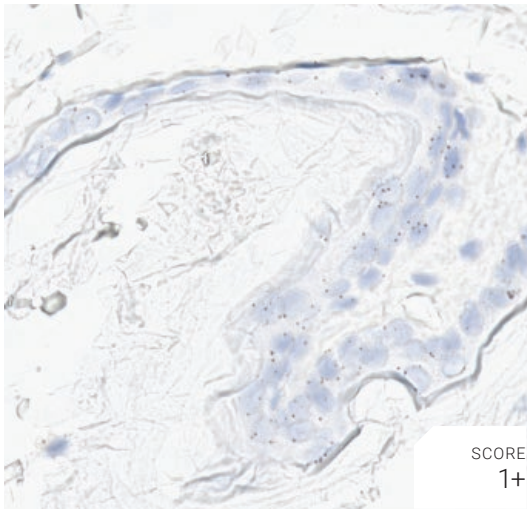
Pretreatment: **Mild**



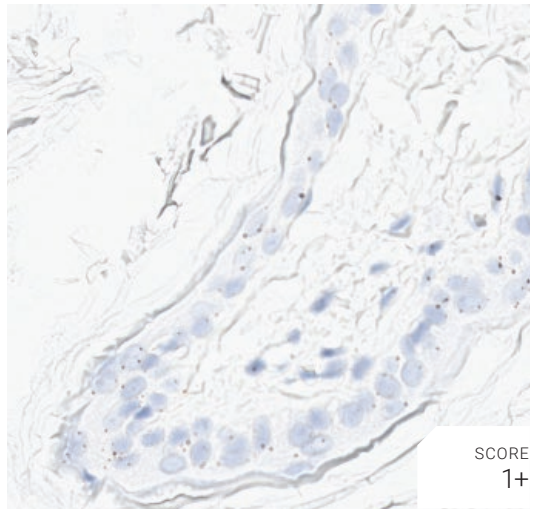
Optimal Pretreatment: Standard

Positive control probe: Cl-POLR2A
Tissue: Skin

Pretreatment: **Standard**



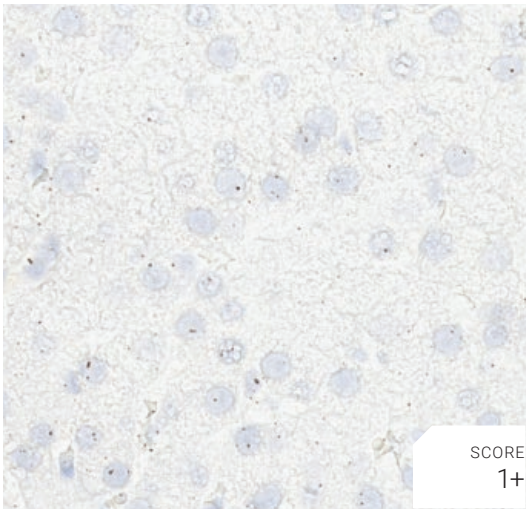
Pretreatment: **Mild**



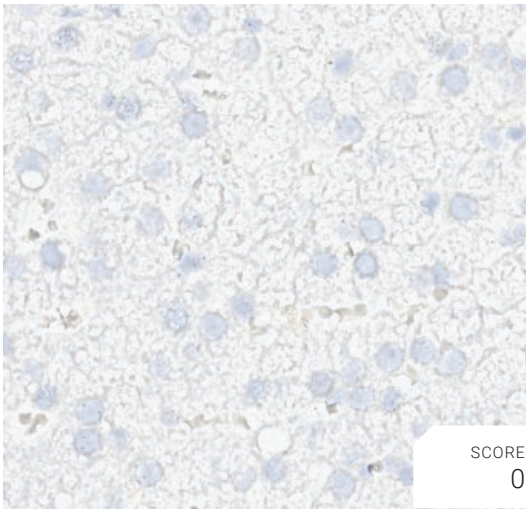
Optimal Pretreatment: Standard

Positive control probe: Cl-POLR2A
Tissue: Liver

Pretreatment: **Standard**



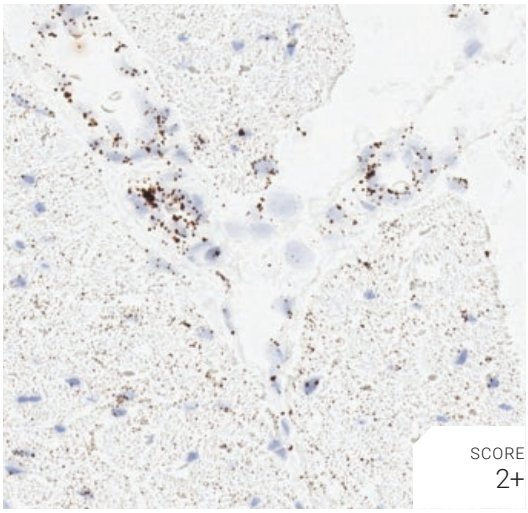
Pretreatment: **Mild**



Optimal Pretreatment: Standard

Positive control probe: Cl-UBC*
Tissue: Heart

Pretreatment: **Standard**



Optimal Pretreatment: Standard

*Low PPIB/POLR2A detection in heart muscle

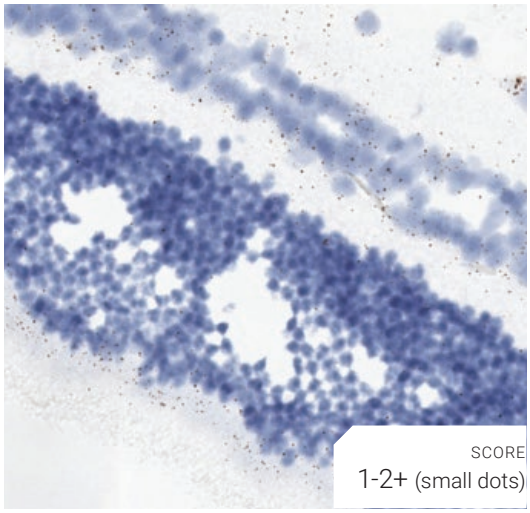
Dog

Canis lupus

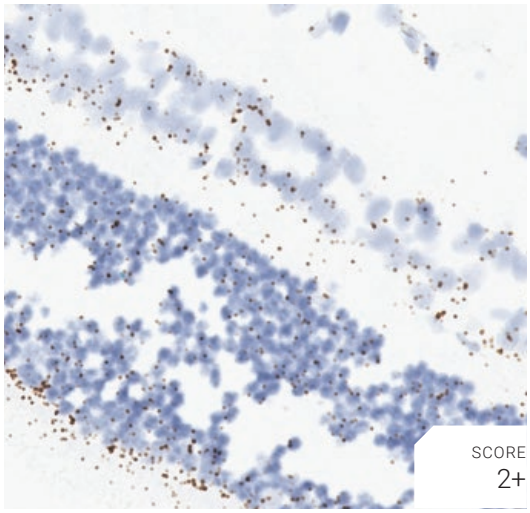
The following pages show the three positive controls (PPIB, POLR2A, UBC) and the negative control images using optimal pretreatment condition. Recommended positive control probe is indicated for each tissue type.

Tissue: Retina (eye)
Pretreatment: Mild

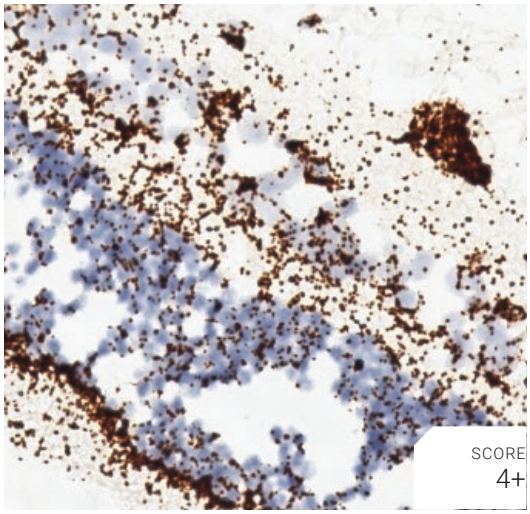
Control probe: **Cl-PPIB**



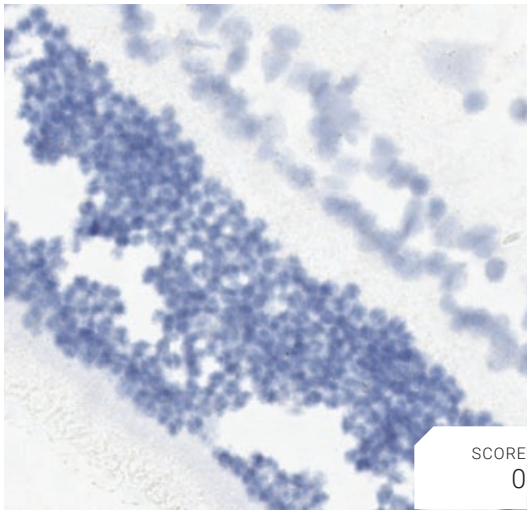
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



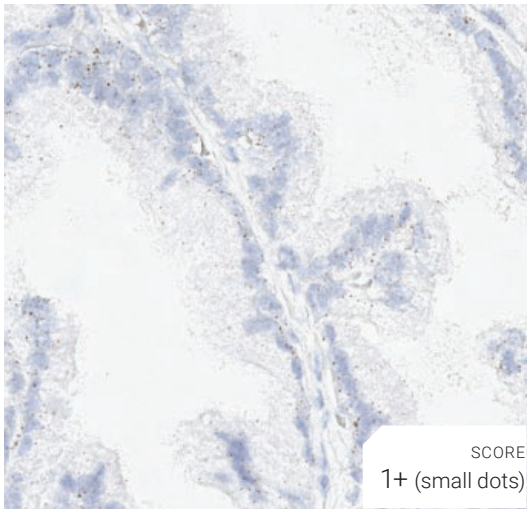
Control probe: **DapB**



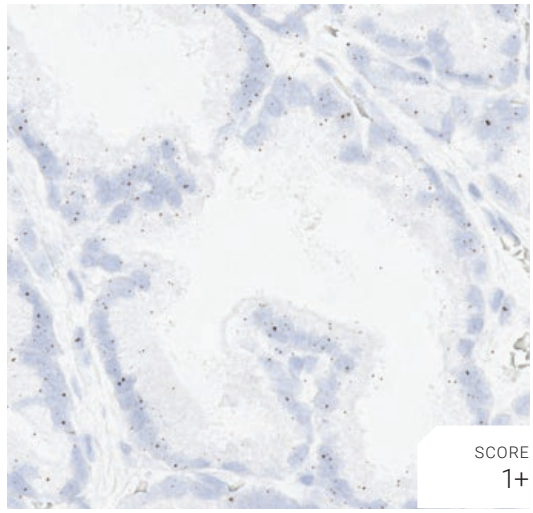
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Prostate gland
Pretreatment: Mild

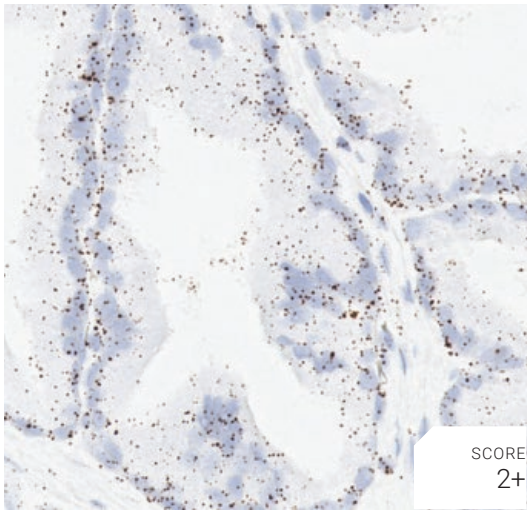
Control probe: **Cl-PPIB**



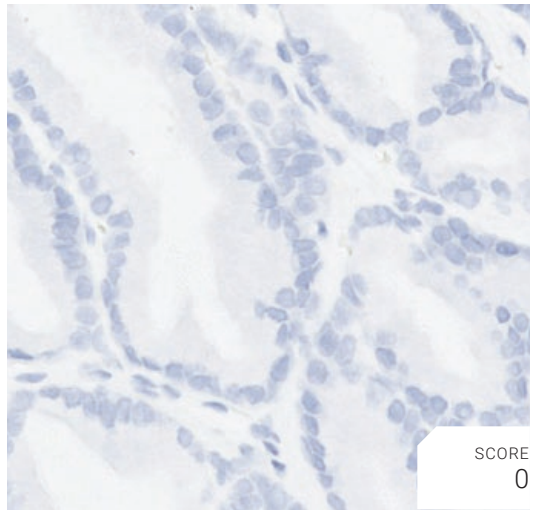
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



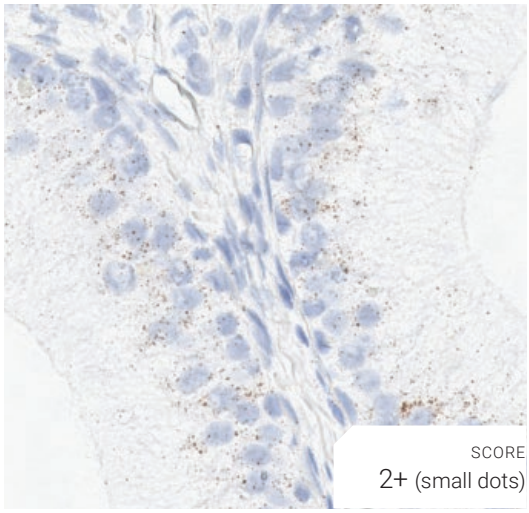
Control probe: **DapB**



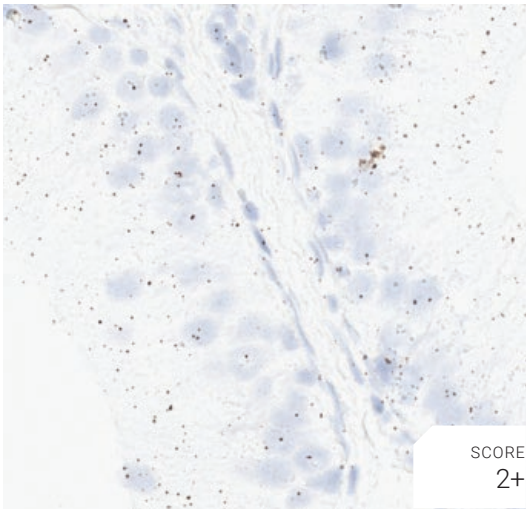
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Epididymis
Pretreatment: Mild

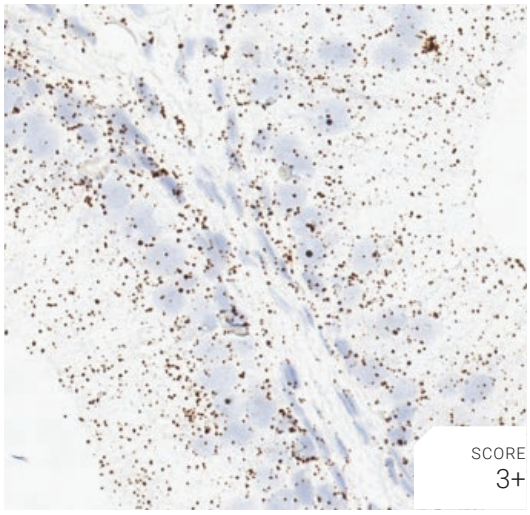
Control probe: **Cl-PPIB**



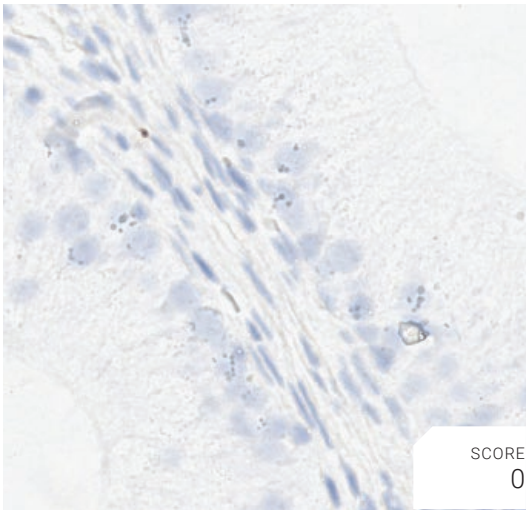
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



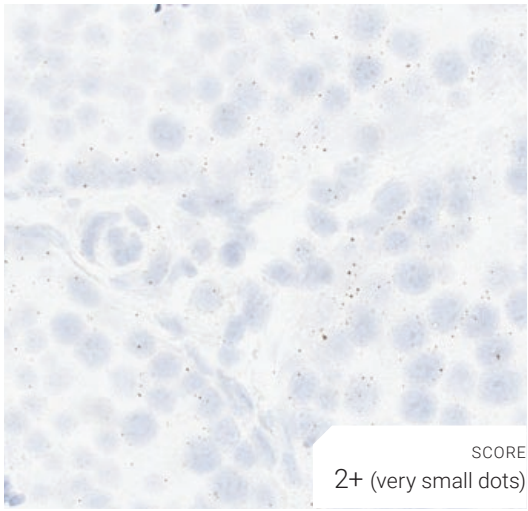
Control probe: **DapB**



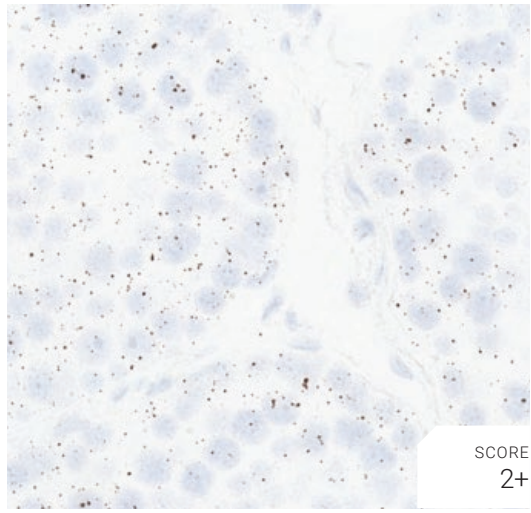
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Testis
Pretreatment: Mild

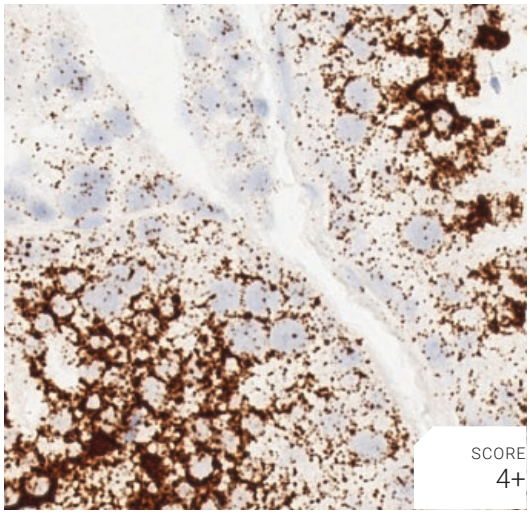
Control probe: **Cl-PPIB**



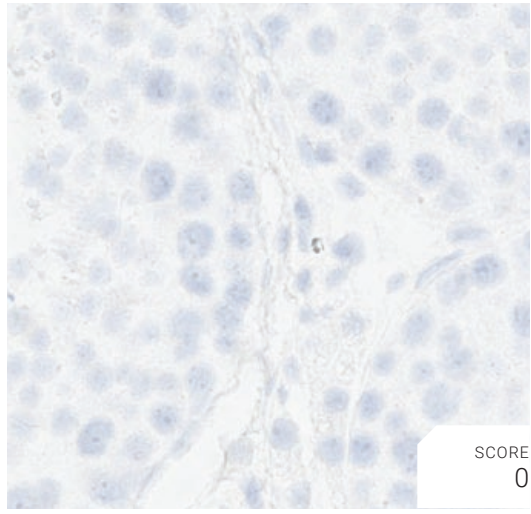
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



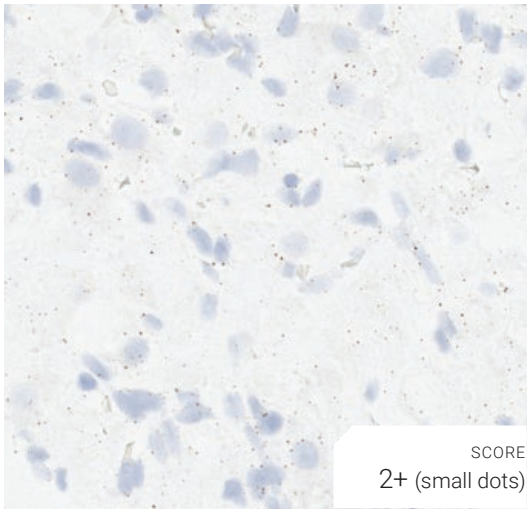
Control probe: **DapB**



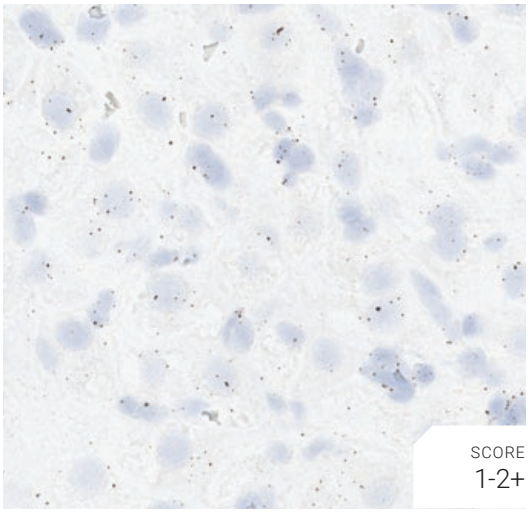
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Ovary
Pretreatment: Standard

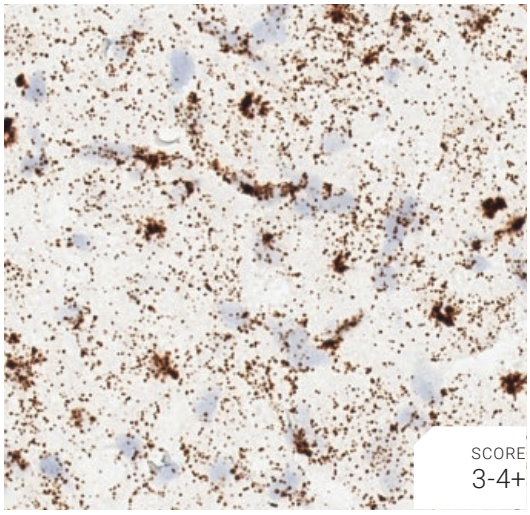
Control probe: **Cl-PPIB**



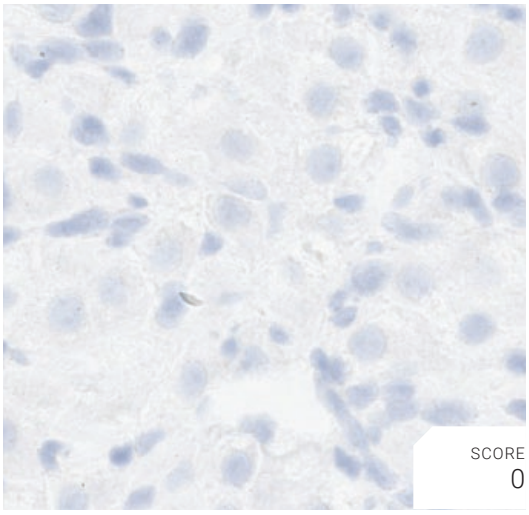
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



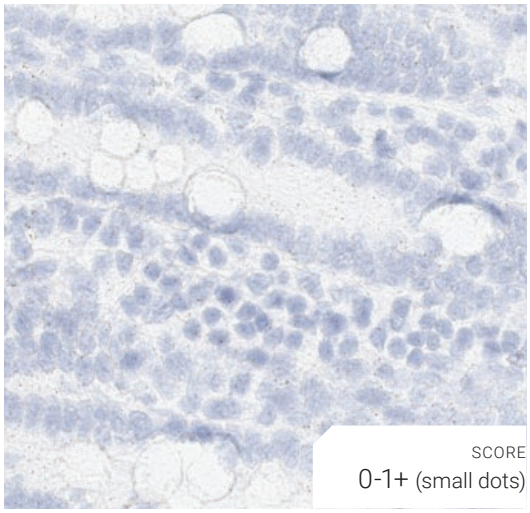
Control probe: **DapB**



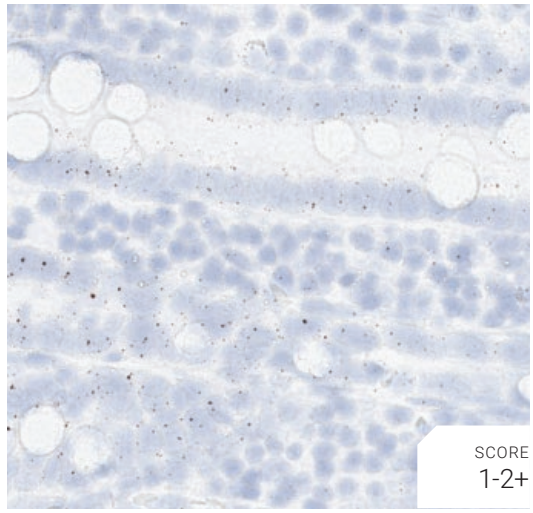
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Duodenum
Pretreatment: Mild

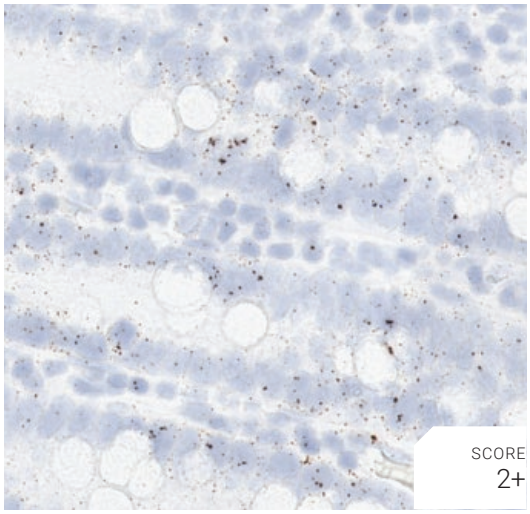
Control probe: **Cl-PPIB**



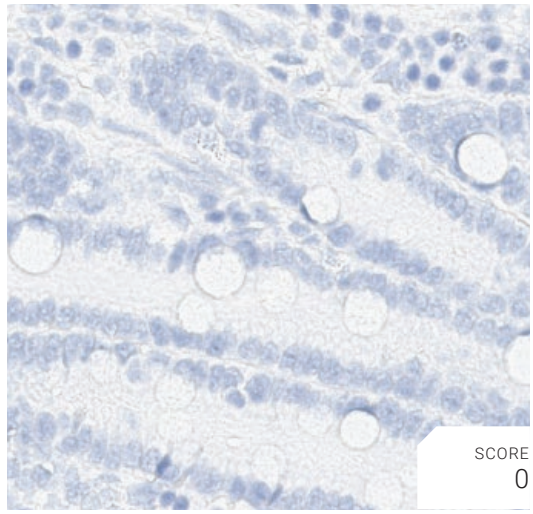
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



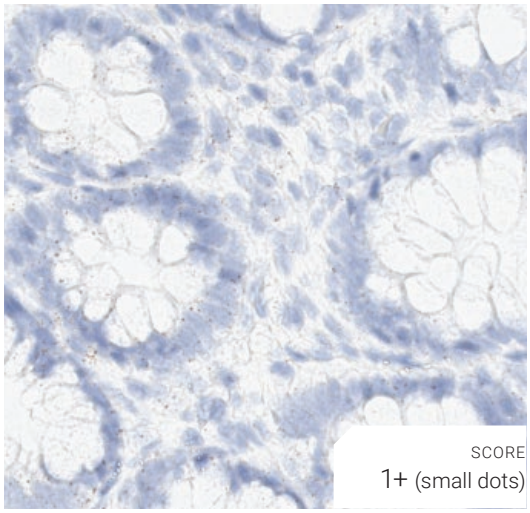
Control probe: **DapB**



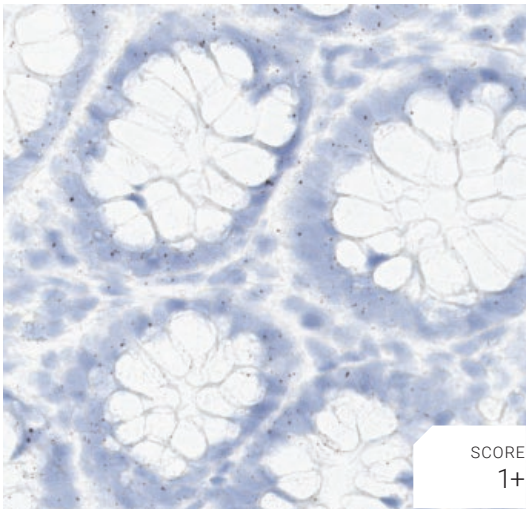
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Colon
Pretreatment: Mild

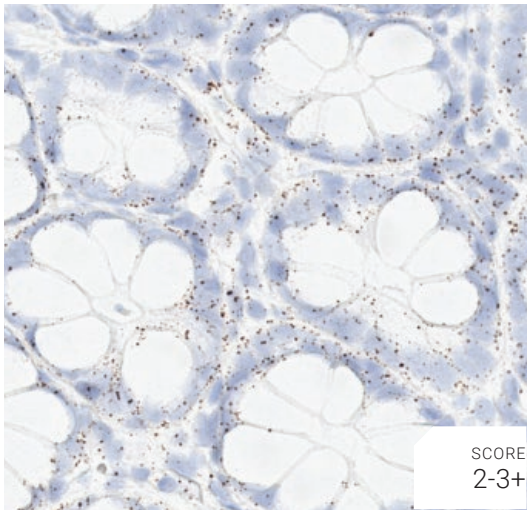
Control probe: **Cl-PPIB**



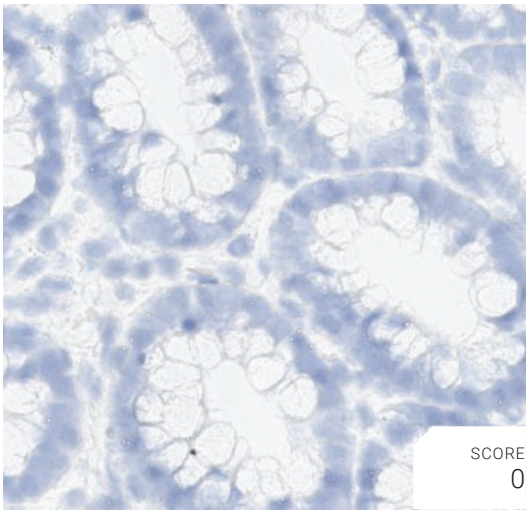
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



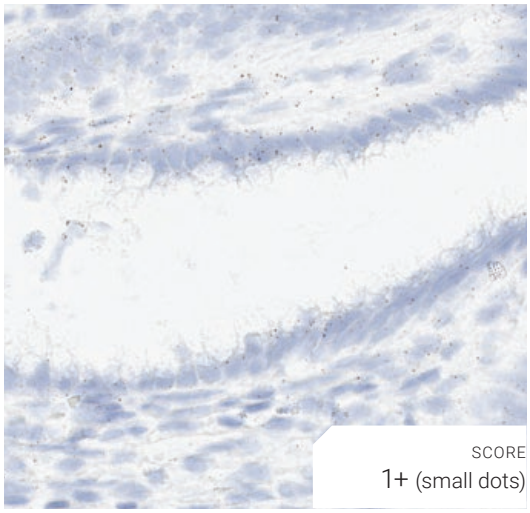
Control probe: **DapB**



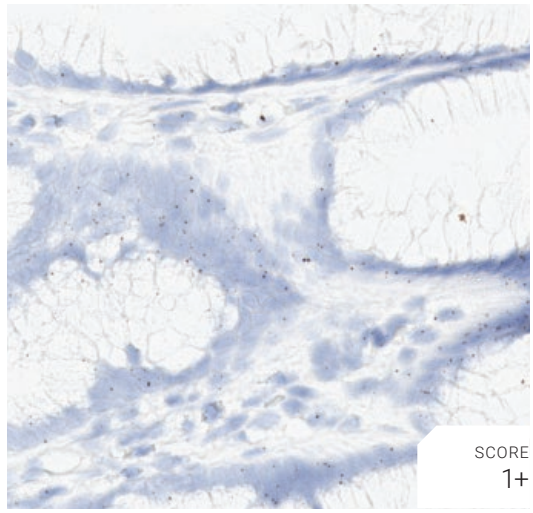
Recommended Positive Control Probe: Cl-POLR2A/Cl-UBC

Tissue: Stomach
Pretreatment: Standard

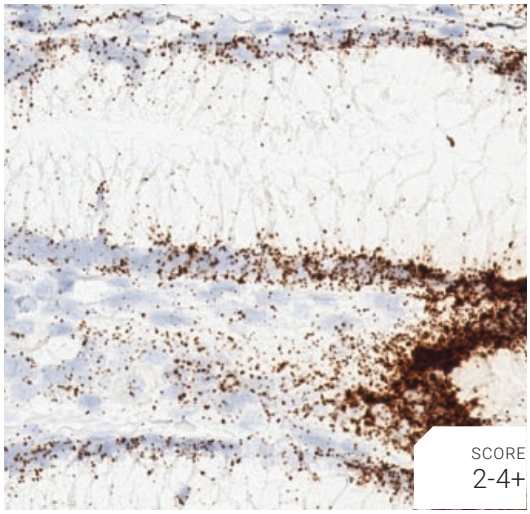
Control probe: **Cl-PPIB**



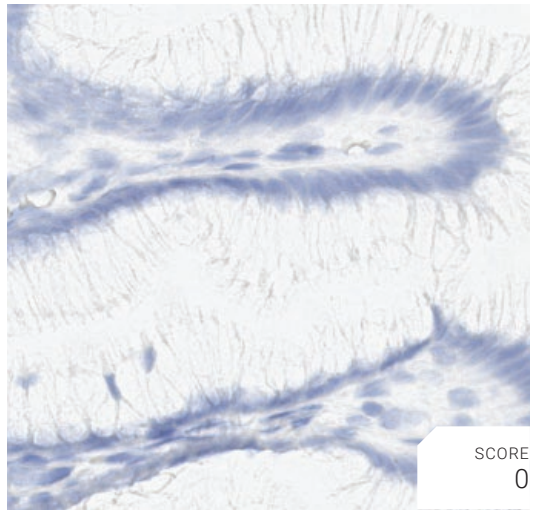
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



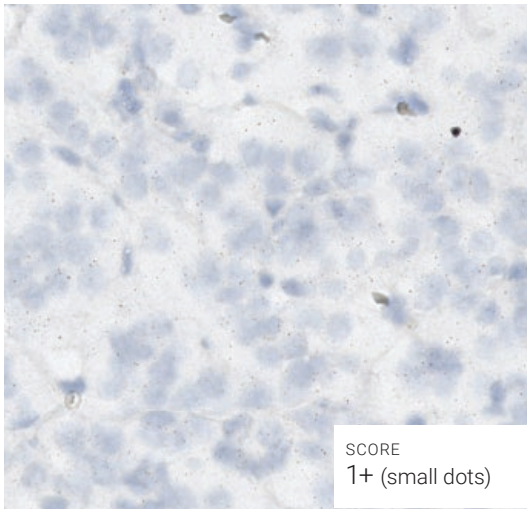
Control probe: **DapB**



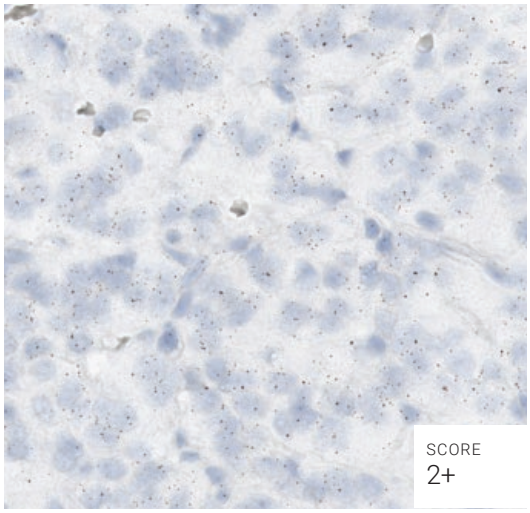
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Adrenal gland–medulla
Pretreatment: Standard

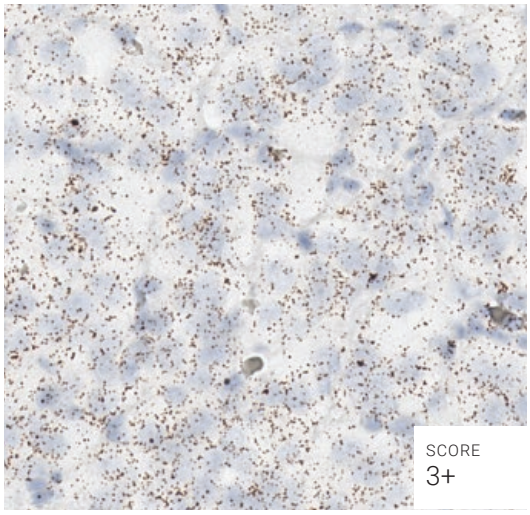
Control probe: **Cl-PPIB**



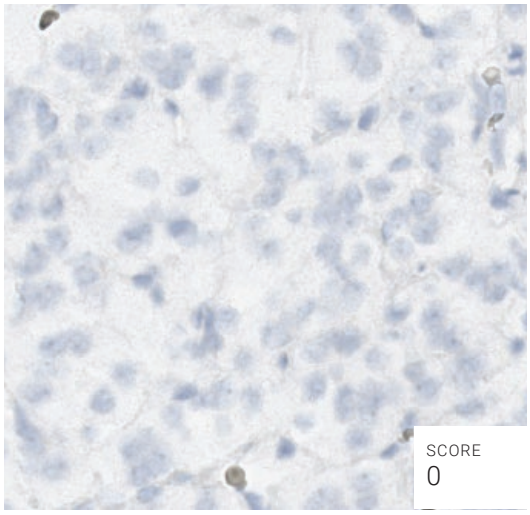
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



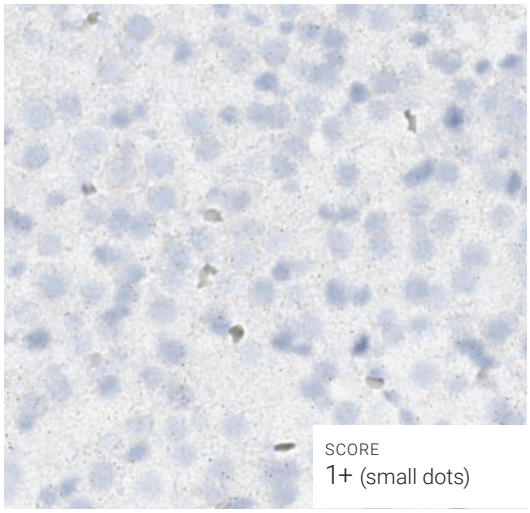
Control probe: **DapB**



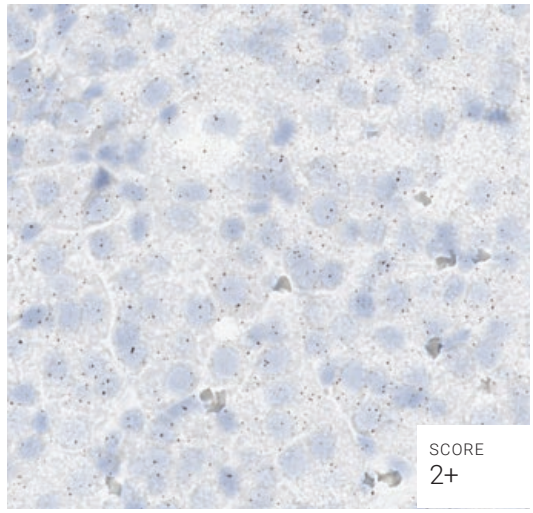
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Adrenal gland–cortex
Pretreatment: Standard

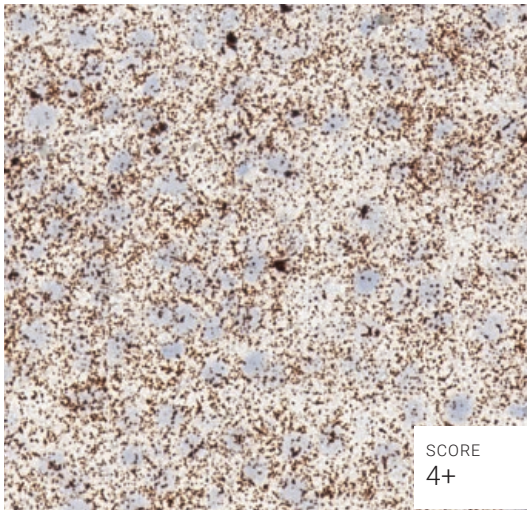
Control probe: **Cl-PPIB**



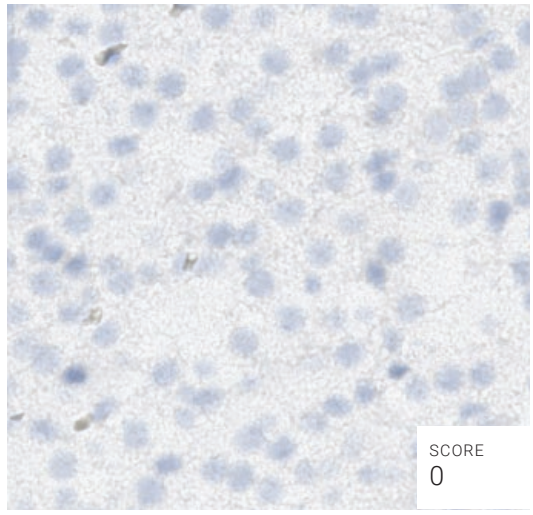
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



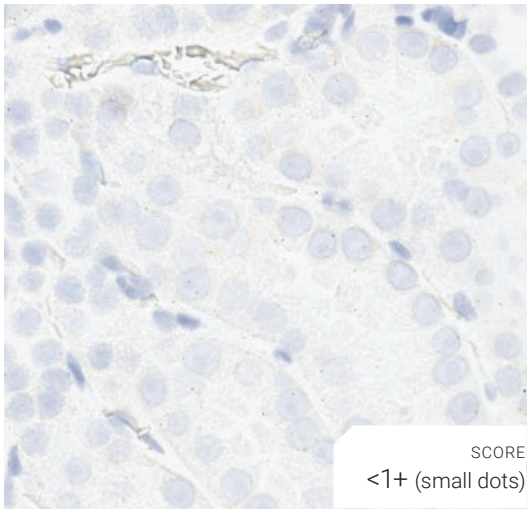
Control probe: **DapB**



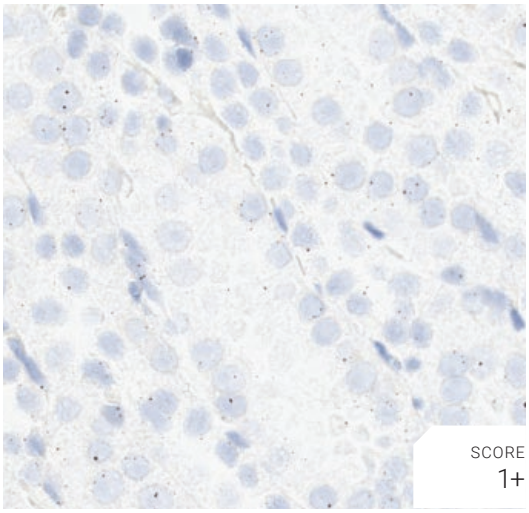
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Kidney
Pretreatment: Standard

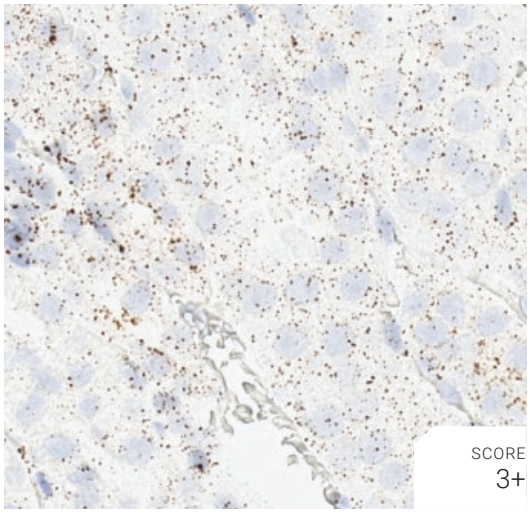
Control probe: **Cl-PPIB**



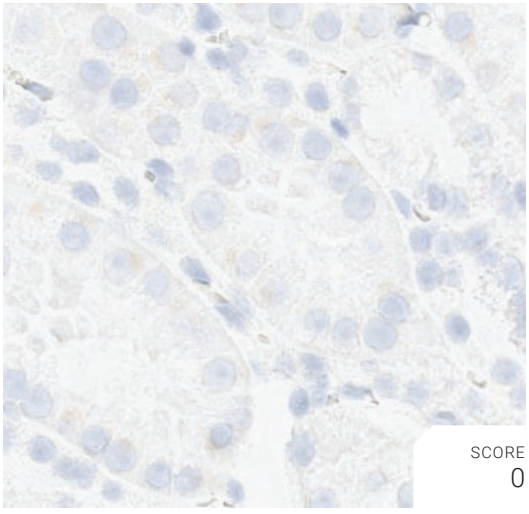
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



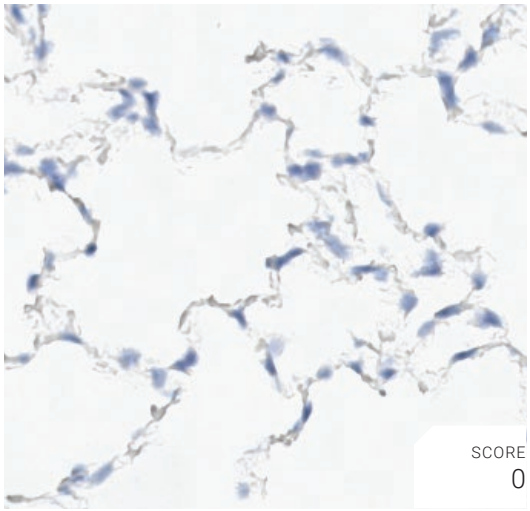
Control probe: **DapB**



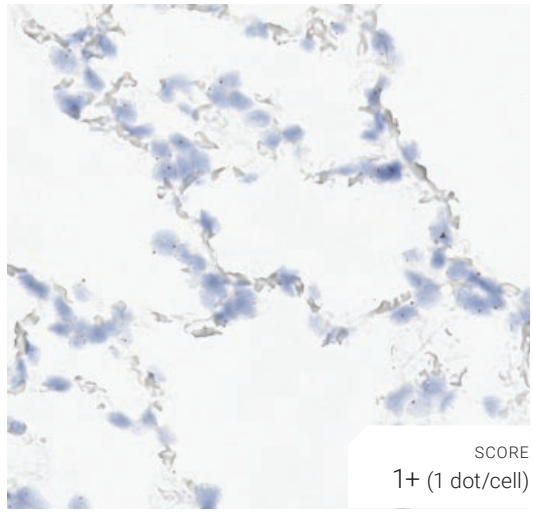
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Lung
Pretreatment: Standard

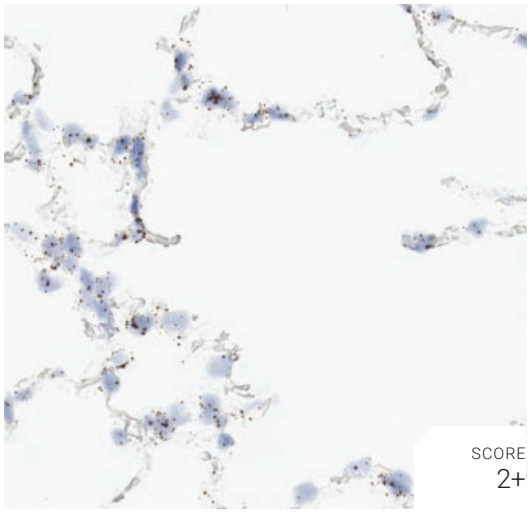
Control probe: **Cl-PPIB**



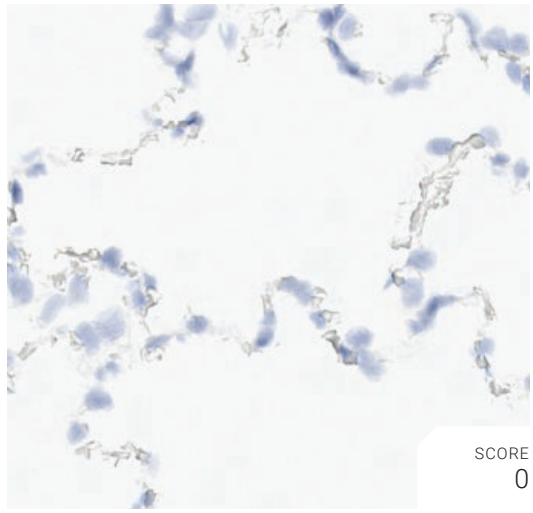
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



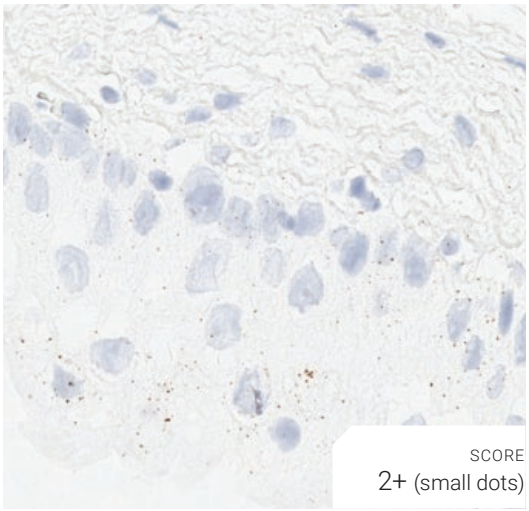
Control probe: **DapB**



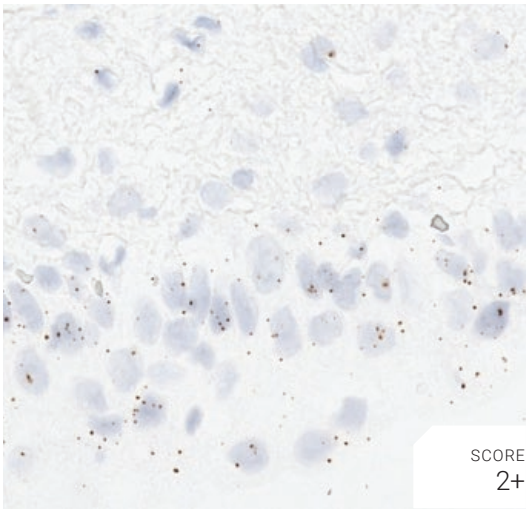
Recommended Positive Control Probe: Cl-UBC

Tissue: Urinary bladder
Pretreatment: Standard

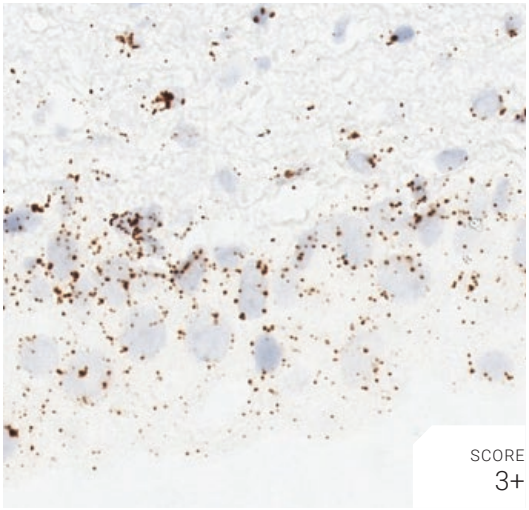
Control probe: **Cl-PPIB**



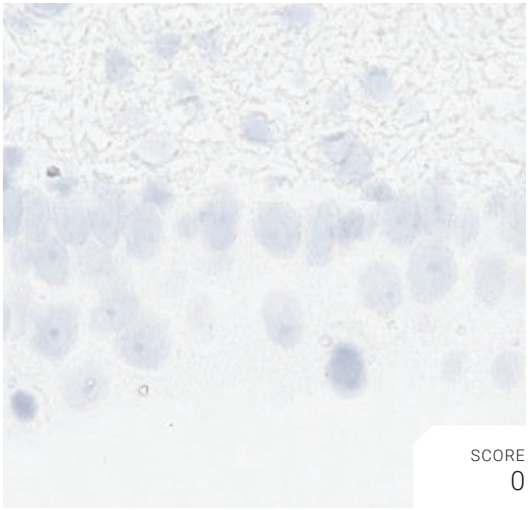
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



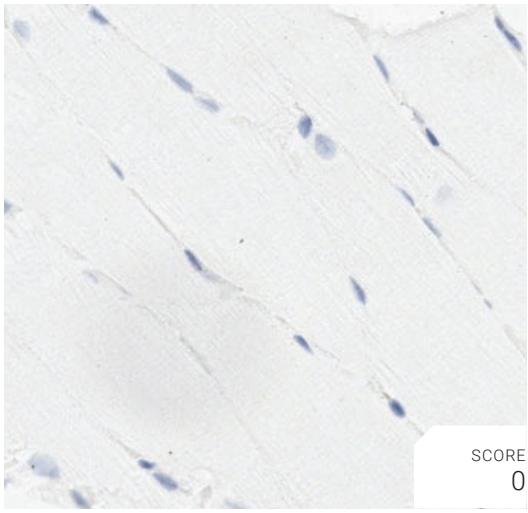
Control probe: **DapB**



Recommended Positive Control Probe: Cl-POLR2A

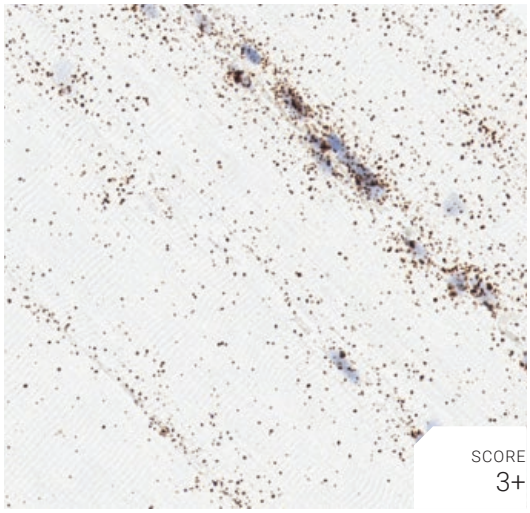
Tissue: Skeletal muscle
Pretreatment: Standard

Control probe: **Cl-PPIB**

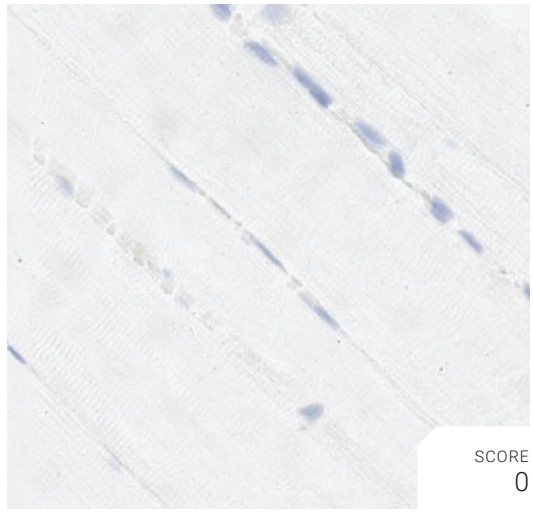


Control probe: **Cl-POLR2A**

Control probe: **Cl-UBC**



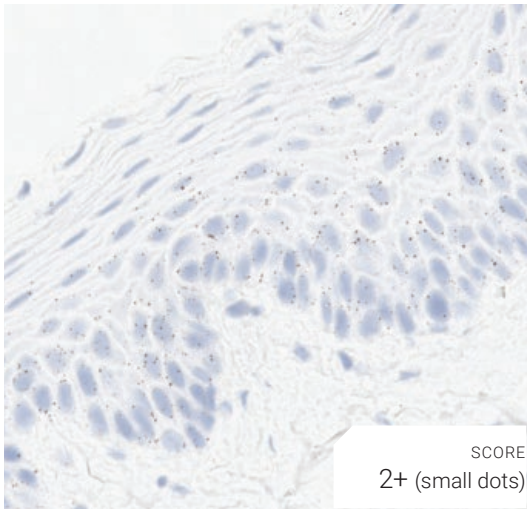
Control probe: **DapB**



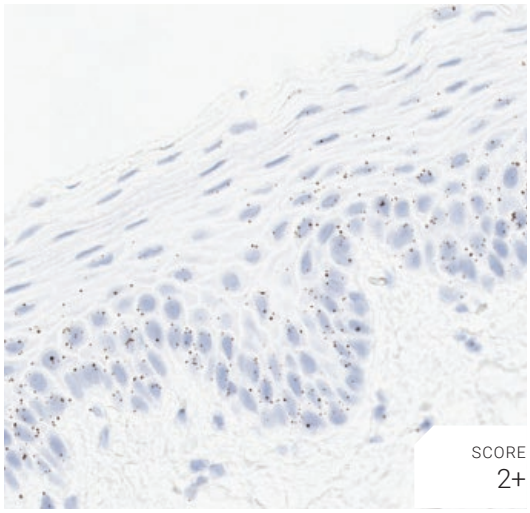
Recommended Positive Control Probe: Cl-UBC

Tissue: Esophagus
Pretreatment: Standard

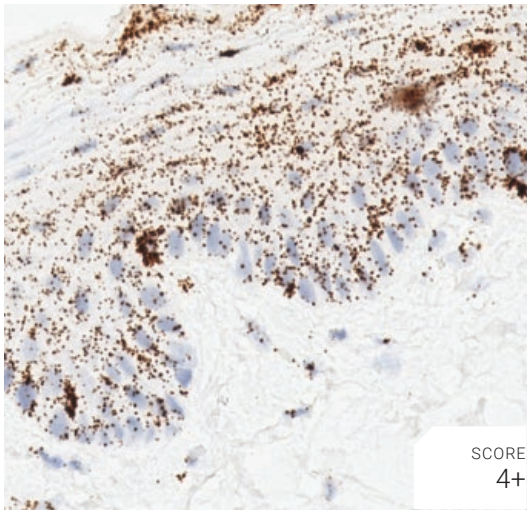
Control probe: **Cl-PPIB**



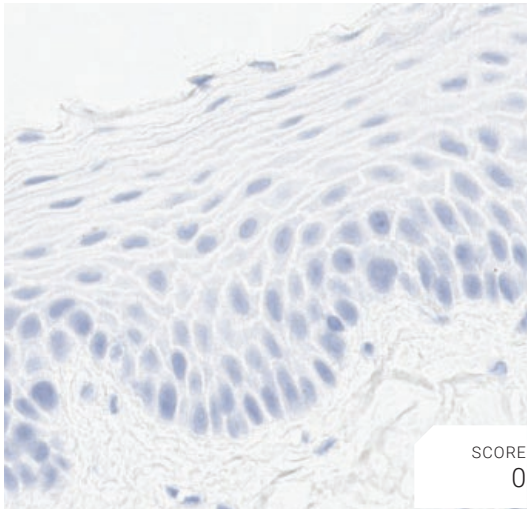
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



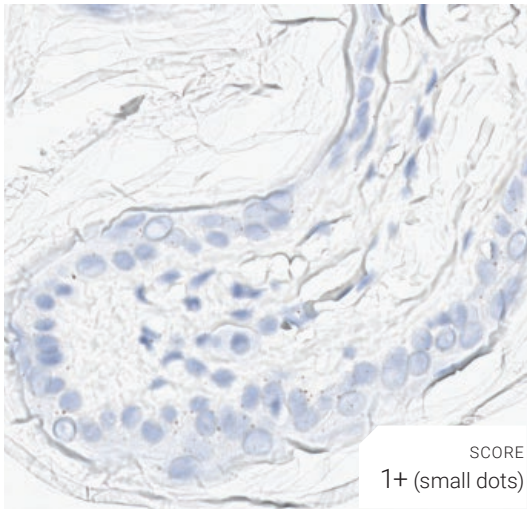
Control probe: **DapB**



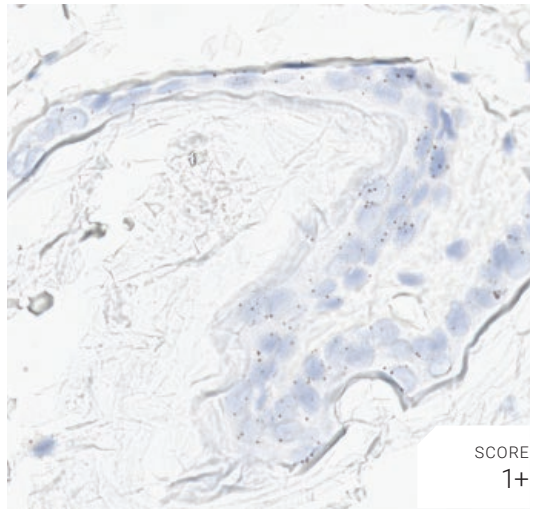
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Skin
Pretreatment: Standard

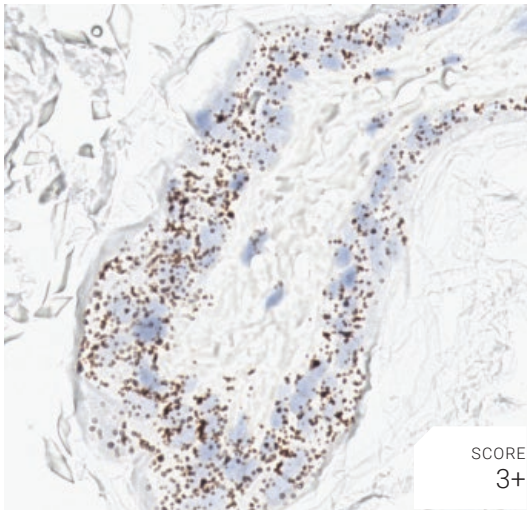
Control probe: **Cl-PPIB**



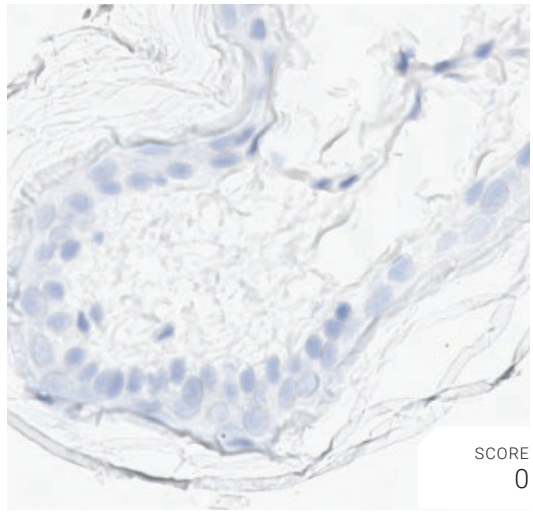
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



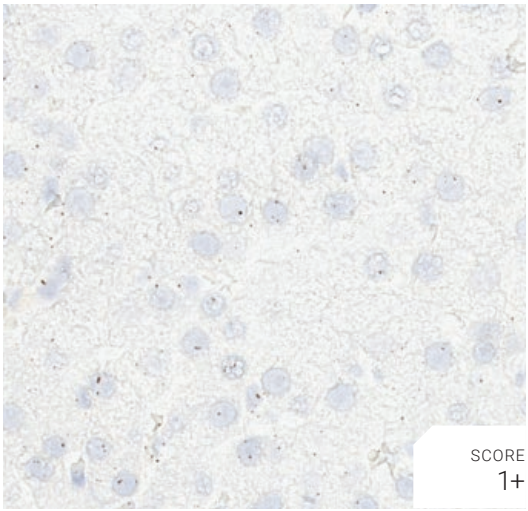
Control probe: **DapB**



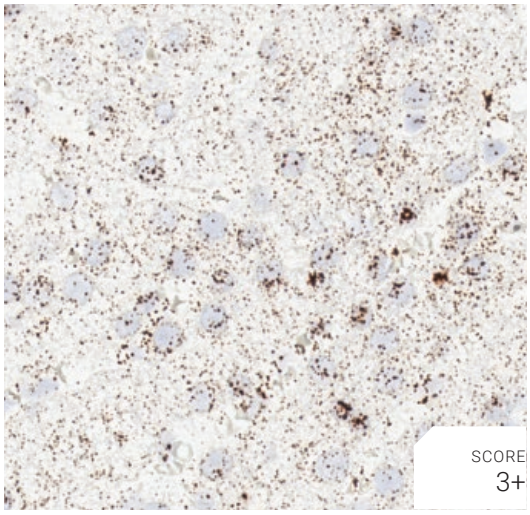
Recommended Positive Control Probe: Cl-POLR2A

Tissue: Liver
Pretreatment: Standard

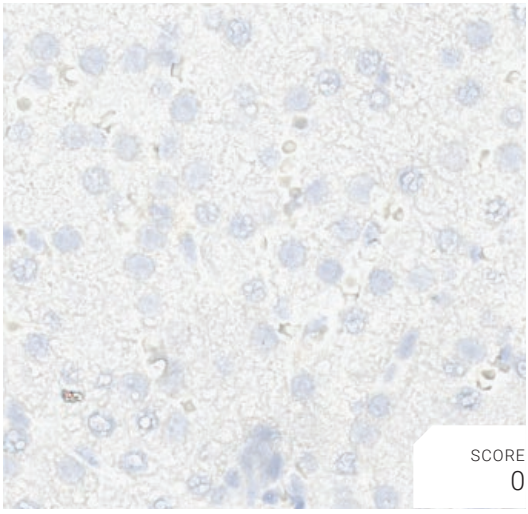
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



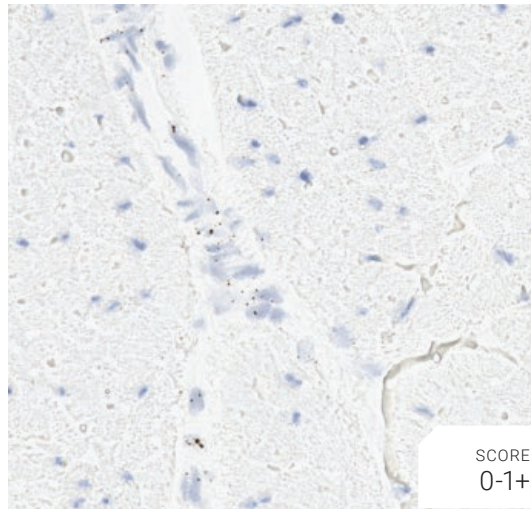
Control probe: **DapB**



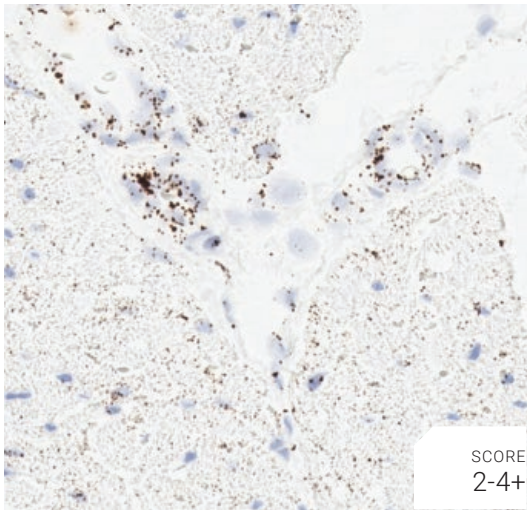
Recommended Positive Control Probe: Cl-POLR/Cl-UBC

Tissue: Heart
Pretreatment: Standard

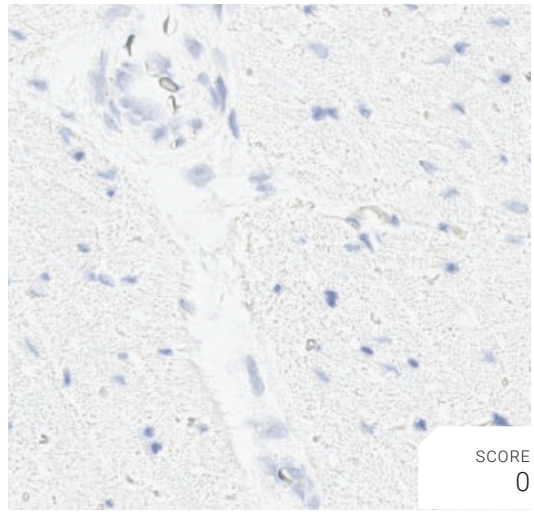
Control probe: **Cl-POLR2A**



Control probe: **Cl-UBC**



Control probe: **DapB**



Recommended Positive Control Probe: Cl-UBC

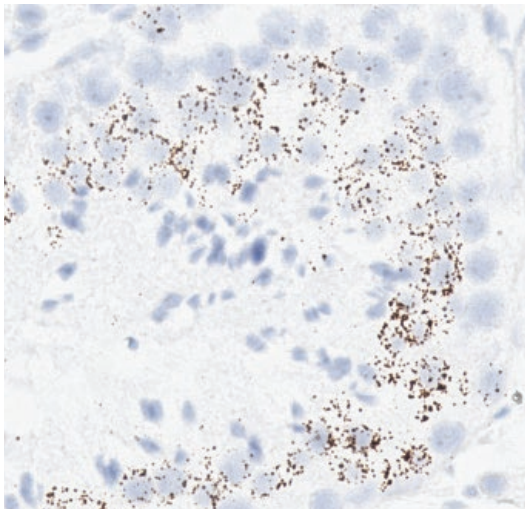
Dog

Canis lupus

The following pages show the proliferation and apoptosis marker images using the optimal pretreatment condition for each tissue.

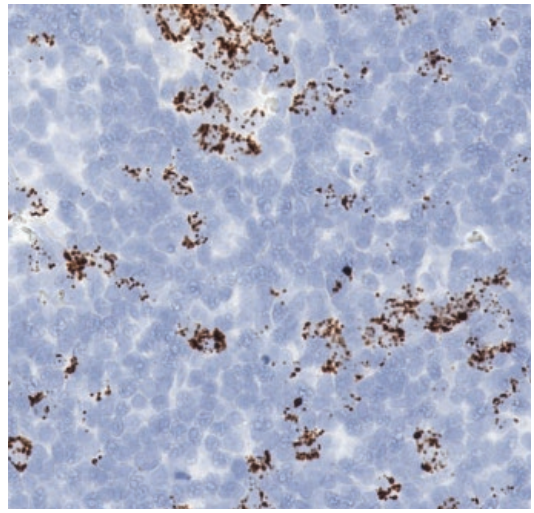
Tissue: Testis

Target probe: **CI-MK167 (Ki-67)**



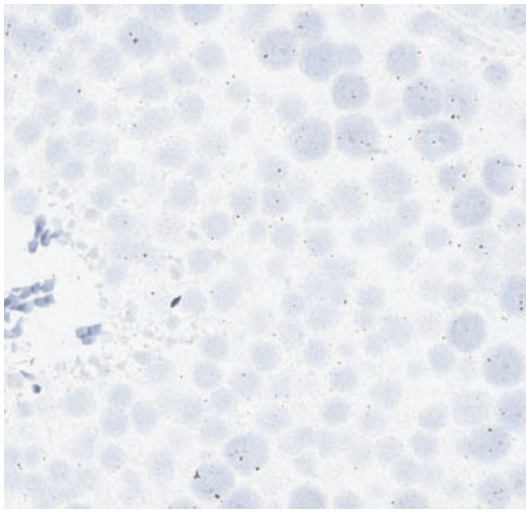
Tissue: Lymph node

Target probe: **CI-MK167 (Ki-67)**



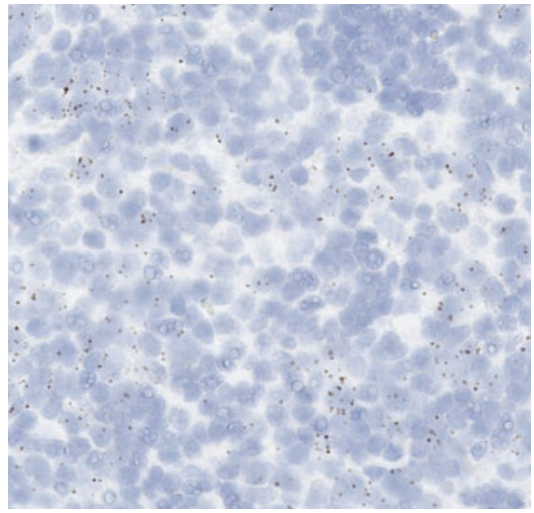
Tissue: Testis

Target probe: **CI-CCNE1 (Cyclin E1)**



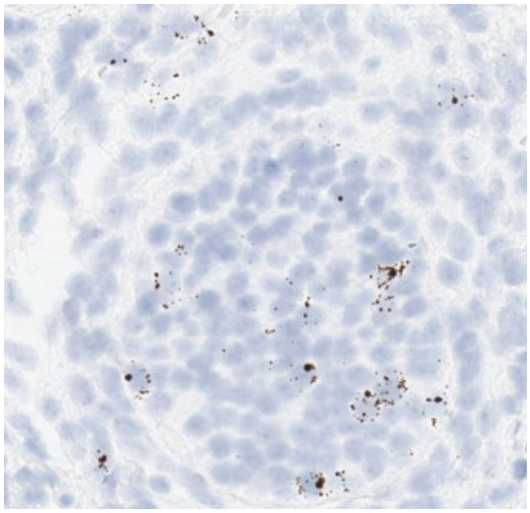
Tissue: Lymph node

Target probe: **CI-CCNE1 (Cyclin E1)**



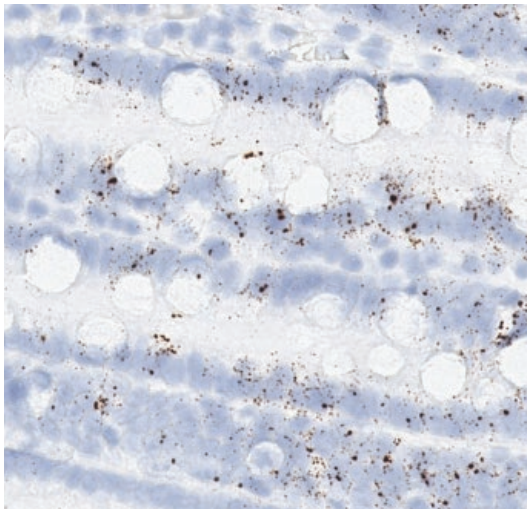
Tissue: Ovary

Target probe: **CI-MK167 (Ki-67)**



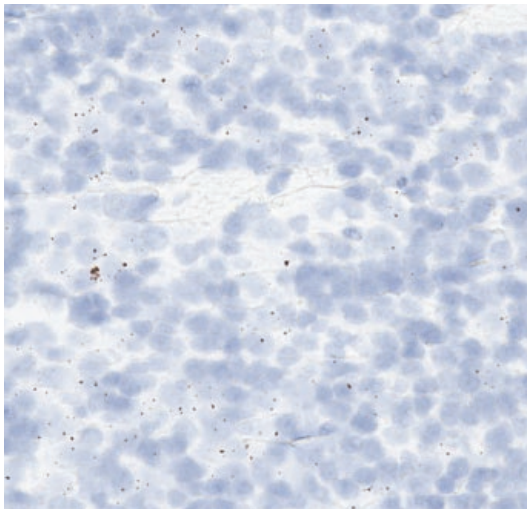
Tissue: Duodenum

Target probe: **CI-MK167 (Ki-67)**



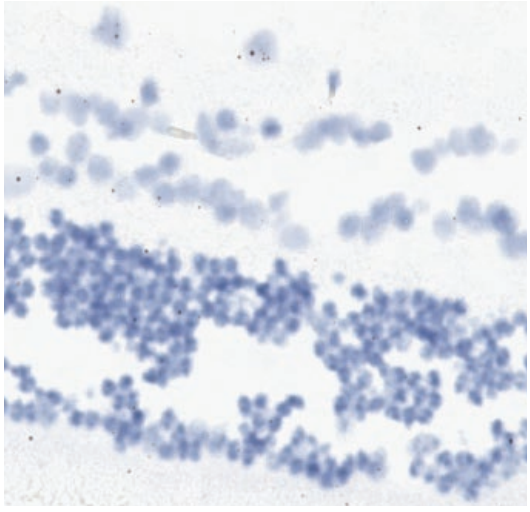
Tissue: Colon (lymphoid tissue)

Target probe: **CI-CCNE1 (Cyclin E1)**



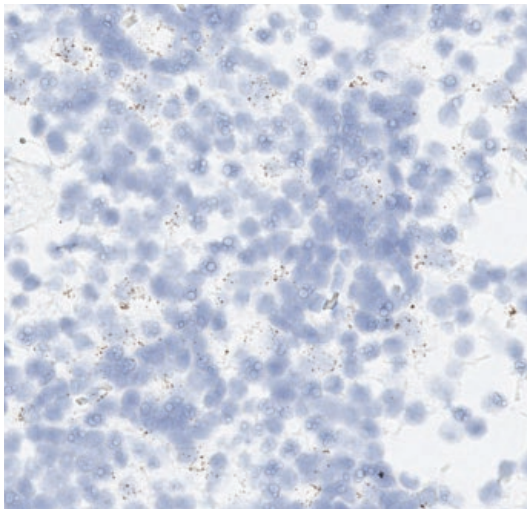
Tissue: Retina

Target probe: **CI-CCNE1 (Cyclin E1)**



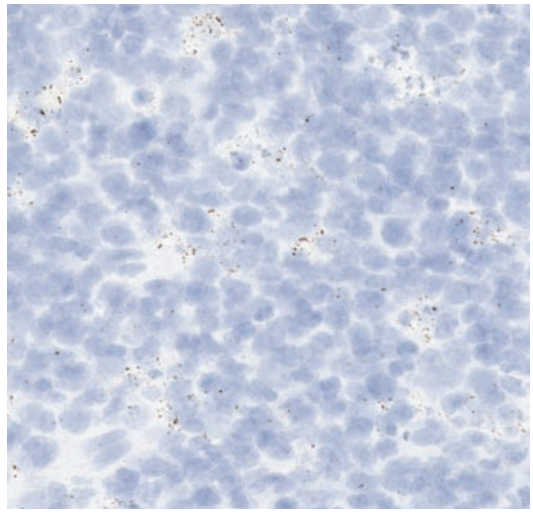
Tissue: Lymph node

Target probe: **CI-CD68**



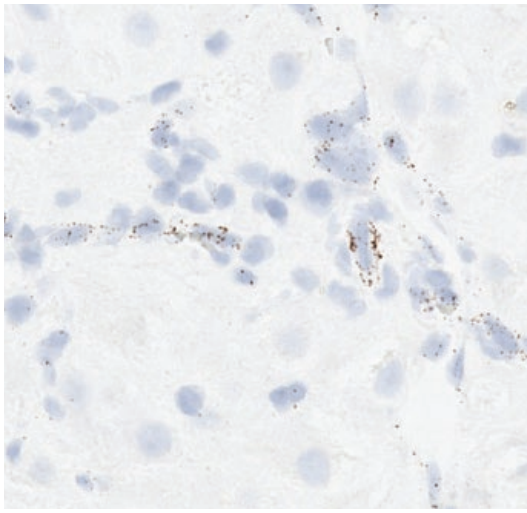
Tissue: Colon (lymphoid tissue)

Target probe: **CI-CD68**



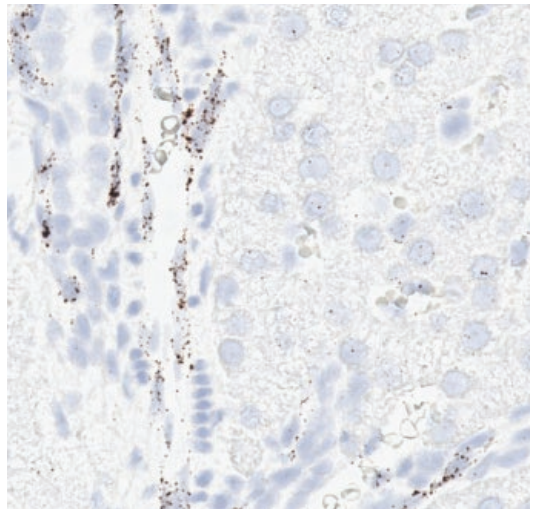
Tissue: Ovary

Target probe: **CI-PECAM1 (CD31)**



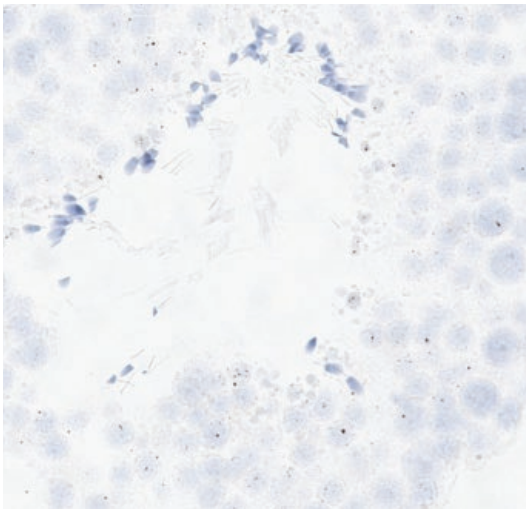
Tissue: Liver

Target probe: **CI-PECAM1 (CD31)**



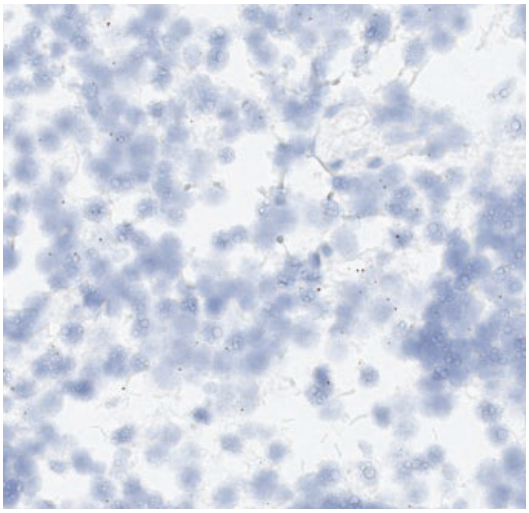
Tissue: Testis

Target probe: **CI-BBC3 (PUMA)**



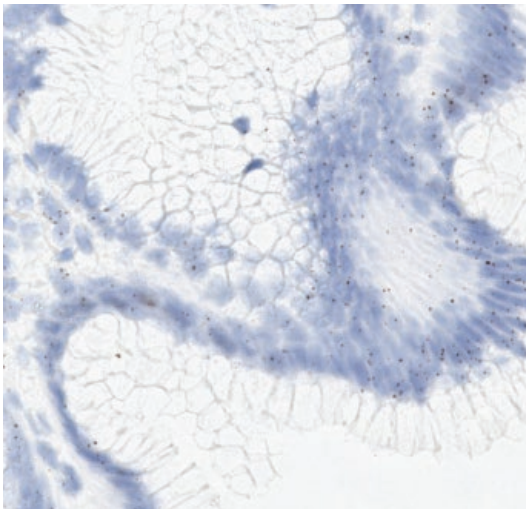
Tissue: Lymph node

Target probe: **CI-BBC3 (PUMA)**



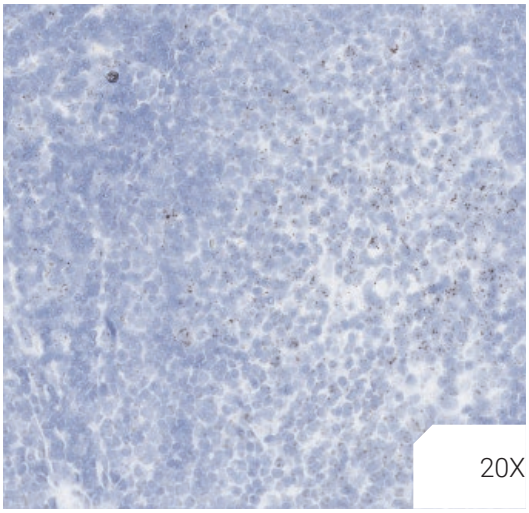
Tissue: Stomach

Target probe: **CI-Fas (CD95)**



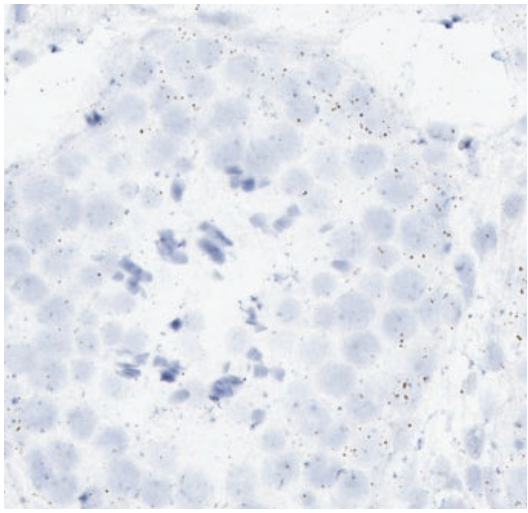
Tissue: Lymph node

Target probe: **CI-Fas (CD95)**



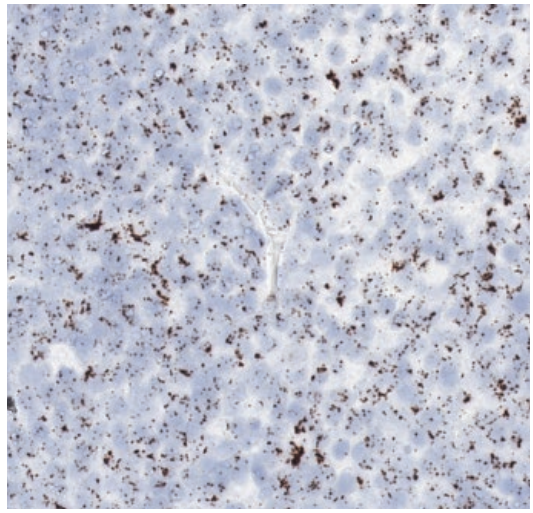
Tissue: Testis

Target probe: **CI-TNFRSF10B (DR5)**



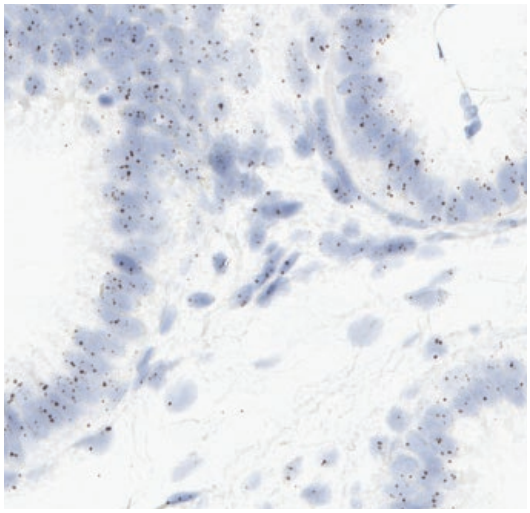
Tissue: Lymph node

Target probe: **CI-TNFRSF10B (DR5)**



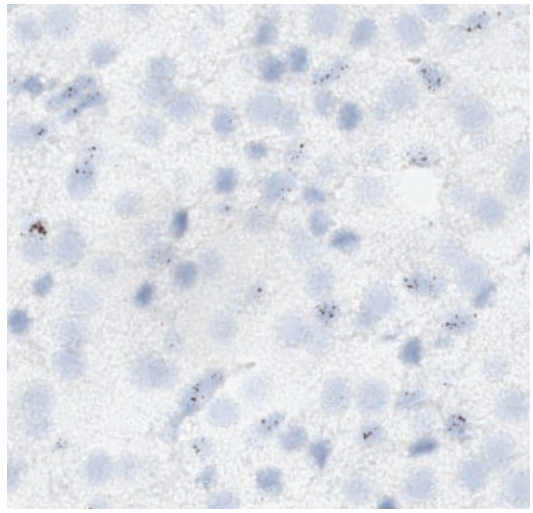
Tissue: Epididymis

Target probe: **CI-TNFRSF10B (DR5)**



Tissue: Adrenal gland

Target probe: **CI-TNFRSF10B (DR5)**



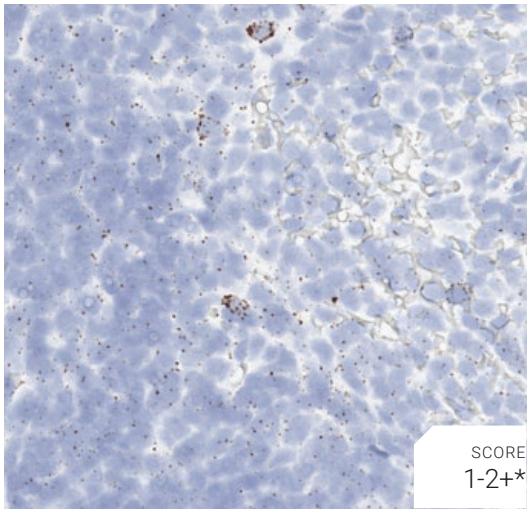
Cynomolgus monkey

Macaca fascicularis

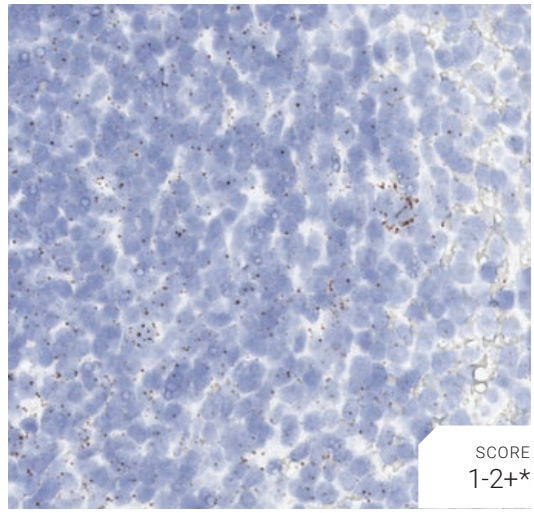
The following pages show positive control probe images with standard and mild pretreatment conditions. Optimal pretreatment condition is indicated for each tissue type.

Positive control probe: Hs-PPIB
Tissue: Spleen

Pretreatment: **Standard**



Pretreatment: **Mild**

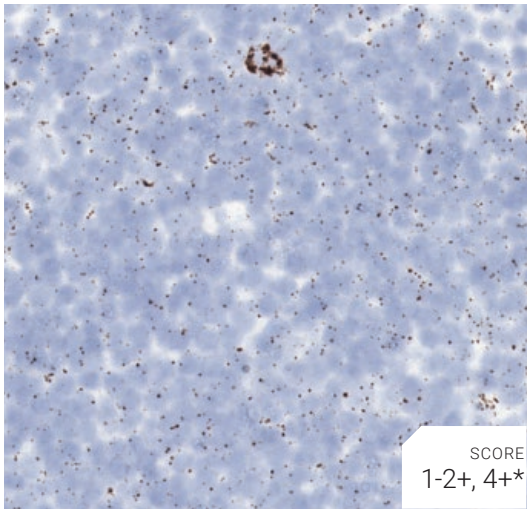


Observation: Loss of cell boundary using standard pretreatment.

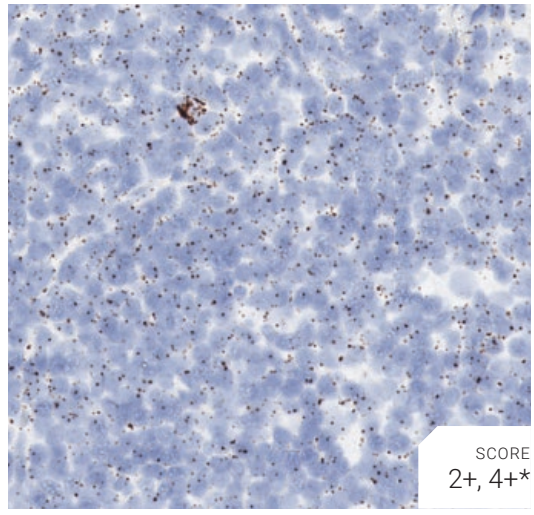
Optimal Pretreatment: Mild

Positive control probe: Hs-PPIB
Tissue: Lymph node

Pretreatment: **Standard**



Pretreatment: **Mild**



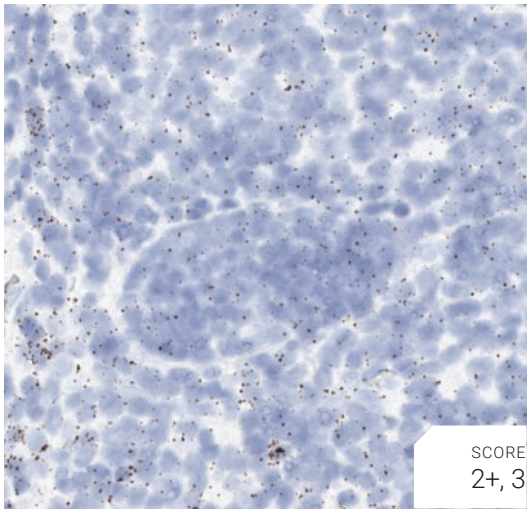
Observation: Loss of cell boundary using standard pretreatment. Signal: Mild ≥ Standard

Optimal Pretreatment: Mild

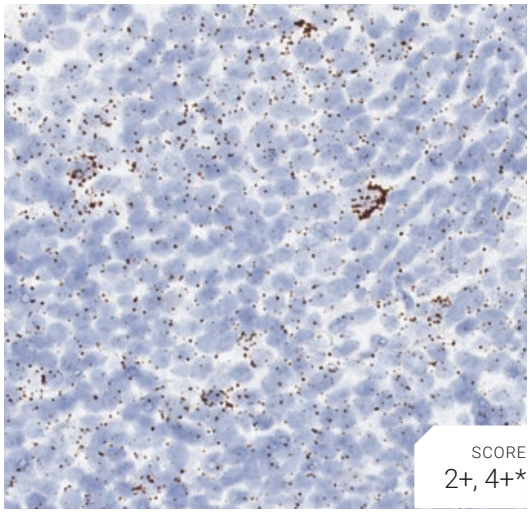
*A few cells have higher Ppib signals

Positive control probe: Hs-PPIB
Tissue: Tonsil

Pretreatment: **Standard**



Pretreatment: **Mild**

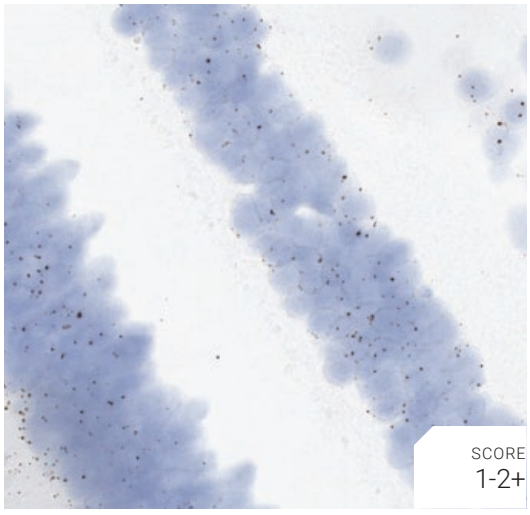


Observation: Loss of cell boundary using standard pretreatment. Signal: Mild > Standard

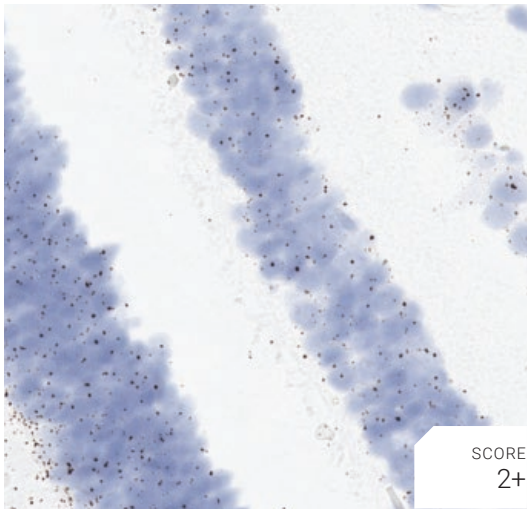
Optimal Pretreatment: Mild

Positive control probe: Hs-POLR2A**
Tissue: Retina

Pretreatment: **Standard**



Pretreatment: **Mild**



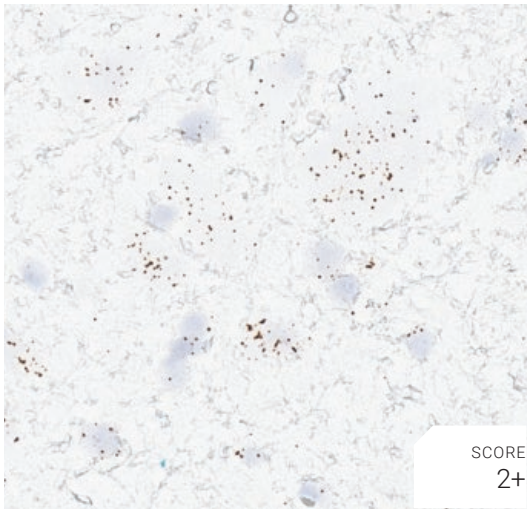
Observation: Loss of cell boundary using standard pretreatment. Signal: Mild ≥ Standard

Optimal Pretreatment: Mild

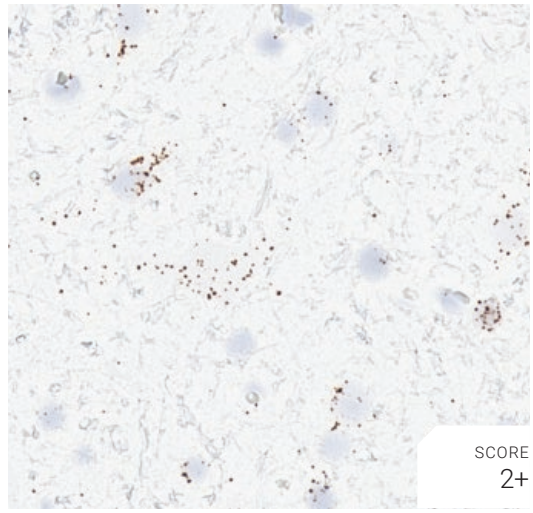
*A few cells have higher PPIB signals **Retina expresses low level of PPIB

Positive control probe: Hs-PPIB
Tissue: Spinal cord–grey matter

Pretreatment: **Standard**



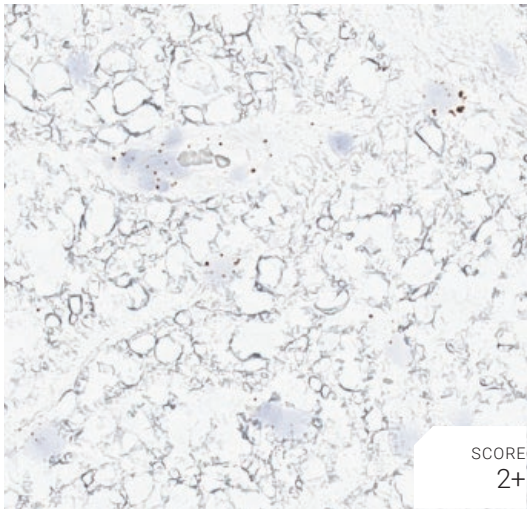
Pretreatment: **Mild**

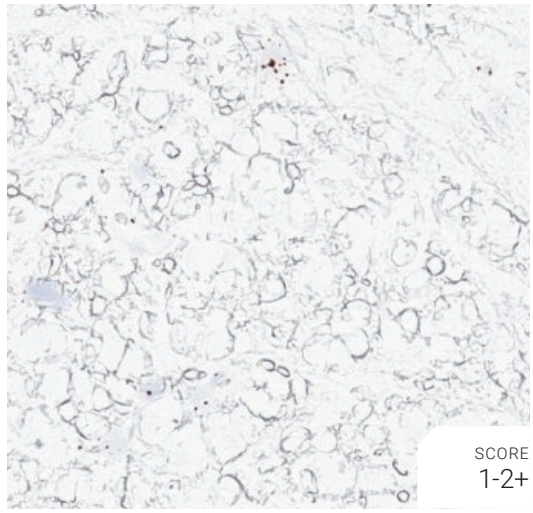


Optimal Pretreatment: Standard

Positive control probe: Hs-PPIB
Tissue: Spinal cord–white matter

Pretreatment: **Standard**



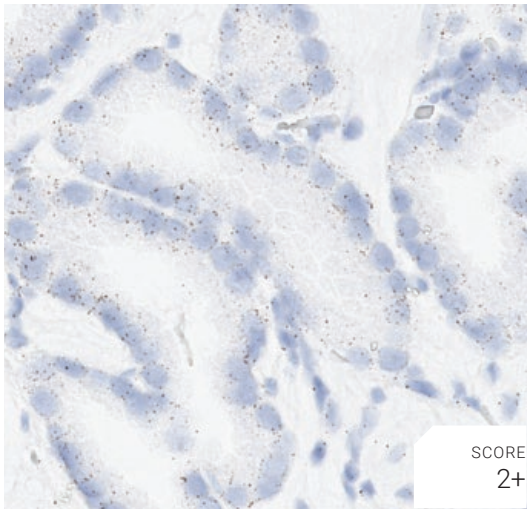


Observation: Standard pretreatment increases staining in white matter.

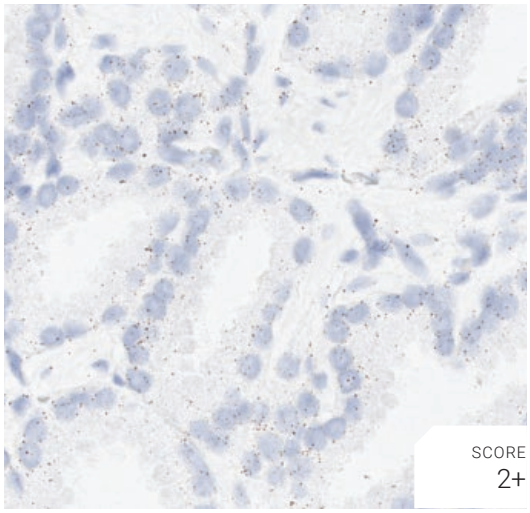
Optimal Pretreatment: Standard

Positive control probe: Hs-PPIB
Tissue: Prostate gland

Pretreatment: **Standard**

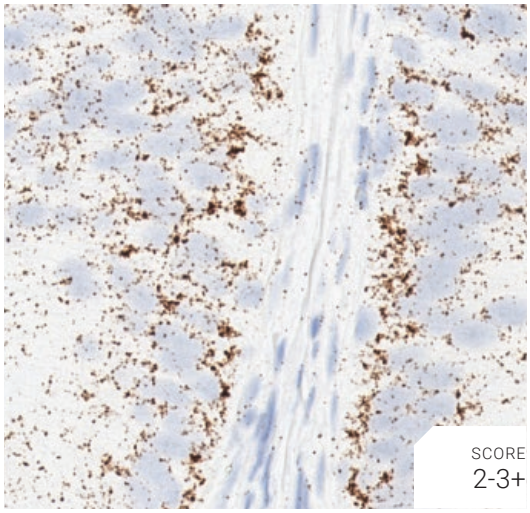


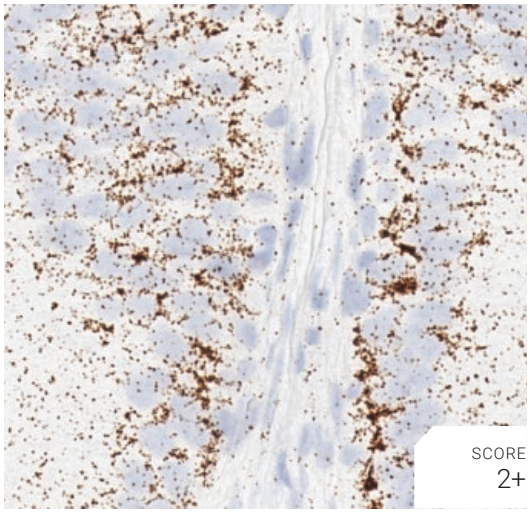
Pretreatment: **Mild**



Optimal Pretreatment: Mild*

Positive control probe: Hs-PPIB
Tissue: Epididymis



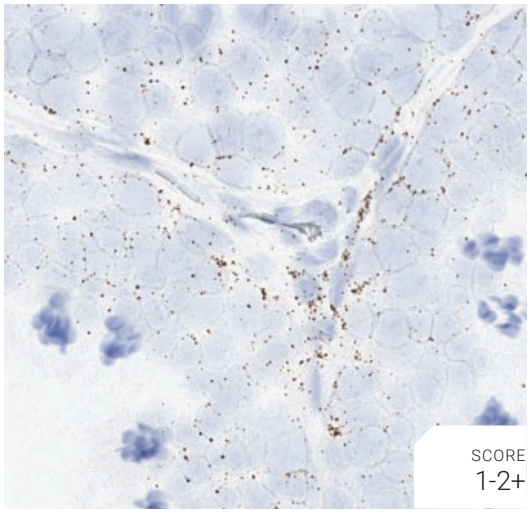


Optimal Pretreatment: Mild*

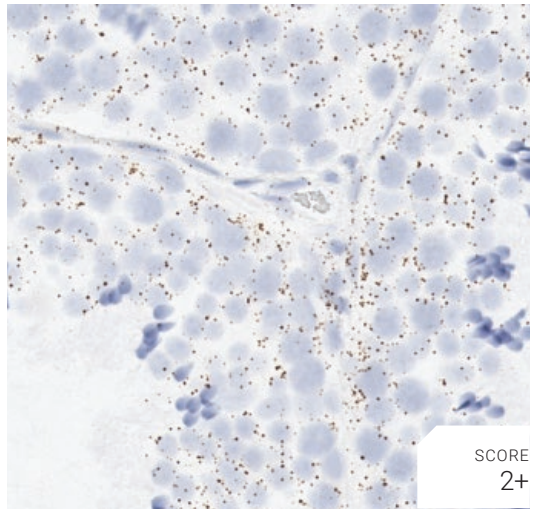
*Standard pretreatment may also be suitable

Positive control probe: Hs-PPIB
Tissue: Testis

Pretreatment: **Standard**



Pretreatment: **Mild**

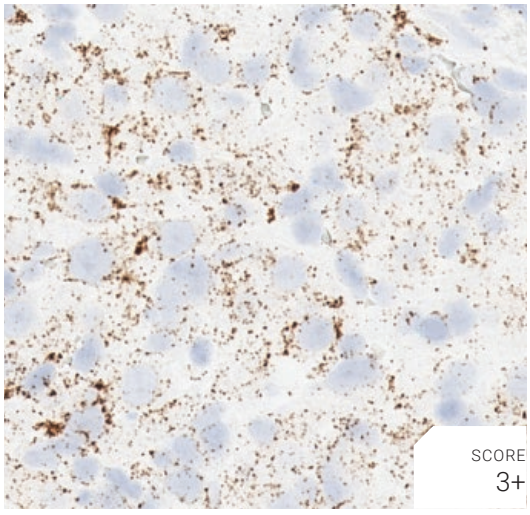


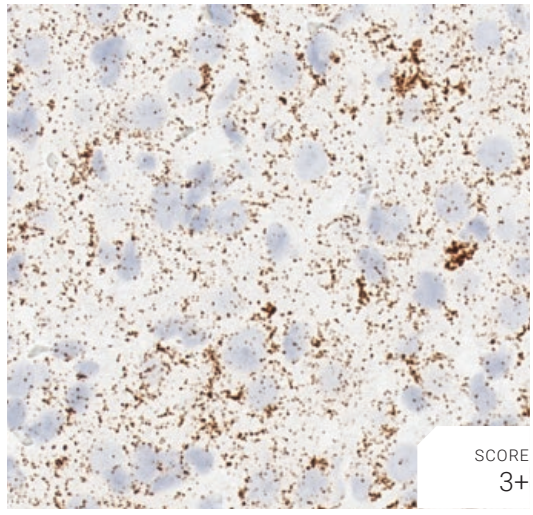
Observation: Loss of cell boundary using standard pretreatment. Signal: Mild > Standard

Optimal Pretreatment: Mild

Positive control probe: Hs-PPIB
Tissue: Ovary

Pretreatment: **Standard**



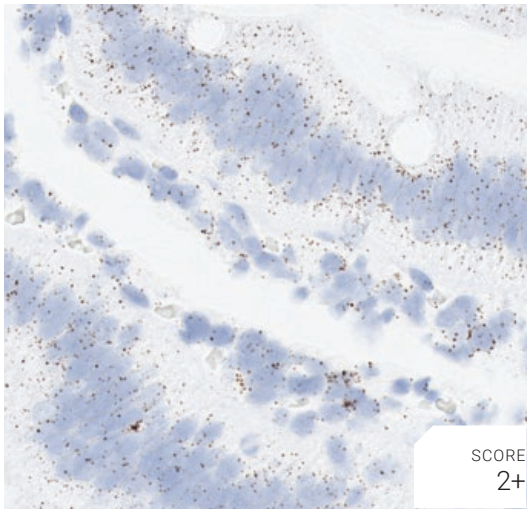


Observation: Loss of cell boundary using standard pretreatment. Signal: Mild > Standard

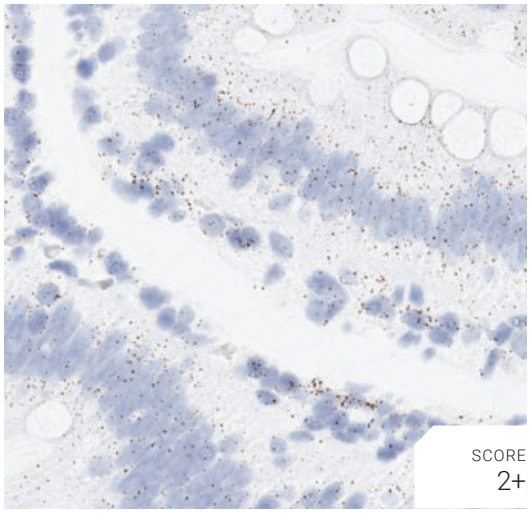
Optimal Pretreatment: Mild

Positive control probe: Hs-PPIB
Tissue: Duodenum

Pretreatment: **Standard**

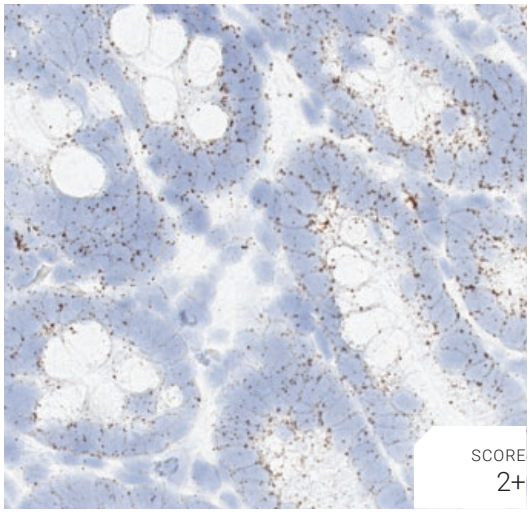


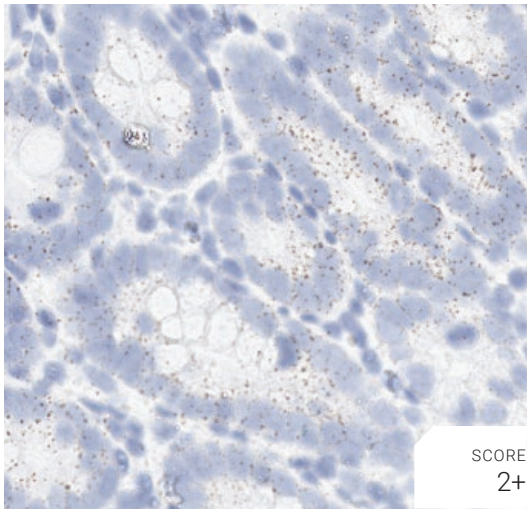
Pretreatment: **Mild**



Optimal Pretreatment: Mild*

Positive control probe: Hs-PPIB
Tissue: Jejunum





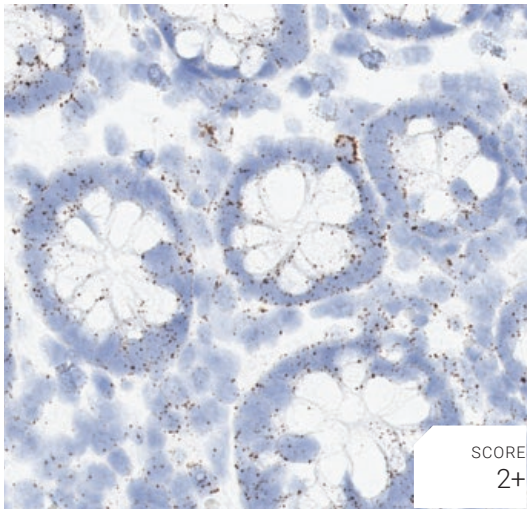
Observation: Loss of cell boundary using standard pretreatment.

Optimal Pretreatment: Mild*

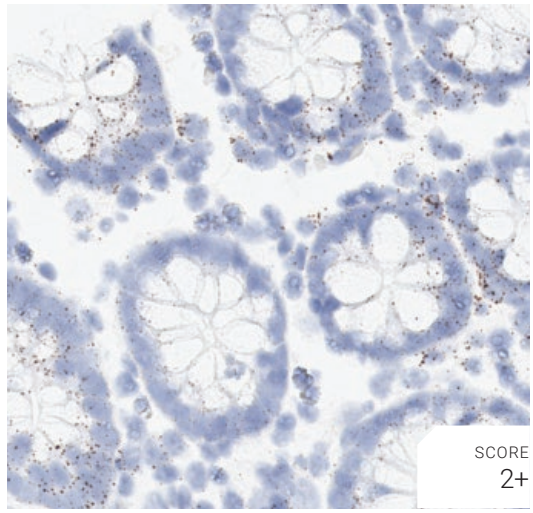
*Standard pretreatment may also be suitable

Positive control probe: Hs-PPIB
Tissue: Colon

Pretreatment: **Standard**

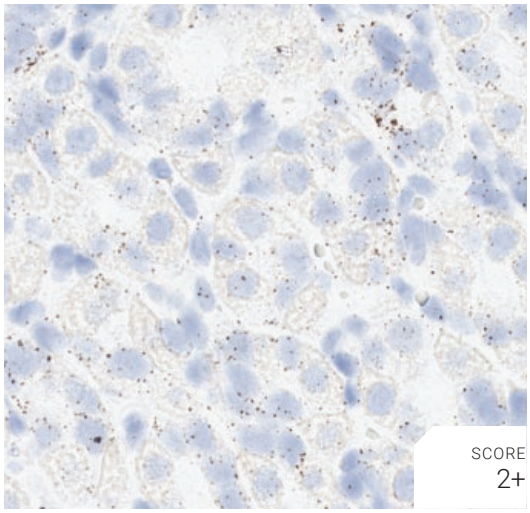


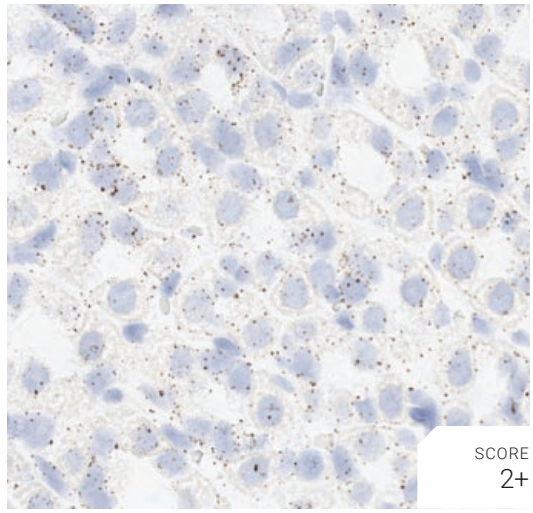
Pretreatment: **Mild**



Optimal Pretreatment: Standard*

Positive control probe: Hs-PPIB
Tissue: Stomach



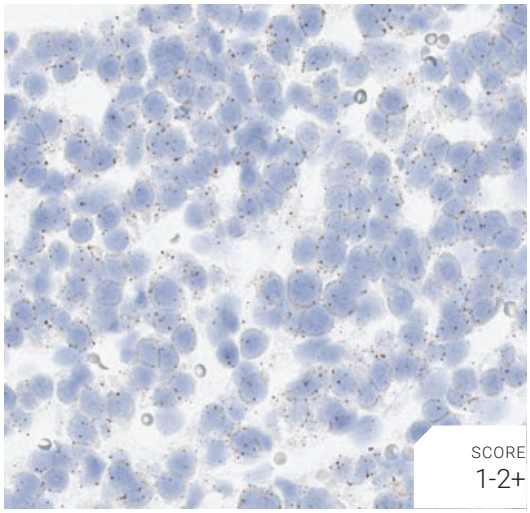


Optimal Pretreatment: Standard*

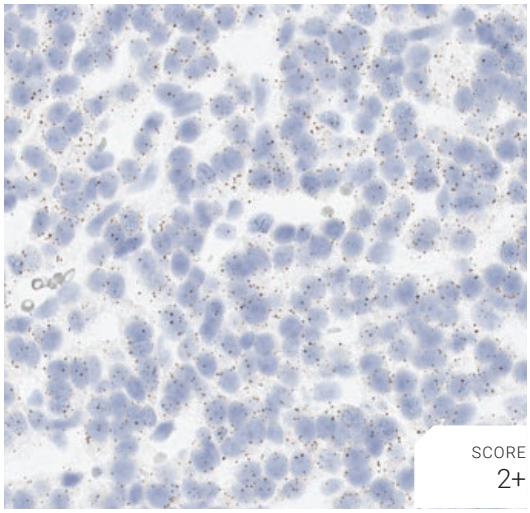
*Mild pretreatment may also suitable

Positive control probe: Hs-PPIB
Tissue: Adrenal gland

Pretreatment: **Standard**



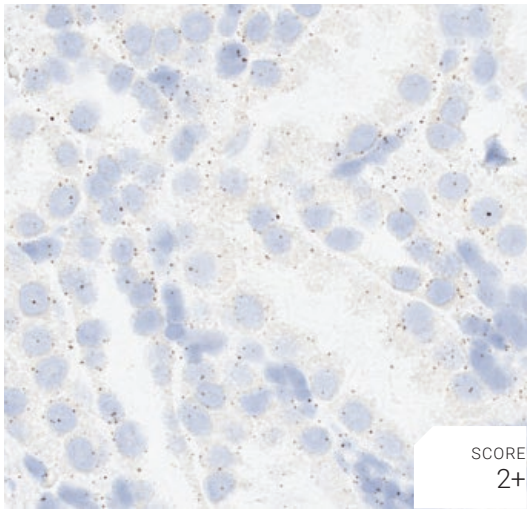
Pretreatment: **Mild**

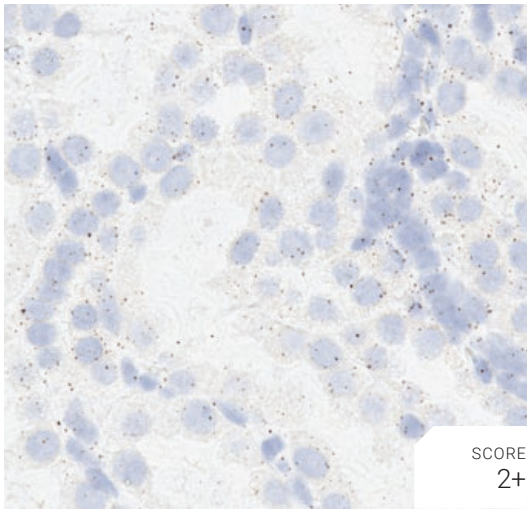


Observation: Loss of cell boundary using standard pretreatment. Signal: Mild > Standard

Optimal Pretreatment: Mild

Positive control probe: Hs-PPIB
Tissue: Kidney

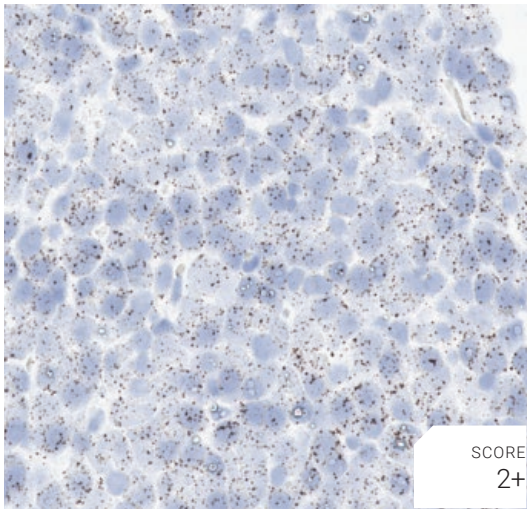




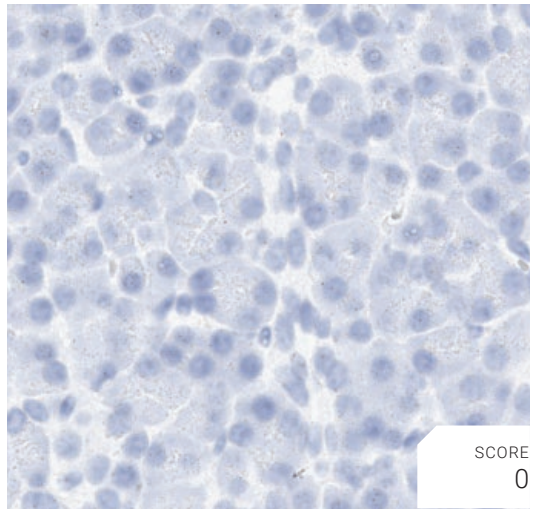
Optimal Pretreatment: Standard

Positive control probe: Hs-PPIB
Tissue: Pancreas

Pretreatment: **Standard**



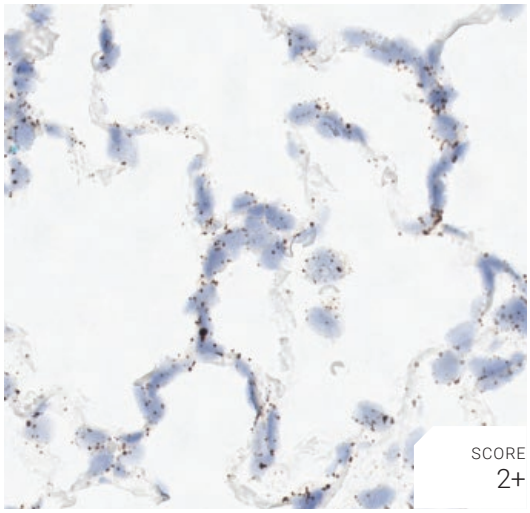
Pretreatment: **Mild**

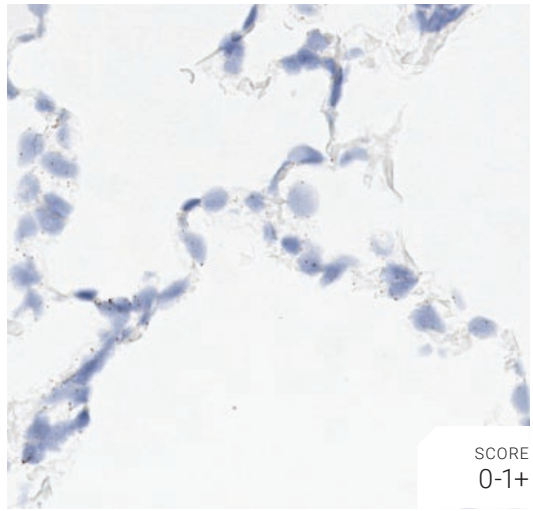


Signal: Standard > Mild

Optimal Pretreatment: Standard

Positive control probe: Hs-UBC*
Tissue: Lung





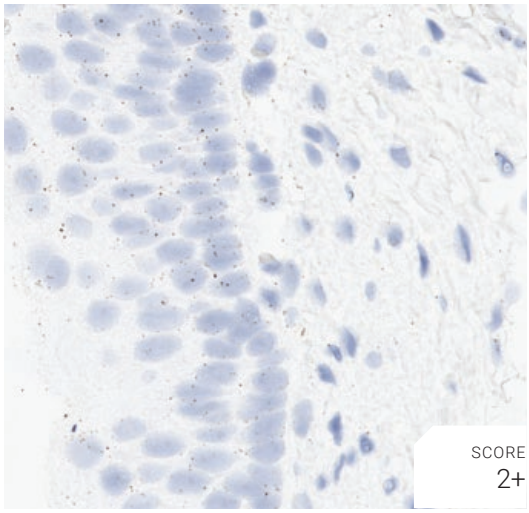
Signal: Standard ≥ Mild

Optimal Pretreatment: Standard

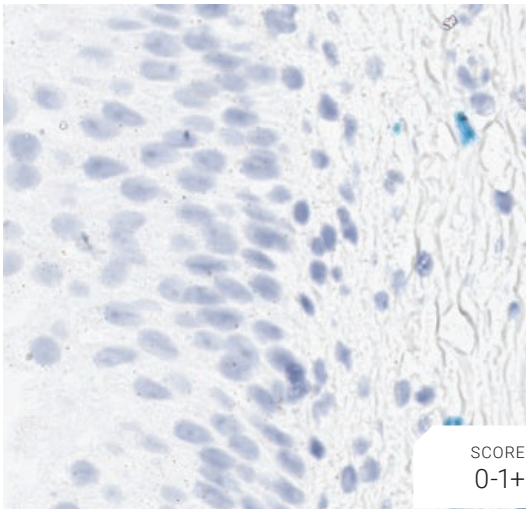
*Low PPIB detection in alveolar cells

Positive control probe: Hs-PPIB
Tissue: Urinary bladder

Pretreatment: **Standard**



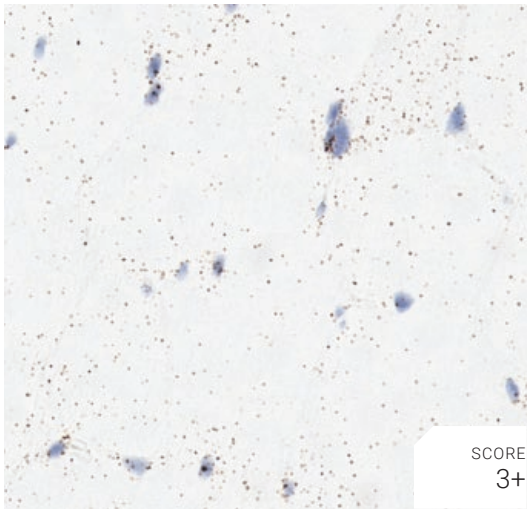
Pretreatment: **Mild**

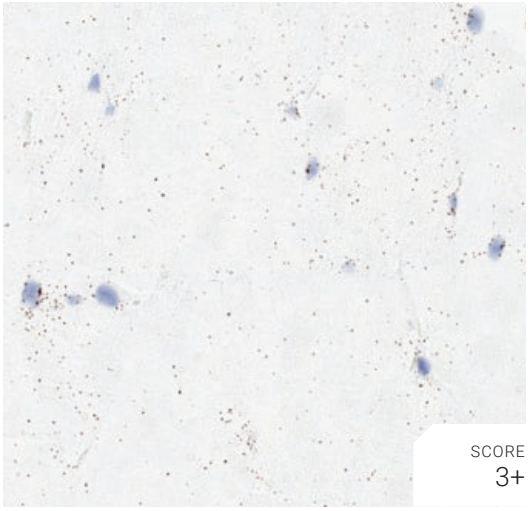


Signal: Standard > Mild

Optimal Pretreatment: Standard

Positive control probe: Hs-UBC*
Tissue: Skeletal muscle





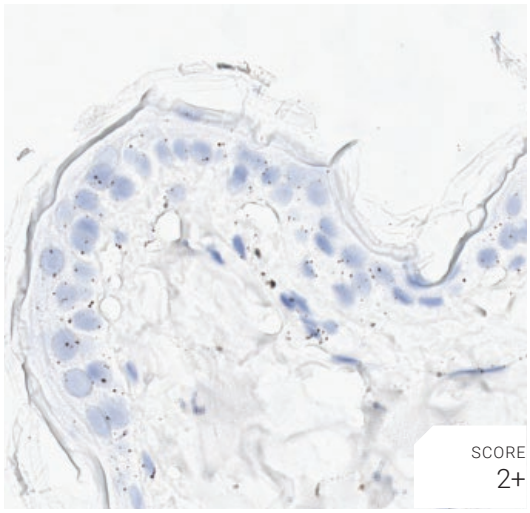
Signal: Standard > Mild

Optimal Pretreatment: Standard

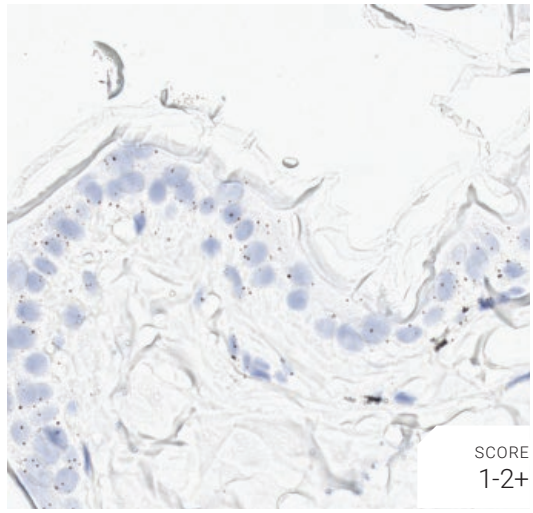
*Low PPIB expression in skeletal muscle cells

Positive control probe: Hs-PPIB
Tissue: Skin

Pretreatment: **Standard**

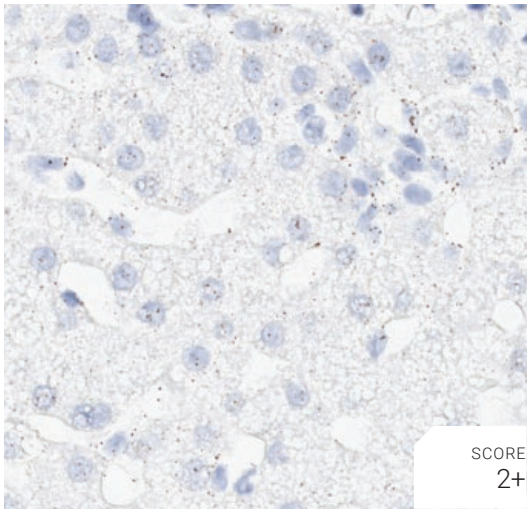


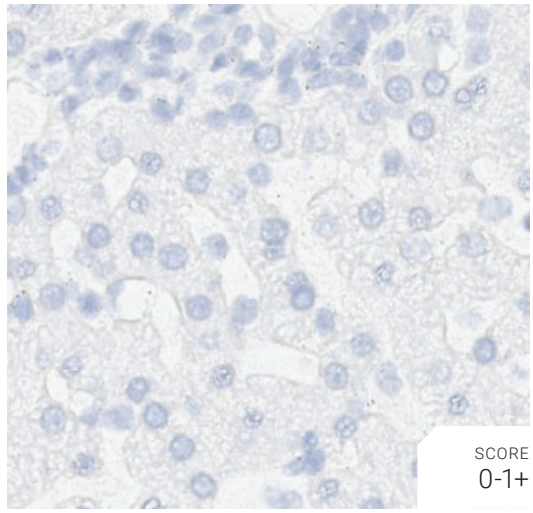
Pretreatment: **Mild**



Optimal Pretreatment: Standard

Positive control probe: Hs-PPIB
Tissue: Liver



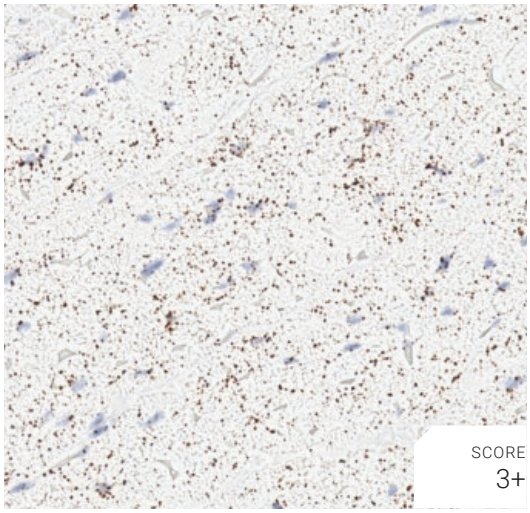


Signal: Standard > Mild

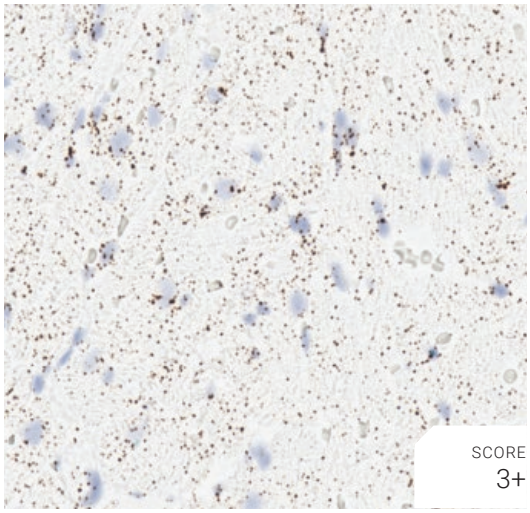
Optimal Pretreatment: Standard

Positive control probe: Hs-UBC*
Tissue: Heart

Pretreatment: **Standard**



Pretreatment: **Mild**



Signal: Standard > Mild

Optimal Pretreatment: Standard

*Low PPIB expression in heart muscle cells

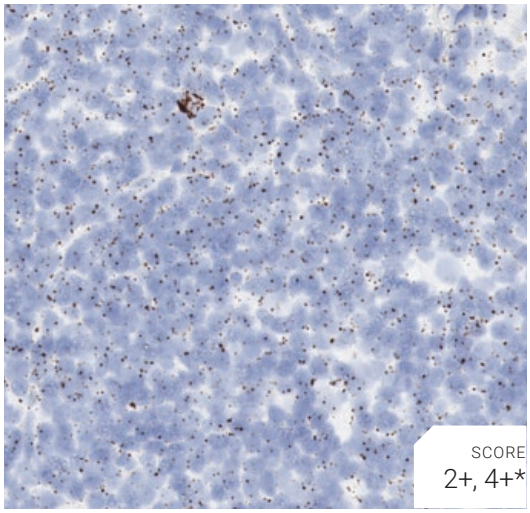
Cynomolgus monkey

Macaca fascicularis

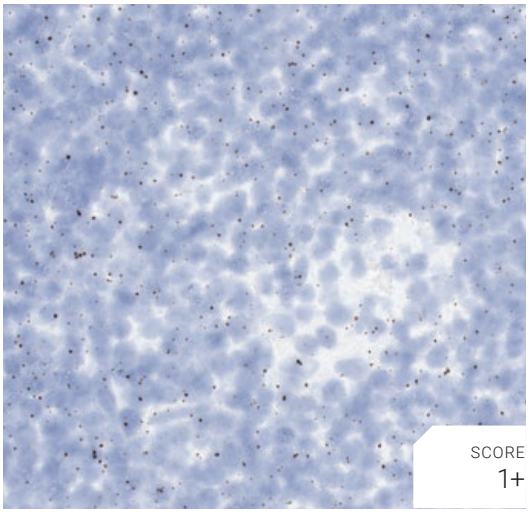
The following pages show the three positive controls (PPIB, POLR2A, UBC) and the negative control images using optimal pretreatment condition. Recommended positive control probe is indicated for each tissue type.

Tissue: Lymph node
Pretreatment: Mild

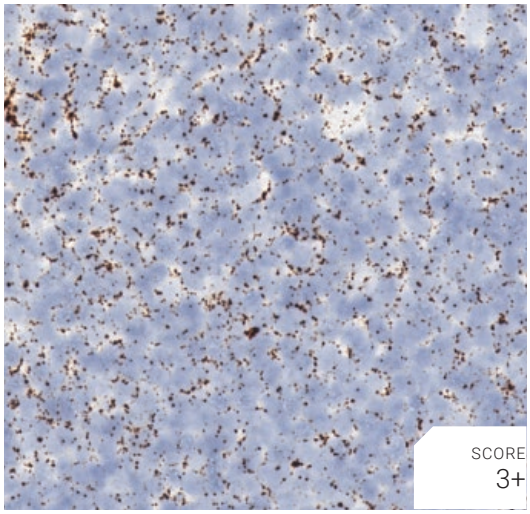
Control **probe: Hs-PPIB**



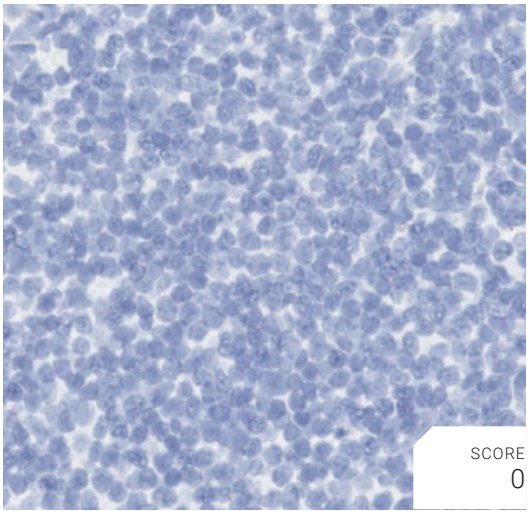
Control **probe: Hs-POLR2A**



Control **probe: Hs-UBC**



Control probe: **DapB**

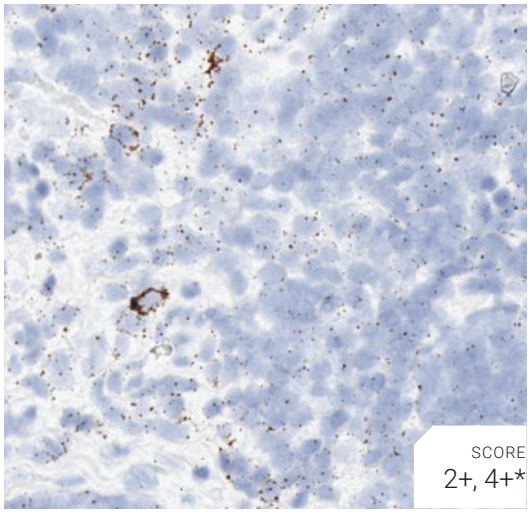


Recommended Positive Control Probe: Hs-PPIB/Hs-POLR2A*

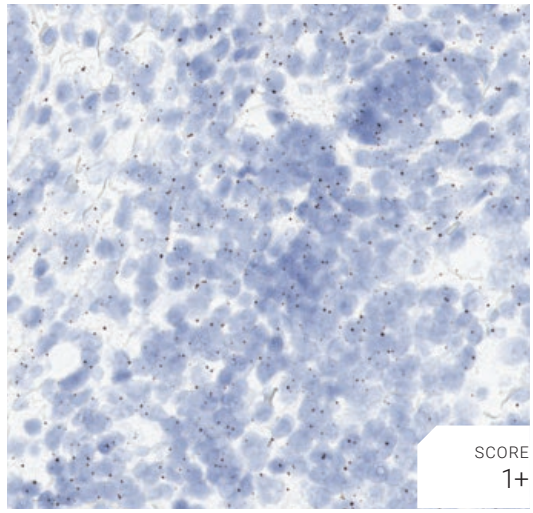
*Hs-POLR2A staining is more evenly distributed

Tissue: Tonsil
Pretreatment: Mild

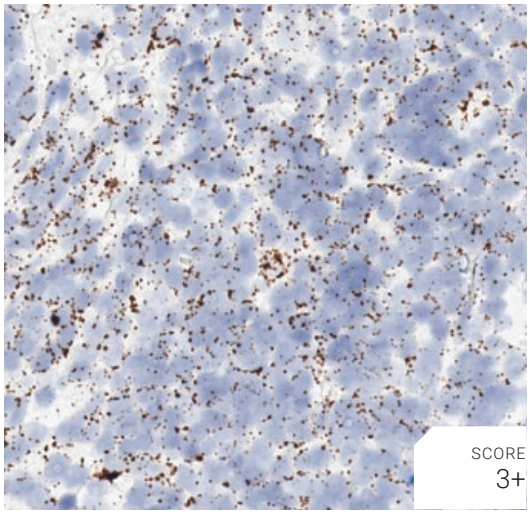
Control probe: **Hs-PPIB**



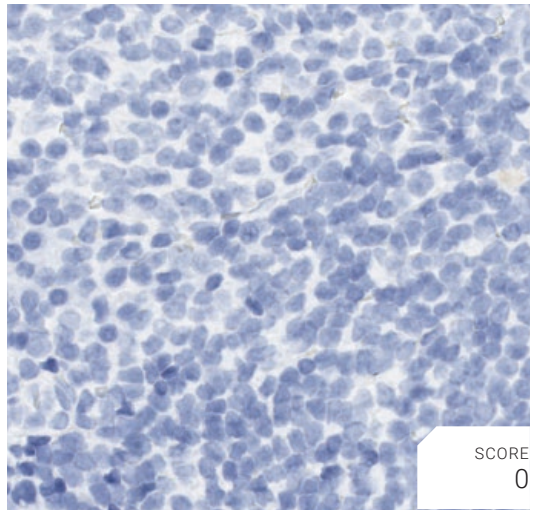
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

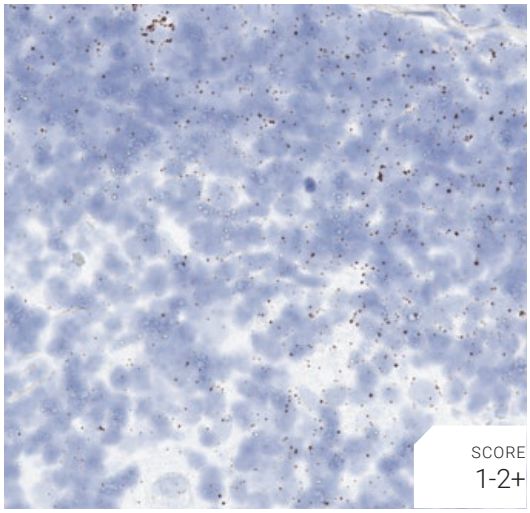


Recommended Positive Control Probe: Hs-PPIB/Hs-POLR2A*

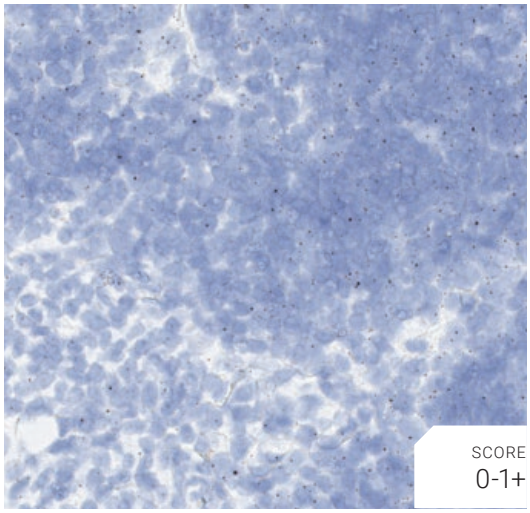
*Hs-POLR2A staining is more evenly distributed

Tissue: Thymus
Pretreatment: Mild

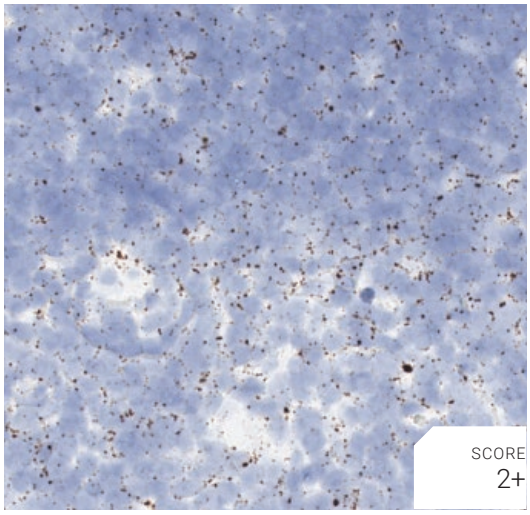
Control probe: **Hs-PPIB**



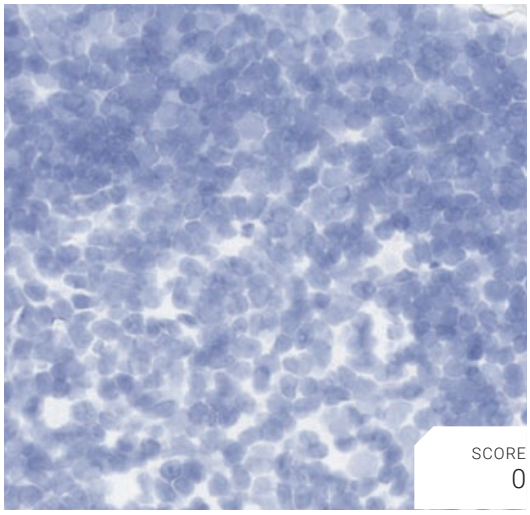
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



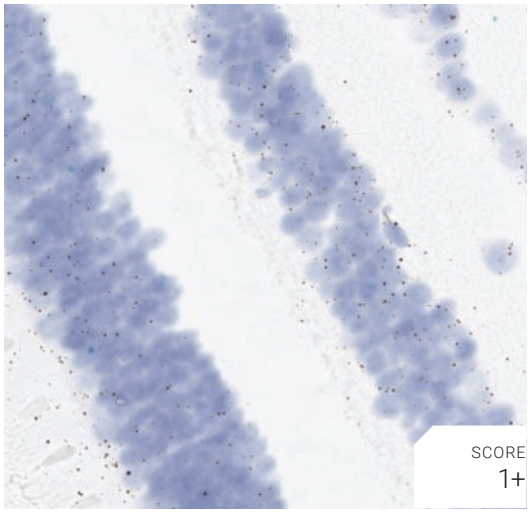
Control probe: **DapB**



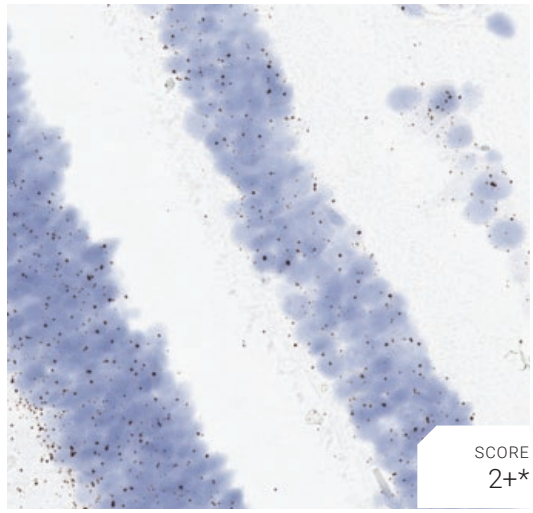
Recommended Positive Control Probe: Hs-PPIB

Tissue: Retina
Pretreatment: Mild

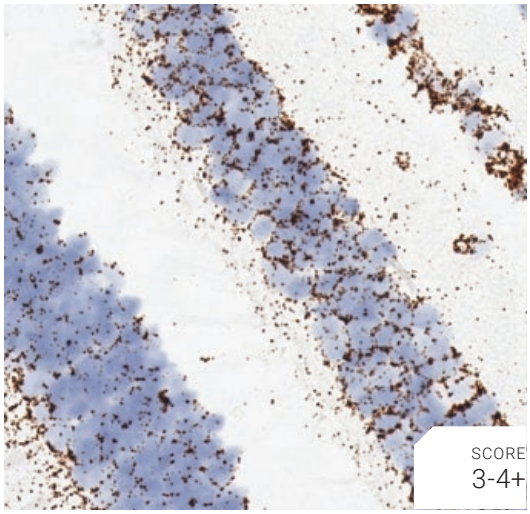
Control probe: **Hs-PPIB**



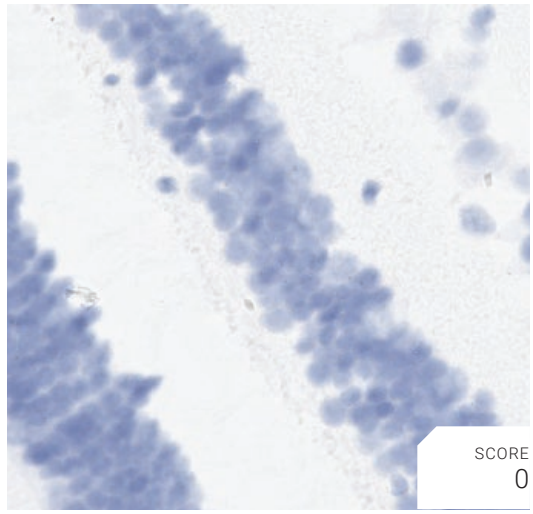
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

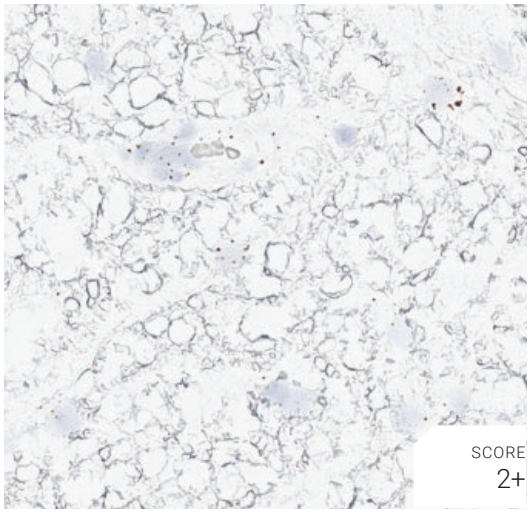


Recommended Positive Control Probe: Hs-POLR2A

*Signal: POLR2A > PPIB

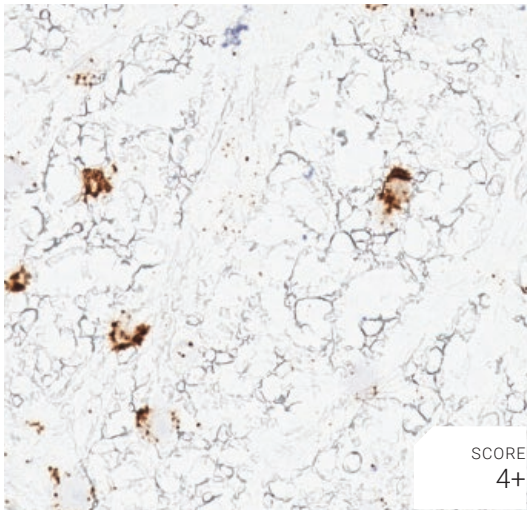
Tissue: Spinal cord–white matter
Pretreatment: Standard

Control probe: **Hs-PPIB**

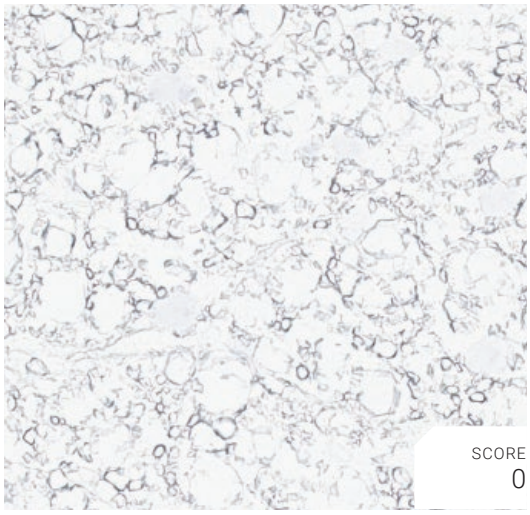


Control probe: **Hs-POLR2A**

Control probe: **Hs-UBC**



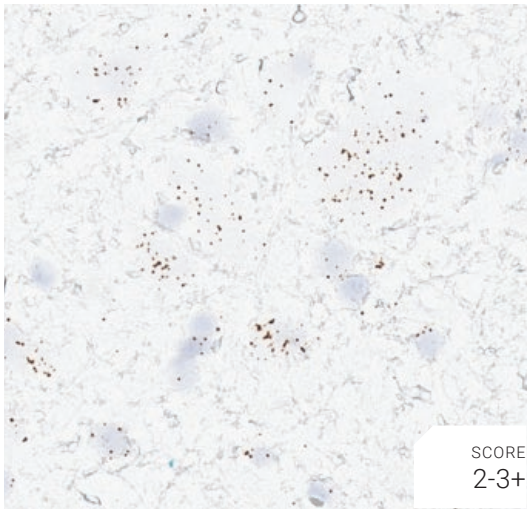
Control probe: **DapB**



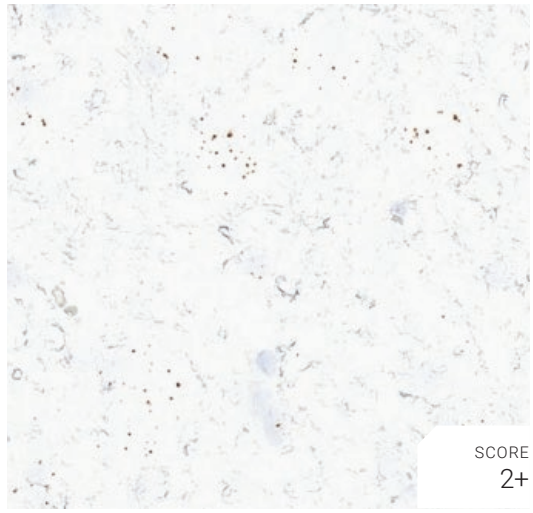
Recommended Positive Control Probe: Hs-PPIB

Tissue: Spinal cord–grey matter
Pretreatment: Standard

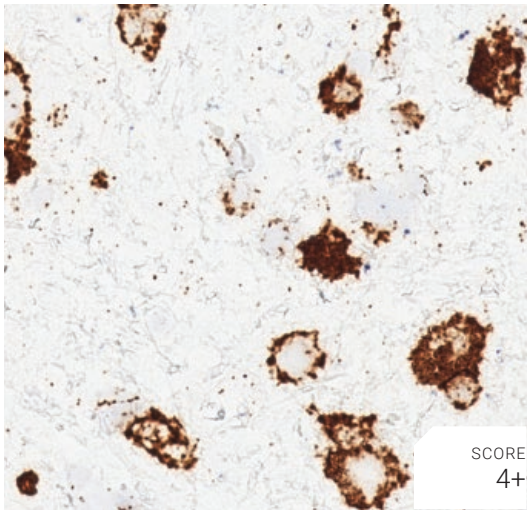
Control probe: **Hs-PPIB**



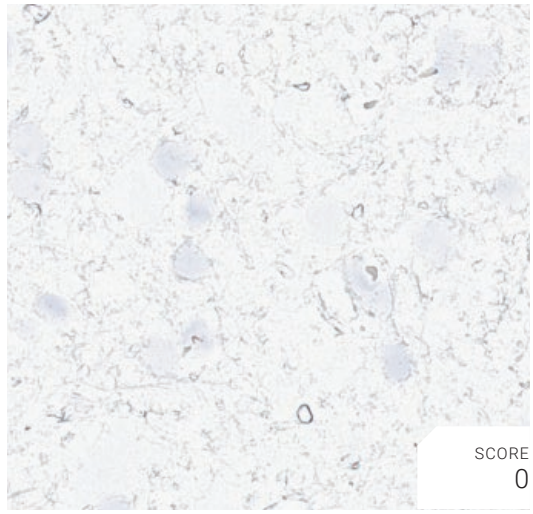
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



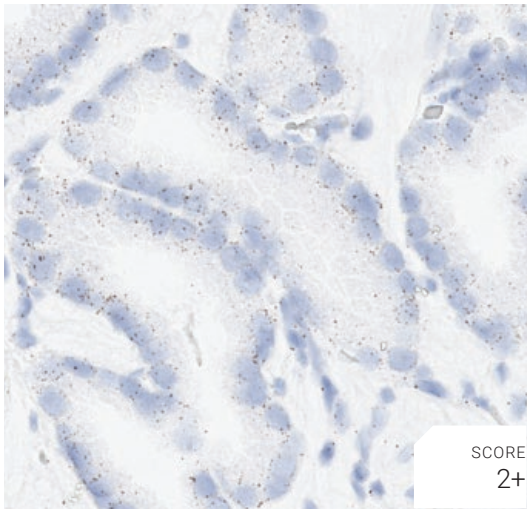
Control probe: **DapB**



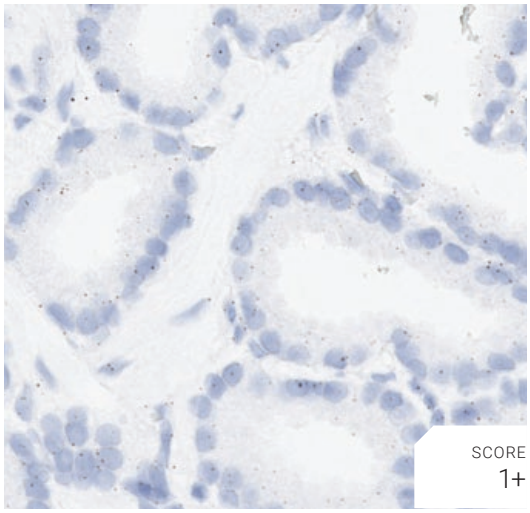
Recommended Positive Control Probe: Hs-PPIB

Tissue: Prostate gland
Pretreatment: Standard

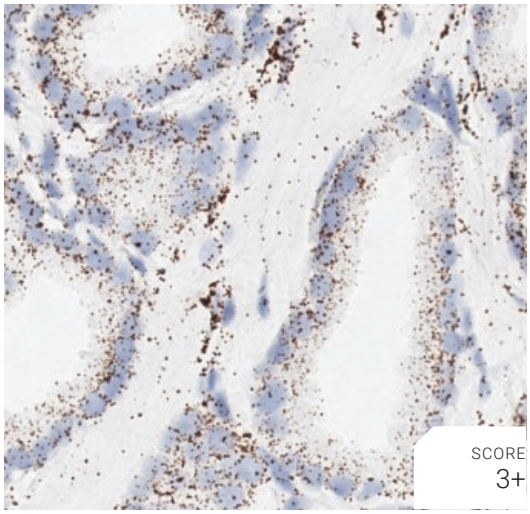
Control probe: **Hs-PPIB**



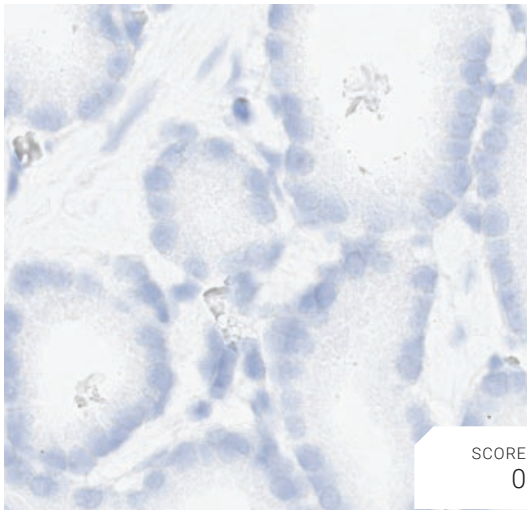
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



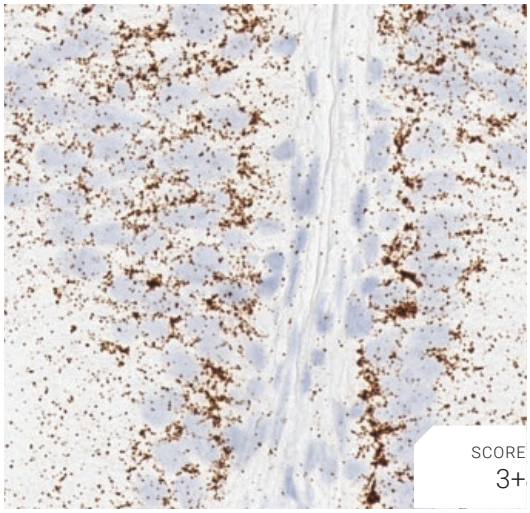
Control probe: **DapB**



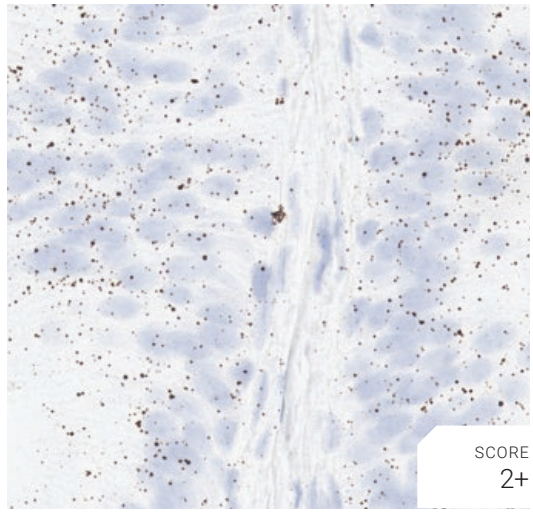
Recommended Positive Control Probe: Hs-PPIB

Tissue: Epididymis
Pretreatment: Mild

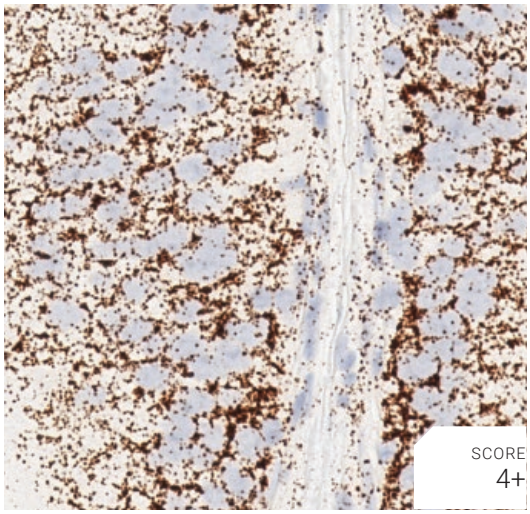
Control probe: **Hs-PPIB**



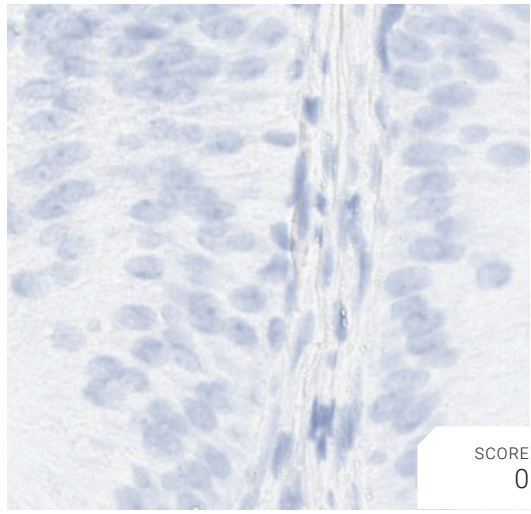
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



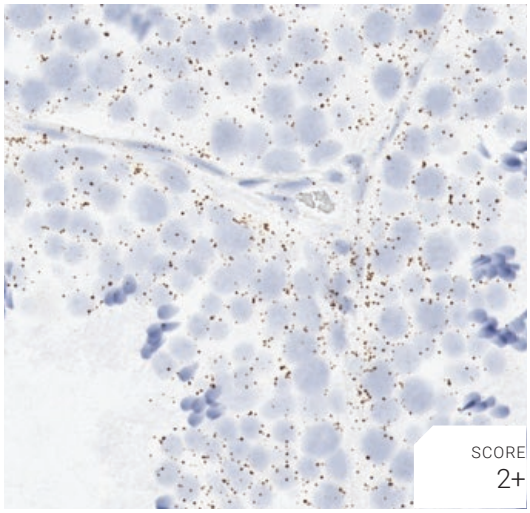
Control probe: **DapB**



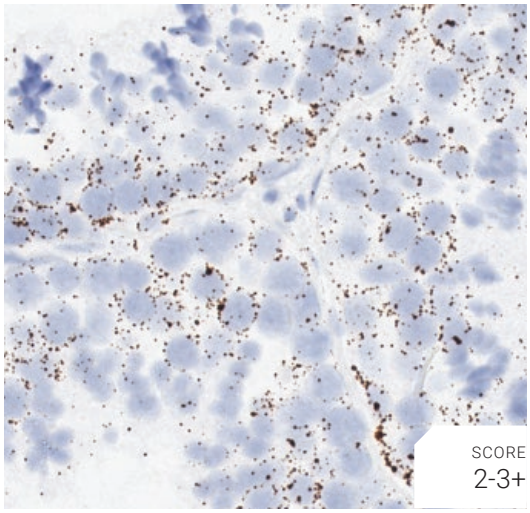
Recommended Positive Control Probe: Hs-PPIB

Tissue: Testis
Pretreatment: Mild

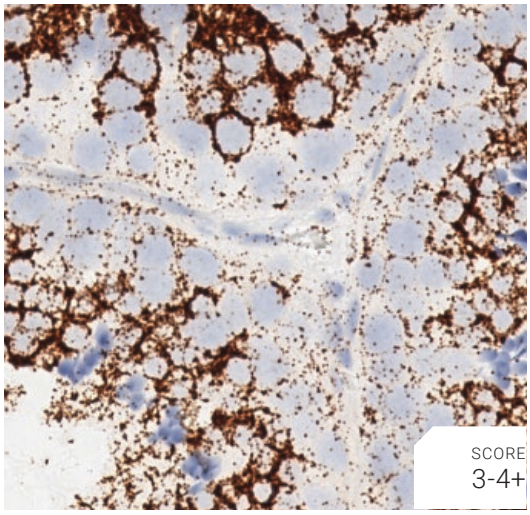
Control probe: **Hs-PPIB**



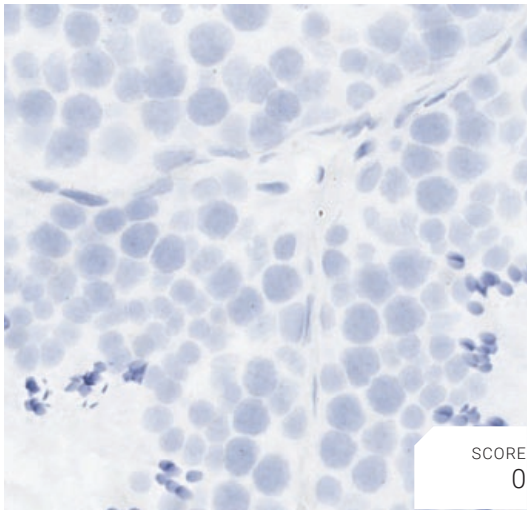
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

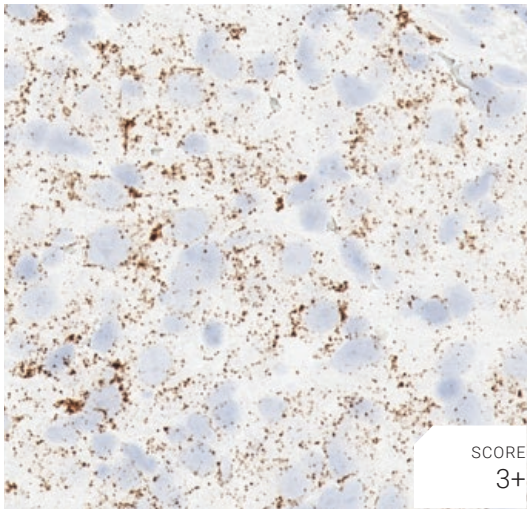


Recommended Positive Control Probe: Hs-POLR2A*/Hs-PPIB

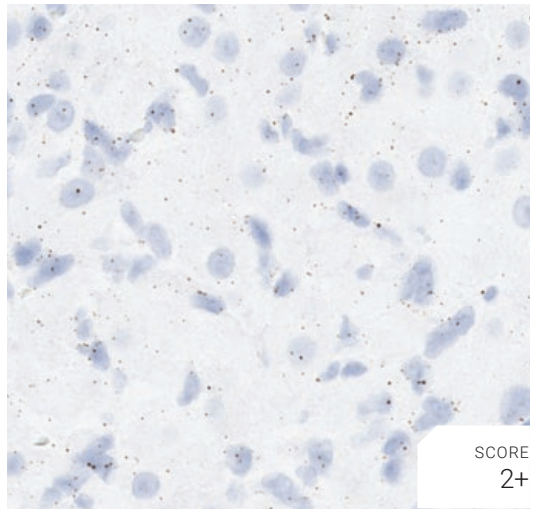
*Signal: POLR2A > PPIB

Tissue: Ovary
Pretreatment: Mild

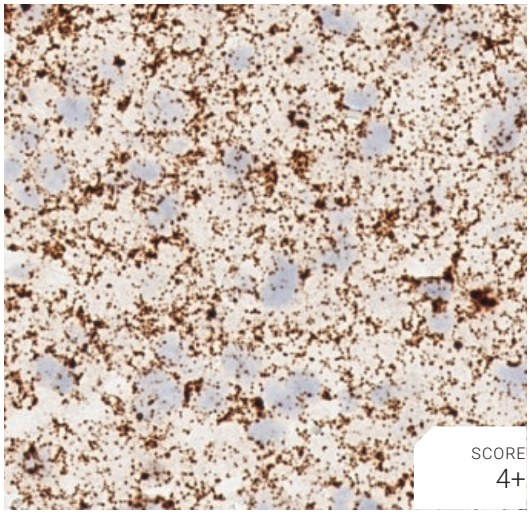
Control probe: **Hs-PPIB**



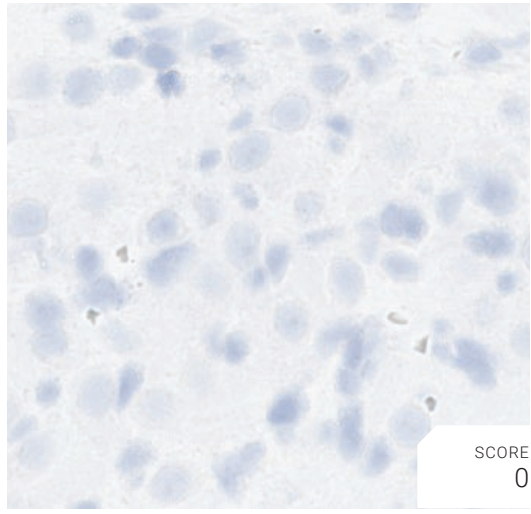
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



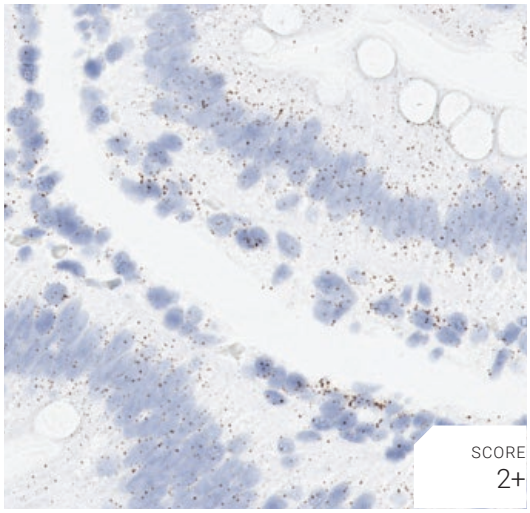
Control probe: **DapB**



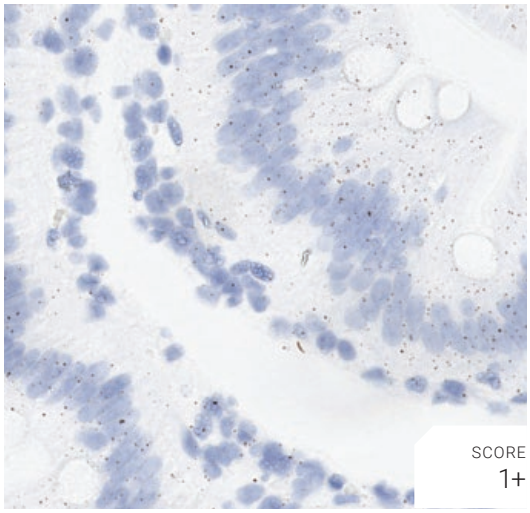
Recommended Positive Control Probe: Hs-PPIB

Tissue: Duodenum
Pretreatment: Mild

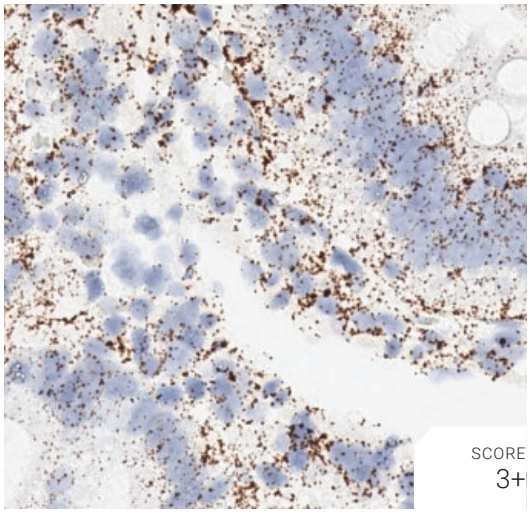
Control probe: **Hs-PPIB**



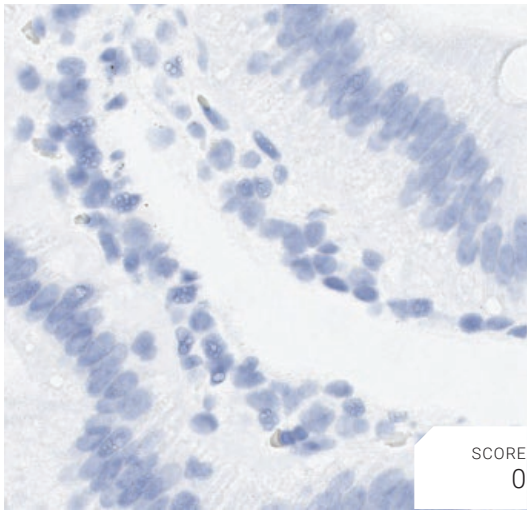
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



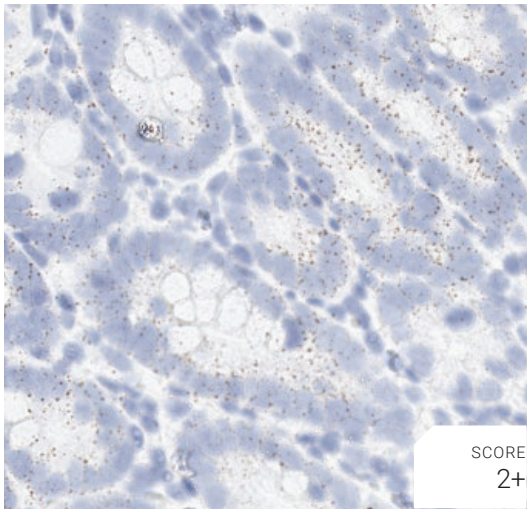
Control probe: **DapB**



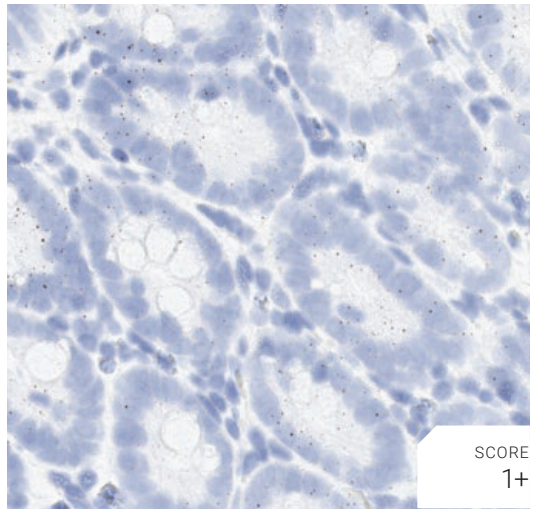
Recommended Positive Control Probe: Hs-PPIB

Tissue: Jejunum
Pretreatment: Mild

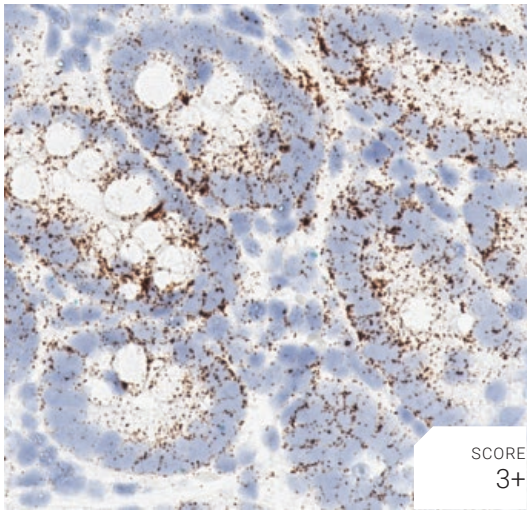
Control probe: **Hs-PPIB**



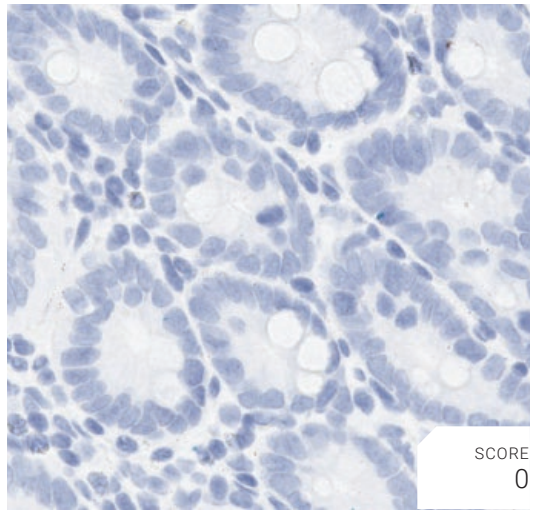
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



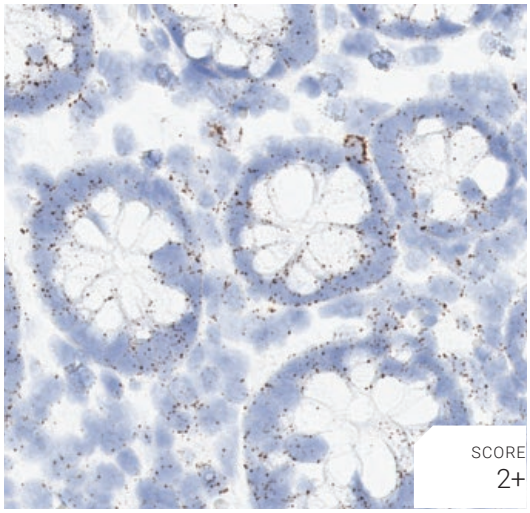
Control probe: **DapB**



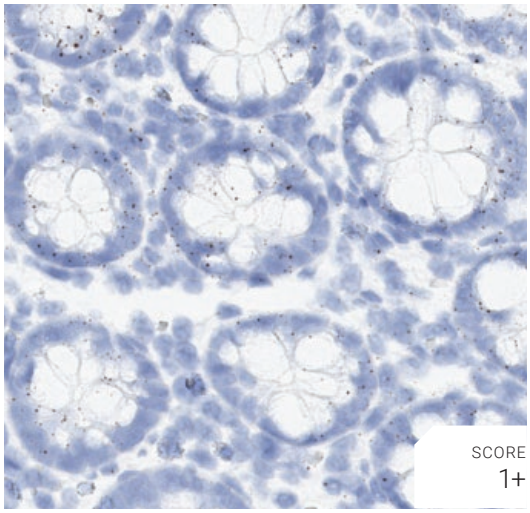
Recommended Positive Control Probe: Hs-PPIB

Tissue: Colon
Pretreatment: Standard

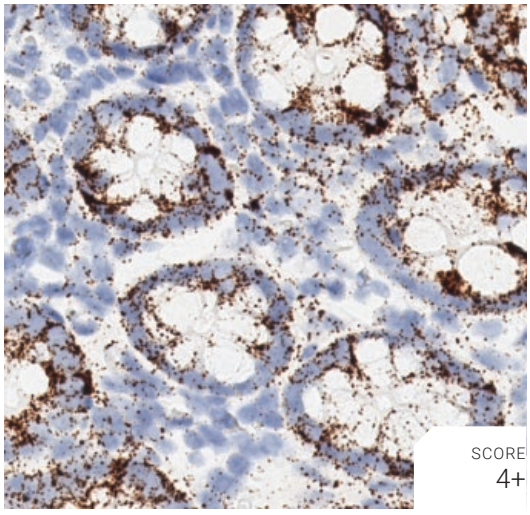
Control probe: **Hs-PPIB**



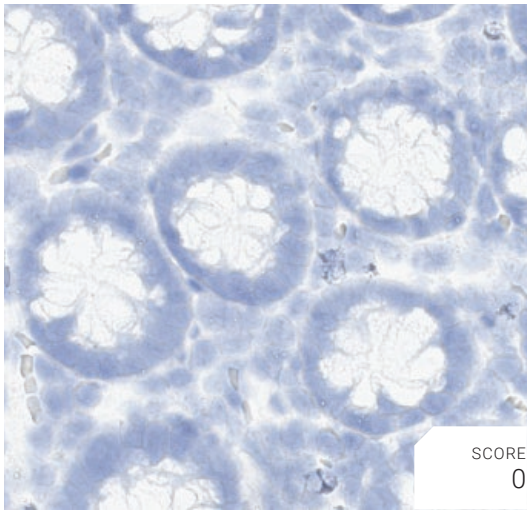
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



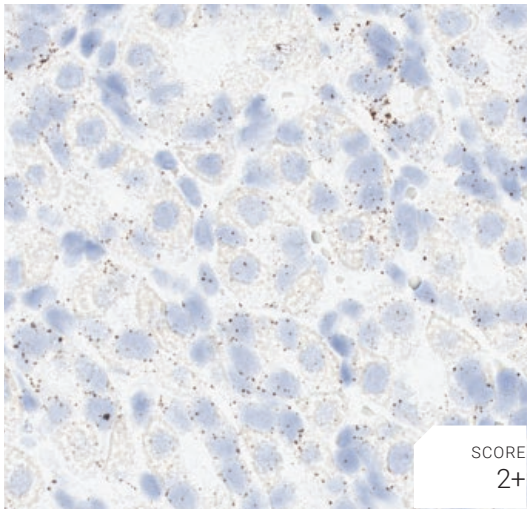
Control probe: **DapB**



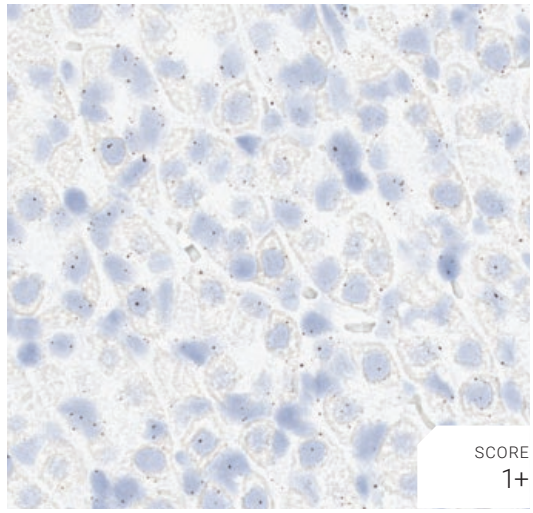
Recommended Positive Control Probe: Hs-PPIB

Tissue: Stomach
Pretreatment: Standard

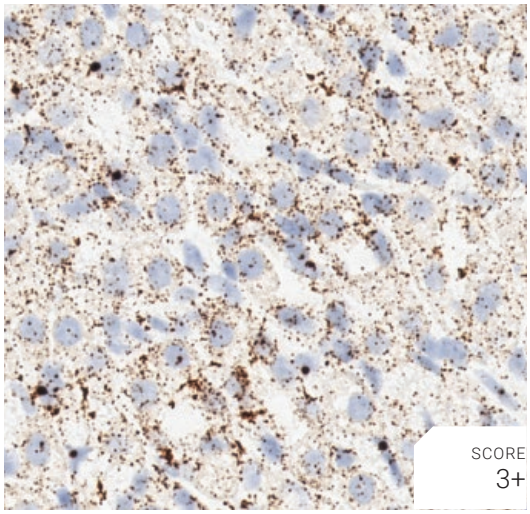
Control probe: **Hs-PPIB**



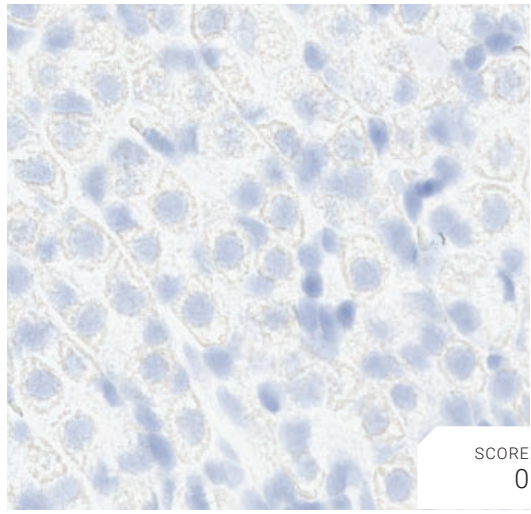
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



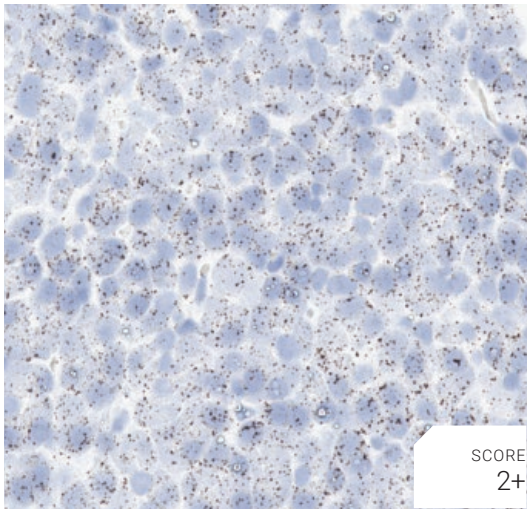
Control probe: **DapB**



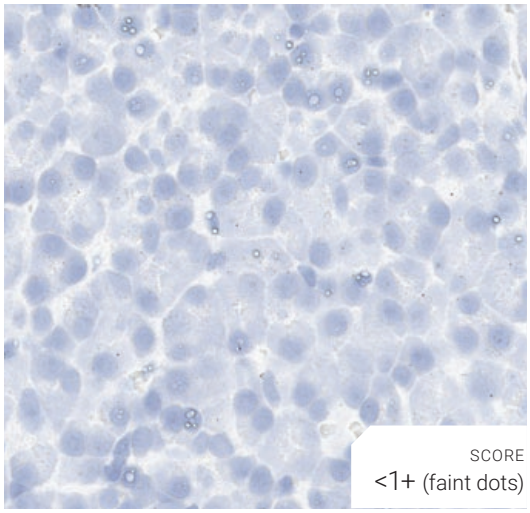
Recommended Positive Control Probe: Hs-PPIB

Tissue: Pancreas
Pretreatment: Standard

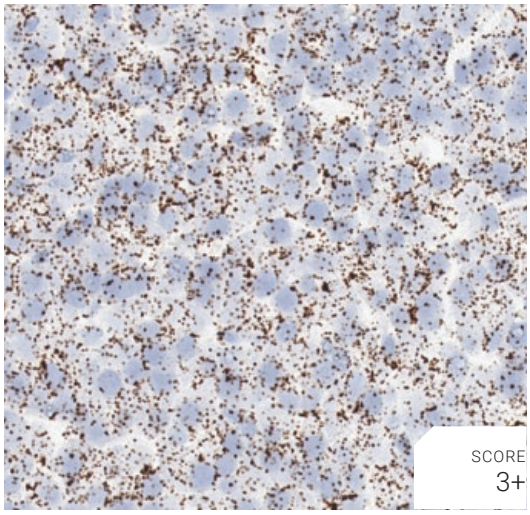
Control probe: **Hs-PPIB**



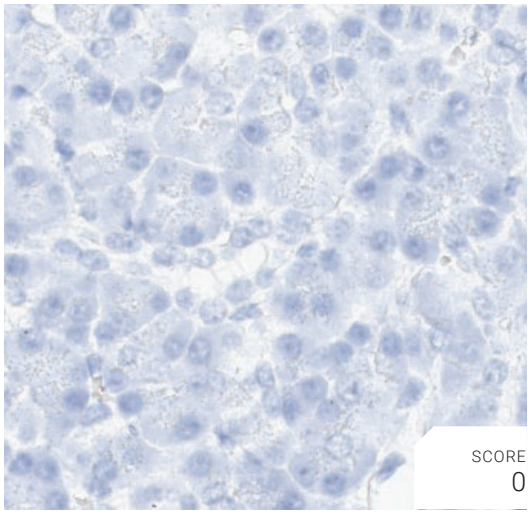
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



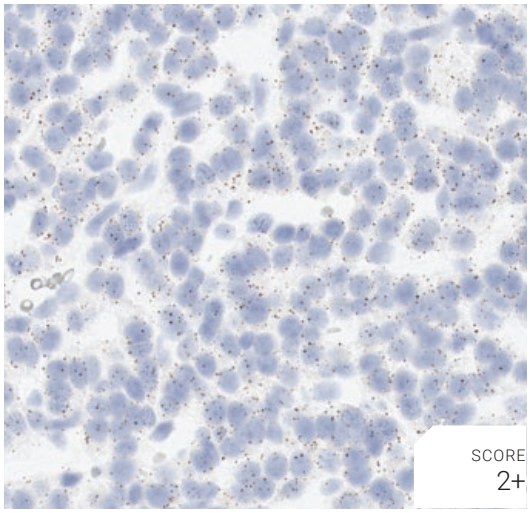
Control probe: **DapB**



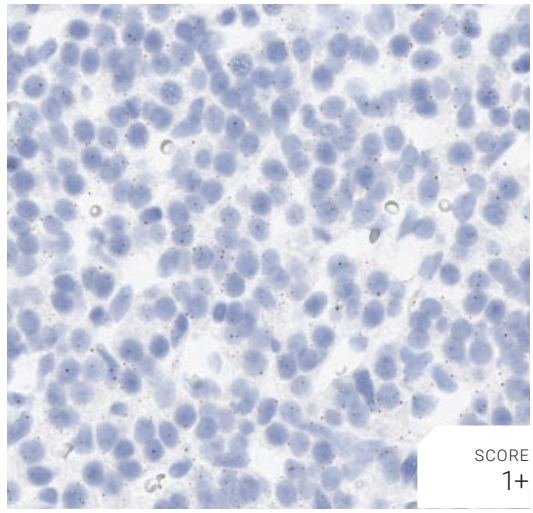
Recommended Positive Control Probe: Hs-PPIB

Tissue: Adrenal gland
Pretreatment: Mild

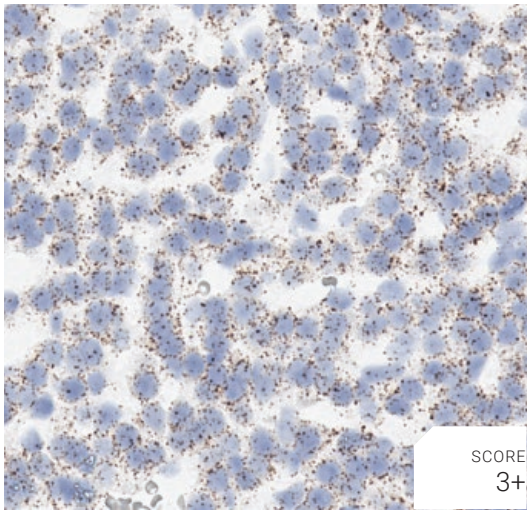
Control probe: **Hs-PPIB**



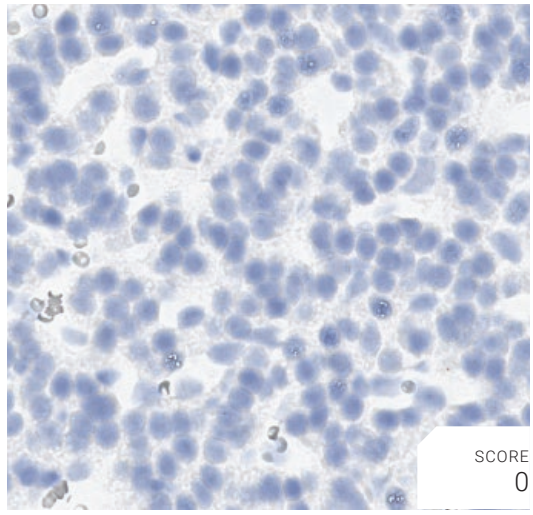
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



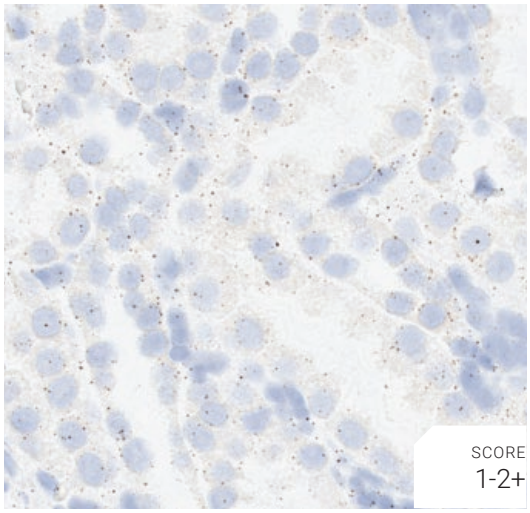
Control probe: **DapB**



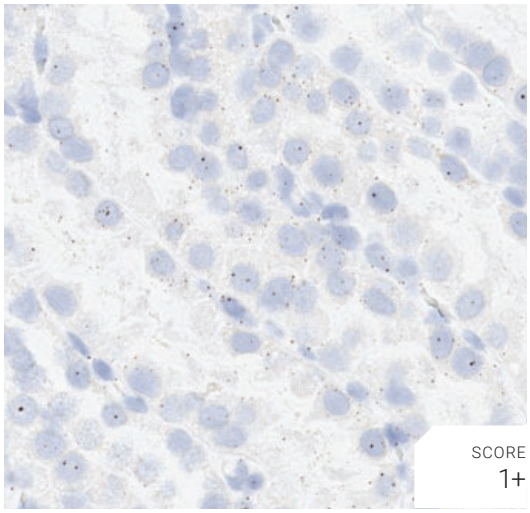
Recommended Positive Control Probe: Hs-PPIB

Tissue: Kidney
Pretreatment: Standard

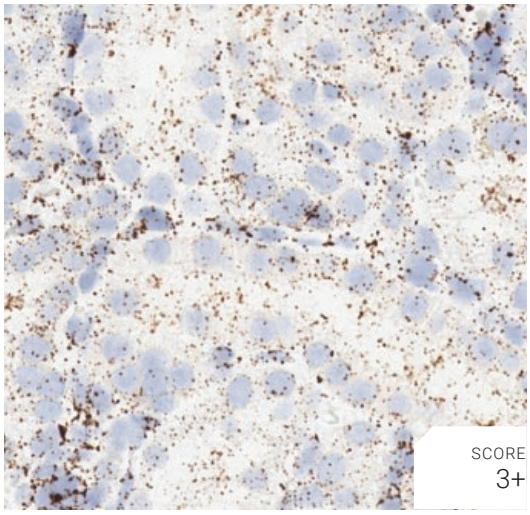
Control probe: **Hs-PPIB**



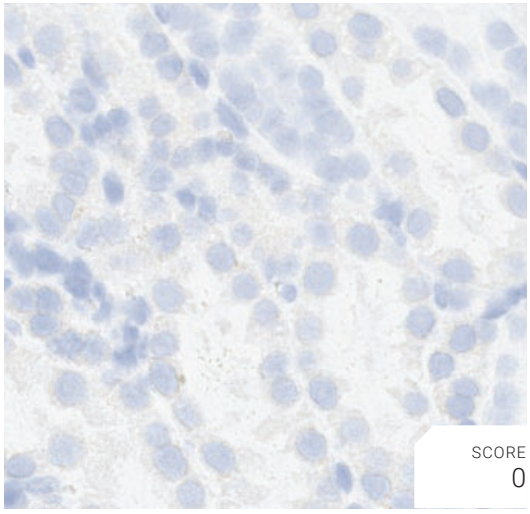
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



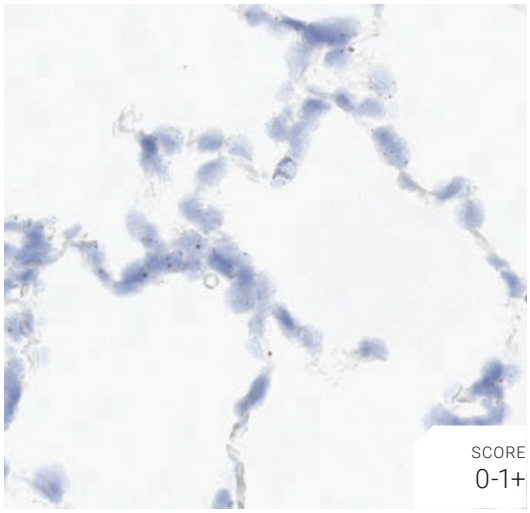
Control probe: **DapB**



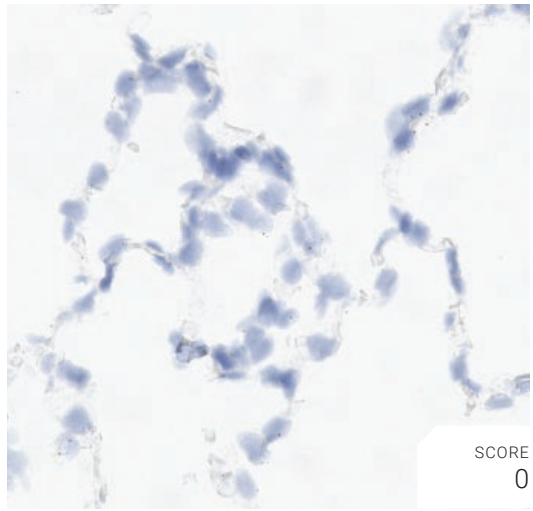
Recommended Positive Control Probe: Hs-PPIB

Tissue: Lung
Pretreatment: Standard

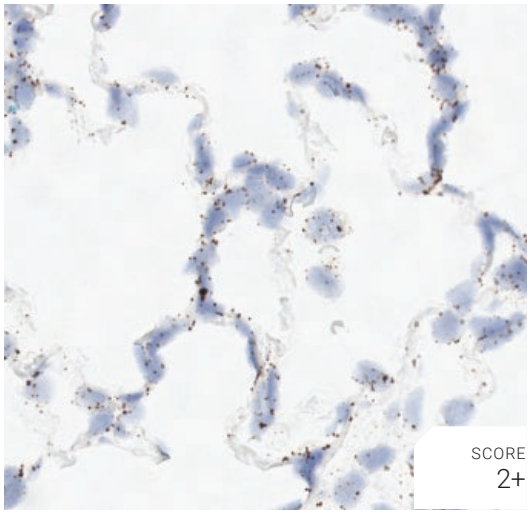
Control probe: **Hs-PPIB**



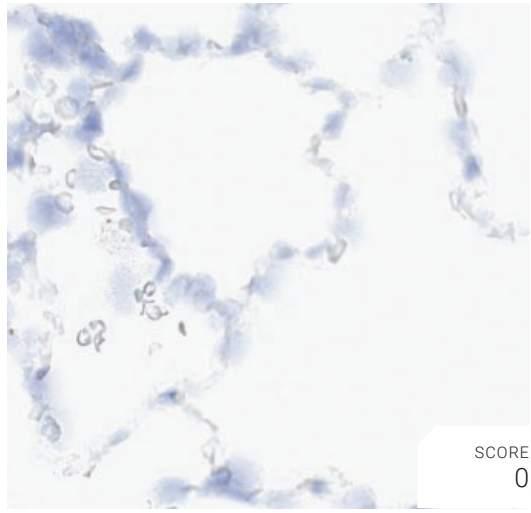
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

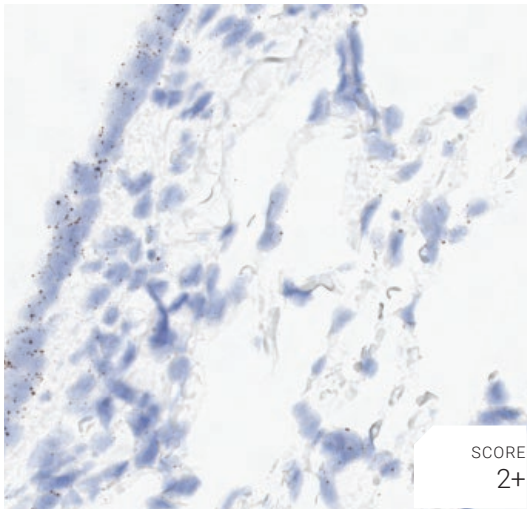


Recommended Positive Control Probe: Hs-UBC*

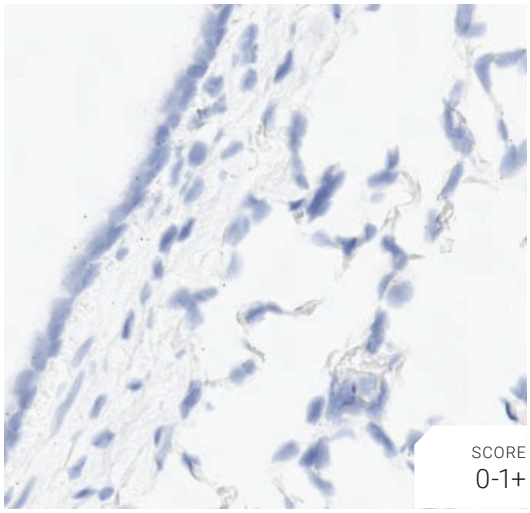
*Staining: Bronchus > alveolar cells; Recommend Hs-UBC to control alveolar cells

Tissue: Lung (bronchus)
Pretreatment: Standard

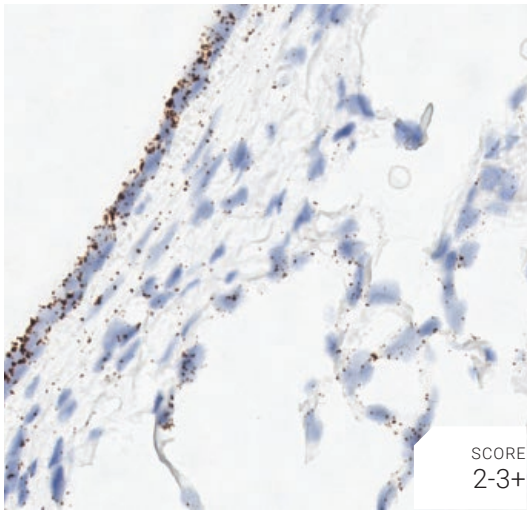
Control probe: **Hs-PPIB**



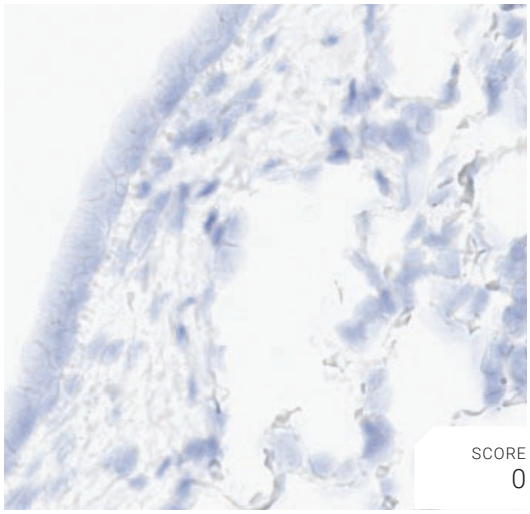
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

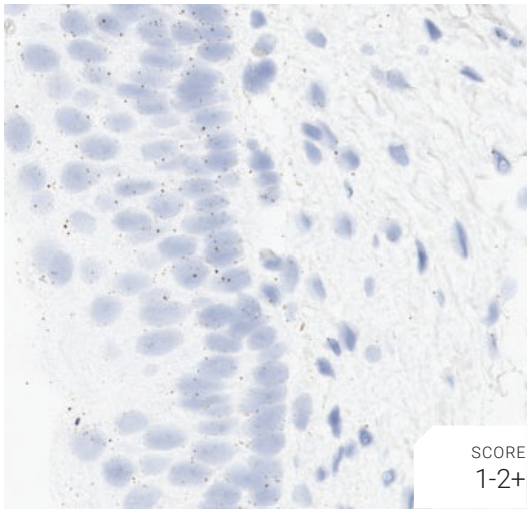


Recommended Positive Control Probe: Hs-PPIB/Hs-UBC*

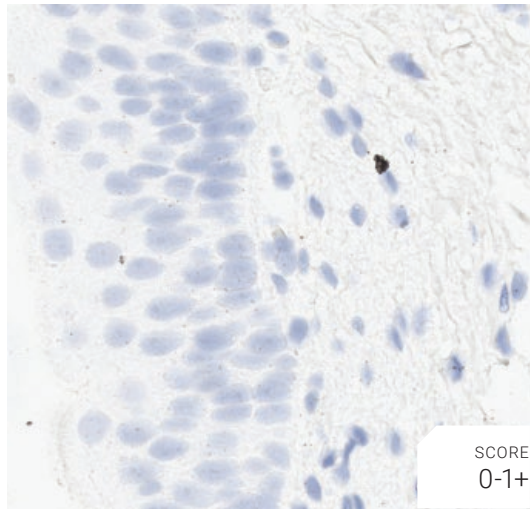
*Staining: Bronchus > alveolar cells; Recommend Hs-UBC to control alveolar cells

Tissue: Urinary bladder
Pretreatment: Standard

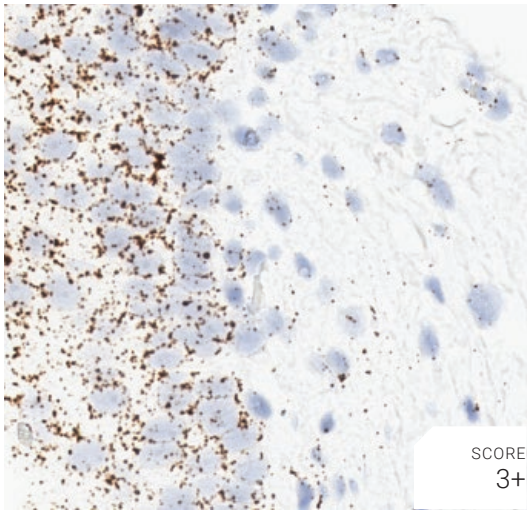
Control probe: **Hs-PPIB**



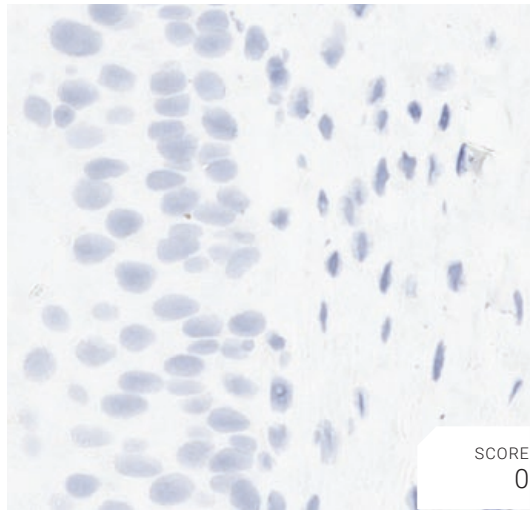
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



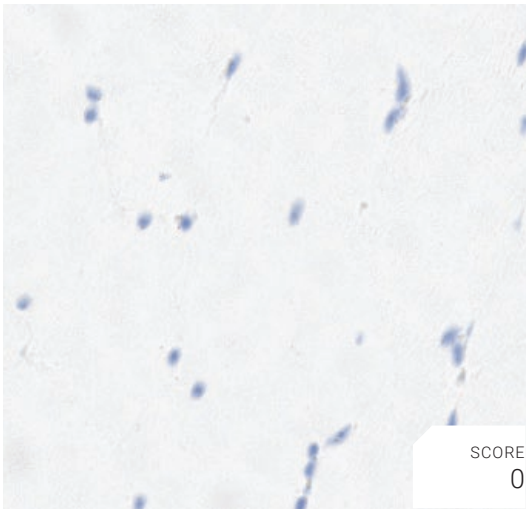
Control probe: **DapB**



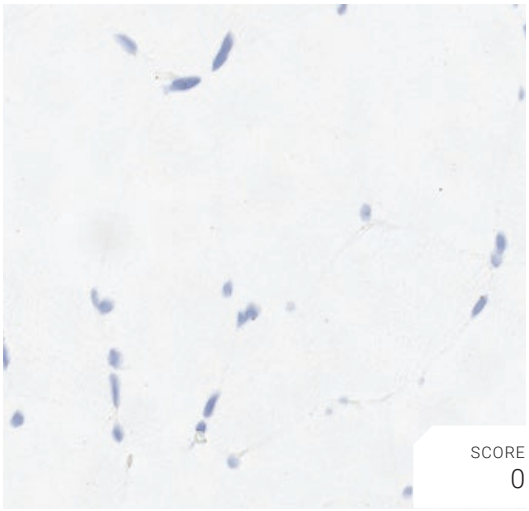
Recommended Positive Control Probe: Hs-PPIB

Tissue: Skeletal muscle*
Pretreatment: Standard

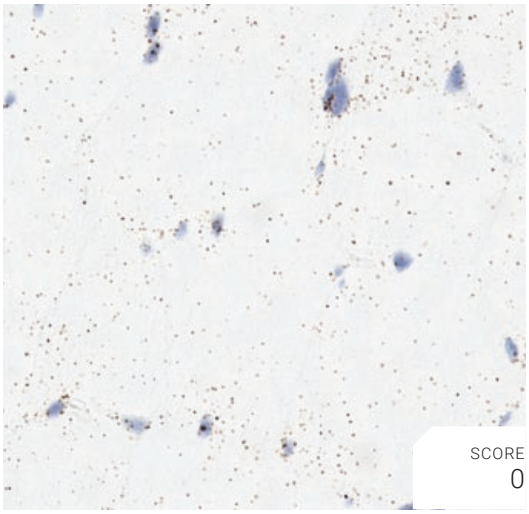
Control probe: **Hs-PPIB**



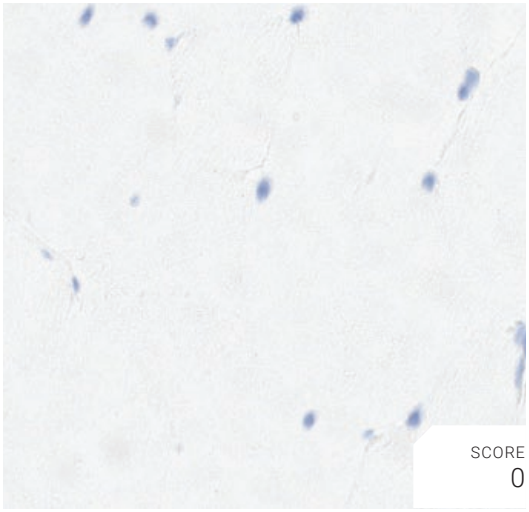
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

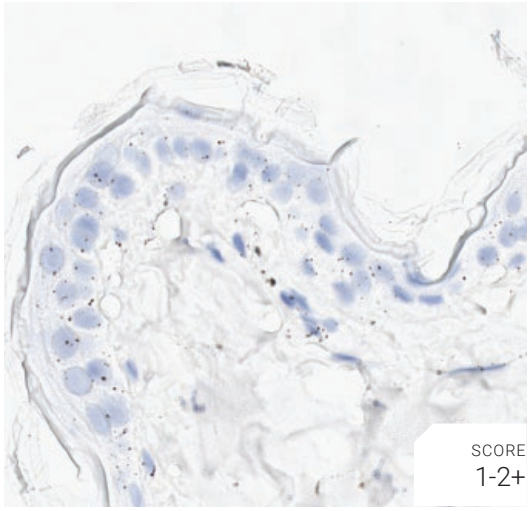


Recommended Positive Control Probe: Hs-UBC

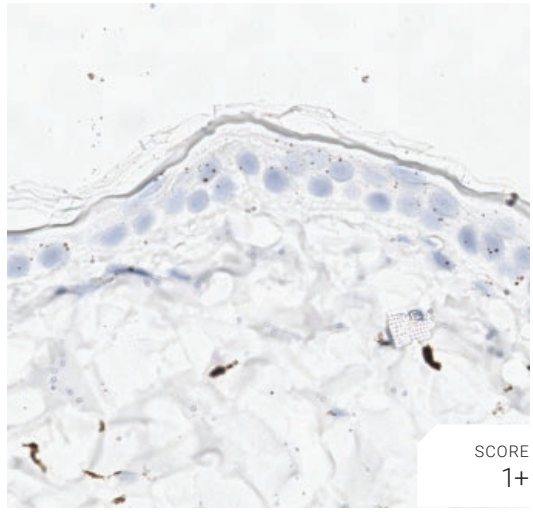
*Skeletal muscle express relatively low level of PPIB

Tissue: Skin
Pretreatment: Standard

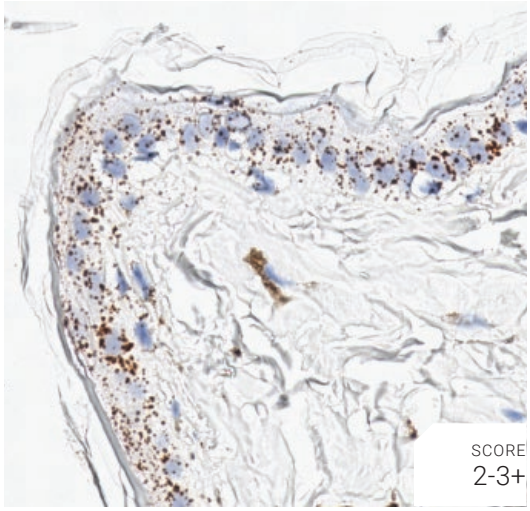
Control probe: **Hs-PPIB**



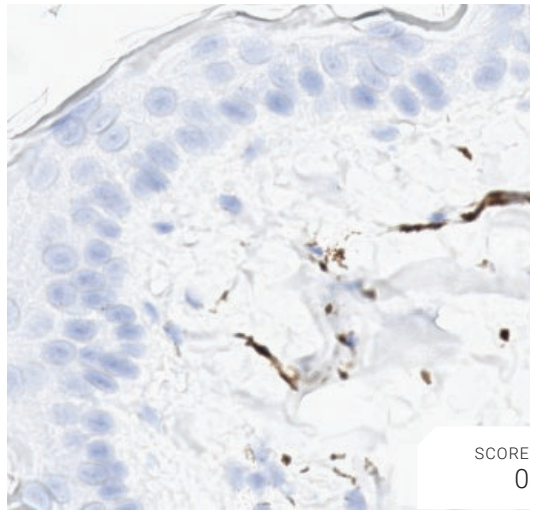
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



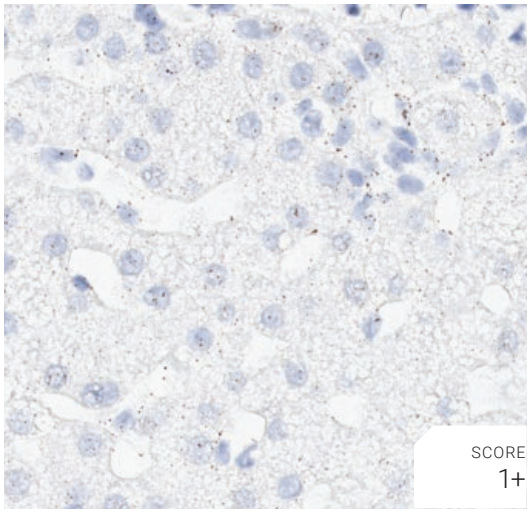
Control probe: **DapB**



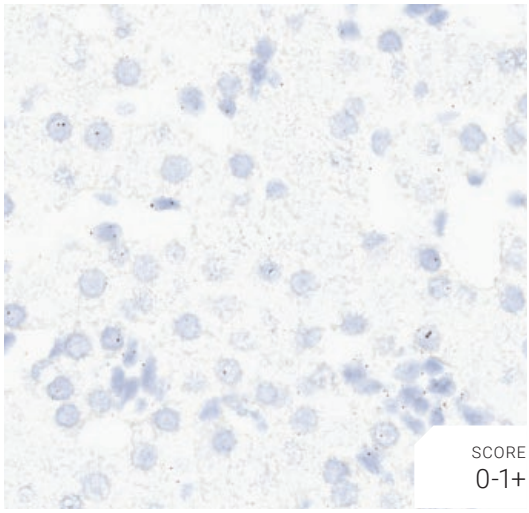
Recommended Positive Control Probe: Hs-PPIB/Hs-UBC

Tissue: Liver
Pretreatment: Standard

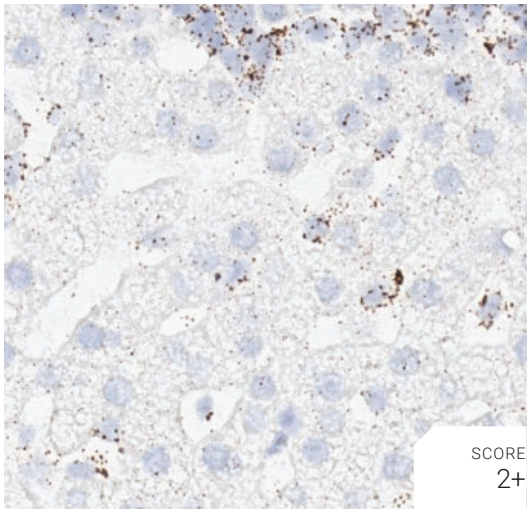
Control probe: **Hs-PPIB**



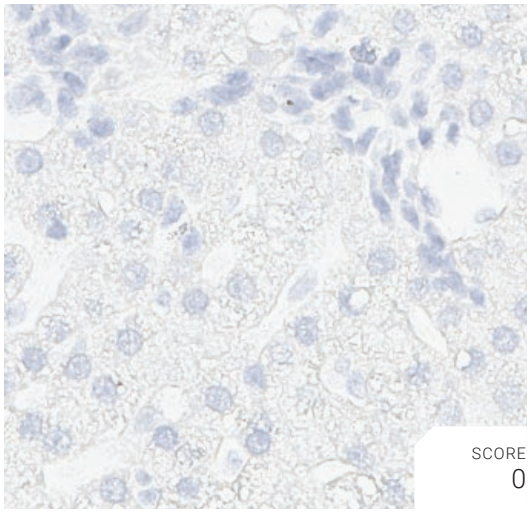
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**

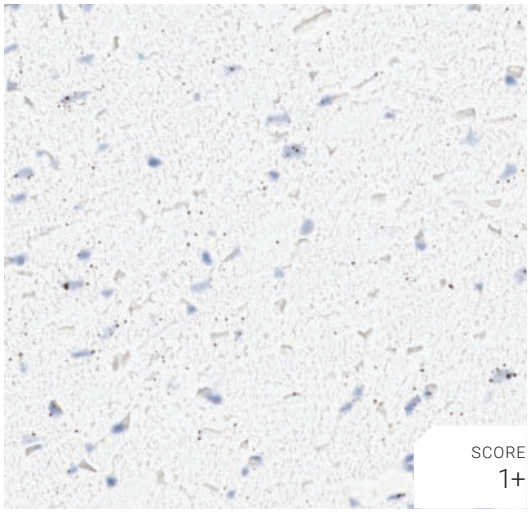


Recommended Positive Control Probe: Hs-UBC/Hs-PPIB*

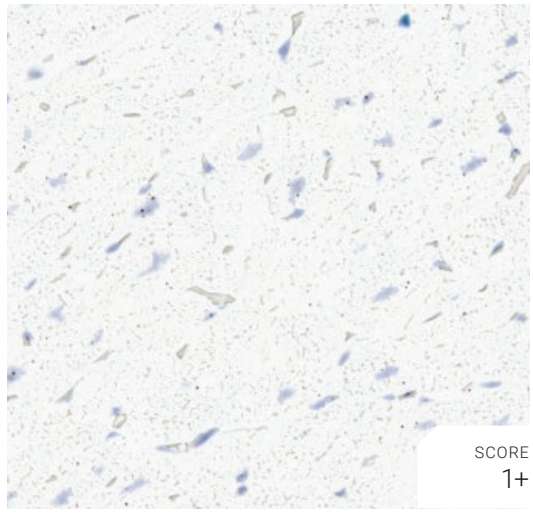
*PPIB staining is low

Tissue: Heart
Pretreatment: Standard

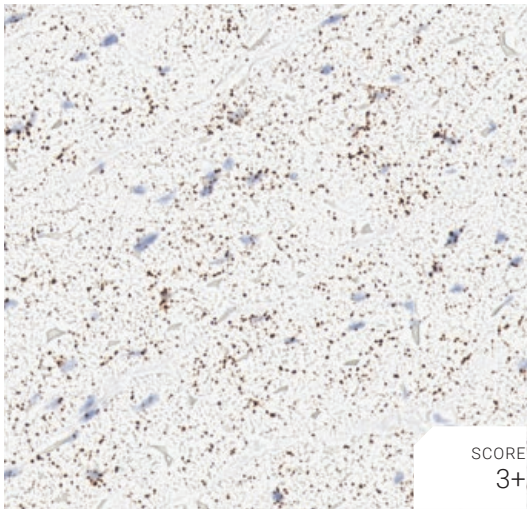
Control probe: **Hs-PPIB**



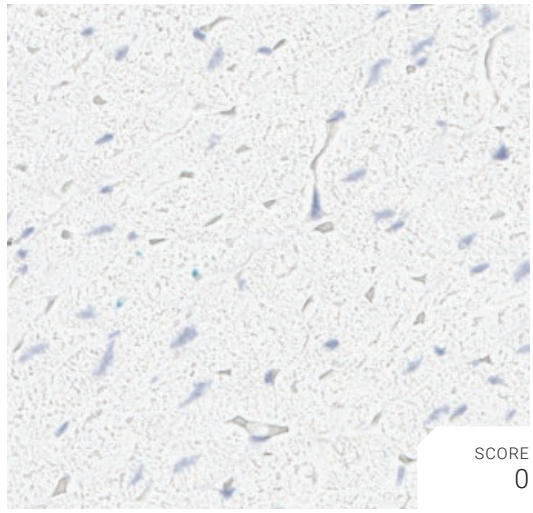
Control probe: **Hs-POLR2A**



Control probe: **Hs-UBC**



Control probe: **DapB**



Recommended Positive Control Probe: Hs-UBC*

*Heart muscle expresses a relatively low level of PPIB

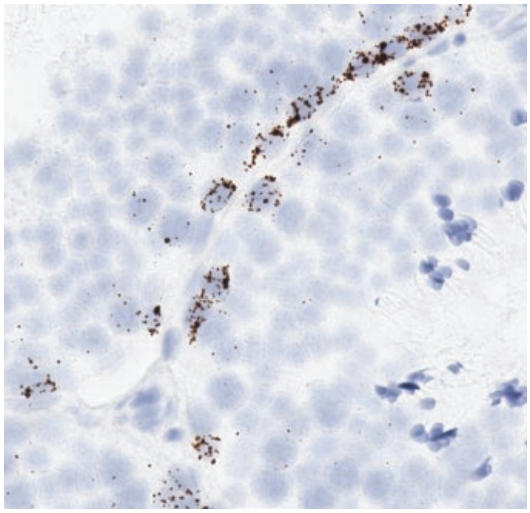
Cynomolgus monkey

Macaca fascicularis

The following pages show the proliferation and apoptosis marker images using the optimal pretreatment condition for each tissue.

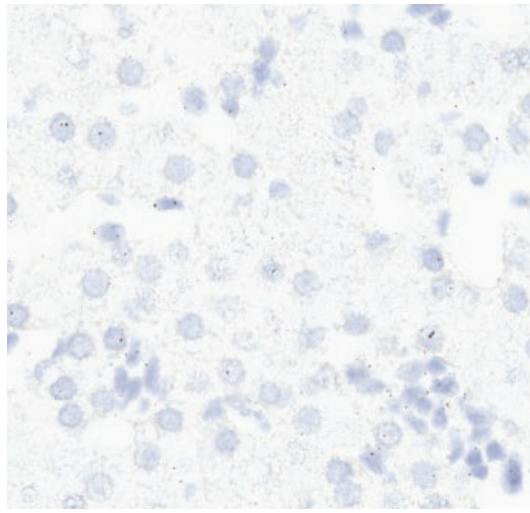
Tissue: Testis

Target probe: **Hs-MKI67 (Ki-67)**



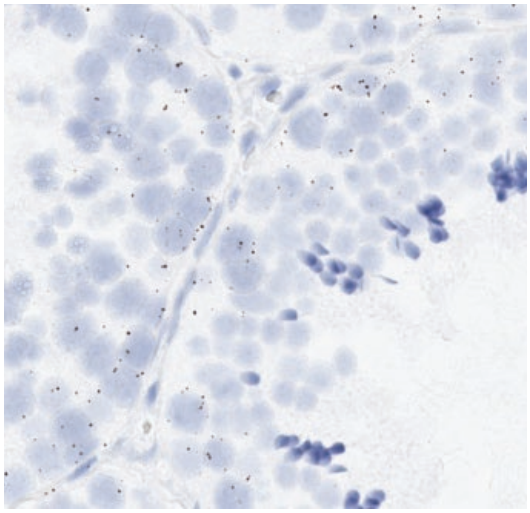
Tissue: Ovary

Target probe: **Hs-MKI67 (Ki-67)**



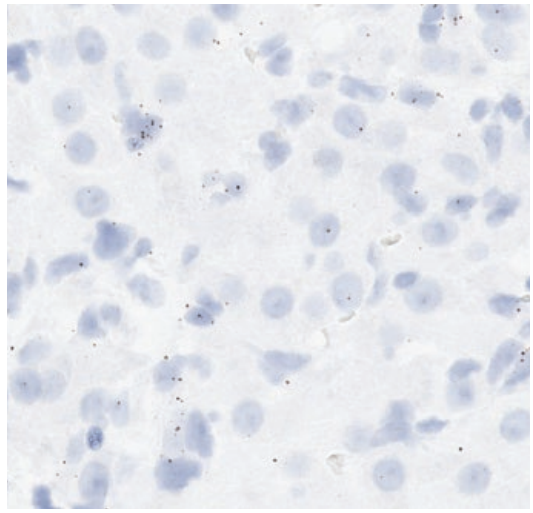
Tissue: Testis

Target probe: **Mfa-CCNE1 (Cyclin E1)**



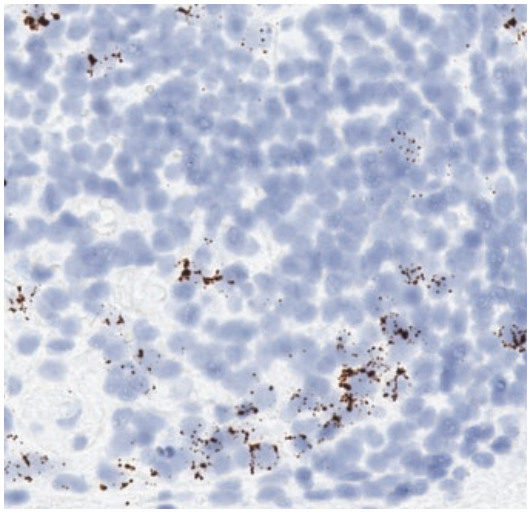
Tissue: Ovary

Target probe: **Mfa-CCNE1 (Cyclin E1)**



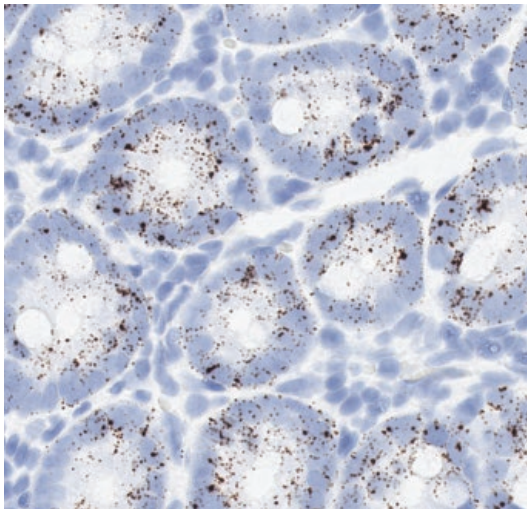
Tissue: Tonsil

Target probe: **Hs-MKI67 (Ki-67)**



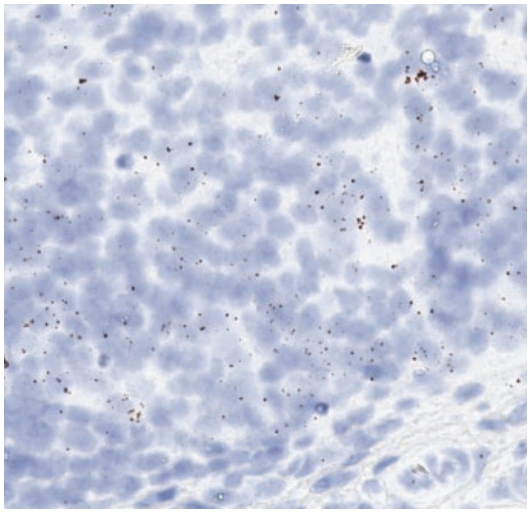
Tissue: Duodenum

Target probe: **Hs-MKI67 (Ki-67)**



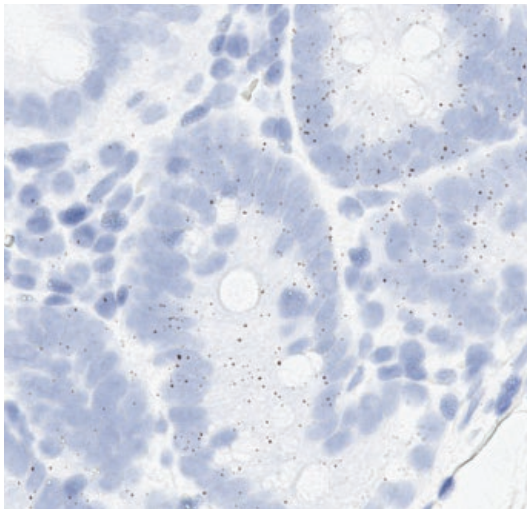
Tissue: Tonsil

Target probe: **Mfa-CCNE1 (Cyclin E1)**



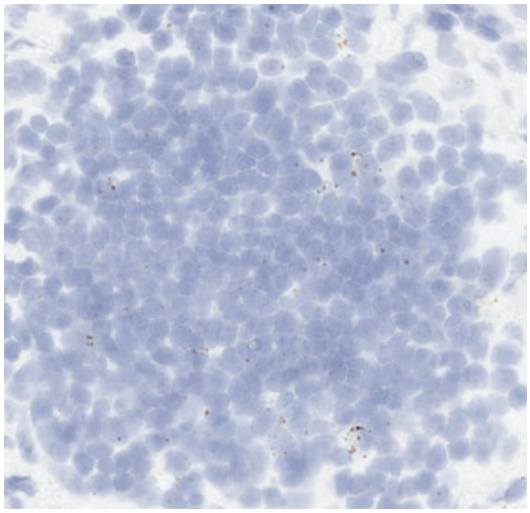
Tissue: Duodenum

Target probe: **Mfa-CCNE1 (Cyclin E1)**



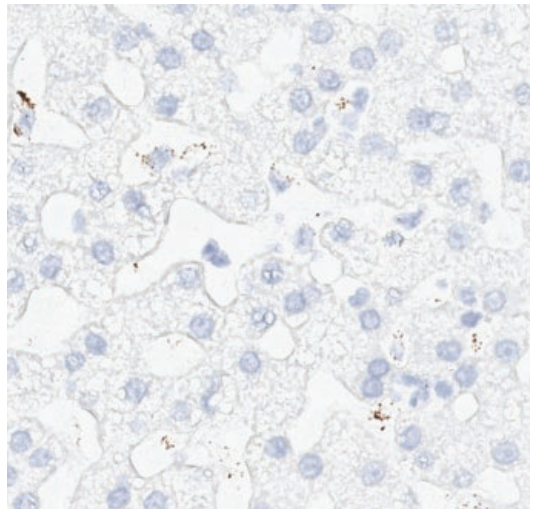
Tissue: Lymph node

Target probe: **Hs-CD68**



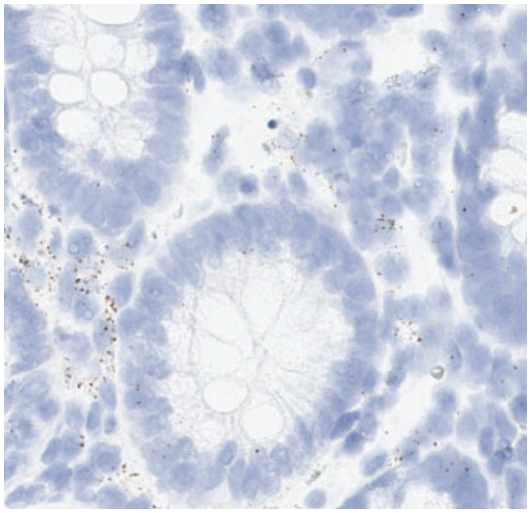
Tissue: Liver

Target probe: **Hs-CD68**



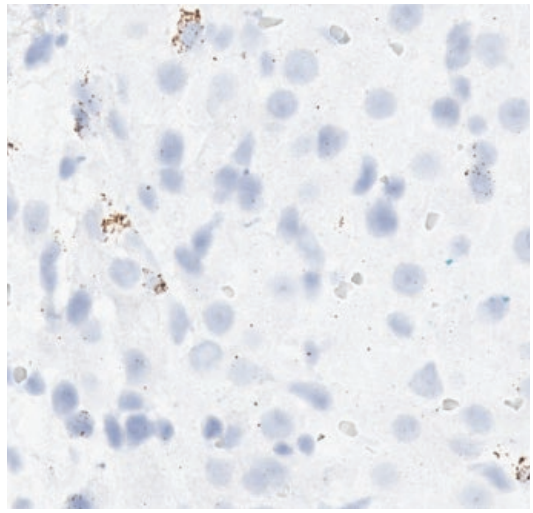
Tissue: Colon

Target probe: **Hs-CD68**



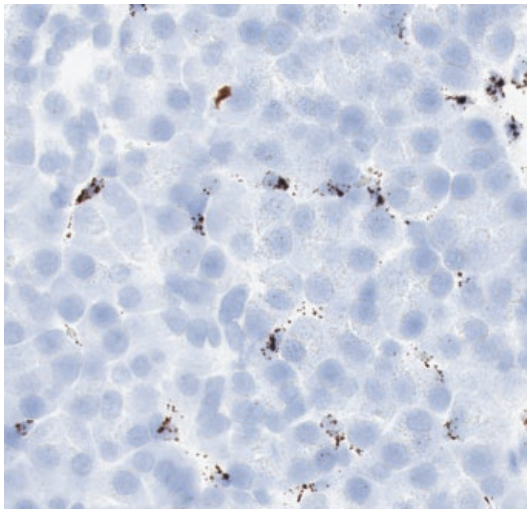
Tissue: Ovary

Target probe: **Hs-CD68**



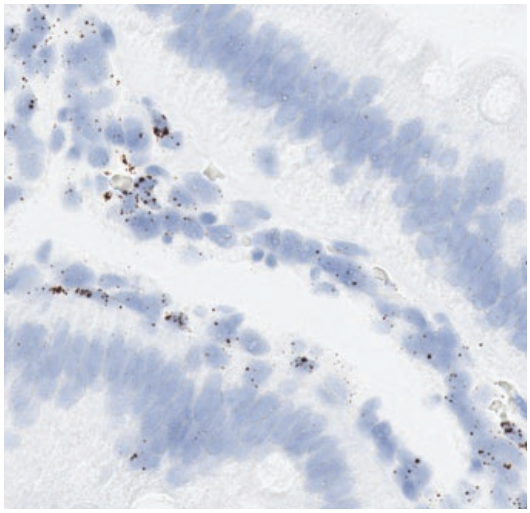
Tissue: Pancreas

Target probe: **Mfa-PECAM1 (CD31)**



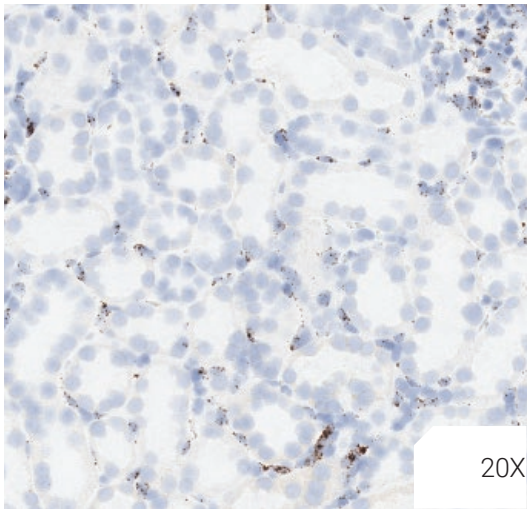
Tissue: Duodenum

Target probe: **Mfa-PECAM1 (CD31)**



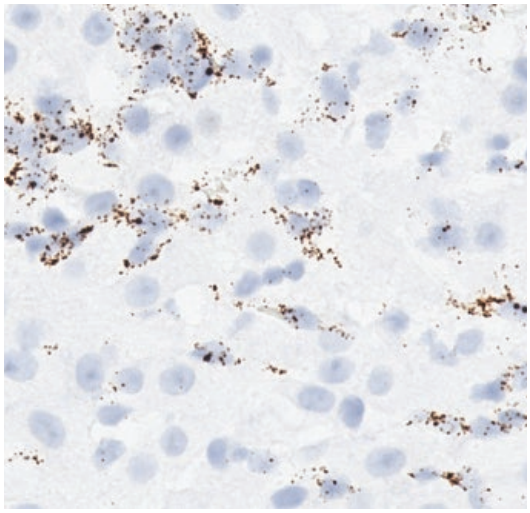
Tissue: Kidney

Target probe: **Mfa-PECAM1 (CD31)**



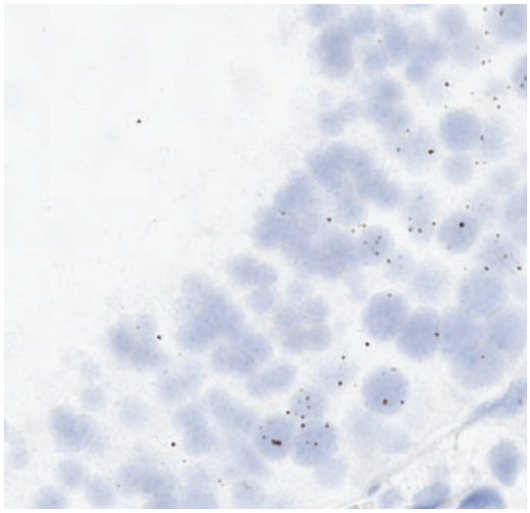
Tissue: Ovary

Target probe: **Mfa-PECAM1 (CD31)**



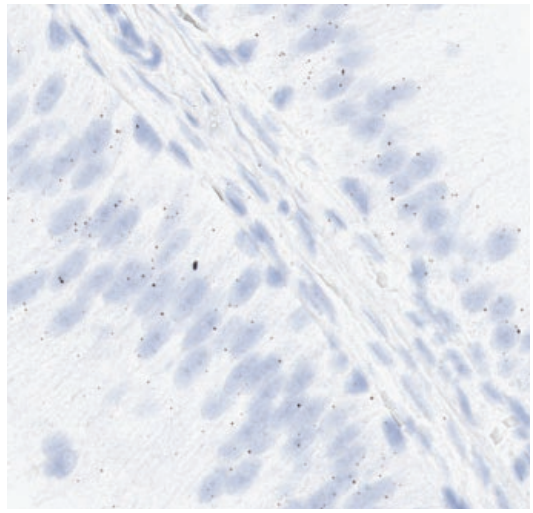
Tissue: Testis

Target probe: **Mfa-Fas (CD95)**



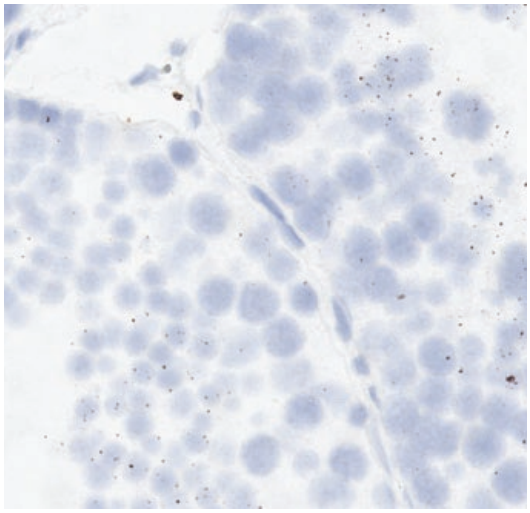
Tissue: Epididymis

Target probe: **Mfa-Fas (CD95)**



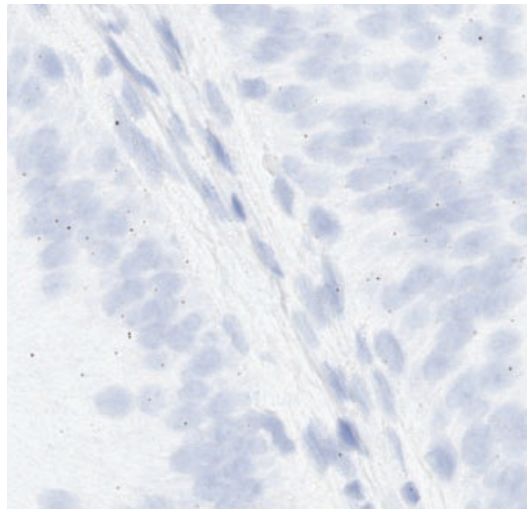
Tissue: Testis

Target probe: **Mfa-BBC3 (Puma)**



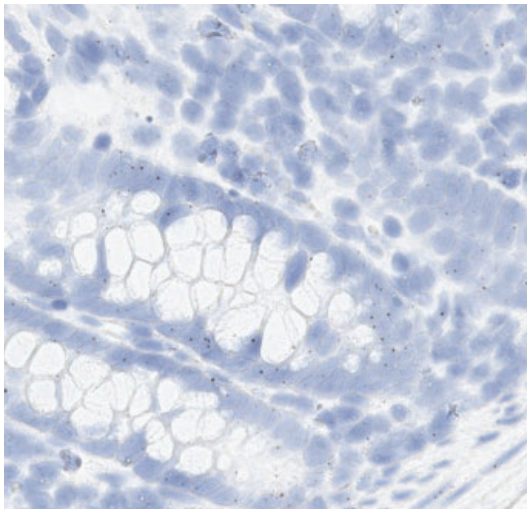
Tissue: Epididymis

Target probe: **Mfa-BBC3 (Puma)**



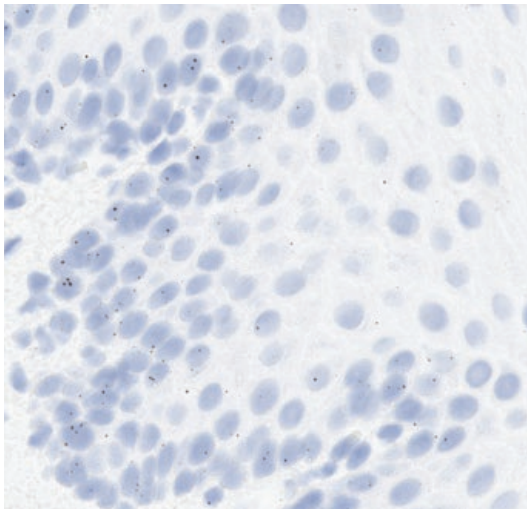
Tissue: Colon

Target probe: **Mfa-TNFRSF10B (DR5)**



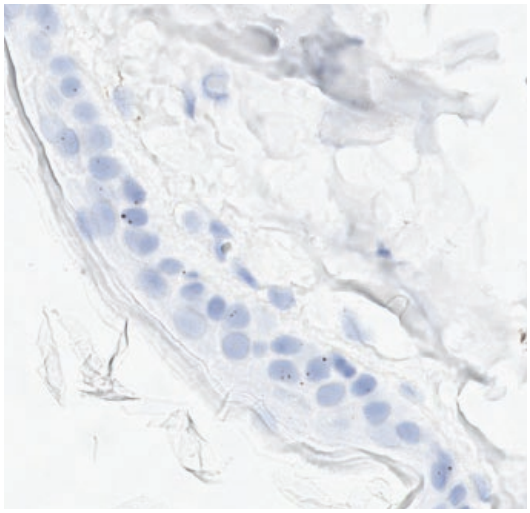
Tissue: Esophagus

Target probe: **Mfa-TNFRSF10B (DR5)**



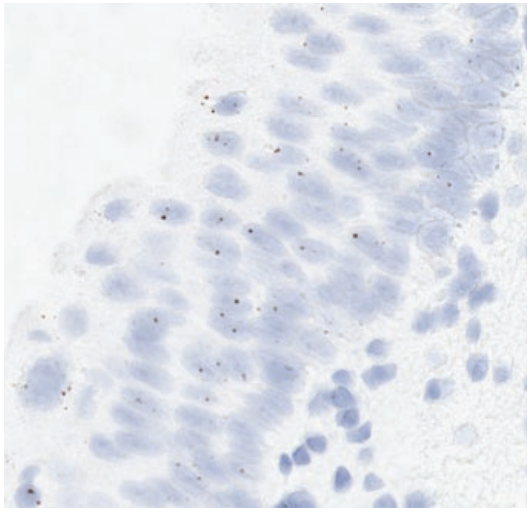
Tissue: Skin

Target probe: **Mfa-TNFRSF10B (DR5)**



Tissue: Bladder

Target probe: **Mfa-TNFRSF10B (DR5)**



Troubleshooting

Sample preparation and optimization

Note: Under-fixation will result in significant RNA loss during storage and may result in low signal when performing the RNAscope® assay.

The most common reason for subpar results with the RNAscope® assay is suboptimal sample preparation. Wherever possible, tissues should be prepared according to standard methods:

1. Tissue specimens should be fixed in fresh 10% NBF for 16-32 hours at room temperature and blocked into a thickness of 3-4 mm.
2. Dehydrate in a graded series of ethanol and xylene, followed by infiltration with melted paraffin held at no more than 60°C.
3. Trim paraffin blocks as needed and cut embedded tissue into 5 ±1 µm sections using a microtome.
4. Place paraffin ribbon in water bath, and mount sections on Superfrost® Plus Slides.
5. Air-dry slides overnight at room temperature. Do not bake slides unless they will be used within one week.

In many situations information on tissue preparation procedures may be unavailable. Tissue optimization steps depend not only on the type of tissue, but also the age of the sample. Optimal conditions are dependent on tissue type, age, and fixation. Simple optimization steps can help obtain quality data.

If sample preparation conditions do not match recommended guidelines or are unknown, qualifying samples prior to performing any experiments is strongly recommended. See Figure 5 for the recommended workflow:

1. Run samples along with the control slides provided by ACD (Cat. No. 310045 for Human Hela Cell Pellet, and Cat. No. 310023 for Mouse 3T3 Cell Pellet) using ACD's positive and negative control probes as previously discussed in section 1.

2. The RNAscope® scoring guidelines should be used to evaluate staining results. Successful PPIB staining should generate a score of ≥2 and UBC ≥3 with relatively uniform PPIB/POLR2A/UBC signals throughout the sample. Samples should display a dapB score of <1 indicating low to no background (see Figure 6 for scoring guidelines).

3. Use the control slides as a reference to determine if the assay was performed correctly.

4. Depending on staining results, further optimization of pretreatment conditions may be required.

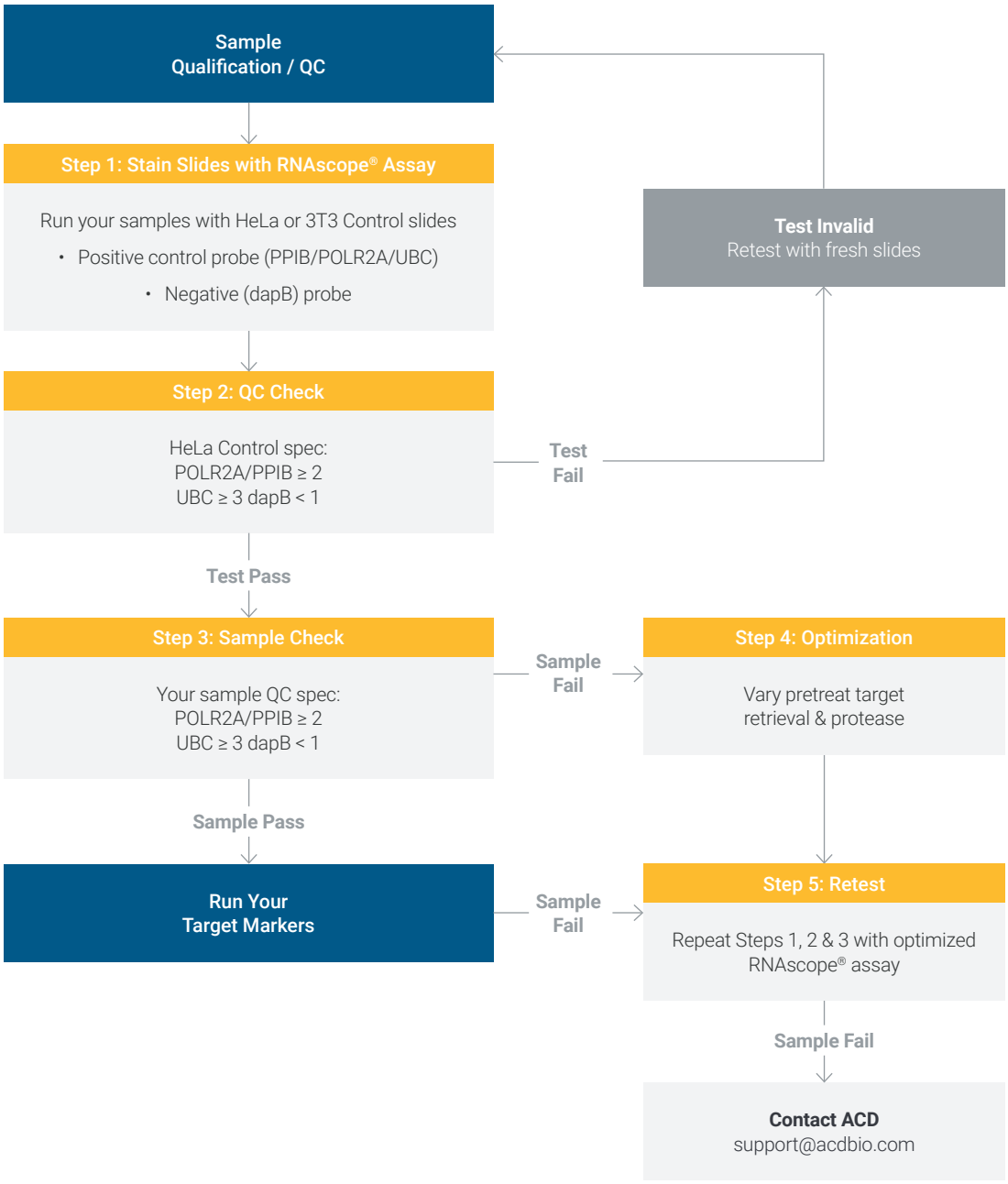
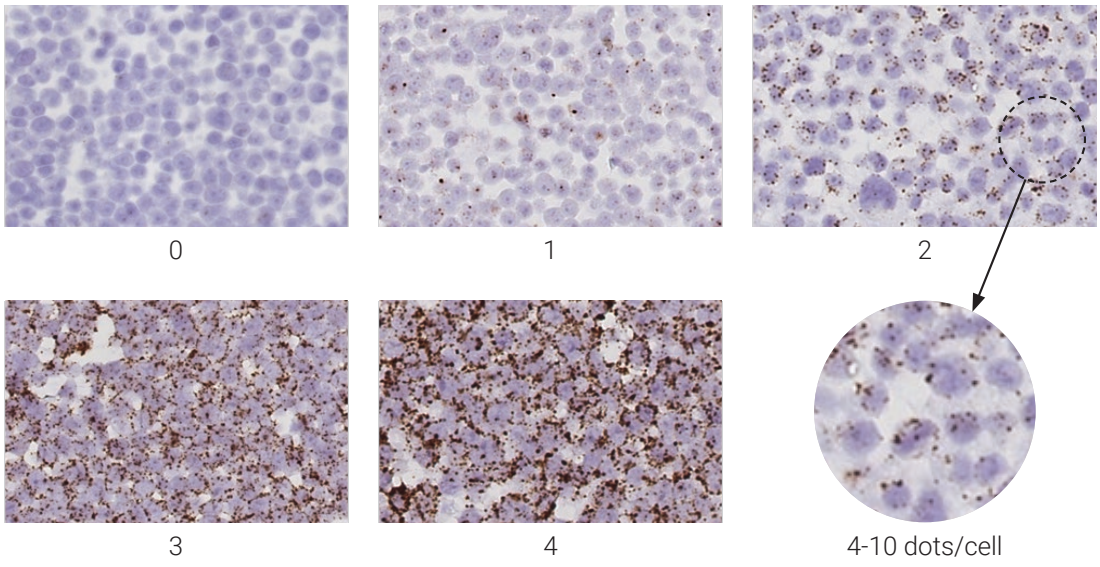


FIGURE 5. Recommended RNAscope® workflow to test samples prior to evaluating target gene expression



Score	Criteria
0	No staining or less than 1 dot to every 10 cells
1	1–3 dots/cell
2	4–10 dots/cell. Very few or no dot clusters
3	10–15 dots/cell, and less than 10% dot clusters
4	>15 dots/cell, and more than 10% dots are in clusters

FIGURE 6. RNAscope® scoring guidelines on HeLa control slides at 20X magnification. If <5% of cells score 1 and >95% of cells score 0, a score of 0 will be given. If 5-30% of cells score 1 and >70% of cells score 0, a score of 0.5 will be given.

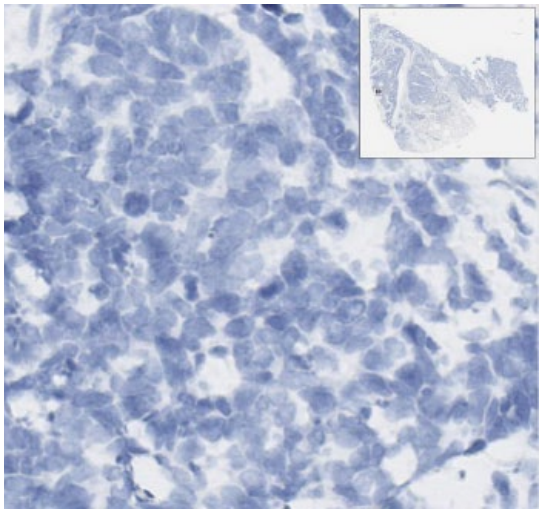
Unexpected staining patterns

Unexpected staining patterns are commonly due to suboptimal digestion conditions. Generally speaking, over-fixed or under-digested tissue will have excellent tissue morphology with weak/no signal and low signal/background ratio due to poor probe accessibility to RNA.

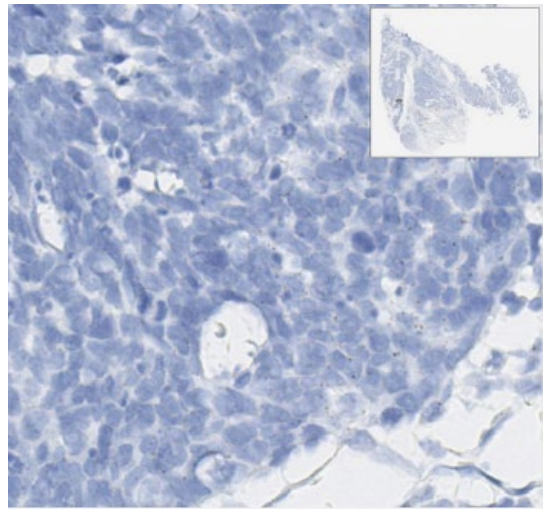
Under-fixed or over-digested tissue will have poor tissue morphology (tissue appears faded with loss of cell borders) and loss of RNA due to protease over-digestion. Use below examples of unexpected staining patterns to determine if your sample is under- or over-digested.

Under-digested

Human Lung Carcinoma: **DapB**
10 min target retrieval at 88°C + 15 min protease



Human Lung Carcinoma: **Hs-PPIB**
10 min target retrieval at 88°C + 15 min protease

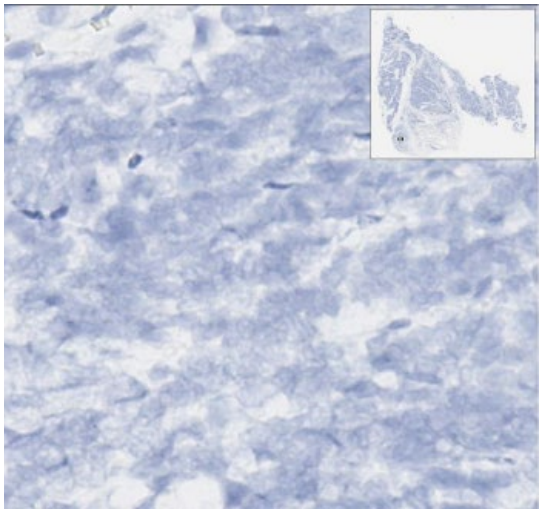


Morphology description: Excellent morphology, strong hematoxylin staining

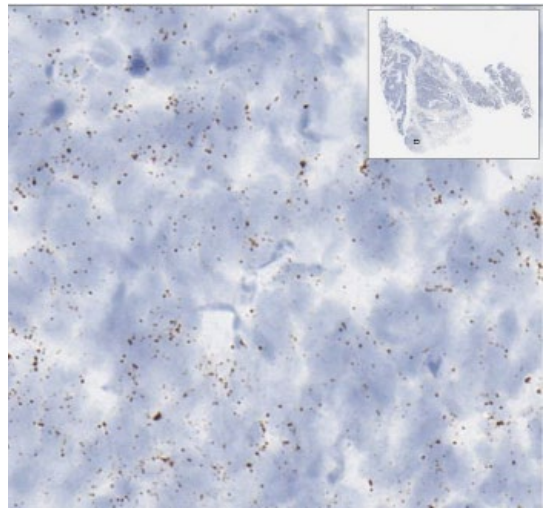
Possible effects:
Weak/no signal due to poor probe accessibility to RNA

Over-digested

Human Lung Carcinoma: **DapB**
20 min target retrieval at 95°C + 15 min protease



Human Lung Carcinoma: **Hs-PPIB**
20 min target retrieval at 95°C + 15 min protease

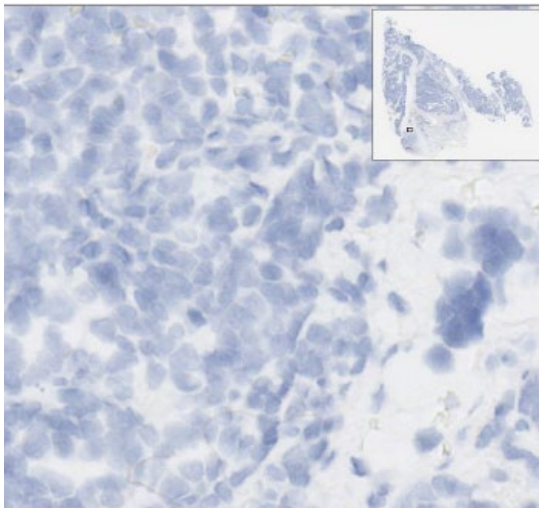


Morphology description: Destroyed tissue morphology, undefined nuclei, weak hematoxylin staining

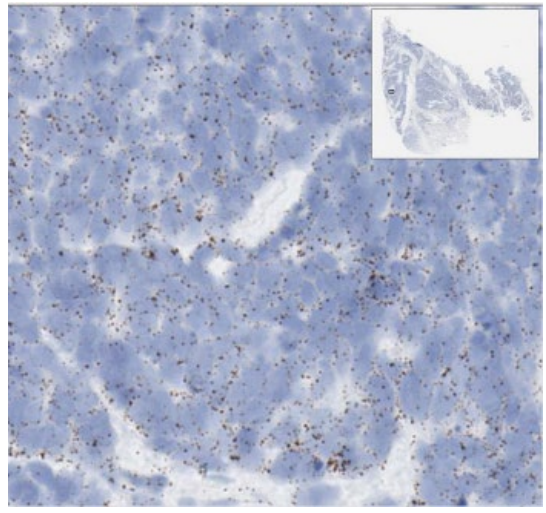
Possible effects:
High background, nonuniform strong/weak signal

Optimal digestion

Human Lung Carcinoma: **DapB**
15 min target retrieval at 95°C + 15 min protease



Human Lung Carcinoma: **Hs-PPIB**
15 min target retrieval at 95°C + 15 min protease



Morphology description: Intact tissue morphology and nuclei. Homogenous hematoxylin staining

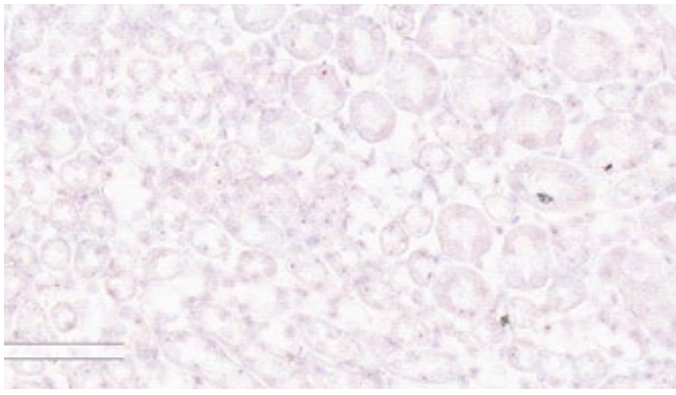
Possible effects:
High signal-to-noise ratio. Strong staining for positive controls with little to no background

High background

Unexpected staining patterns can commonly be due to suboptimal digestion conditions. Use examples below to determine possible issue and solution.

Nuclear background

Standard pretreatment (15 min target retrieval + 30 min protease) on Mm kidney

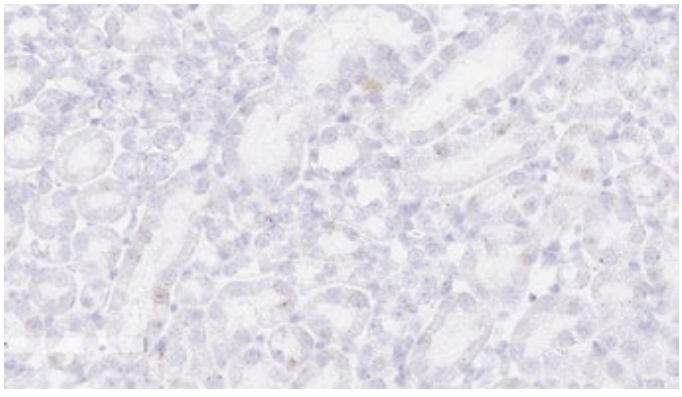


Problem

Pretreatment conditions not optimal

- Tissue is over-digested
- Tissue is under-digested

7 min target retrieval + 30 min protease on Mm kidney



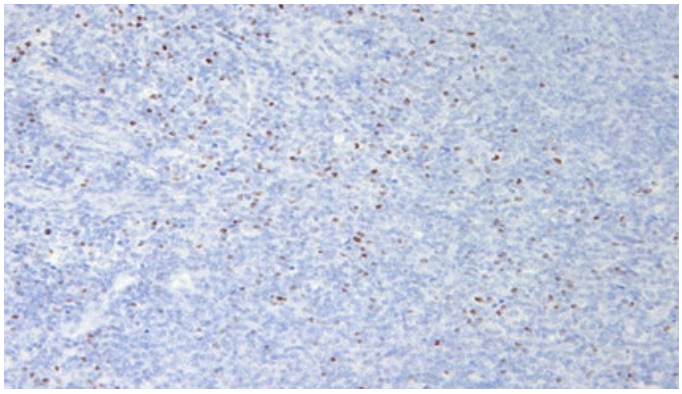
Solution

Optimize pretreatment conditions:

- Decrease boiling and/or protease if tissue is over-digested
- Increase boiling and/or protease if tissue is under-digested

Nuclear hazy background (Leica BOND RX)

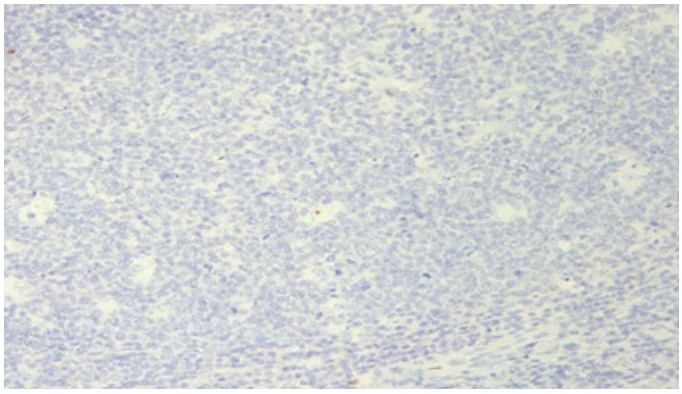
Human Tonsil: 15 min ER2 + 15 min protease



Problem

Under-pretreatment

Human Tonsil: 20 min ER2, 30 min protease



Solution

The presence of nuclear background staining (entire nuclei stained brown) can sometimes be removed by increasing pretreatment times. Increase the ER2 time in increments of 5 min and increase the protease time in increments of 10 min while keeping the temperature constant. You may also apply this process to over-fixed tissues.

Extracellular background

Problem

Incomplete paraffin removal

Solution

Use fresh/unused EtOH and xylene and agitate slides during incubation steps

Problem

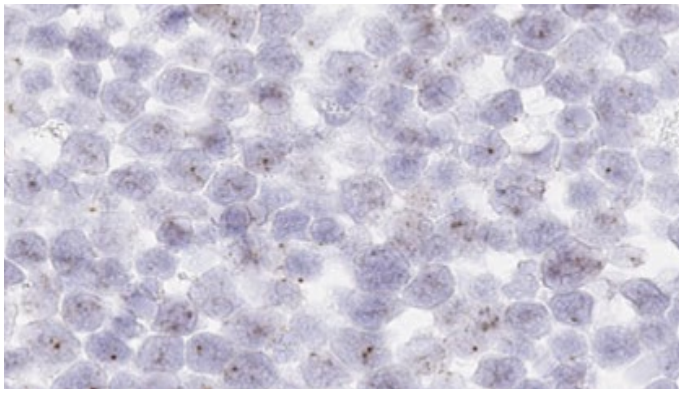
Suboptimal tissue preparation

Solution

Prepare tissue samples according to ACD's recommended procedures

Cytoplasmic background

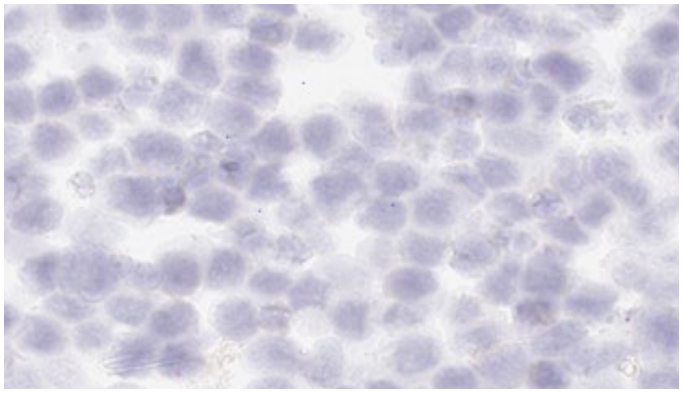
HeLa cell pellet, DapB; Dried-out tissue



Problem

Tissue dries up during assay

HeLa cell pellet, DapB; No drying between Amp steps



Solution

- Completely cover tissue when applying reagents
- Keep humidifying paper moist inside the HybEZ™ Slide Tray
- Process slides one at a time to prevent drying
- Make sure slides are kept level during incubations
- Ensure HybEZ™ Oven is at the appropriate temperature

Tissue detachment

Various samples undergo detachment during reparation for RNAscope® assay. Based on the sample type certain steps can be implemented. The following figures illustrate the steps to be altered.

FFPE Samples

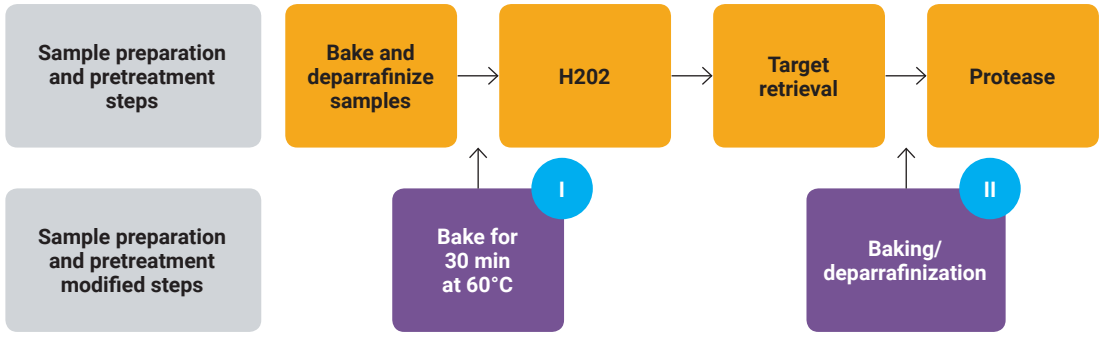
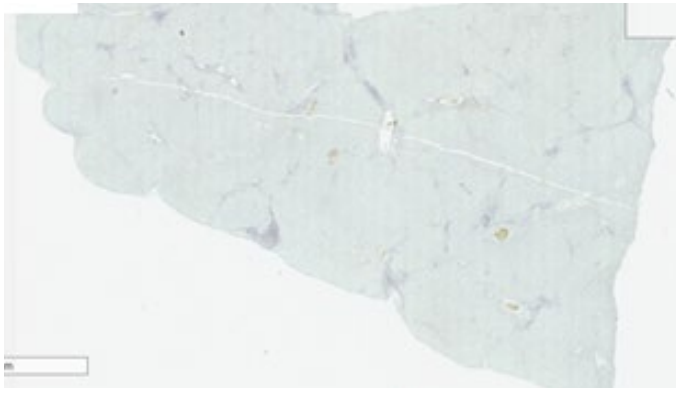


FIGURE 7. FFPE samples. Additional baking steps are added to the sample preparation workflow. For sample detachment after baking/deparaffinizaton step or after target retrieval step, a baking step is added for 30 min at 60°C (I) or (II), respectively.

Before

Suboptimal tissue preparation

Baked for 1 hour at 60°C



After

- Prepare tissue samples according to recommended procedures
- Bake slides for a longer time (up to overnight)
- Reduce boiling time

Tips for troubleshooting tissue detachment in FFPE samples:

- Always use Superfrost Plus Slides® (Fisher Scientific, Cat #12-550-15)
- Bake/dry slides in active air circulating oven (NOT HybEZ™ oven)
- Maintain mild boiling of target retrieval at ~100°C

Fixed Frozen Samples

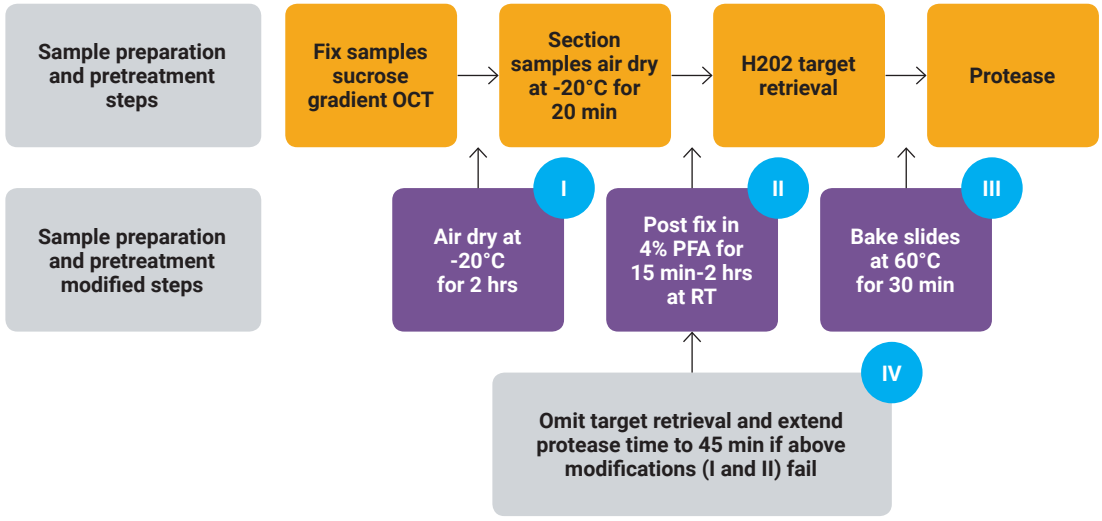


FIGURE 8. An extended air drying step is added to the sample preparation protocol at the air dying step (I) to prevent sample detachment. For sample detachment before H_20_2 step or protease digestion step, a post-fixation protocol is added; 4% PFA for 15 min-2 hr (II) or a baking step at 60°C for 30 min (III). Omit target retrieval and extend protease time to 45 min if the above modifications fail (IV).

Tips for troubleshooting tissue detachment in fixed frozen samples:

- Recommended section thickness 7-15µm
- Always use Superfrost® Plus Slides (Fisher Scientific, Cat #12-550-15)
- Bake/dry slides in active air circulating oven (NOT HybEZ™ oven)
- Maintain mild boiling of target retrieval at ~100°C

Fresh Frozen Samples

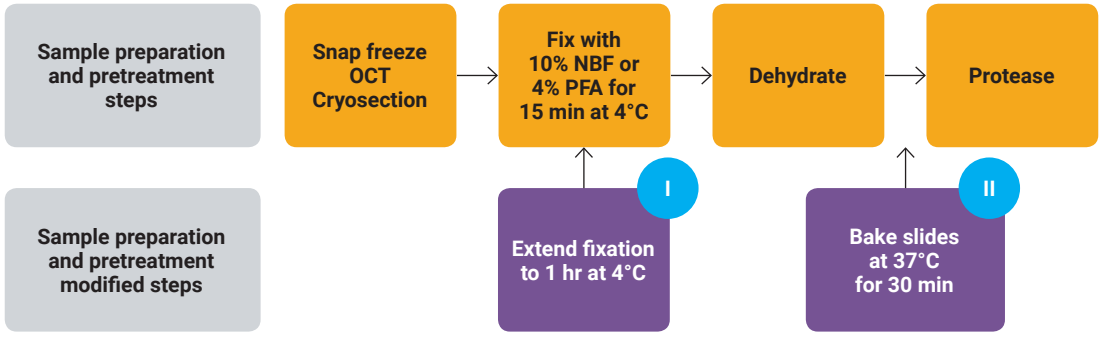


FIGURE 9. An extended fixation step is added to the sample preparation protocol to prevent sample detachment during fixation (I). For sample detachment after dehydration step and before the protease digestion step, slides are baked at 37°C for 30 min (II).

Tips for troubleshooting tissue detachment in fresh frozen samples:

- Recommended section thickness 10-20µm
- Always use Superfrost® Plus Slides (Fisher Scientific, Cat #12-550-15)
- Bake/dry slides in active air circulating oven (NOT HybEZ™ oven)

Other issues

Successful implementation of the RNAscope® assay is directly linked to hybridization environment. The ACD HybEZ™ Hybridization System and its ability to accurately keep the temperature stable is essential to success of the RNAscope® assay.

The HybEZ™ Oven is a simple, easy-to-use, low-profile benchtop hybridization oven that provides superior conditions for RNA-ISH, and is the only hybridization oven for which ACD can provide our customers with our RNAscope® assay performance guarantee. The HybEZ™ Oven provides a gasket-sealed, temperature-controlled humidifying chamber necessary for optimized RNAscope® assay performance.

No staining observed

Problem

Hybridzation conditions not optimal

Solution

Use HybEZ™ Hybridization System

Corrected Staining

No staining or less than 1 dot to every 10 cells

Hazy red staining

Problem

Not using appropiate mounting medium

Solution

Use EcoMount when mounting slides for RNAscope® Red Assays

Corrected Staining

1-3 dots/cell

No staining observed

Problem

Hybridzation conditions not optimal

Solution

Use HybEZ™ Hybridization System

Corrected Staining

10–15 dots/cell, and less than 10% dot clusters

Acknowledgements: ACD would like to thank Ming-Xiao He, PhD, and Bingqing Zhang, PhD, for generating the data and performing the analysis.

Thank you for taking the time to review Advanced Cell Diagnostics' (ACD) RNAscope® Reference Guide.

Rob Monroe, MD, PhD
Chief Medical Officer and Pathologist, Advanced Cell Diagnostics

We hope that it will help you achieve high-quality results from your RNAscope® assays and lead you to novel insights and discoveries.

As a result of its superior sensitivity and specificity over standard RNA ISH methods, RNAscope® technology has become the leading platform for detection of RNA in its morphological context at single molecule resolution. The technology is universal and can be used for detection of any RNA in any tissue in any species. RNAscope® technology has been employed in a wide variety of applications in research, drug development, and diagnostics with over 600 publications to date, leveraging the technology across the fields of oncology, immunology, infectious disease, inflammation, neuroscience, stem cell biology, developmental biology, pathology, and many others.

Since its launch in 2010, ACD has continued to enhance RNAscope® technology and expand RNAscope® ISH product offerings, including specially designed singleplex, duplex and multiplex kits and protocols for manual, automated, chromogenic, and fluorescent assays. Additionally, to date we have more than 11,000 unique off-the-shelf target probes available, addressing many popular targets and pathways that have been run at more than 3,000 labs worldwide. While ACD strives to make RNAscope® technology as accessible and easy to use, achieving high-quality results often requires sample and protocol optimization depending on the fixation method, tissue type, and species. To this end, we have aggregated and distilled the combined knowledge and experience of ACD's R&D and support scientists (including customer feedback) into this guide to help you optimize and troubleshoot your RNAscope® assays for the most common species and tissues. We have also included a discussion of control strategy and recommendations to ensure accurate interpretation of your RNAscope® data. As you start to run RNAscope® assays in your lab, we strongly encourage you to reach out to ACD's support team anytime to help you become successful faster. We have a special program designed to assist our new users. Additionally, for customers that want increased capacity or throughput, our Pharma Assay Services (PAS) team can be leveraged to run your projects, samples, and analysis here at ACD.

At ACD, we strive to serve your needs for RNA *in situ* detection through RNAscope® technology. We are continually amazed by the novel ways you are using RNAscope® technology and the exciting applications you have developed. Thank you for teaching us new applications, and we look forward to supporting and enabling your research with RNAscope® technology!

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