In Situ Hybridization (ISH) – Novel techniques

Technical aspects of Branched DNA ISH Technology

RNAscope/Basescope/ViewRNA

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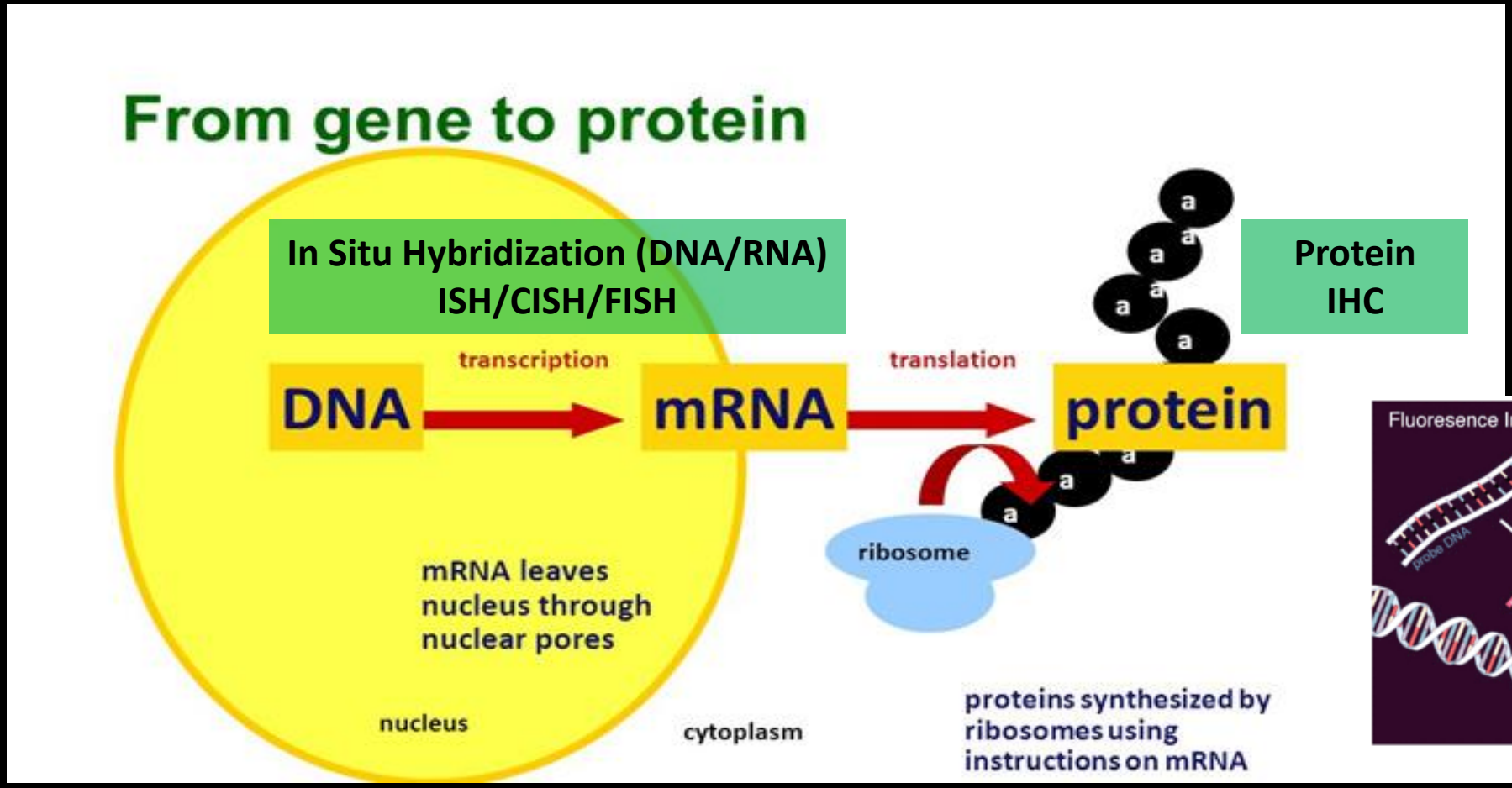
In Situ Hybridization (ISH)

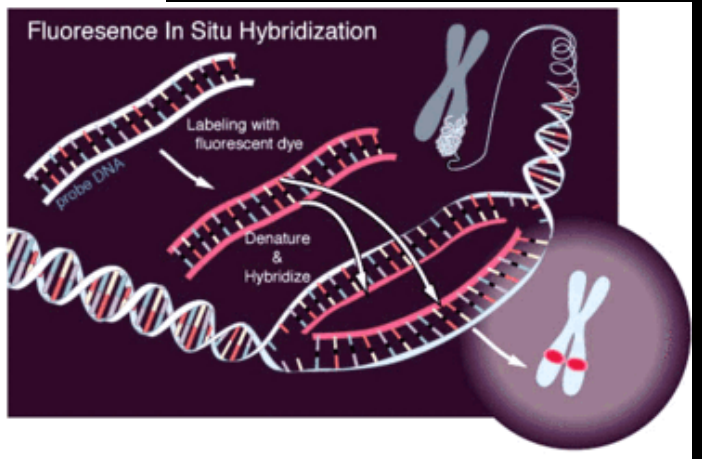
In situ hybridization (ISH) is a method using labeled complementary DNA, RNA or modified nucleic acids sequences (probes) annealing to specific target DNA or RNA molecules in cells or tissue sections.

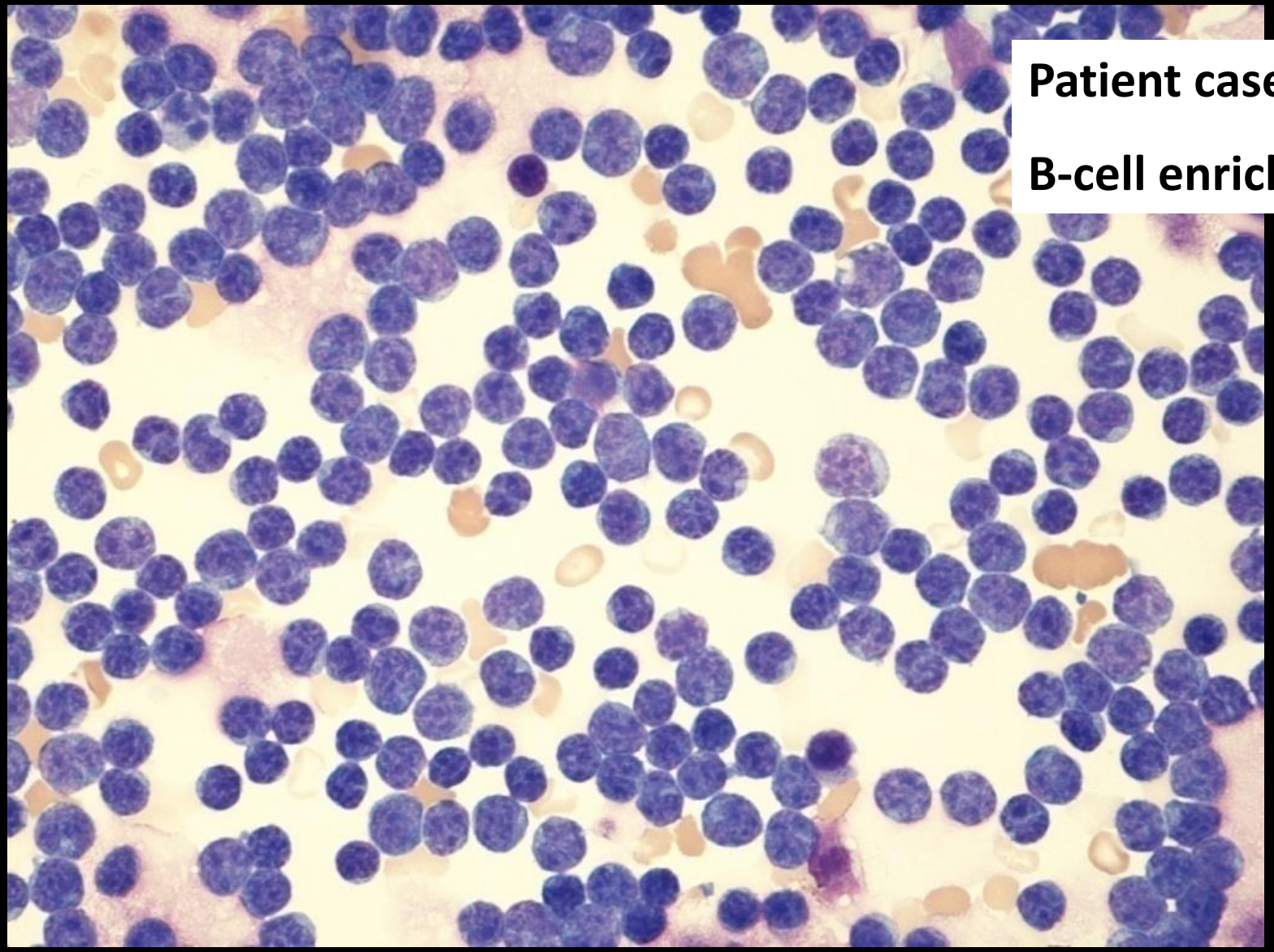


Fluorescence (FISH)

Chromogenic (CISH)

……





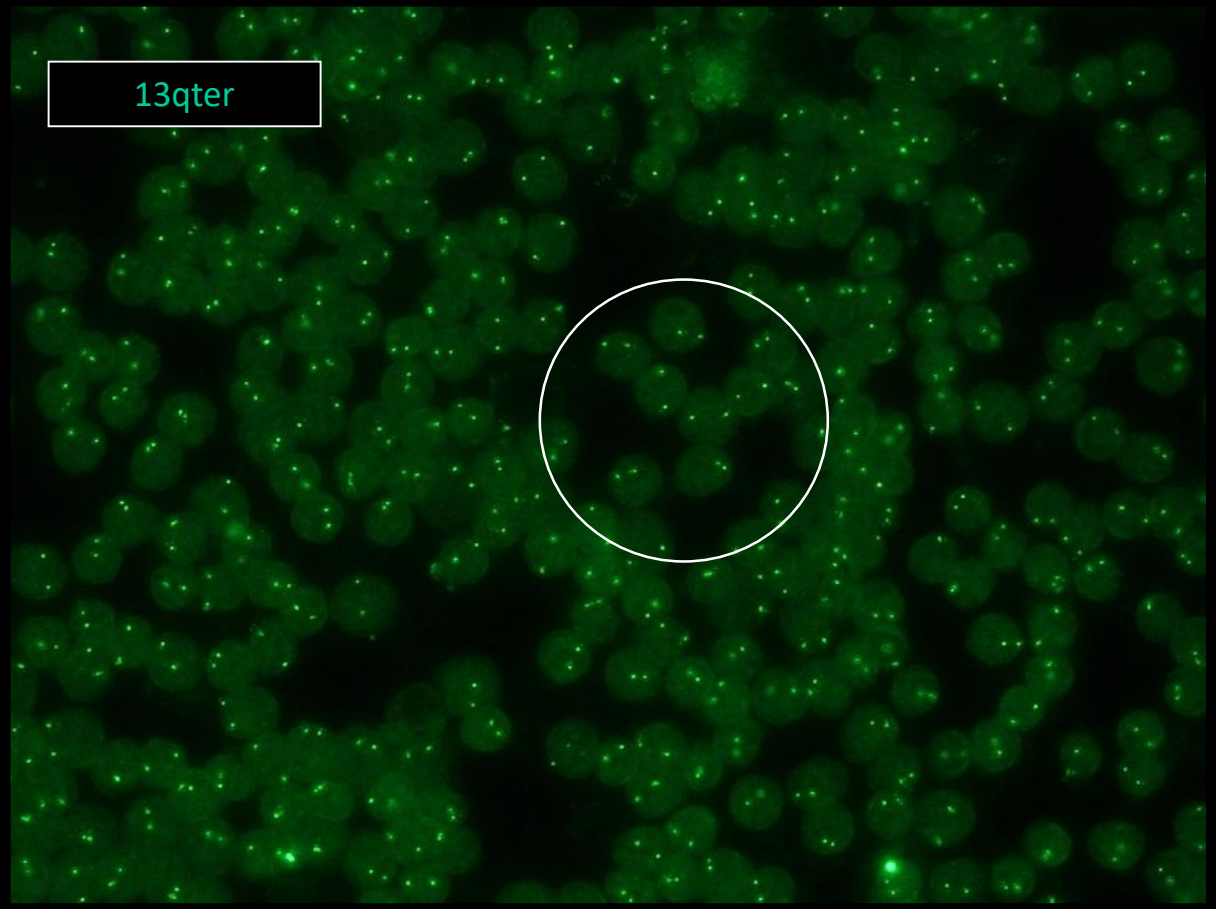
Patient case: CLL

B-cell enrichment using Rosette Sep

Note: Nearly 100% neoplastic B-cells (cytospun preparation)

Patient case: CLL

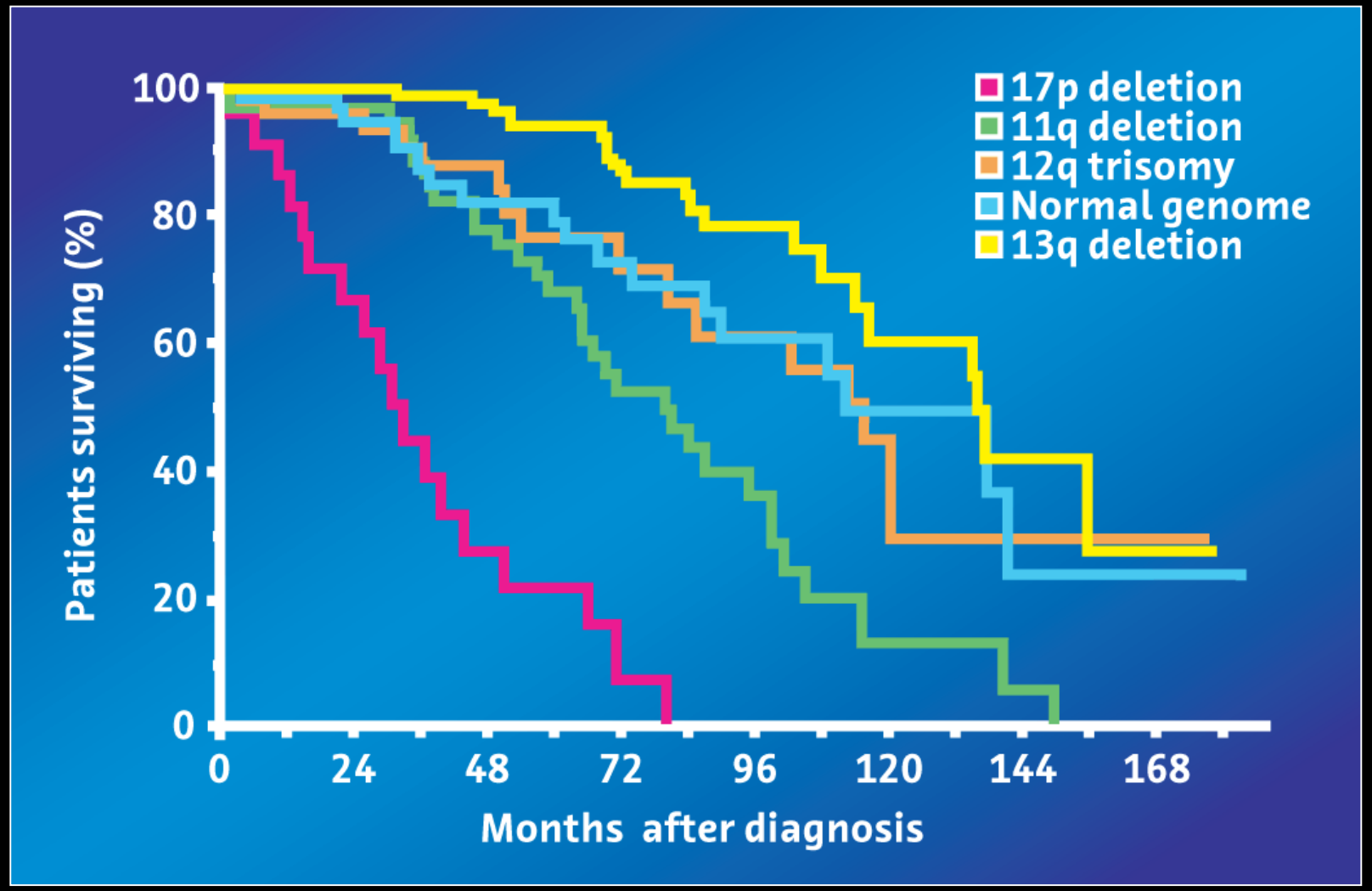
B-cell enrichment using Rosette Sep



Standard FISH technology

In addition: Deletion of ATM 11q22.3

Effects of genetic abnormalities on survival in patients with CLL (N=325)



Döhner H, et al. *N Engl J Med*. 2000;343:1910-1916.

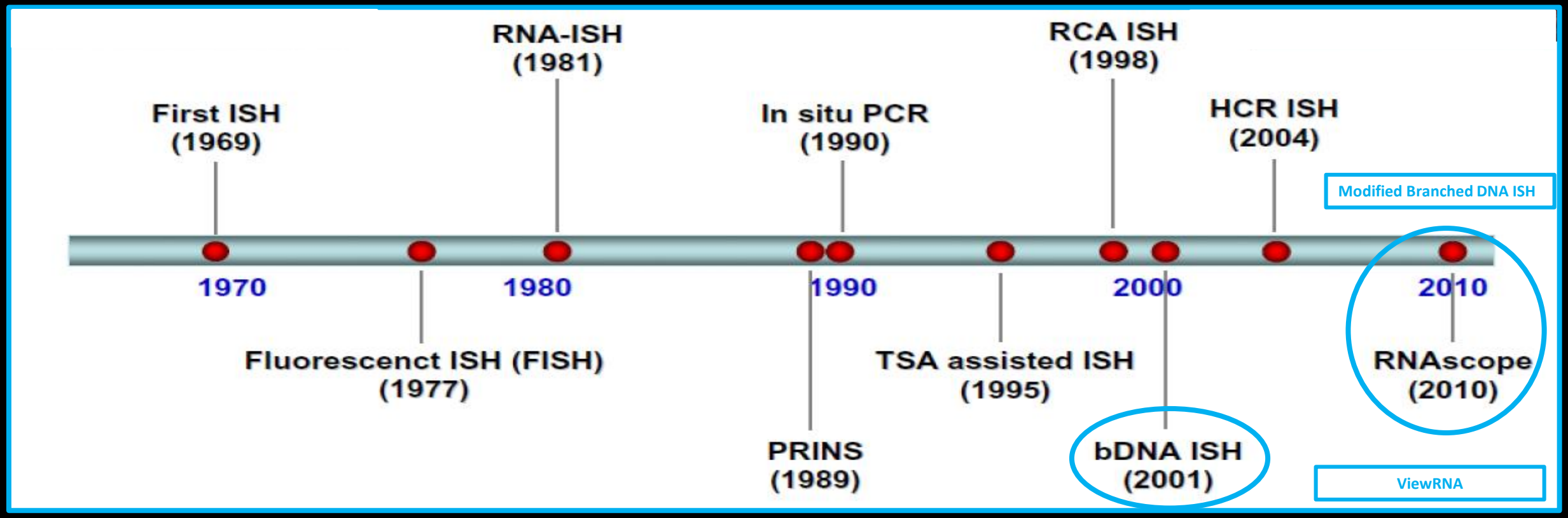
ISH: Typically use in the routine Pathology Departments:

Chromogenic ISH (CISH)	Fluorescent ISH (FISH)	Research ISH (several techniques)
▪ Human Papilloma Virus (DNA) ▪ Epstein Barr Virus encoded RNA´s (EBER - small nuclear RNA) ▪ Cytomegalovirus (DNA) ▪ IGK/IGL (mRNA) ▪ HER-2/CEP17 (DNA) ▪ ……	▪ Foetal Pathology ▪ Haematology ▪ Carcinomas ▪ Sarcomas ▪ …… Numeric abnormalities (e.g., +21/Downs Syndrome) Structural abnormalities Deletions e.g., del 17p13 (P53/CLL) Amplifications e.g., 17q12 (HER2/Breast Ca.) Translocations e.g., t(9;22)(q34;q11) (CML) Inversions e.g., inv(2)(p21;p23) (ALK/EML4) ……	▪ mRNA **(Base/RNA scope or ViewRNA)** ▪ Long non coding RNA´s (LncRNA) ▪ Small non coding RNA`s (regulatory)  - mikro RNA (miRNA) - small nucleolar RNA`s (snoRNA) - small nuclear RNA´s (snRNA) - small-interfering RNA`s (siRNA) - PIWI-interacting RNA`s (piRNA) ▪ Other - e.g. circular RNA

mRNA (Base/RNA scope) ?

LncRNA > 200 nucleotides
Regulatory small non coding RNA´s < 200 nuclotides

History of In Situ Hybridization (mRNA)



Conventional *in situ* RNA detection methodologies lack the sensitivity and specificity required to reliably detect rare or low-expressing RNA biomarkers within the tissue context

The Journal of Molecular Diagnostics, Vol. 14, No. 1, January 2012
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DOI: 10.1016/j.jmoldx.2011.08.002

Technical Advance

RNAscope

A Novel in Situ *RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues*

Wang F et al.

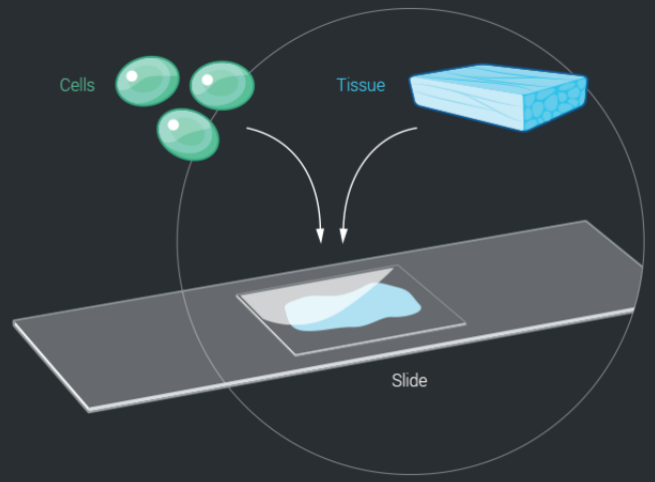
The first paper describing the use of Branched DNA ISH technology on formalin fixed and paraffin embedded tissue.

RNAscope® In Situ Hybridization Assay Workflow

Step 1:

Permeabilize

Tissue sections or cells are fixed onto slides and pretreated with RNAscope® Pretreatment Kit to unmask target RNA and permeabilize cells.

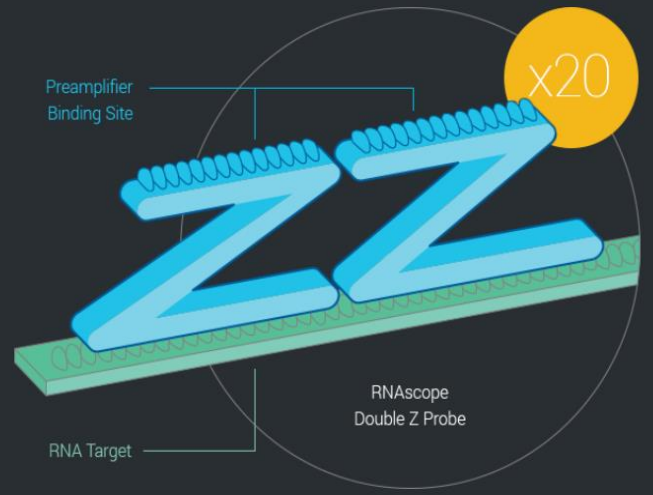


RNAscope® In Situ Hybridization Assay Workflow

Step 2:

Hybridize

Designed with ~20 target-specific double Z probes, RNAscope® Probes hybridize to target RNA molecules.



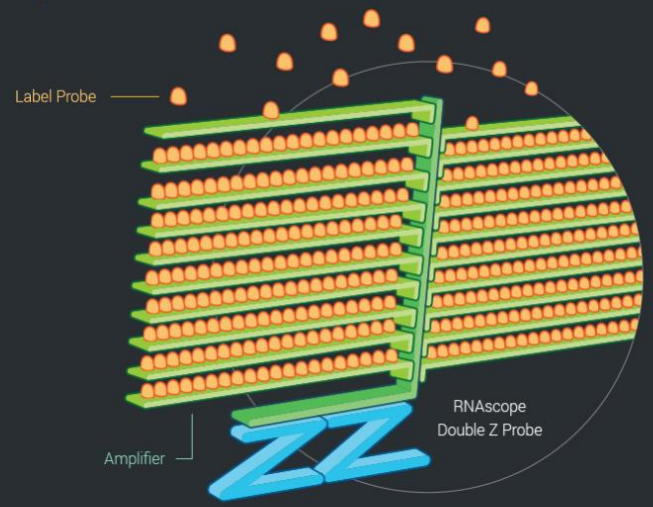
Modified Branched DNA ISH

RNAscope® In Situ Hybridization Assay Workflow

Step 3:

Amplify

RNAscope® Detection Reagents amplify the hybridization signals via sequential hybridization of amplifiers and label probes.



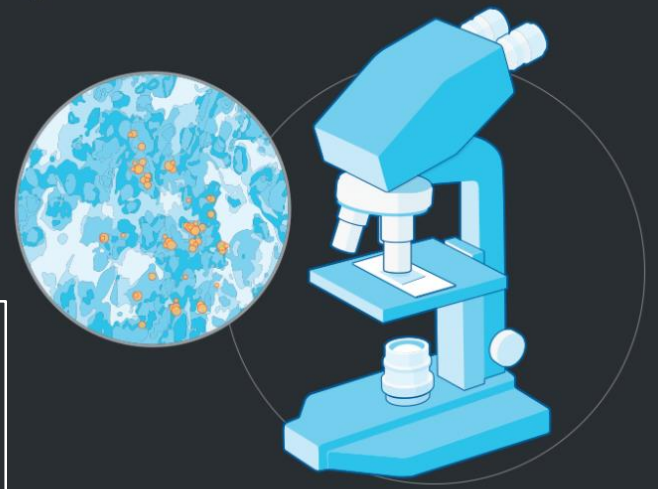
Modified Branched DNA ISH

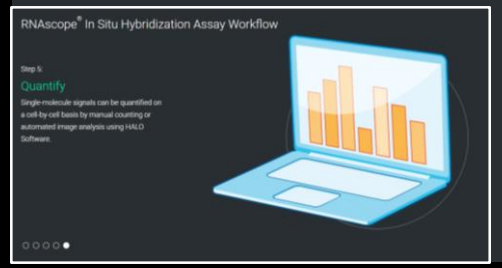
RNAscope® In Situ Hybridization Assay Workflow

Step 4:

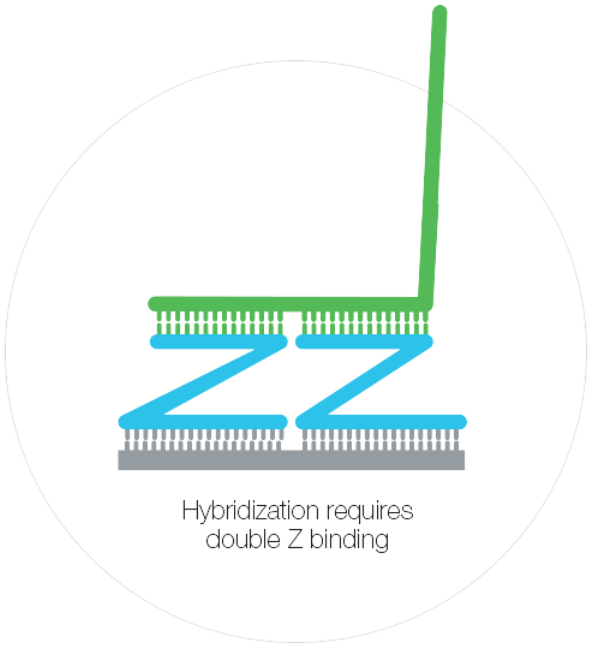
Visualize

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





SinglePlex /Advance Cell Diagnostic (ACD)



RNAscope® Probe Design and Signal Amplification Strategy

In order to substantially improve the signal-to-noise ratio of RNA ISH, RNAscope® employs a probe design strategy much akin to fluorescence resonance energy transfer (FRET), in which two independent probes (double Z probes) have to hybridize to the target sequence in tandem in order for signal amplification to occur. As it is highly unlikely that two independent probes will hybridize to a non-specific target right next to each other, this design concept ensures selective amplification of target-specific signals.

For each target RNA species, ~20 double Z target probe pairs are designed to specifically hybridize to the target molecule, but not to non-targeted molecules.

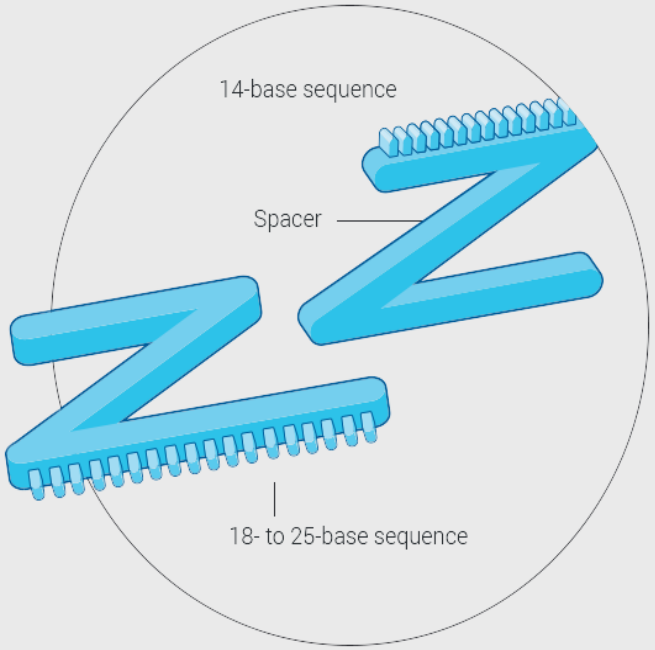
- > 45000 catalog target probes.
- > 140 species (human, mouse, rat…..).
- New customer probes in two weeks (development/manufacturing).

Each Target Z Probe Contains Three Elements

The lower region of the Z is an 18-to 25-base region that is complementary to the target RNA. This sequence is selected for target specific hybridization and uniform hybridization properties.

A spacer sequence that links the two components of the probe. The upper region of the Z is a 14-base tail sequence.

The two tails from a double Z probe pair forms a 28 base binding site for the pre-amplifier.

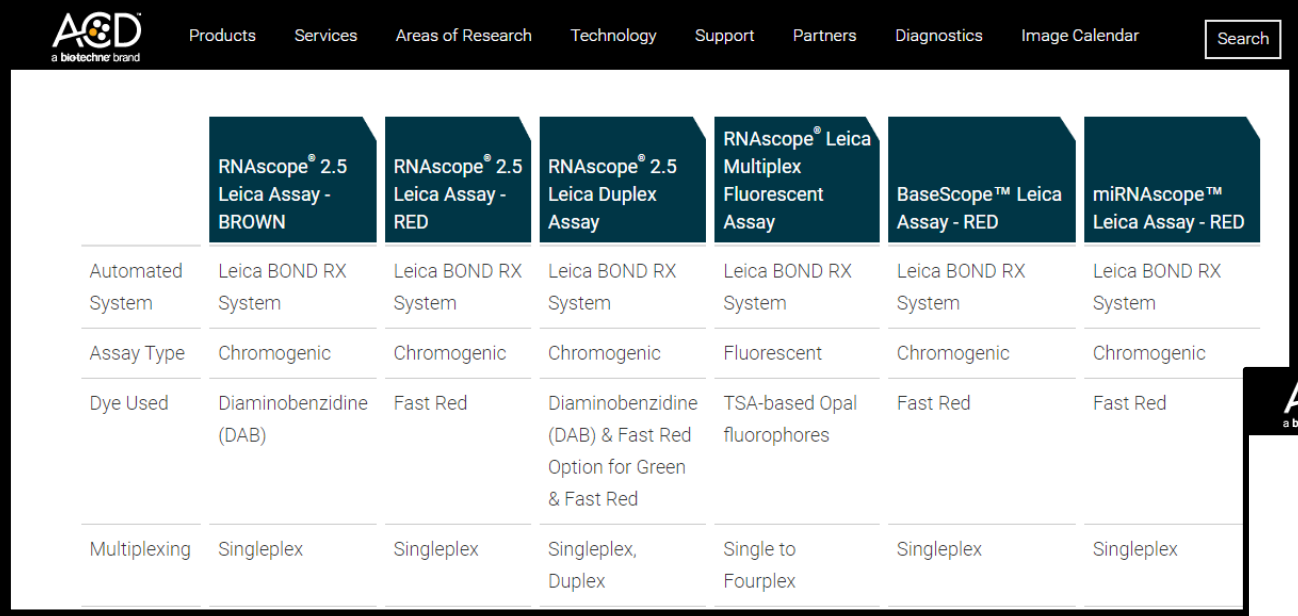


BaseScope vs RNAscope (mRNA ISH)

	BaseScope	RNAscope
Size of target RNA	▪ RNA 50-300 nt (bases)	▪ mRNA > 300 nt ▪ lncRNA > 300 nt
Number of ZZ pairs pr. target	▪ 1-4 ZZ pairs depending on application	▪ Standard 20 ZZ pairs (minimum 6 ZZ pairs)
Application	▪ Single RNA molecule detection ▪ Exon Junction/splice variants, point mutation and short RNA sequences ▪ Other (e.g., gene fusion at mRNA level)	▪ Single RNA molecule detection
Detection options	▪ Single (Chromogenic Red) ▪ Duplex (Chromogenic Green/Red)	▪ Single (Chromogenic or fluorescent) ▪ Duplex (Chromogenic) ▪ Multiplex Fluorescent (up to 4 RNA targets) ▪ HiPlex (up to 12 RNA targets)
Automation	▪ Bond Rx (Leica): Single/Fast Red ▪ Ventana Discovery: Single/Fast Red	▪ Bond Rx (Leica): Single, Duplex and Multiplex ▪ Ventana Discovery (Roche): Single and Duplex ▪ Lunaphore (Single or HiPlex/Multiomics and Proteomics)
Workflow length	▪ 8.5 Hours (Manual/Single Staining)	▪ 8 Hours (Manual/Single staining)
Probes	▪ C1 (HRP)/C2 (AP) Channels	▪ C1/C2/C3/C4…… ?

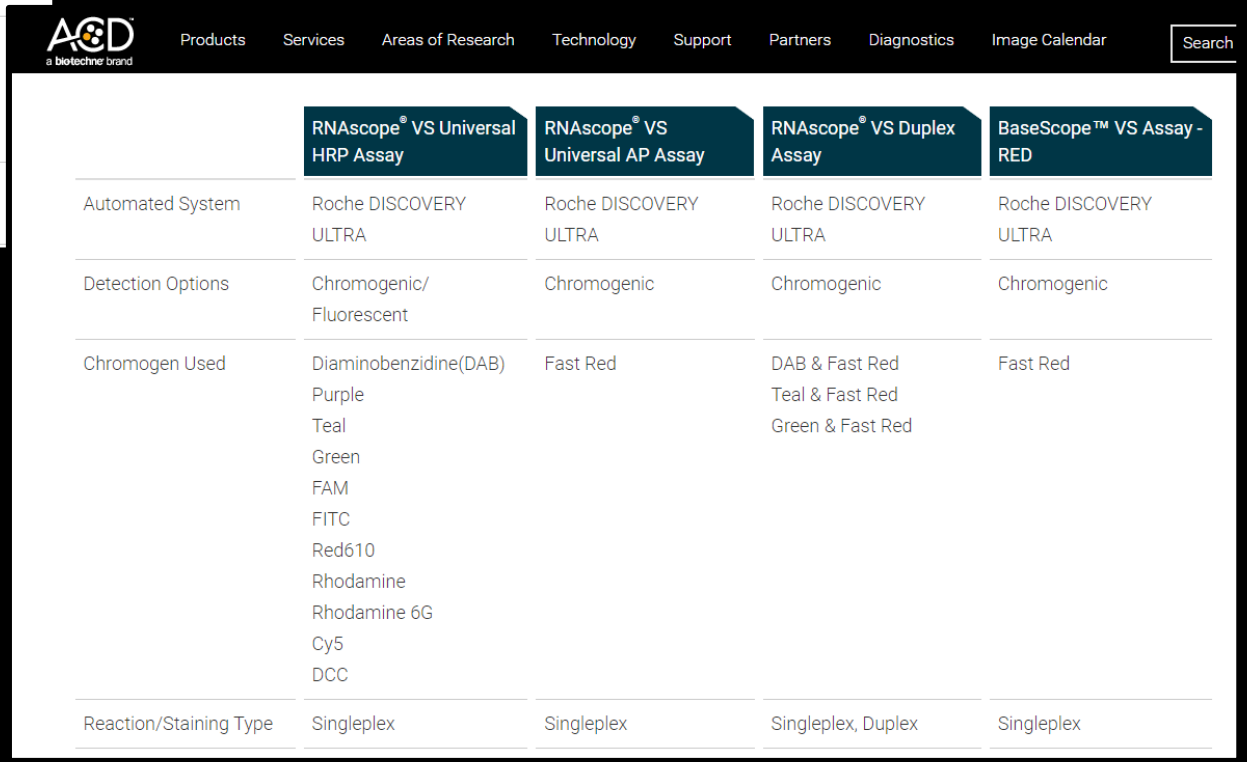
Long non-coding RNAs (lncRNAs) are a large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides that do not encode proteins (or lack > 100 amino acid open reading frame). lncRNAs are important regulators of gene expression, and lncRNAs are thought to have a wide range of functions in cellular and developmental processes.

Base/RNAScope (mRNA ISH) - Automation



ACD a biotechne brand | Products | Services | Areas of Research | Technology | Support | Partners | Diagnostics | Image Calendar | Search

	RNAscope® 2.5 Leica Assay - BROWN	RNAscope® 2.5 Leica Assay - RED	RNAscope® 2.5 Leica Duplex Assay	RNAscope® Leica Multiplex Fluorescent Assay	BaseScope™ Leica Assay - RED	miRNAscope™ Leica Assay - RED
Automated System	Leica BOND RX System	Leica BOND RX System	Leica BOND RX System	Leica BOND RX System	Leica BOND RX System	Leica BOND RX System
Assay Type	Chromogenic	Chromogenic	Chromogenic	Fluorescent	Chromogenic	Chromogenic
Dye Used	Diaminobenzidine (DAB)	Fast Red	Diaminobenzidine (DAB) & Fast Red Option for Green & Fast Red	TSA-based Opal fluorophores	Fast Red	Fast Red
Multiplexing	Singleplex	Singleplex	Singleplex, Duplex	Single to Fourplex	Singleplex	Singleplex



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	RNAscope® VS Universal HRP Assay	RNAscope® VS Universal AP Assay	RNAscope® VS Duplex Assay	BaseScope™ VS Assay - RED
Automated System	Roche DISCOVERY ULTRA	Roche DISCOVERY ULTRA	Roche DISCOVERY ULTRA	Roche DISCOVERY ULTRA
Detection Options	Chromogenic/ Fluorescent	Chromogenic	Chromogenic	Chromogenic
Chromogen Used	Diaminobenzidine(DAB) Purple Teal Green FAM FITC Red610 Rhodamine Rhodamine 6G Cy5 DCC	Fast Red	DAB & Fast Red Teal & Fast Red Green & Fast Red	Fast Red
Reaction/Staining Type	Singleplex	Singleplex	Singleplex, Duplex	Singleplex

Automation – Highly recommended

Manual – Labor intensive (many hybridization/protocol steps).

Molecular Diagnosis & Therapy (2022) 26:19–37
https://doi.org/10.1007/s40291-021-00570-2

SYSTEMATIC REVIEW

Evaluation of the Suitability of RNAscope as a Technique to Measure Gene Expression in Clinical Diagnostics: A Systematic Review

Sameeha Atout[1] · Shaymaa Shurrab[2] · Carolyn Loveridge[1]

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Abstract

Objective To evaluate the application of RNAscope in the clinical diagnostic field compared to the current 'gold standard' methods employed for testing gene expression levels, including immunohistochemistry (IHC), quantitative real time PCR (qPCR), and quantitative reverse transcriptase PCR (qRT-PCR), and to detect genes, including DNA in situ hybridisation (DNA ISH).

Methods This systematic review searched CINAHL, Medline, Embase and Web of Science databases for studies that were conducted after 2012 and that compared RNAscope with one or more of the 'gold standard' techniques in human samples. QUADAS-2 test was used for the evaluation of the articles' risk of bias. The results were reviewed narratively and analysed qualitatively.

Results A total of 27 articles (all retrospective studies) were obtained and reviewed. The 27 articles showed a range of low to middle risk of bias scores, as assessed by QUADAS-2 test. 26 articles studied RNAscope within cancer samples. RNAscope was compared to different techniques throughout the included studies (IHC, qPCR, qRT-PCR and DNA ISH). The results confirmed that RNAscope is a highly sensitive and specific method that has a high concordance rate (CR) with qPCR, qRT-PCR, and DNA ISH (81.8–100%). However, the CR with IHC was lower than expected (58.7–95.3%), which is mostly due to the different products that each technique measures (RNA vs. protein).

Discussion This is the first systematic review to be conducted on the use of RNAscope in the clinical diagnostic field. RNAscope was found to be a reliable and robust method that could complement gold standard techniques currently used in clinical diagnostics to measure gene expression levels or for gene detection. However, there were not enough data to suggest that RNAscope could stand alone in the clinical diagnostic setting, indicating further prospective studies to validate diagnostic accuracy values, in keeping with relevant regulations, followed by cost evaluation are required.

1 Introduction

1.1 The Developmental History of RNAscope

Gene expression involves transcription of DNA into messenger RNA (mRNA) followed by translation of mRNA to protein. Other important RNA molecules, such as microRNAs and long non-coding RNAs, can also play a role in

Key Points

RNAscope is a novel technology that can be used to measure gene expression (RNA).

RNAscope could be used as a complementary technique alongside existing procedures to enhance the diagnosis of disease that occurs as a result of abnormal gene expression, for example to confirm any unclear results from gold standard methods.

For RNAscope to be used as a tool to diagnose disease, further research is required to fully validate the technique so that it complies with regulatory standards and to assess cost implications for the health service.

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[2] Division of Biochemical Diseases, Department

Systematically review of the RNAscope technique based on retrospective studies (27 articles)

- Advantages
- Disadvantages
- Clinical use ?

Table 2 Advantages and disadvantages of RNAscope technique

Factor	Study no.[a]
Advantages	
Identify gene expression at a single-cell level within a morphological context	13, 18
Does not depend on antibodies	13
Allows the detection of mRNA as a single gene copy	12, 20
High analytical accuracy, sensitivity and specificity	1, 4, 7–9, 11,15, 17, 19–24
More reliable than IHC	3
Suppress background noise and produce better resolution than IHC	8, 15, 17, 20, 27
Reduce the risk of false-positive results	17
Its results are easy to interpret	5, 6, 8, 15, 17, 21
It is a robust and quantitative technique	11, 16, 27
It can detect tissue heterogeneity and partially degraded RNA	2, 27
Quick to perform	9, 11, 18
It can be performed automatically and manually and saves time	1, 14
Disadvantages	
It is not suitable to discriminate between viral RNA transcripts and viral DNA	7
The stain will not take place well if the samples are with poor fixation quality and the cost is much higher compared to IHC	11
In cervical intraepithelial neoplasia (CIN) cases, the negativity of RNAscope does not guarantee the absence of HR-HPV	16
RNAscope was less specific differentiating AdCC from high grade basaloid sinonasal tumors	22
In the automated system, some areas in the slides need manual selection during the scoring process	26

[a]The study numbers (Study no.) in this table are used throughout the article to refer to the papers. See Table 1 for references

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This paper also reviewed that there was :

A close concordance rates between the results of RNAscope and molecular techniques as qPCR /DNA ISH.

A high level of variability between the results of RNAscope and IHC

Details that could account for the high level of variability (RNAscope vs IHC)

- **Gene regulations: Transcriptional (mRNA) and post transcriptional (protein) levels**
- **Content (difference) between RNA and protein e.g., mutations might alter protein content**
- **Phophorylation/glycosylation (post-translation) can affect protein but not RNA expression**
- **Raw mRNA molecules (coding and non-coding sequences) might be translated differently into several proteins, and thus, could explain for the relative low CR between RNAscope and IHC.**
- **Other variables ?**

RNAscope and use in clinical diagnostic

Am J Clin Pathol. 2013 Nov;140(5):736-46

Ultrasensitive RNA in situ hybridization for detection of restricted clonal expression of low-abundance immunoglobulin light chain mRNA in B-cell lymphoproliferative disorders.

JCI Insight. 2020 5(12); e139042

Molecular Detection of SARS-CoV-2 in Formalin Fixed Paraffin Embedded Specimens

J Vis Exp. 2014 Mar 11;(85):51426

RNAscope for in situ detection of transcriptionally active human papillomavirus in head and neck squamous cell carcinoma.

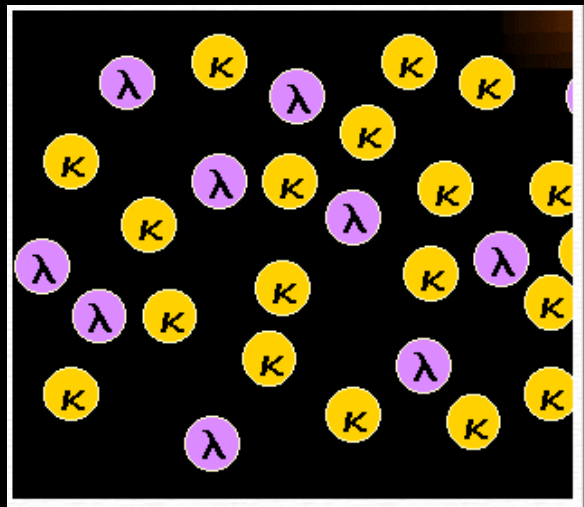
Few publications ?

Why should we use RNAscope in a Pathology Department

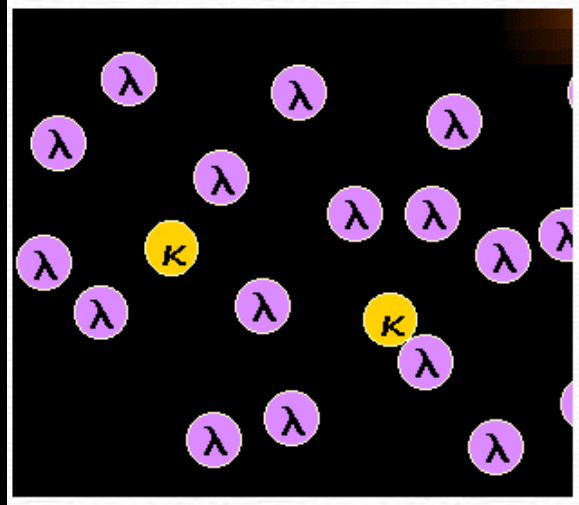
- Helpful in challenging diagnostic situations e.g., detection of light chain restrictions (kappa/lambda) in B-cell Lymphomas
- Confirming mRNA findings (Nanostring profiling) – which cells are positive
- Validation/verification of reaction patterns obtained with antibodies
- Lack of valid primary antibodies
- BaseScope

e.g. point mutation (BRAF V600E in melanomas or colon adenocarcinomas) or gene fusion products

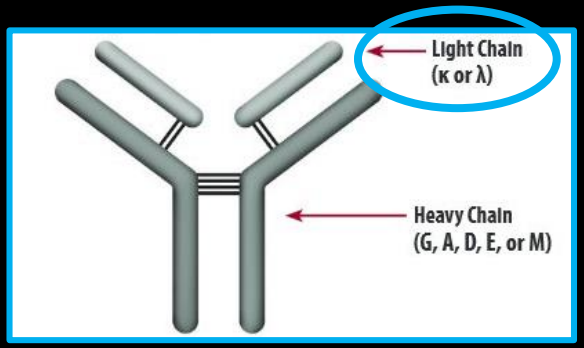
B-cell lymphomas and plasma cell disorders are characterized by showing immunoglobulin light chain restrictions and is the hallmark of discriminating reactive conditions from malignant transformation .



Normal, polyclonal B-cells are a mixture of kappa-B-cells and lambda-B-cells.
Our B-cells have a special feature letting us detect their clonality easily: A B-cell carries either kappa- or lambda-light chains on its surface. And normal polyclonal B-cells are a mixture of kappa-B-cells and lambda B-cells as can be seen in the left-hand figure.



Monoclonal mature B-cells are either kappa or lambda.
If a malignant B-cell clone proliferates this will result in a B-cell population consisting of either only kappa- or only lambda-B-cells. The latter case (i.e. lambda-monoclonal B-cells) is symbolized in the left-hand figure.
Note: in rare cases we find no light chain expressed on the B-cell surface, even in a mature B-cell neoplasm. This makes the diagnosis a little bit more difficult.



<u>In general:</u>

B-cells express membranous Ig`s

Plasma cells express cytoplasmic Ig´s and secret Ig`s to the surrounding tissue

Demonstrations of immunoglobulin light chain restrictions in B-cell lymphomas

Challenges:

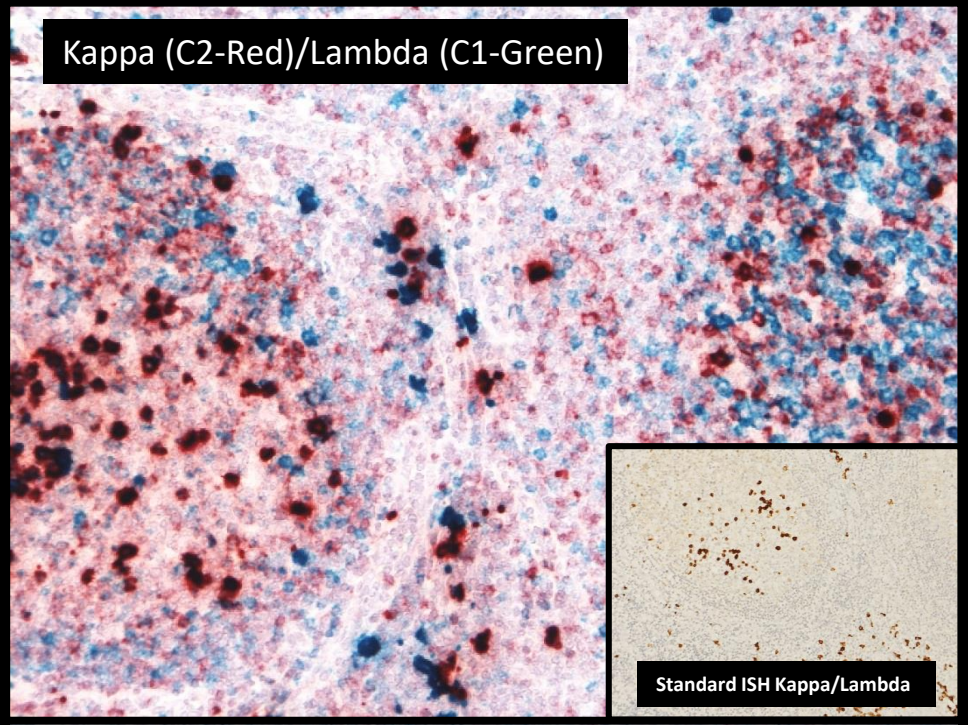
- **Fresh and unfixed material unavailable for Flowcytometric investigations (Standard method).**
  - Kappa/lambda antibodies are used in panels with other hematolymphoid markers
- **Immunohistochemistry have the tendency to be confounded by background staining.**
  - Serum immunoglobulin
  - Require carefully calibrated protocol (difficult) and “optimal” pre-analytic conditions
  - Risk of false positive /negative results due suboptimal fixation
- **Lack of sensitive and robust mRNA ISH technology for FFPE tissue**
  - Mature B-cell lymphomas often express low level of membranous immunoglobulin (protein) and thus, low level of mRNA K/L

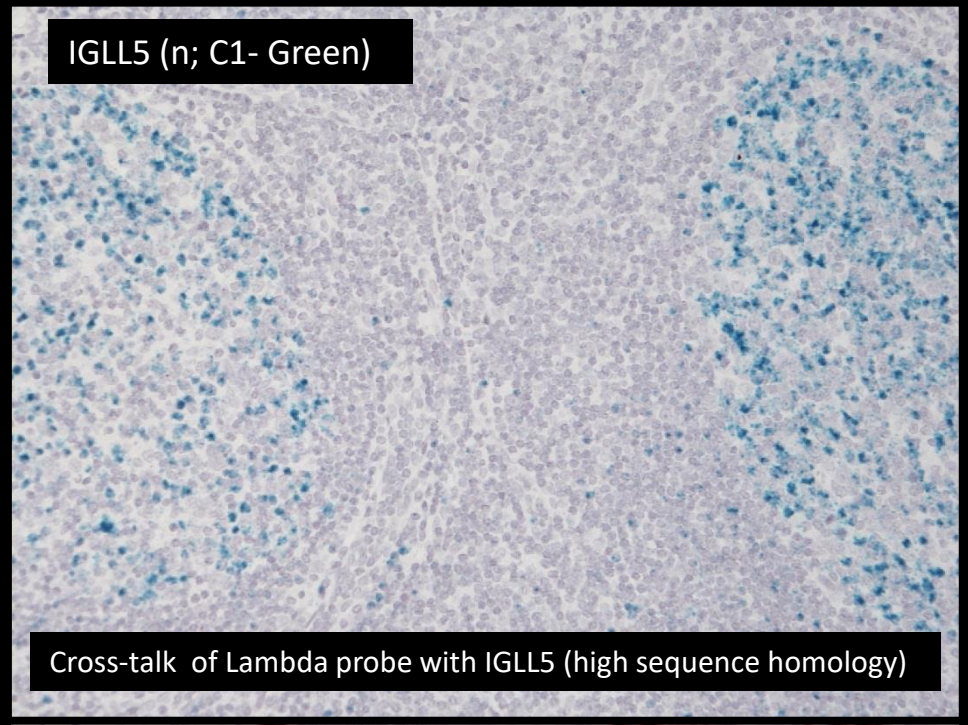
Test: RNAscope for light chain restriction (Kappa/Lambda)

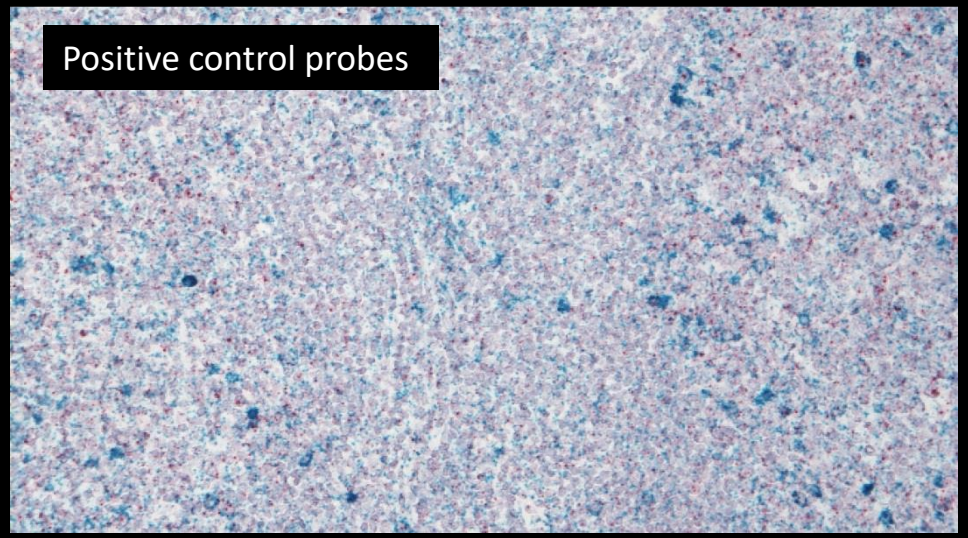
TMA`s /Diagnosis	Clinical info Light chain restriction (standard methods)	
Lymphoplasmacytoid lymphoma (LPL) (1) Nae	Lambda^{+}/Kappa^{-} (IHC^{+}/ISH^{+})	
Lymphoplasmacytoid lymphoma (LPL) (2)/Nae	Lambda^{+}/Kappa^{-} (IHC^{+}/ISH^{+})	
Lymphoplasmacytoid lymphoma (LPL) (3)/Nae	Kappa^{+}/Lambda^{-} (IHC^{+}/ISH^{+})	
Lymphoplasmacytoid lymphoma (LPL) (4)/Ros	Kappa^{+}/Lambda^{-} (FC)	
Lymphoplasmacytoid lymphoma (LPL) (5)/Ros	Kappa^{+}/Lambda^{-} (FC)	High mRNA level
Lymphoplasmacytoid lymphoma (LPL) (6)/Ros	Kappa^{+}/Lambda^{-} (FC)	
Lymphoplasmacytoid lymphoma (LPL) (7)/Ros	Unknown	
Lymphoplasmacytoid lymphoma (LPL) (8)/Ros	Unknown	
Myeloma/Næ	Kappa^{+}/Lambda^{-} (IHC^{+}/ISH^{+})	
Mantle cell lymphoma (MCL) (1)/Nae	Kappa^{+}/Lambda^{-} (IHC^{+}/ISH^{-})	
Mantle cell lymphoma (MCL) (2)/Nae	Kappa^{+}/Lambda^{-} (IHC^{+}/ISH^{-})	
Mantle cell lymphoma (MCL) (3)/Nae	Lambda^{+}/Kappa^{-} (IHC^{+}/ISH^{-})	
Mantle cell lymphoma (MCL) (4)/Nae	Lambda^{+}/Kappa^{-} (IHC^{+}/ISH^{-})	
Follicular Lymphoma (FL) (1)/Ros	Kappa^{+}/Lambda^{-} (FC)	Low mRNA level
Follicular Lymphoma (FL) (2)/Ros	Unknown	
Follicular Lymphoma (FL) (3)/Ros	Kappa^{+}/Lambda^{-} (FC)	
Diffuse Large B-Cell Lymphoma (DLBCL) (1)/Ros	Lambda^{+}/Kappa^{-} (FC)	
Diffuse Large B-Cell Lymphoma (DLBCL) (2)/Ros	Unknown	
Tonsil (Fix time 6-168h)	Poly	
Negative control tissue (Appendix, Kidney and placenta)	Negative	

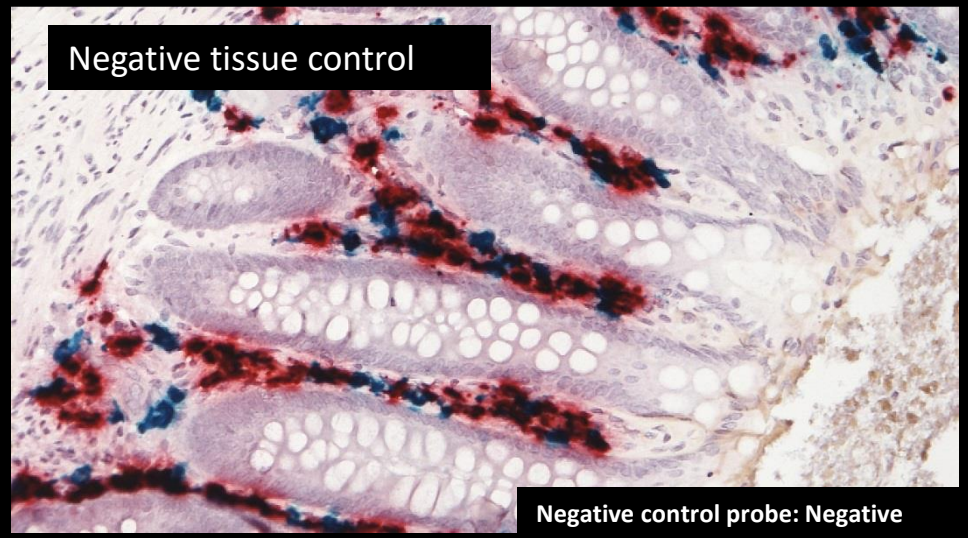
RNA Scope Duplex

Tonsil fix 96h (NBF)









IGLL5 = Immunoglobulin Lambda Like Polypeptid 5

(H15/P10-20-30)

Mantle cell B-cell Lymphomas

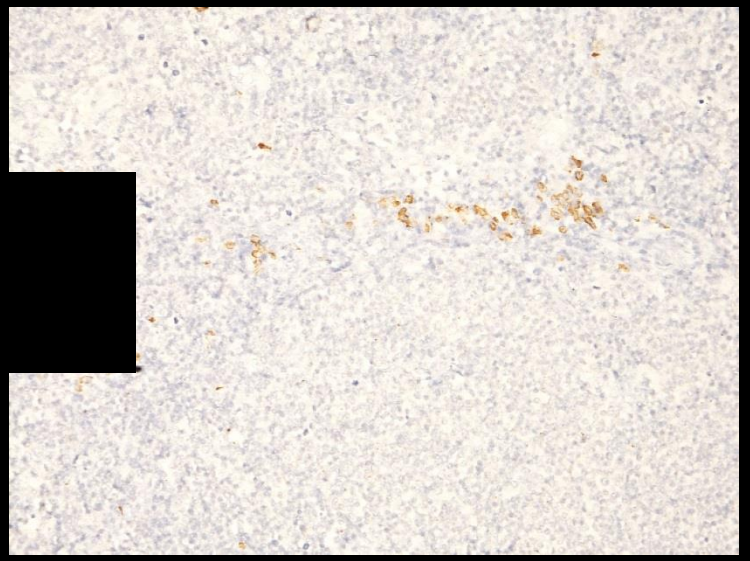
Standard ISH (Kappa)/DAB

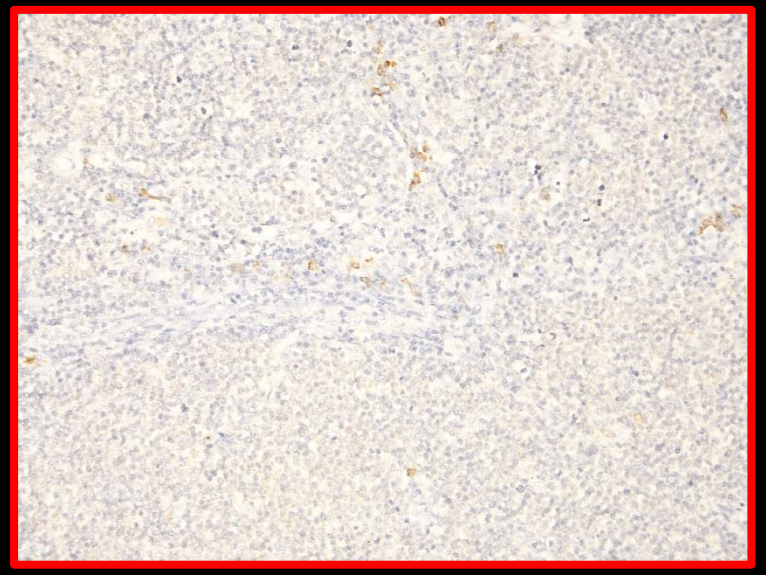
Standard ISH (Lambda)/DAB

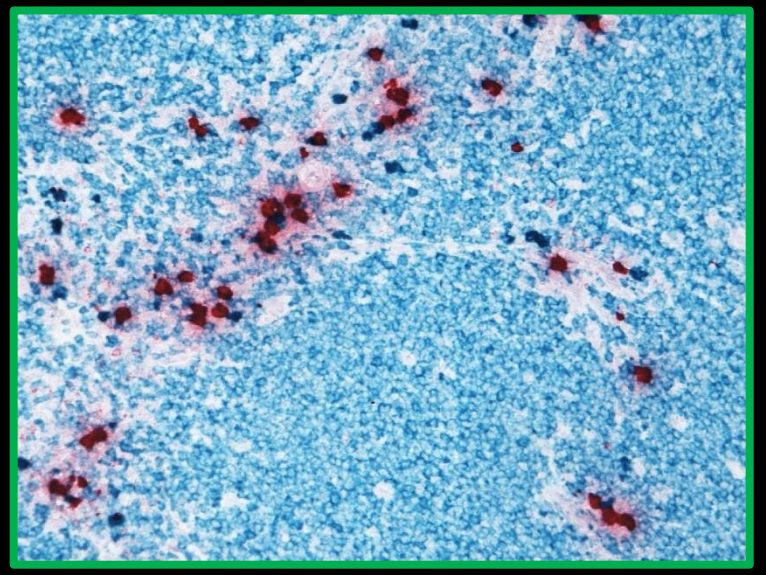
RNAscope Kappa (C2-Red)/Lambda(C1-Green)

Mantle cell Lymphoma

Lambda +

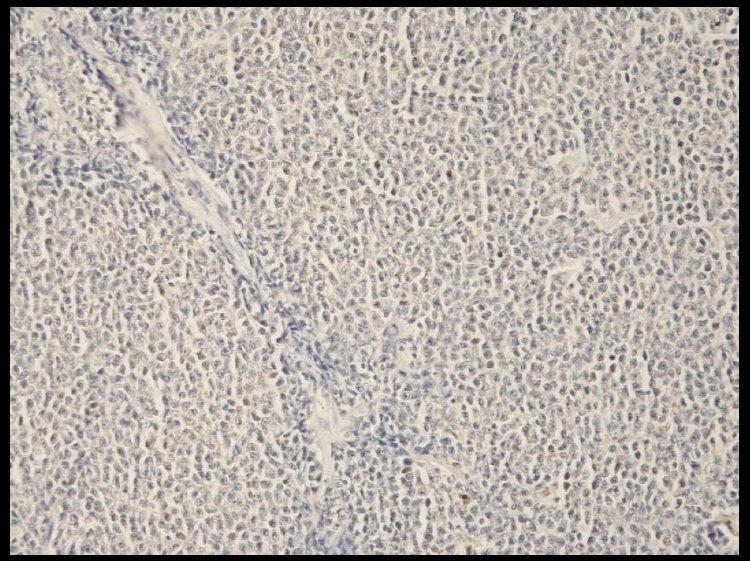


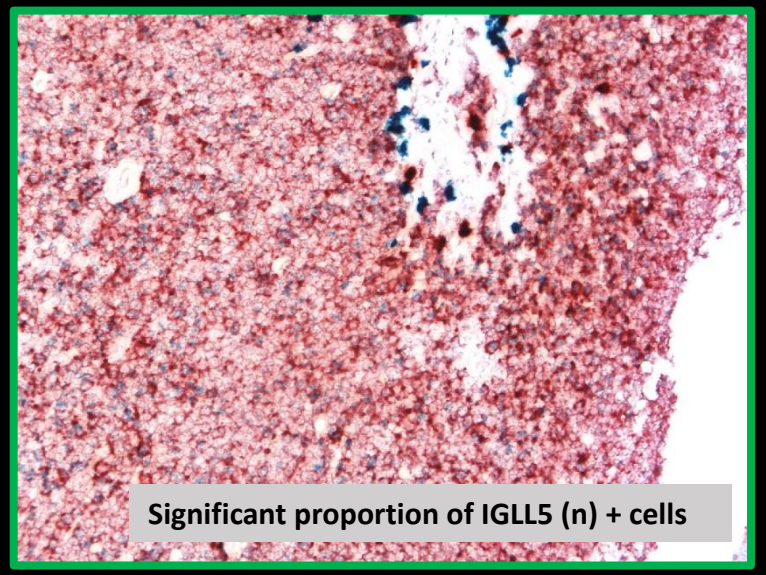




Mantle cell Lymphoma

Kappa +



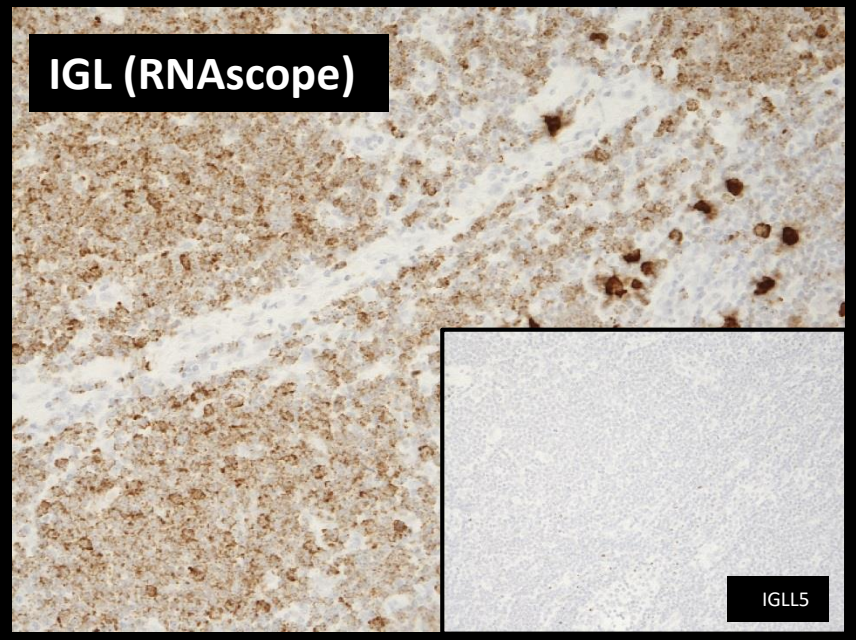


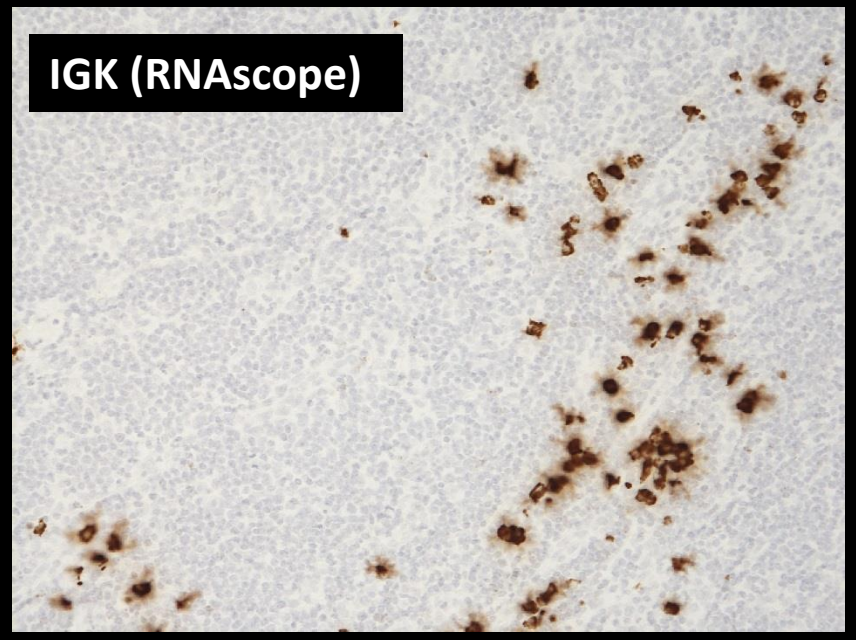
Mantle cell B-cell Lymphomas

RNAscope (SinglePlex/DAB) K/L

Mantle cell Lymphoma

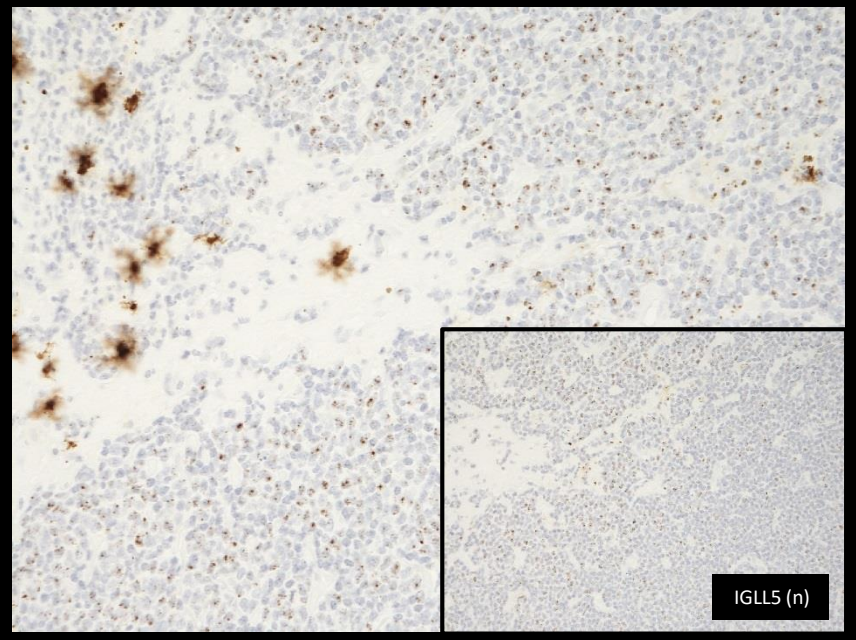
Lambda +

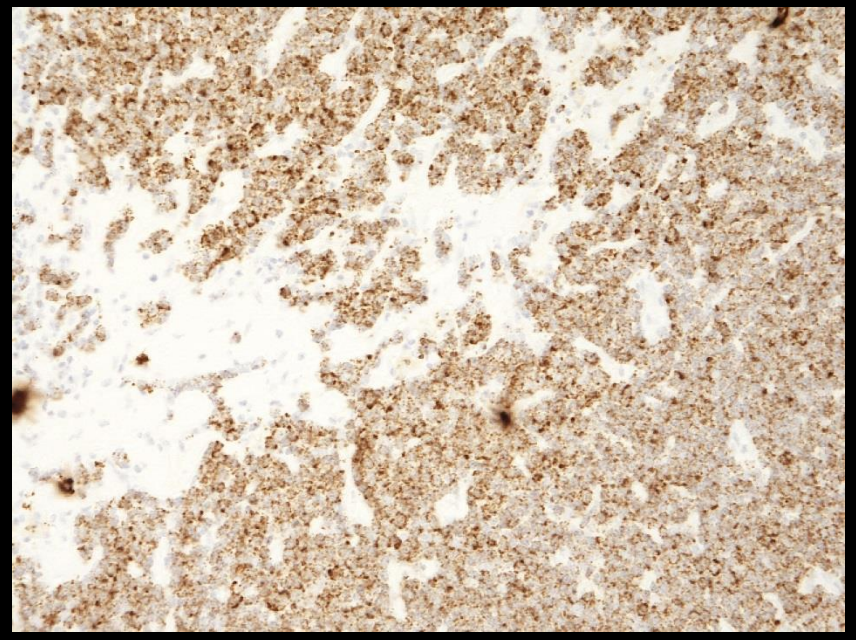




Mantle cell Lymphoma

Kappa +



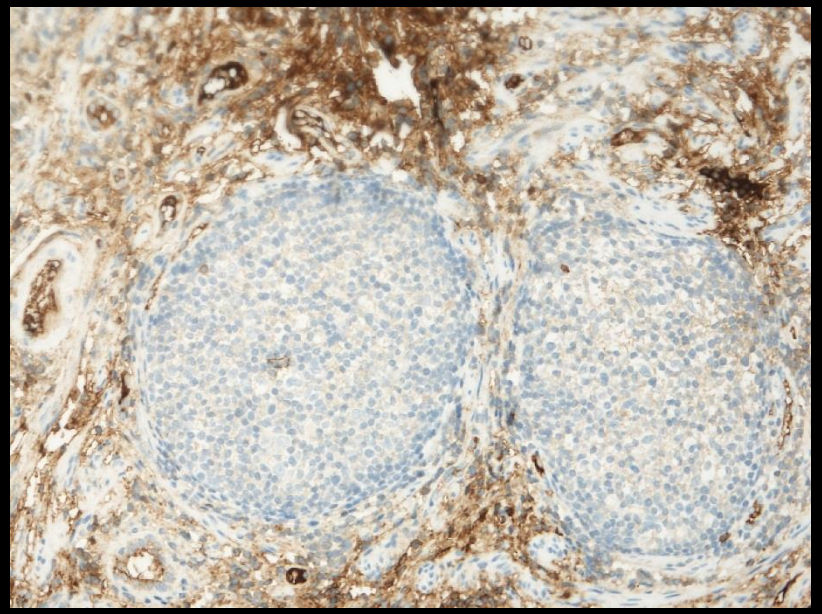


Low grade B-cell Lymphoma

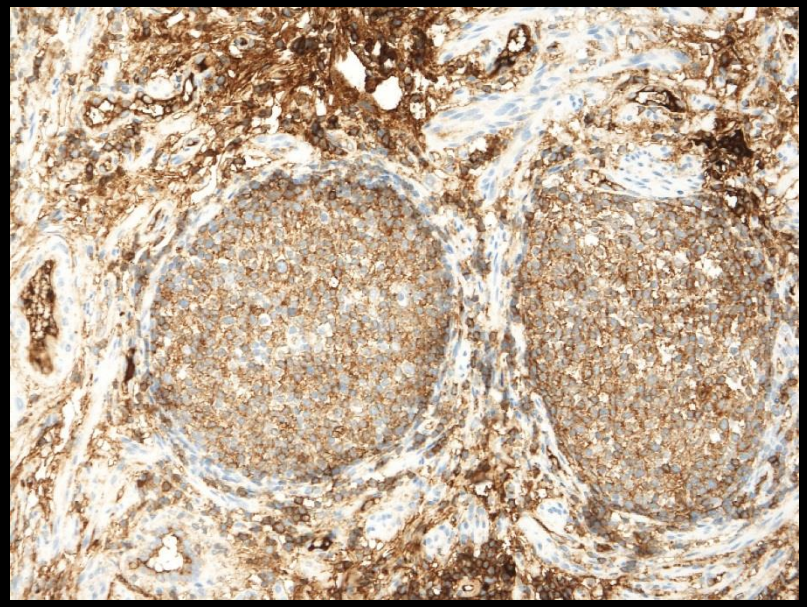
RNA Scope (Duplex)

Follicular Lymphoma (2)/Ovary

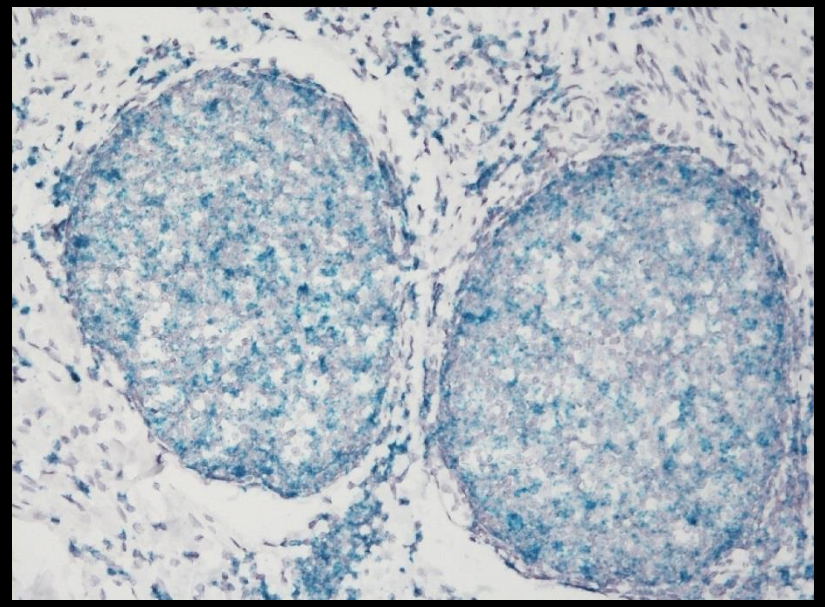
Clinical info: Unknown Kappa/Lambda status (Ros)



IHC Kappa (re-test)



IHC Lambda (re-test)



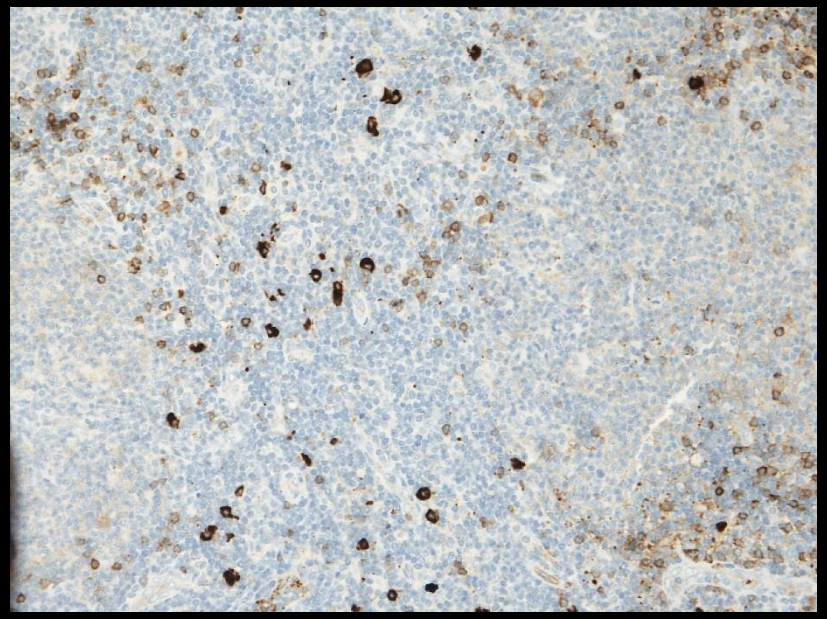
RNA scope Kappa(C2-Red)/Lambda (C1-Green)

(H15/P10-20-30)

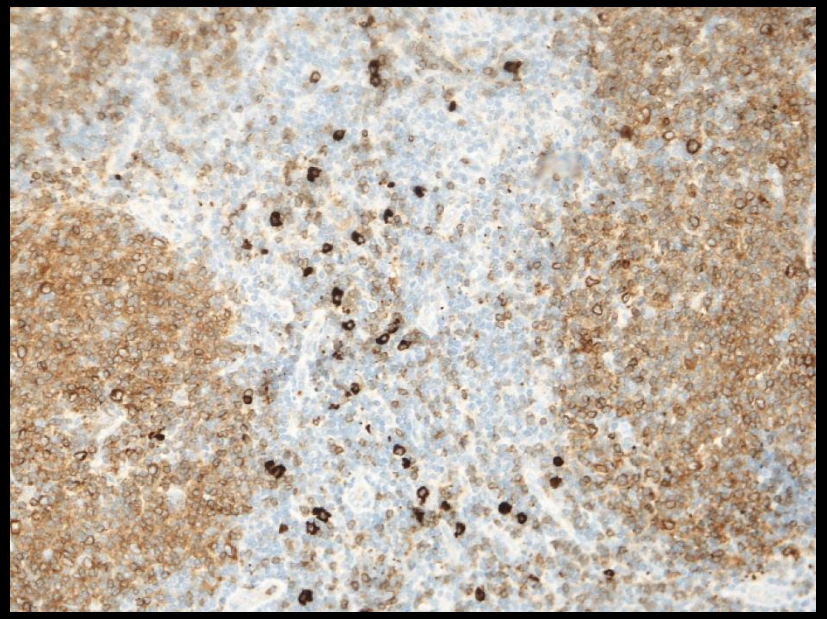
Low grade B-cell Lymphoma
RNA Scope (Duplex)

Follicular Lymphoma (1)

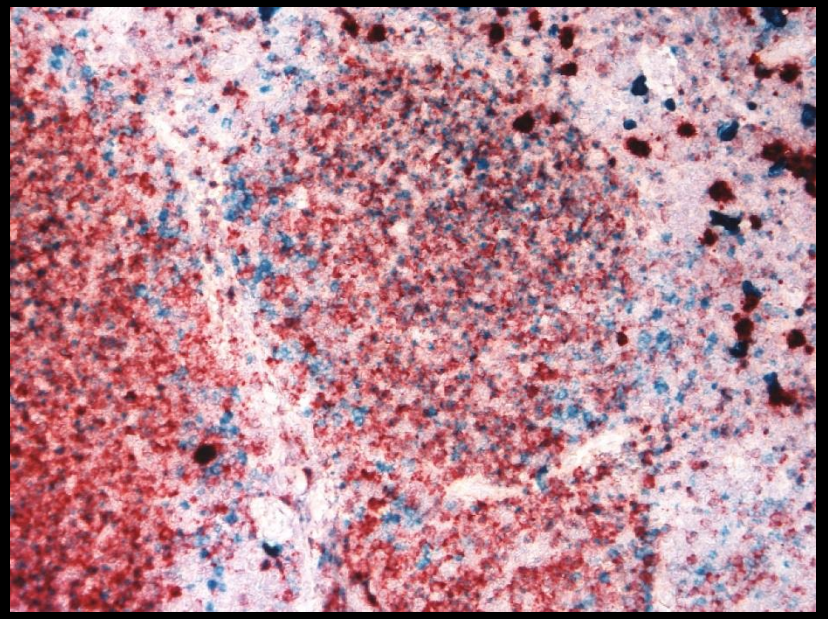
Clinical info: Kappa positive (Ros)



IHC Lambda (re-test)



IHC Kappa (re-test)



RNA scope Kappa-(C2-Red) Lambda-(C1-Green)

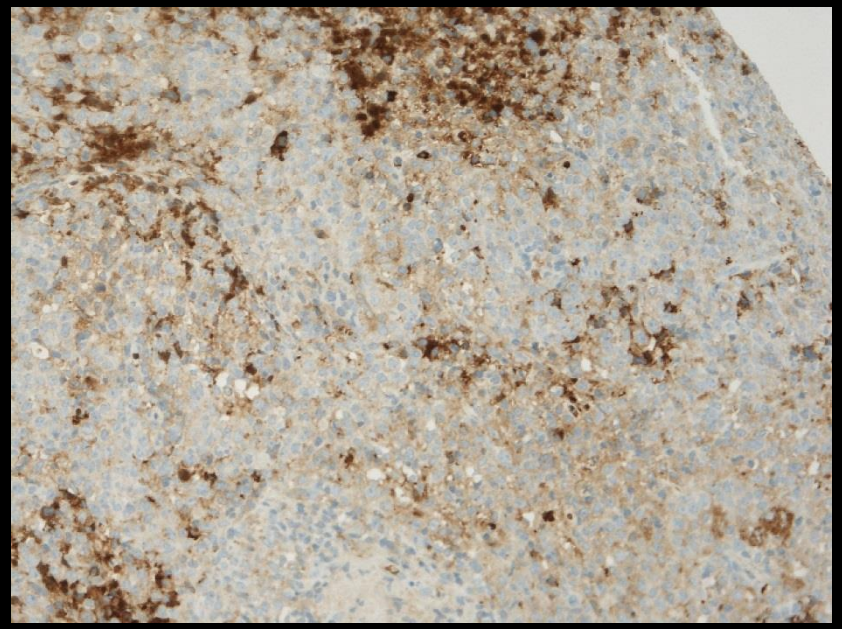
Significant proportion of IGLL5 (n) + cells

H15/P10-20-30

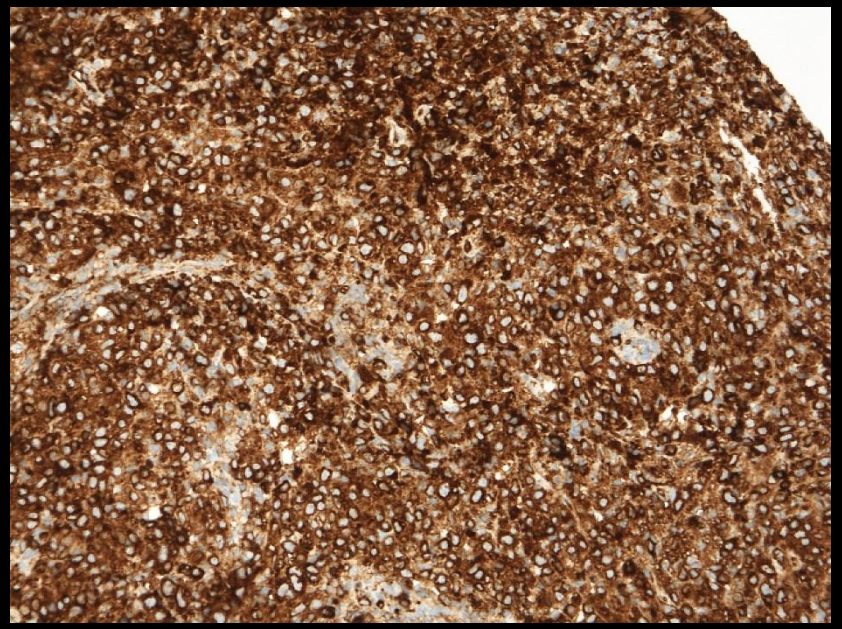
RNA Scope

Diffuse Large B-Cell Lymphoma (1)

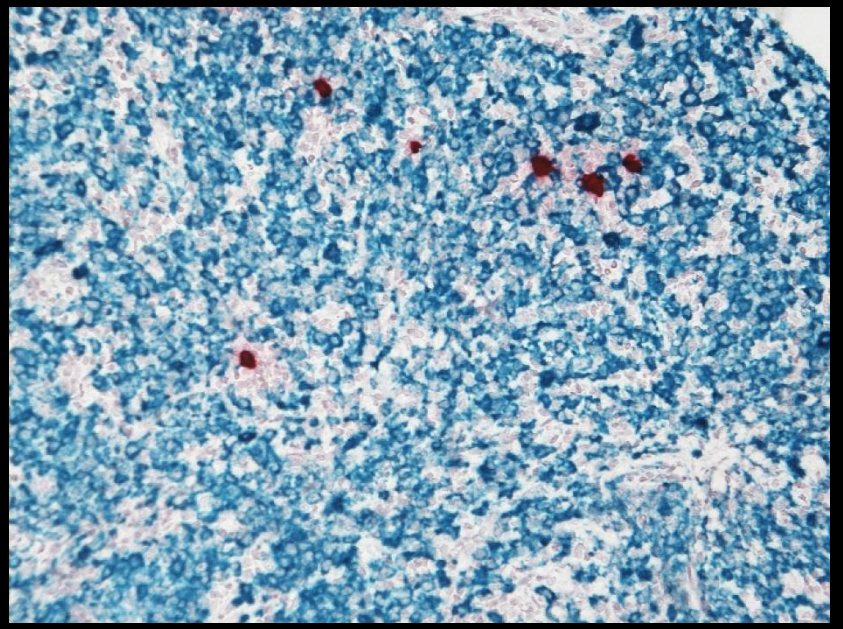
Clinical info: Lambda positive (Ros)



IHC Kappa (re-test)



IHC Lambda (re-test)



RNA scope Kappa (C2-Red)/Lambda (C1-Green)

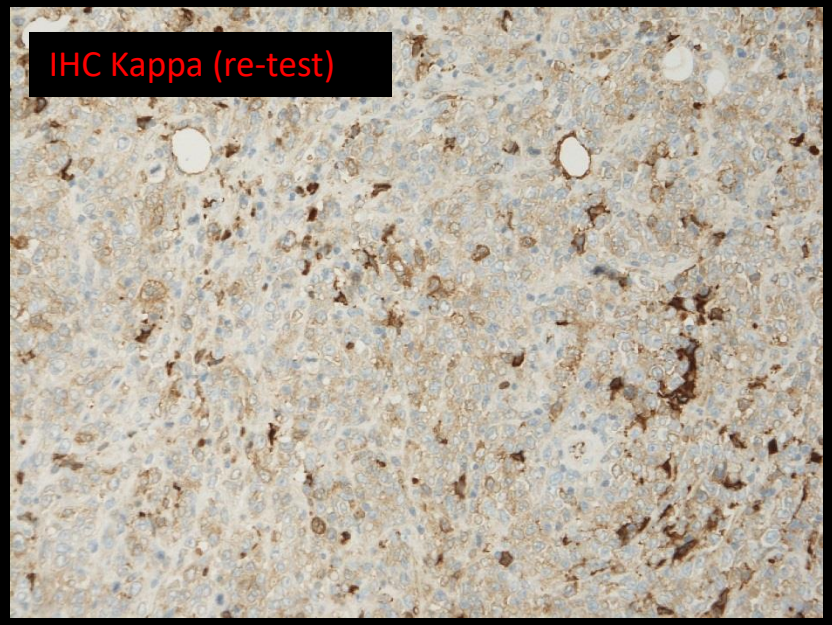
H15/P10-20-30

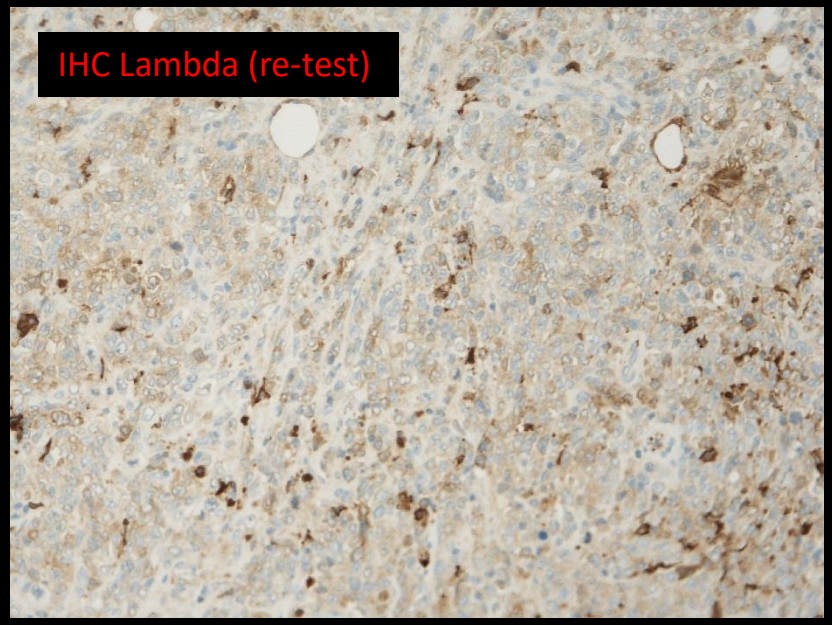
RNA Scope

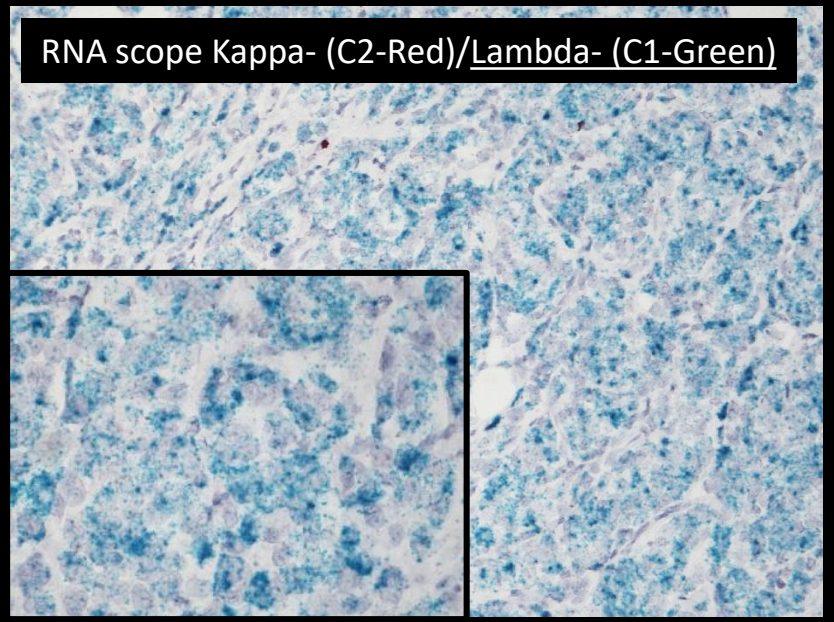
Diffuse Large B-Cell Lymphoma (2)

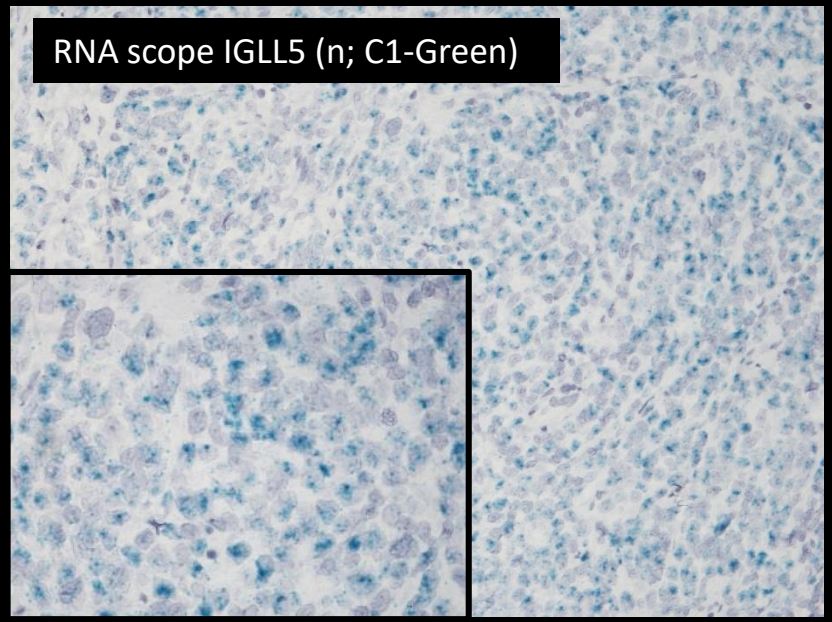
Clinical info: Unknown

Difficult case (Lambda + ?)







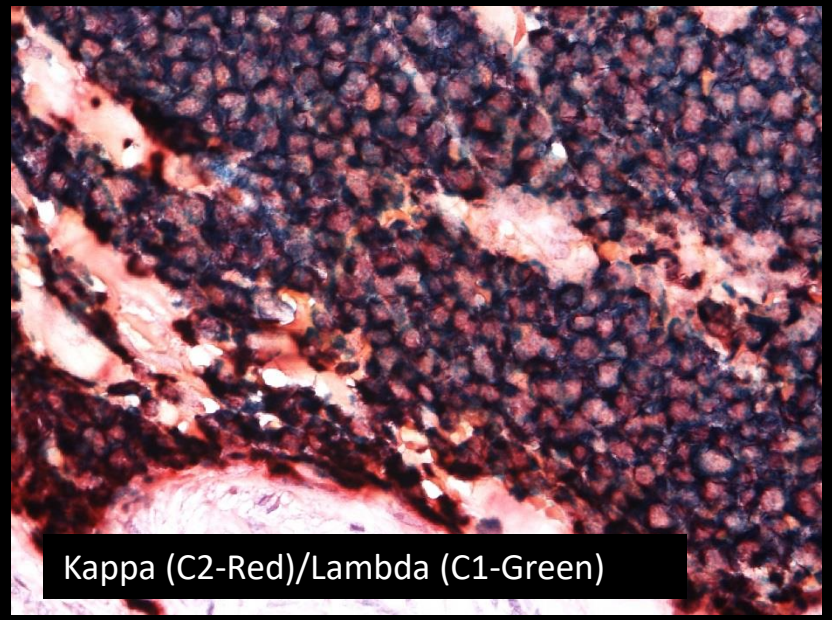


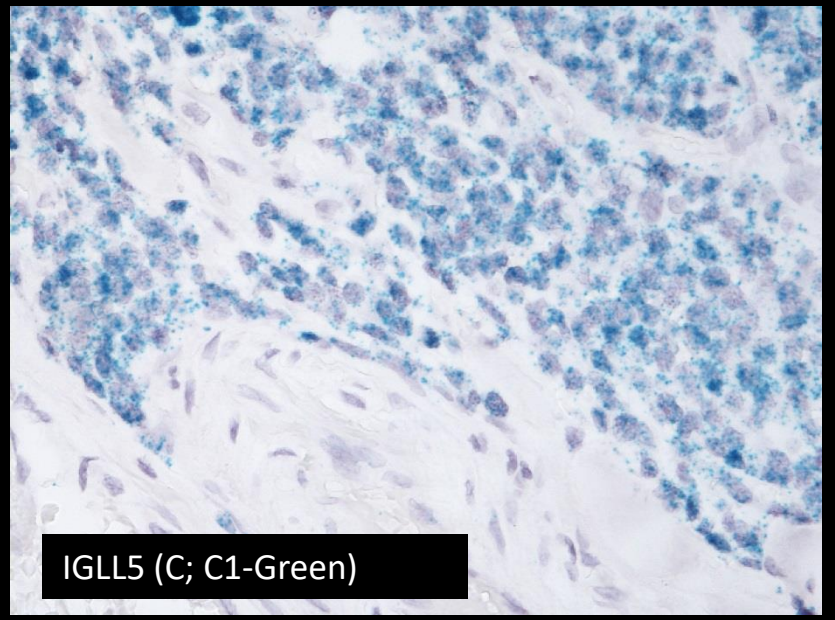
H15/P15

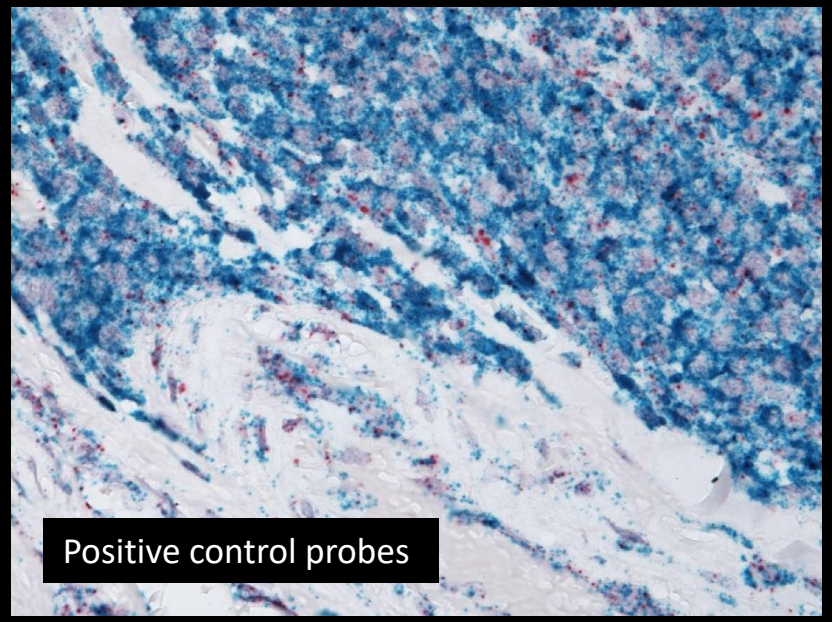
The problems !

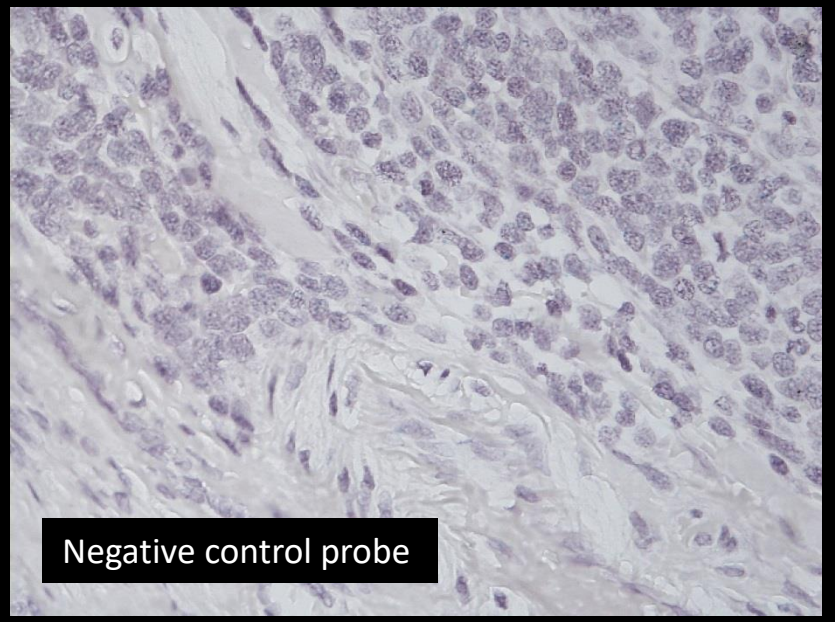
RNA Scope Duplex

Myeloma (Kappa +)









“Cross-reactivity” IGLL5+

H15/P10-20-30

RNA Scope Duplex

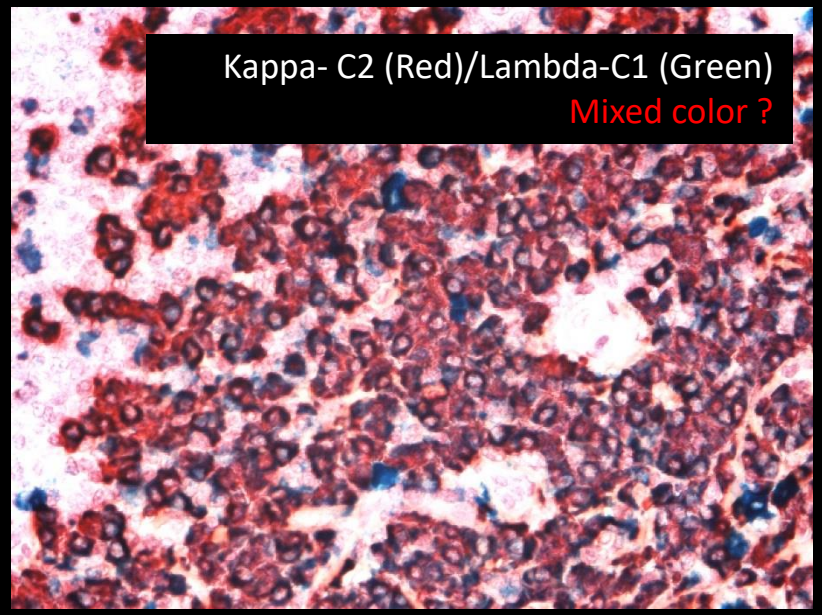
With/Without Lambda probe

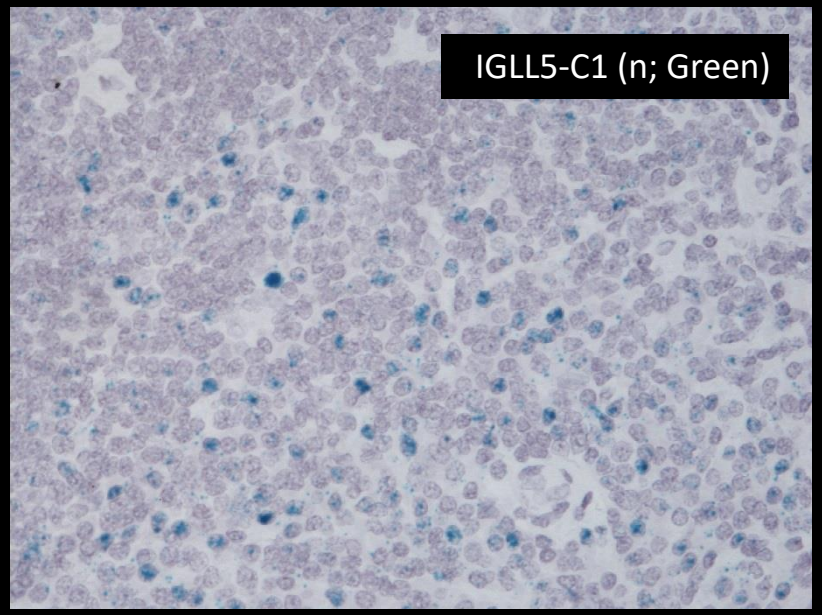
LPL (case 3)/ Kappa +

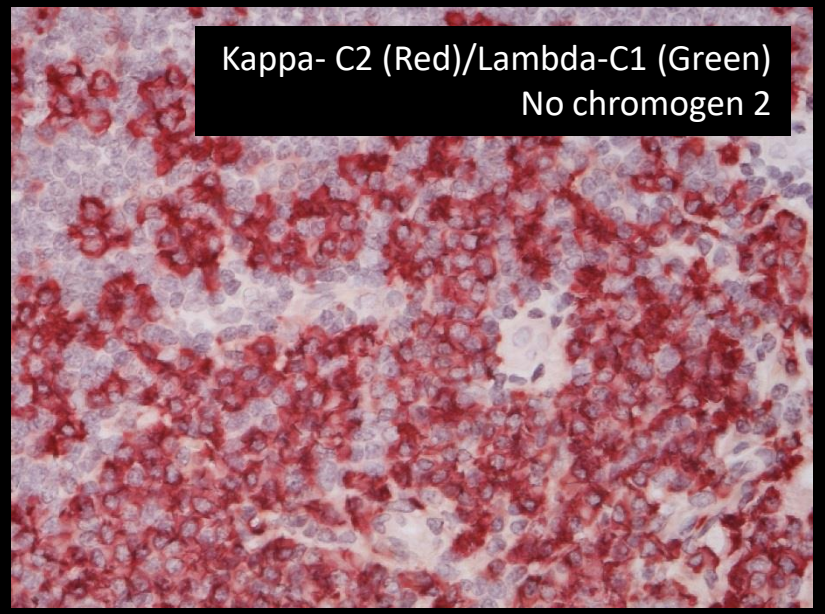
Cross-reactivity with Lambda probe ?

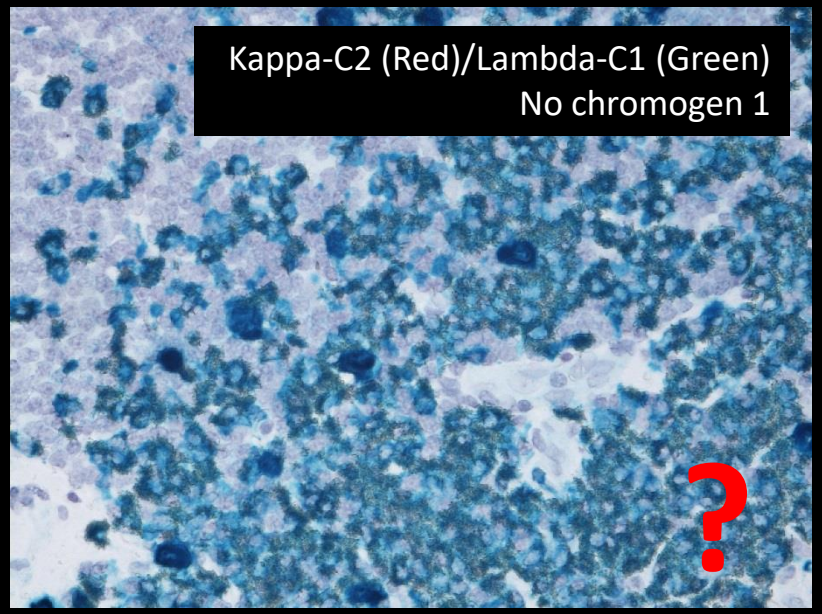
Detection system – cross talk ?

No reaction were seen with Kappa/Lambda/IGLL5 probe in non-lymphoid tissue e.g. trophoblastic cells of the placenta, epithelial/stromal cells of the all specimens. Positive and negative controls displayed the expected reaction pattern in all specimens.





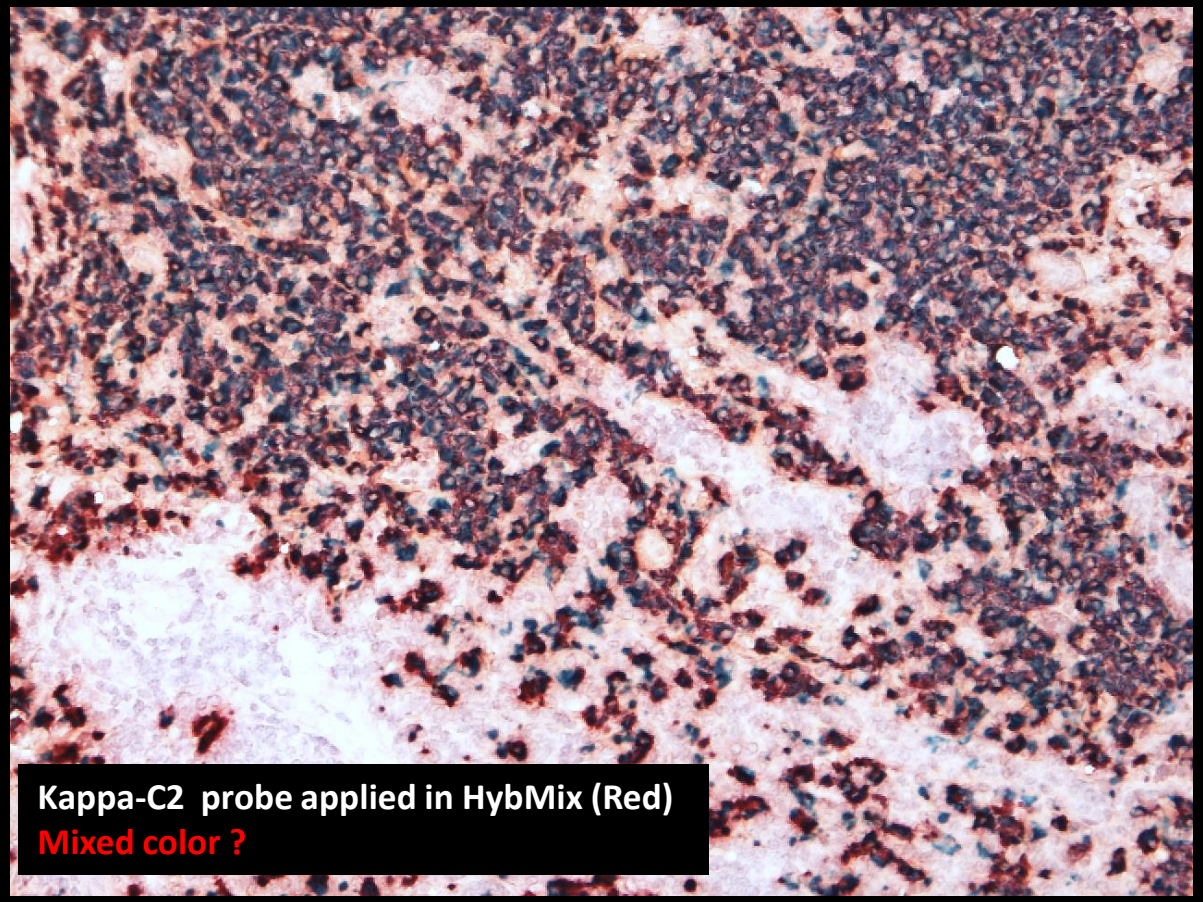


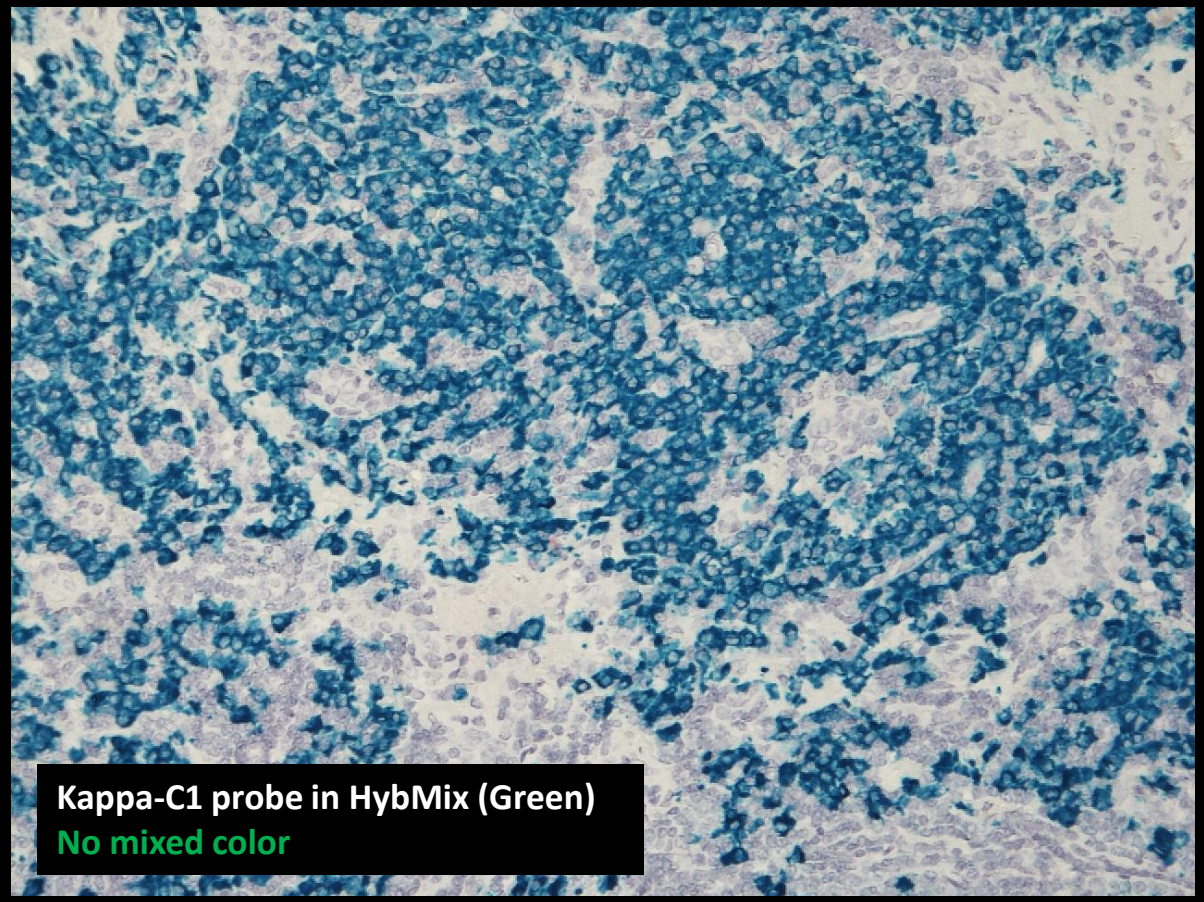


H15/P10-20-30

RNA Scope Duplex: Kappa-C2 versus Kappa-C1 (assays run without lambda probes)

LPL (case 3)/ Kappa +





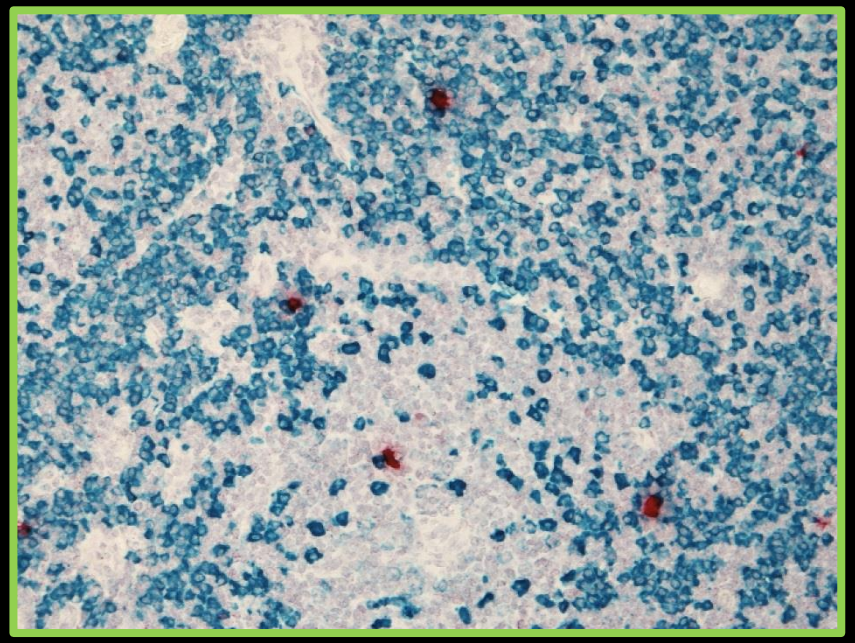
Problems related to abundant expression of a given target mRNA type, e.g, Kappa positive LPL cases, and application of corresponding C2 probe to the same target.

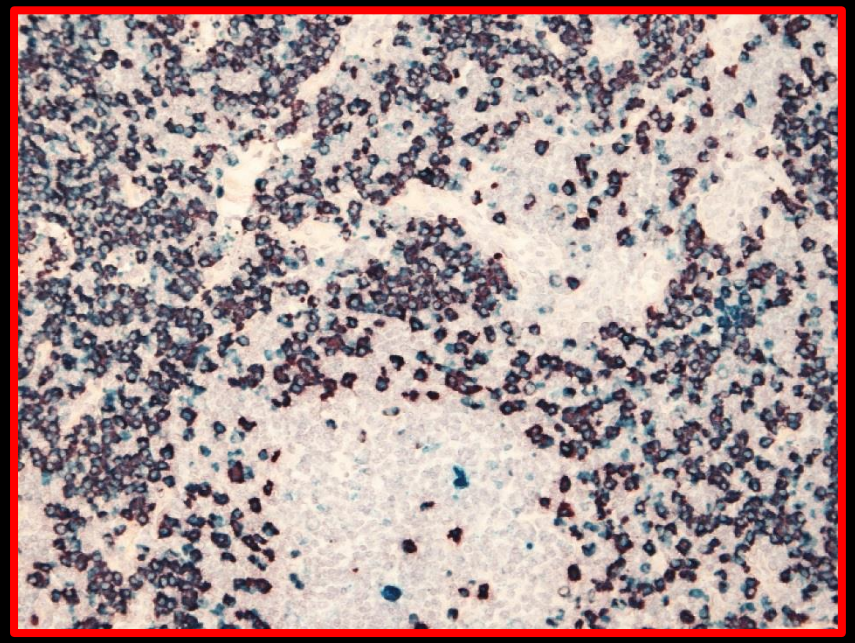
RNA Scope Duplex

IGL (C1) + Kappa (C2)

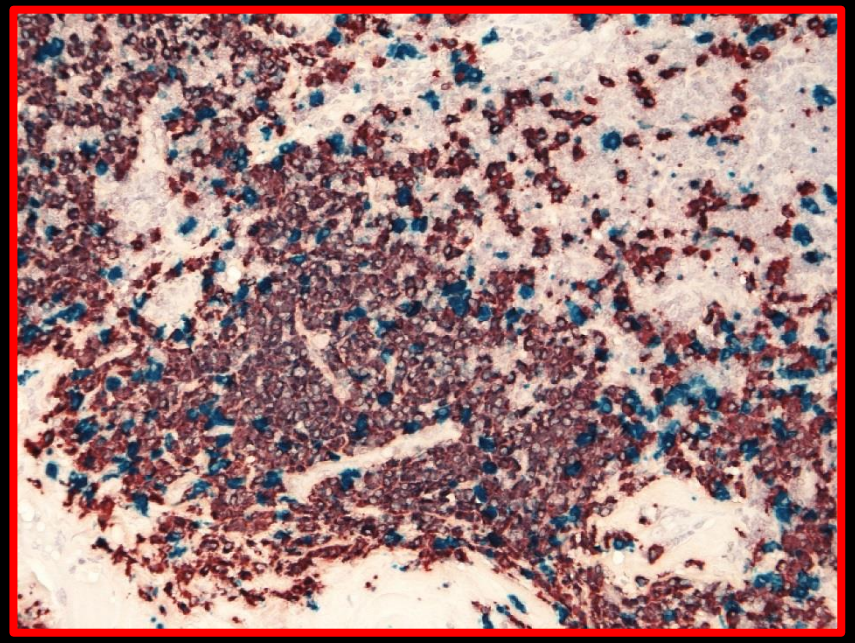
IGL (C2) + Kappa (C1)

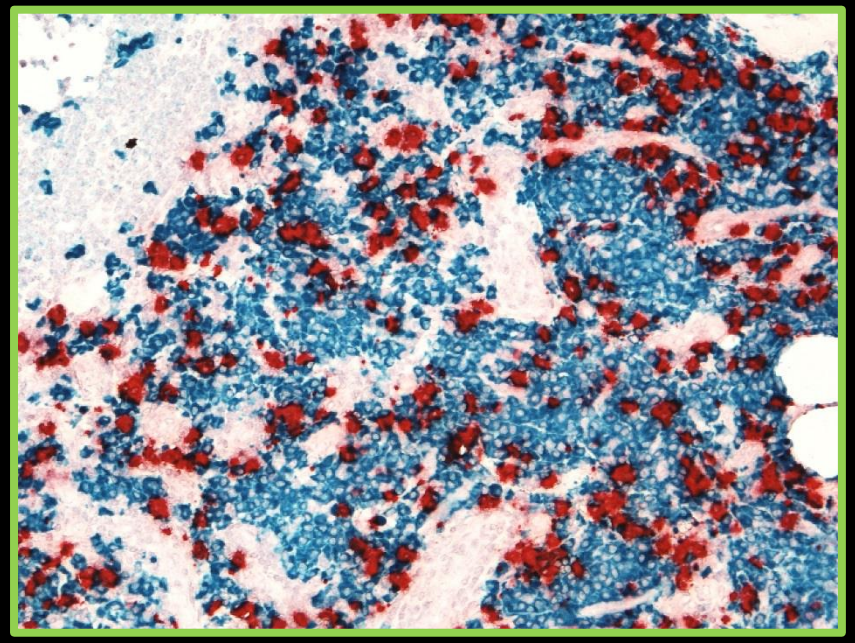
LPL (Lambda +)





LPL (Kappa +)





Advance Cell Diagnostic (RNAscope) respond:

This means the problem is with high C2 signal that creates unspecific green signal overlapping with the red, which follows the expected pattern of the C2 (red) target.

And it turns out that this is actually something we expect for the RNAscope and BaseScope duplex assays. We always recommend to put the highest expressor in C1, because we know that a lot of red signal can interfere with the green signal.

However, we rarely see any problem even if customers pick C2 for a target that is a bit higher than that in C1 and we understand that it is not always possible to know this in advance.
But, Kappa and Lambda tissues are the “extreme” of this situation, where Kappa or Lambda can be very very high. And it is exactly for cases like this that we have this rule.

So, fundamentally there is nothing wrong, but we are dealing simply with a limit of the RNAscope duplex chemistry and there is no way around it if not switching the probes for samples where you see this happening.

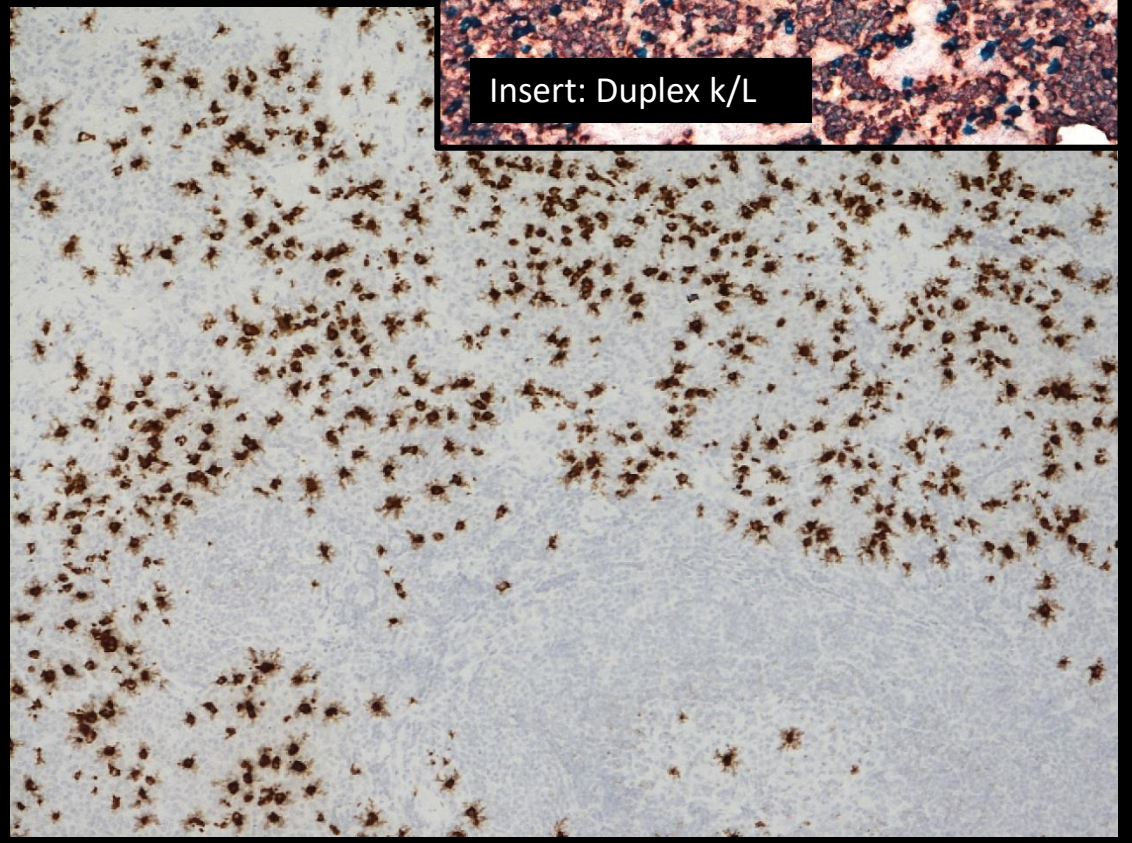
Trouble shooting guide ?

RNA Scope (Single Plex)

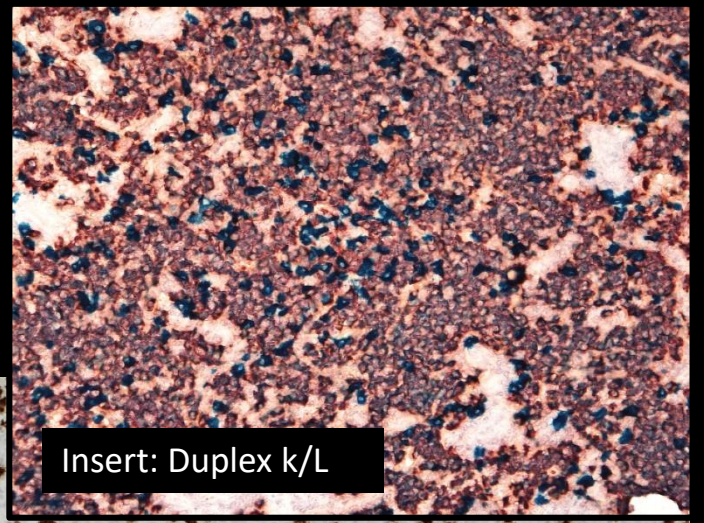
LPL (case 3)

Kappa +

Kappa-C1 (Single Plex)



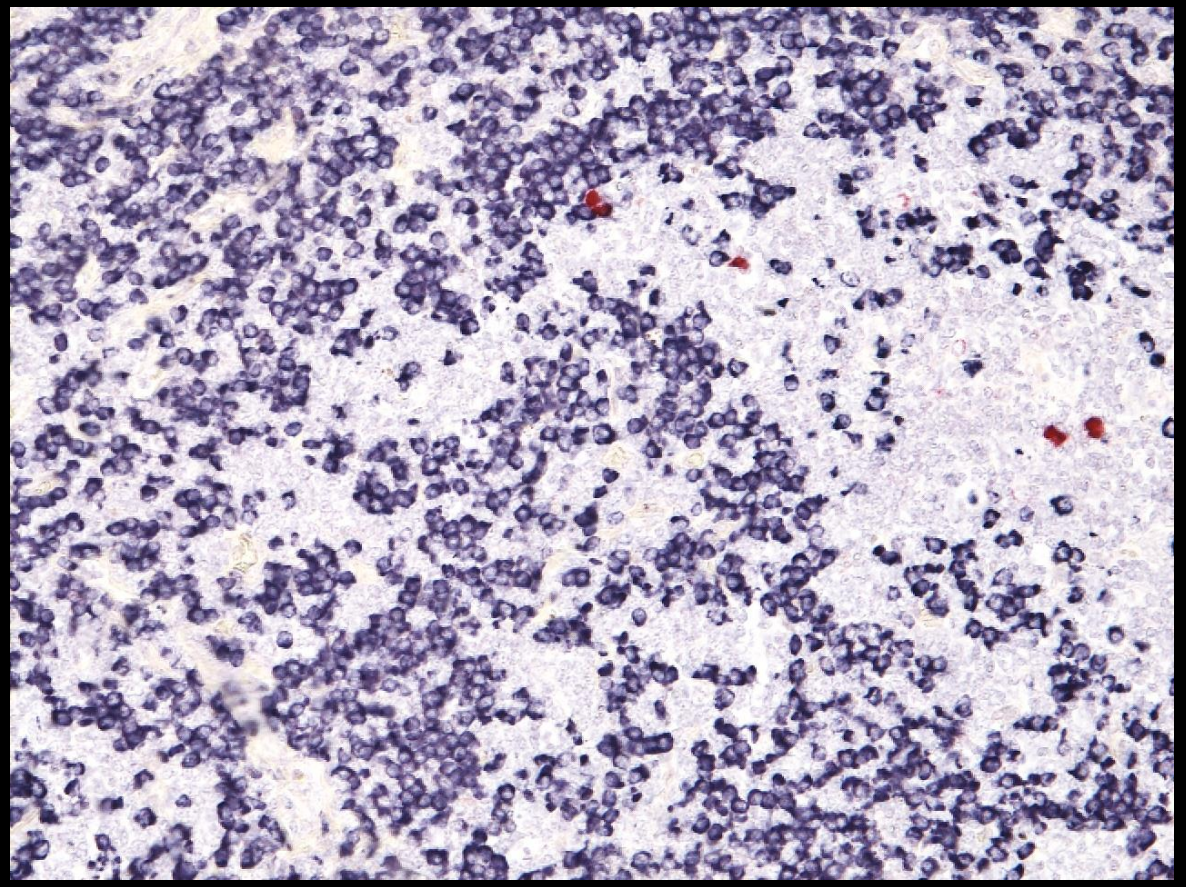
Lambda-C1 (Single Plex)



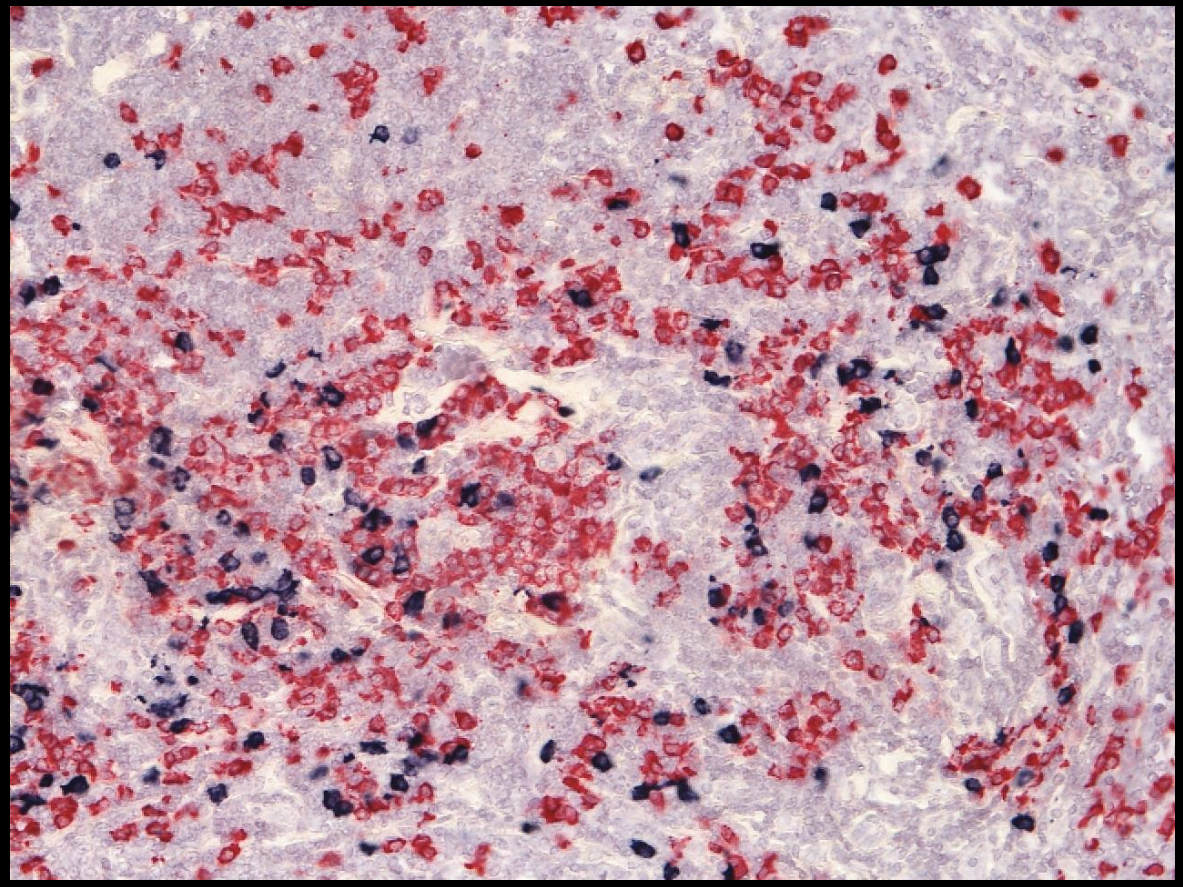
No cross-reactivity

ViewRNA 2-Plex: Lambda (Type 6 probe/Blue) and Kappa (Type 1 probe/Red)

LPL (Lambda positive)



LPL (Kappa positive)



Preliminary result for the ViewRNA: Assay needs optimization

No cross talk

Diagnosis	Clinical info Light chain restriction (standard methods)	RNA Scope Duplex: C1 probe Lambda/ C2 probe Kappa Light chain restriction
Lymphoplasmacytoid lymphoma (LPL) (1) Næ	Lambda$^+$/Kappa$^-$ (IHC$^+$/ISH$^+$)	Lambda$^+$/Kappa$^-$
Lymphoplasmacytoid lymphoma (LPL) (2)/Næ	Lambda$^+$/Kappa$^-$ (IHC$^+$/ISH$^+$)	Lambda$^+$/Kappa$^-$
Lymphoplasmacytoid lymphoma (LPL) (3)/Næ	Kappa$^+$/Lambda$^-$ (IHC$^+$/ISH$^+$)	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Lymphoplasmacytoid lymphoma (LPL) (4)/Ros	Kappa$^+$/Lambda$^-$	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Lymphoplasmacytoid lymphoma (LPL) (5)/Ros	Kappa$^+$/Lambda$^-$	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Lymphoplasmacytoid lymphoma (LPL) (6)/Ros	Kappa$^+$/Lambda$^-$	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Lymphoplasmacytoid lymphoma (LPL) (7)/Ros	Unknown (re-test displayed Kappa IHC$^+$/ISH$^+$ result)	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Lymphoplasmacytoid lymphoma (LPL) (8)/Ros	Unknown (re-test displayed Kappa IHC$^+$/ISH$^+$ result)	Kappa$^+$/Lambda$^-$ (Cross-reactivity ?)
Myeloma/Næ	Kappa$^+$/Lambda$^-$ (IHC$^+$/ISH$^+$)	Kappa$^+$/Lambda$^-$ (difficult IGLL5 reaction)
Mantle cell lymphoma (MCL) (1)/Næ	Kappa$^+$/Lambda$^-$ (IHC$^+$/ISH$^-$)	Kappa$^+$/Lambda$^-$
Mantle cell lymphoma (MCL) (2)/Næ	Kappa$^+$/Lambda$^-$ (IHC$^+$/ISH$^-$)	Kappa$^+$/Lambda$^-$
Mantle cell lymphoma (MCL) (3)/Næ	Lambda$^+$/Kappa$^-$ (IHC$^+$/ISH$^-$)	Lambda$^+$/Kappa$^-$
Mantle cell lymphoma (MCL) (4)/Næ	Lambda$^+$/Kappa$^-$ (IHC$^+$/ISH$^-$)	Lambda$^+$/Kappa$^-$
Follicular Lymphoma (FL) (1)/Ros	Kappa$^+$/Lambda$^-$	Kappa$^+$/Lambda$^-$ (difficult IGLL5 reaction)
Follicular Lymphoma (FL) (2)/Ros	Unknown (re-test displayed Lambda IHC$^+$/ISH$^-$ result)	Lambda$^+$/Kappa$^-$
Follicular Lymphoma (FL) (3)/Ros	Kappa$^+$/Lambda$^-$	Interpretation difficult (pre-analytic problems/IGLL5)
Diffuse Large B-Cell Lymphoma (DLBCL) (1)/Ros	Lambda$^+$/Kappa$^-$	Lambda$^+$/Kappa$^-$
Diffuse Large B-Cell Lymphoma (DLBCL) (2)/Ros	Unknown (re-test displayed IHC$^-$/ISH$^-$ result)	Lambda$^+$/Kappa$^-$
Tonsil (Fix time 6-168h)	Poly	Poly/ Germinal centre B-cells (strong IGGL5)
Negative control tissue (Appendix, Kidney and placenta)	Negative	Negative

In general, there is a good correlation between RNA scope results and In House test (Standard ISH, IHC and Flowcytometry). However, ……..

SARS-CoV-2 virus (Covid-19)

ACE2 and TMPRSS2

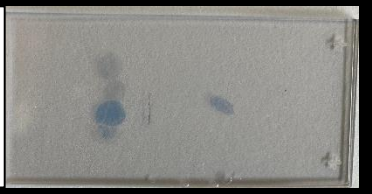
Tissue and tissue controls

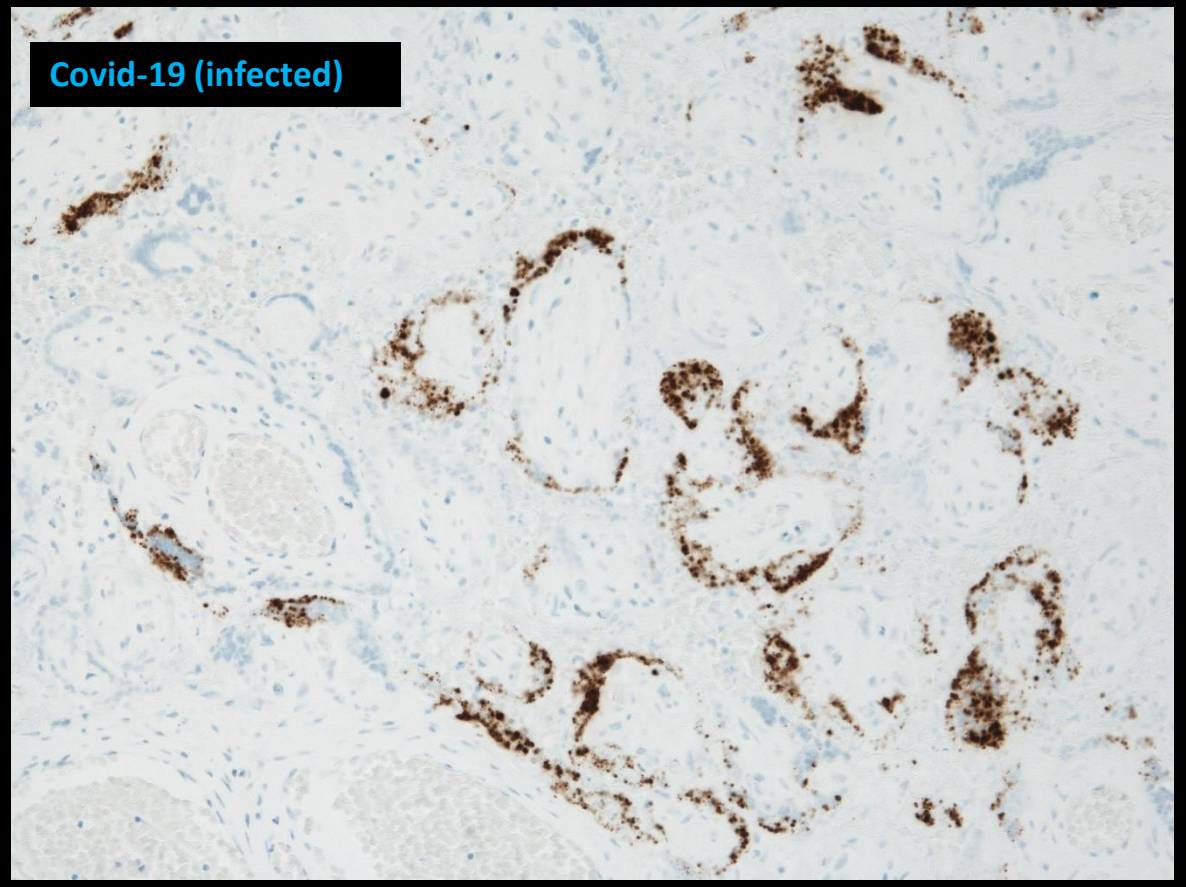
Sensitivity, Specificity and Reproducibility

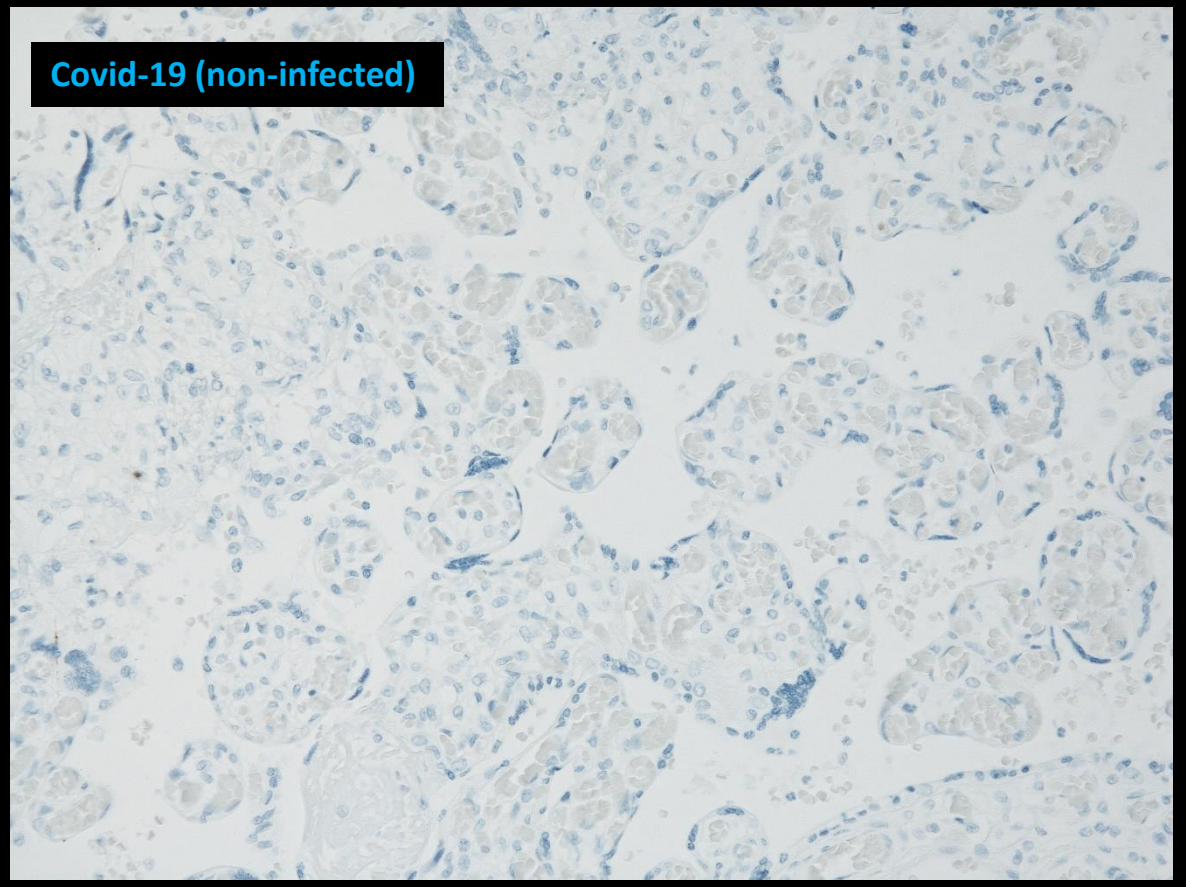
RNAscope Covid-19

Placenta

On-slide control:
Tonsil
Appendix
Placenta
Placenta (infected)



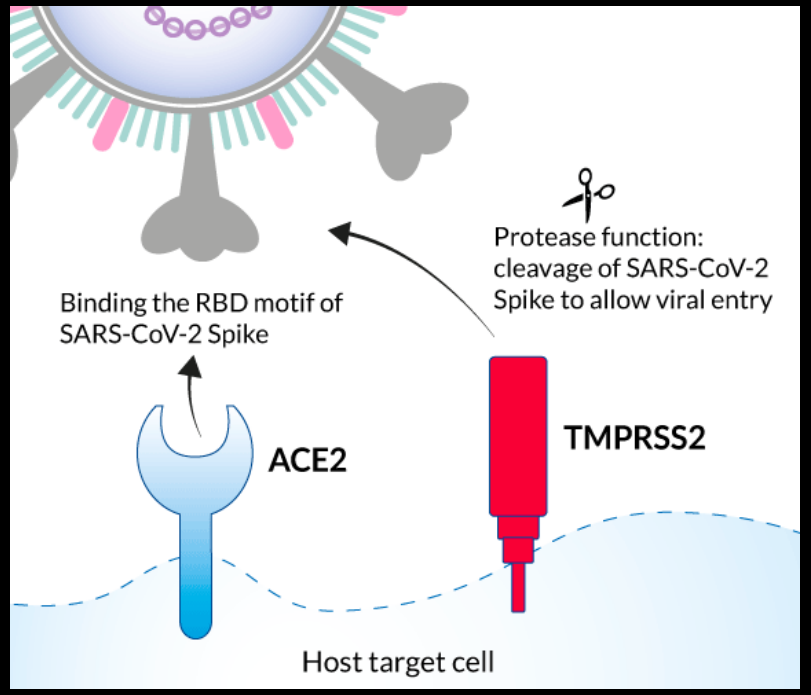




(Discovery standard protocol, CC1 16` (97°C)/ P 16` (37°C), AMP5 12 ` (37°C))

Transmembrane serine protease 2 (TMPRSS2)

(Covid-19)



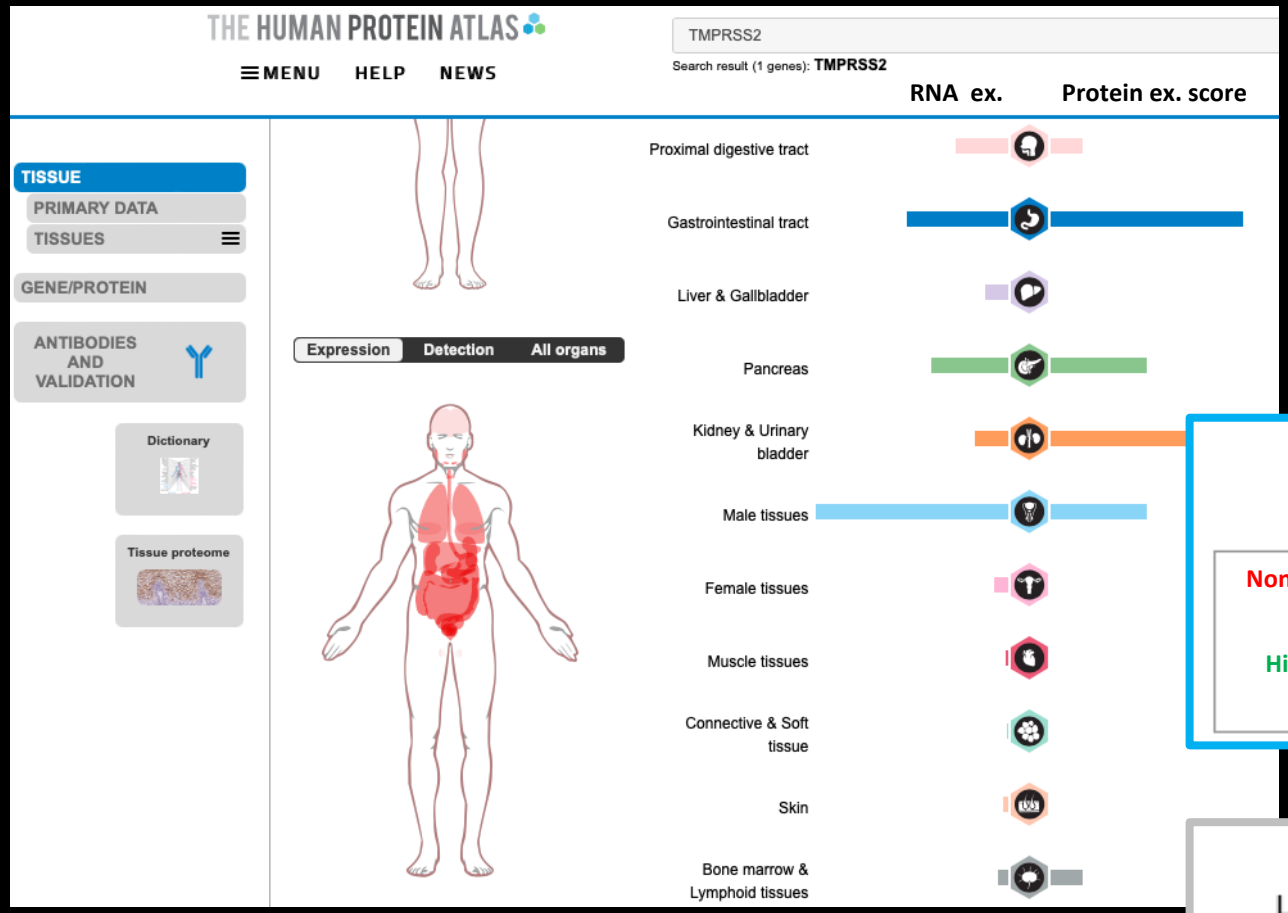
TMPRSS2 is an enzyme involved in the 'priming' of many viruses including coronaviruses such as SARS-CoV-2, allowing them to enter the body to cause disease (such as COVID-19).

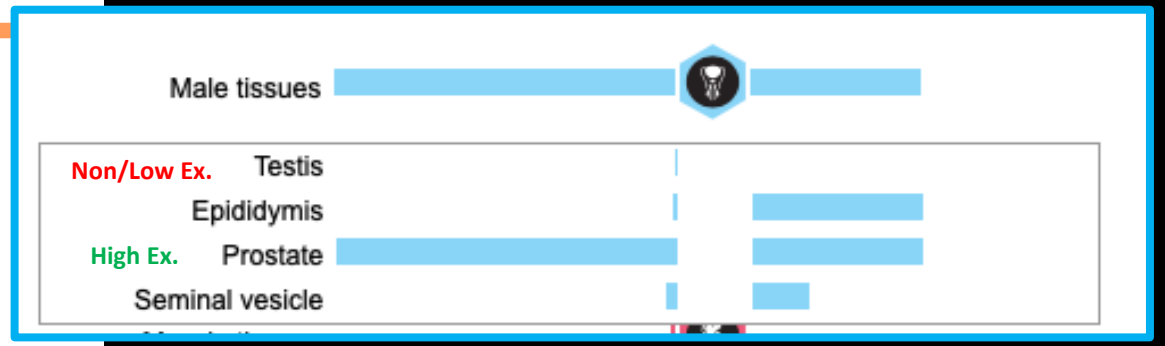
Following binding of ACE2, the Spike protein is subsequently cleaved by the host transmembrane serine protease 2 (TMPRSS2) to release the spike fusion peptide, promoting virus entry into target cells

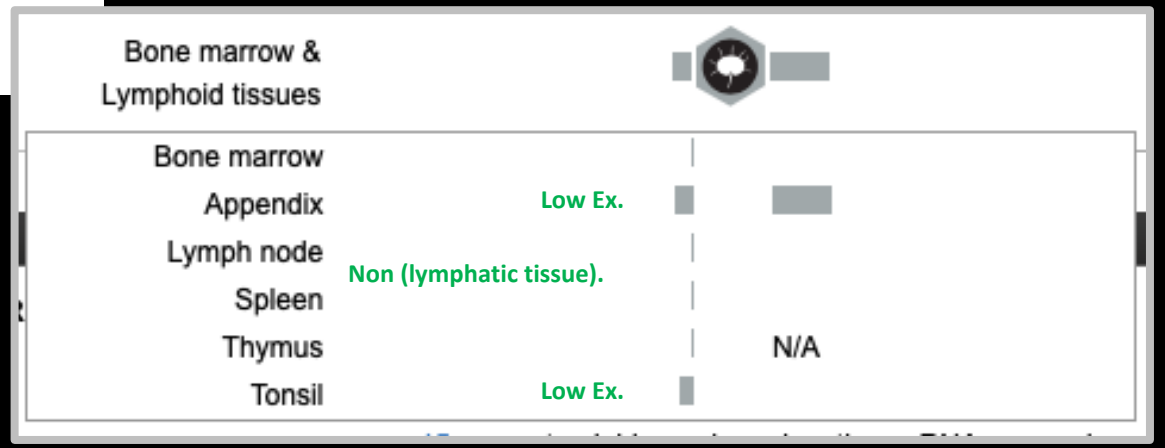
TMPRSS2 is expressed in e.g., prostate, nasal, bronchial, and gastrointestinal epithelium

Drugs/Inhibitors of TMRSS2

TMPRSS2 (RNA levels) – What to expect (normal tissue) ?







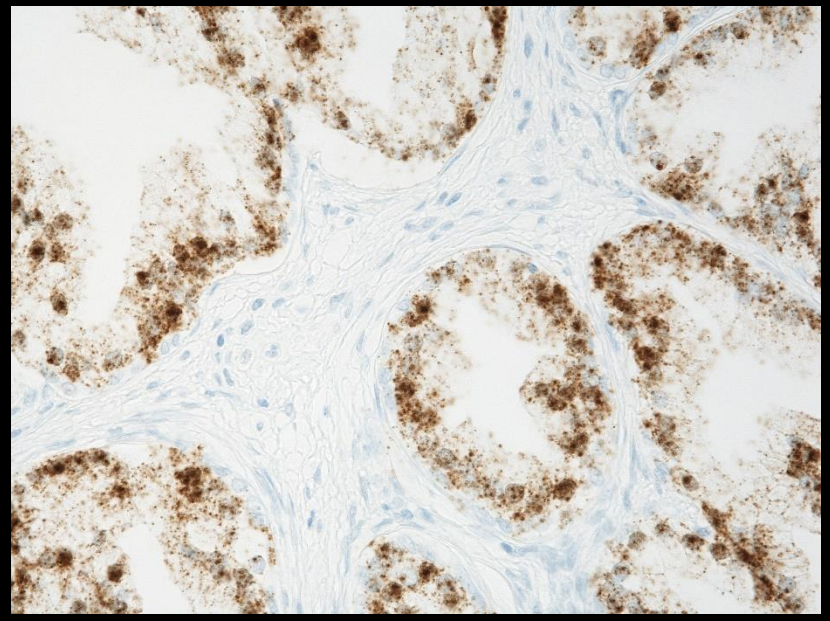
HPA: Might give an idea of the RNA expression levels in different tissue specimens.

However, be critical.

RNAscope TMPRSS (Discovery standard protocol, CC1 16` (97°C)/ P 16` (36°C), AMP5 12 ` (36°C))

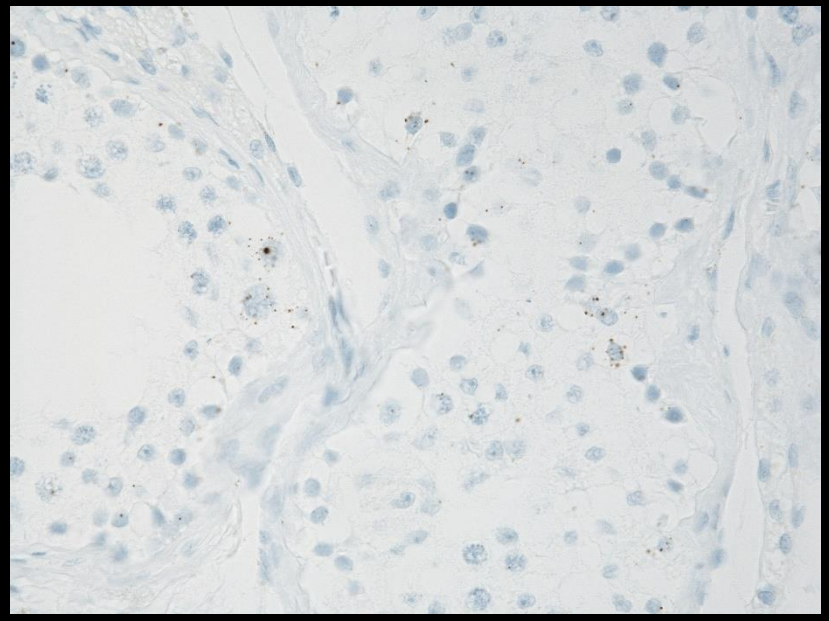
Assay performance characteristic in relation to mRNA findings of the Human Protein Atlas (HPA)

Prostate



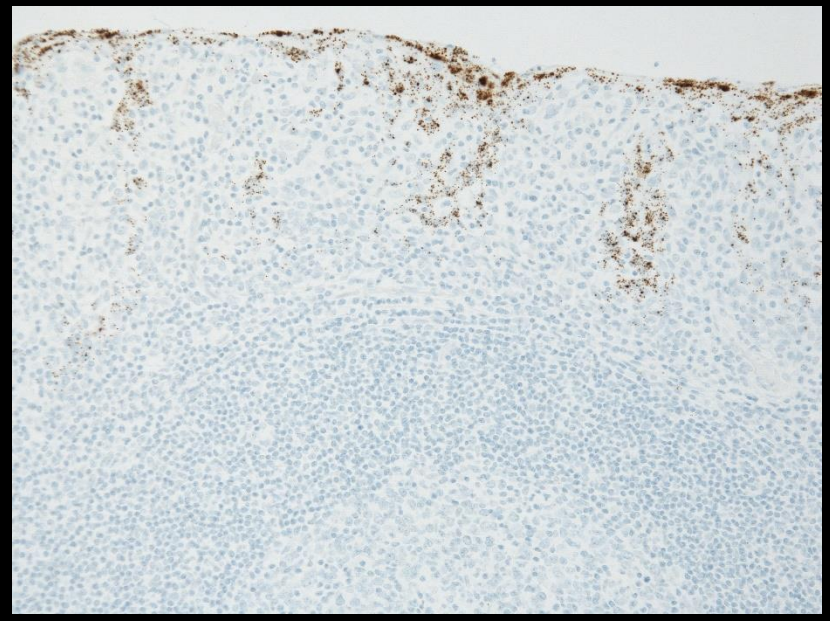
TMPRSS2/ High expressor (HPA)

Testis



TMPRSS2/ Low expressor (HPA)

Tonsil (Lymphatic tissue)

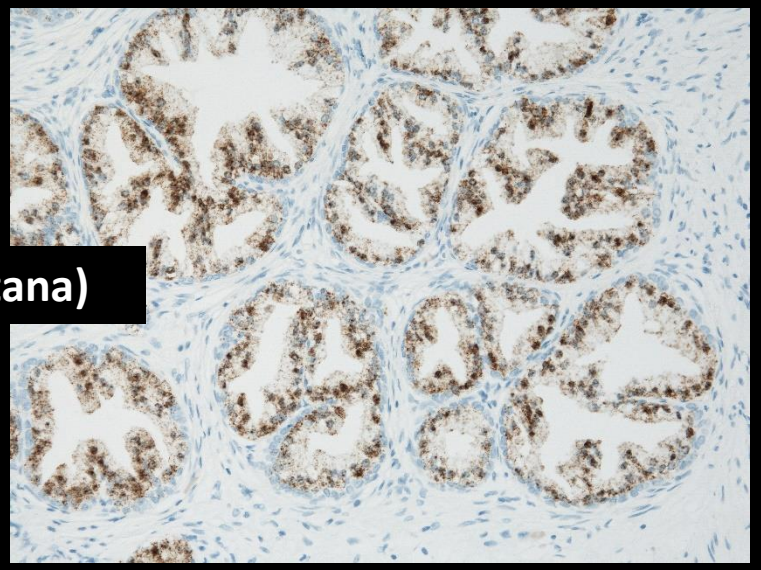


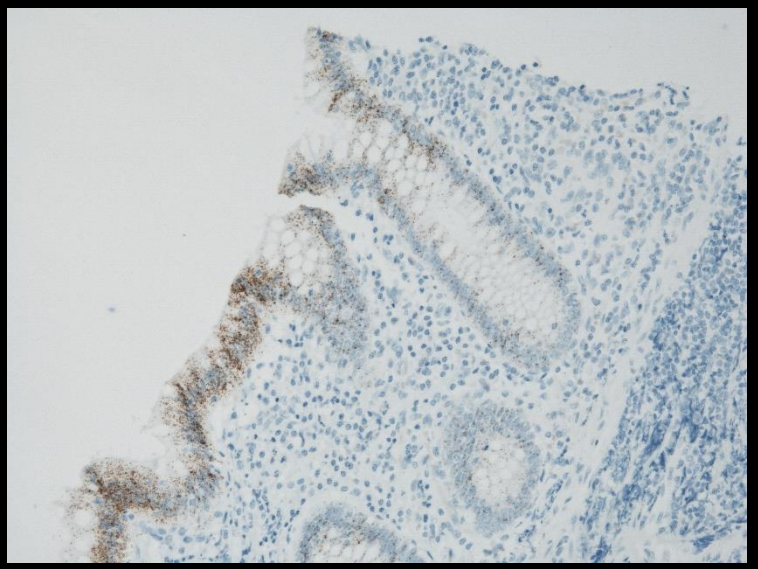
TMPRSS2/ Non expressor (HPA)

RNAscope TMPRSS2: The importance of on-slide control material

<u>On-slide control</u>:
Prostate
Testis
Kidney
Tonsil
Appendix
Placenta
Seminoma

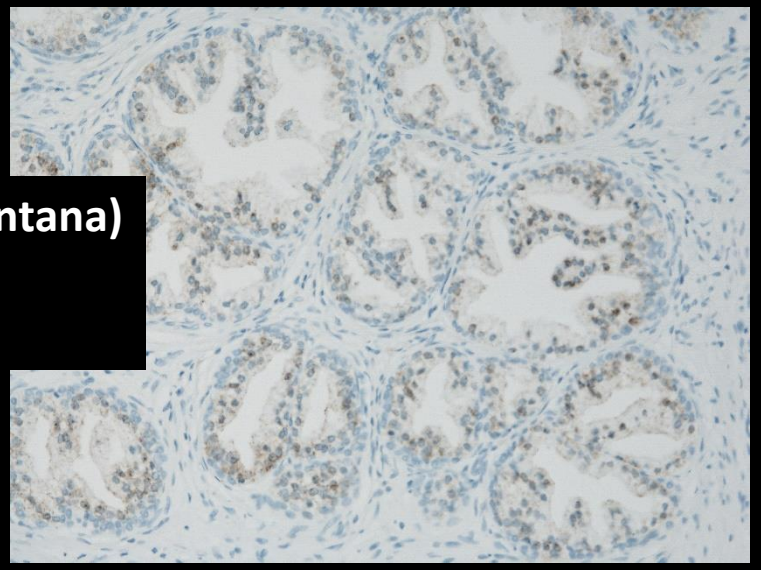
Optimal run on the Discovery (Ventana)

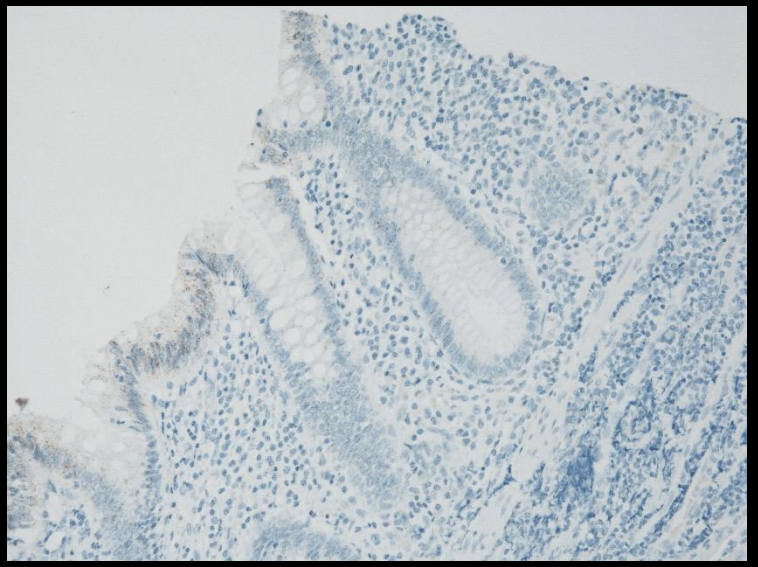




Suboptimal run on the Discovery (Ventana)

Re-Run required



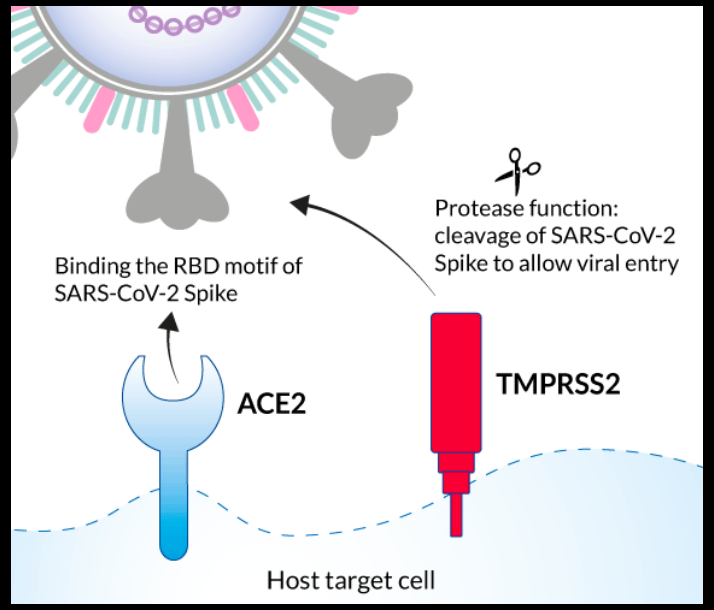


Same day, same run and same reagents

(Discovery standard protocol, CC1 16` (97°C)/ P 16` (37°C), AMP5 12 ` (37°C))

Angiotensin-Converting Enzyme 2 (ACE2 “receptor”)

(Covid-19)



SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as the receptor to mediate viral entry into host cells

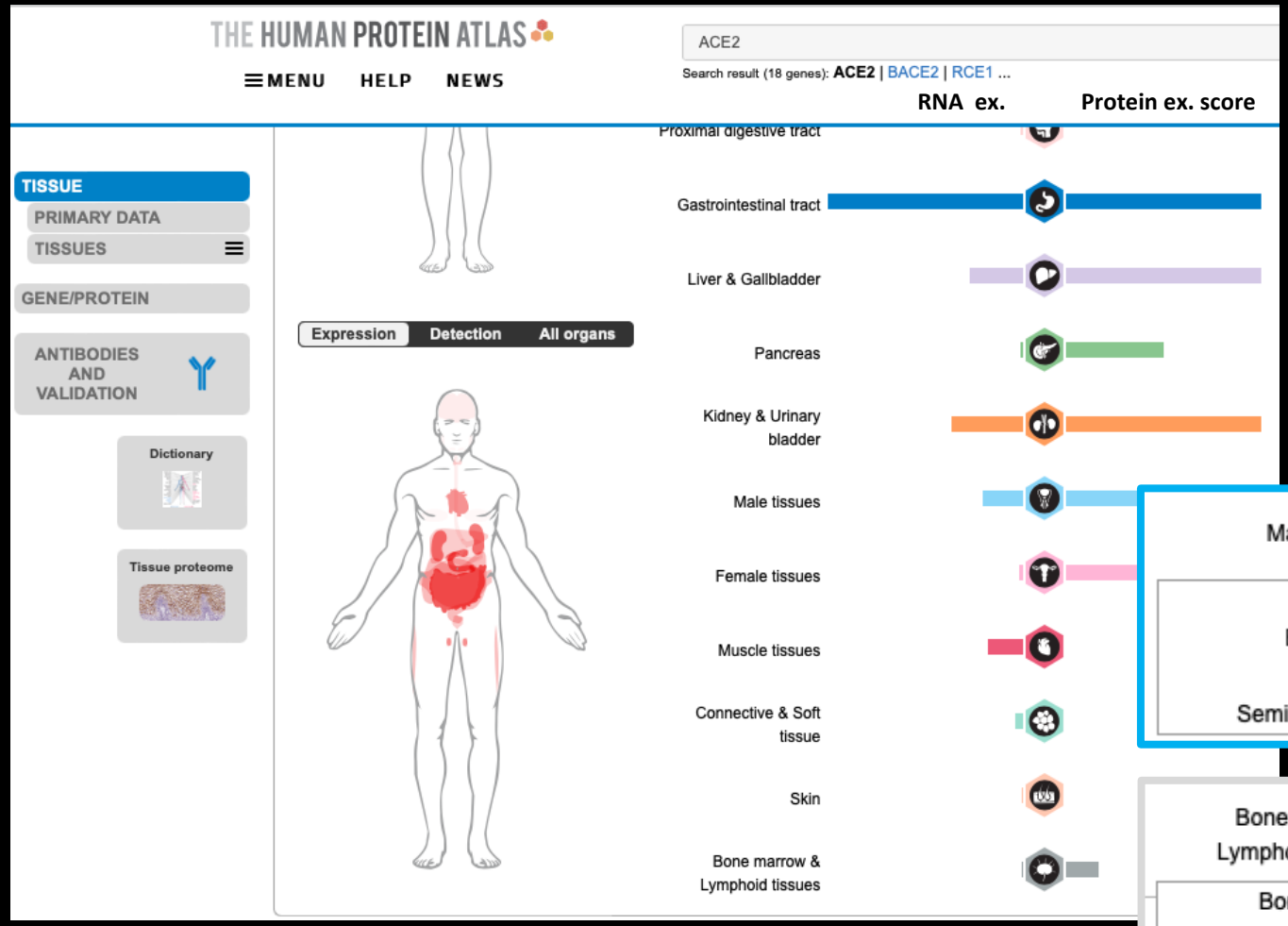
Using the spike-like protein on its surface, the SARS-CoV-2 virus binds to ACE2. Hence, ACE2 acts as a cellular doorway for the virus that causes COVID-19.

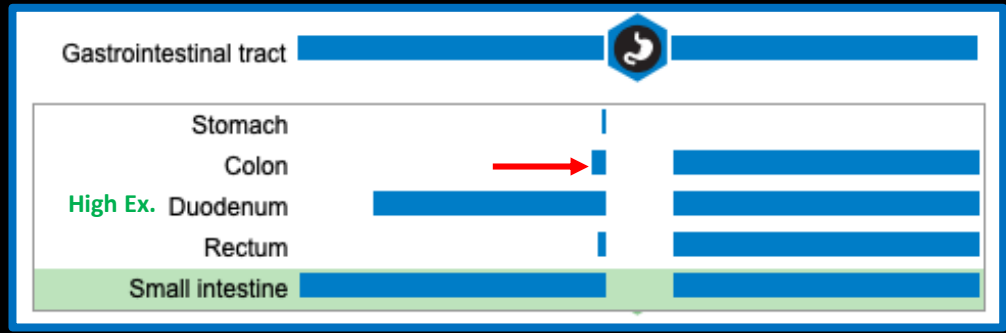
ACE2 is present in many cell types and tissues including the lungs, heart, blood vessels, kidneys, liver and gastrointestinal tract. It is present in epithelial cells, which line certain tissues and create protective barriers.

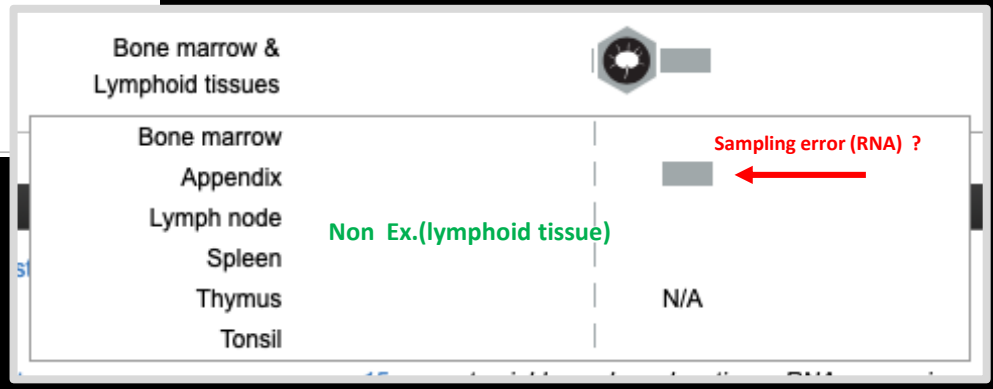
- ACE2 is highly abundant on type 2 pneumocytes, an important cell type present in chambers within the lung called alveoli, where oxygen is absorbed and waste carbon dioxide is released.

Drugs/Inhibitors of ACE2

ACE2 (RNA levels) – What to expect (normal tissue) ?



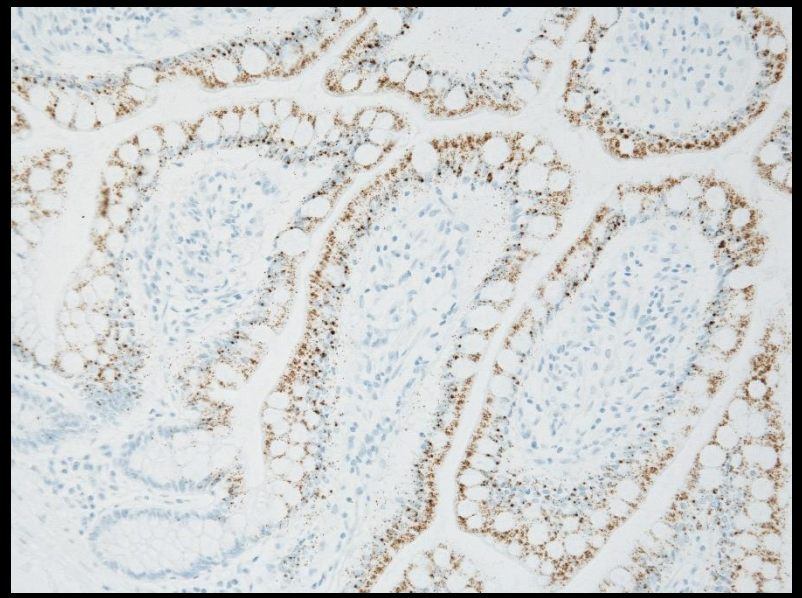




RNAscope ACE2 (Discovery standard protocol, CC1 16` (97°C)/ P 16` (37°C), AMP5 12 ` (37°C))

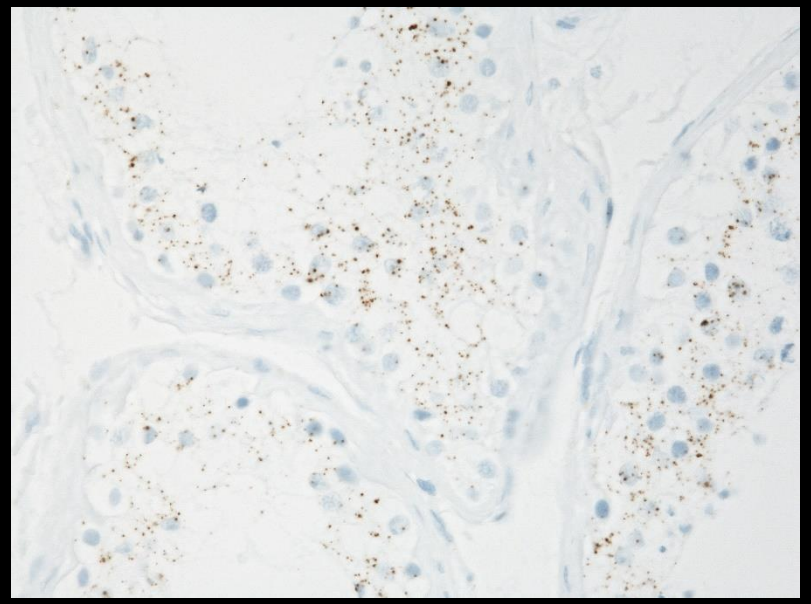
Assay performance characteristic in relation to mRNA findings of the Human Protein Atlas (HPA)

Small intestine



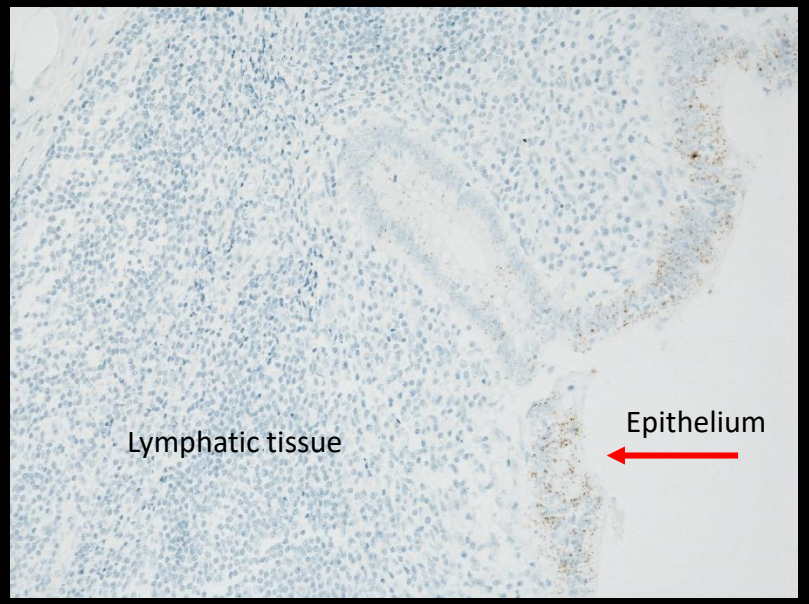
ACE2/ High expressor (HPA)

Testis



ACE2/ Low to medium expressor (HPA)

Appendix (Lymphatic tissue)

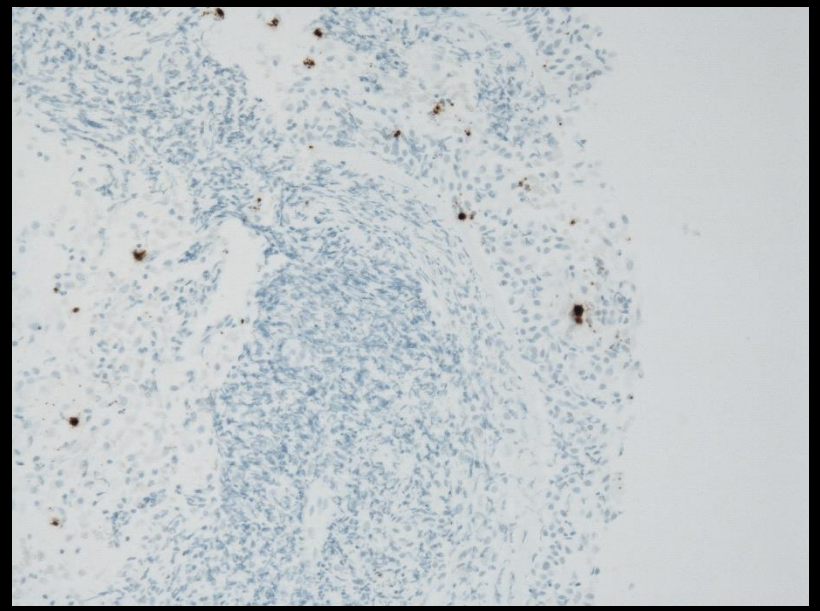


ACE2/ Non expressor – Lymphatic tissue (HPA)

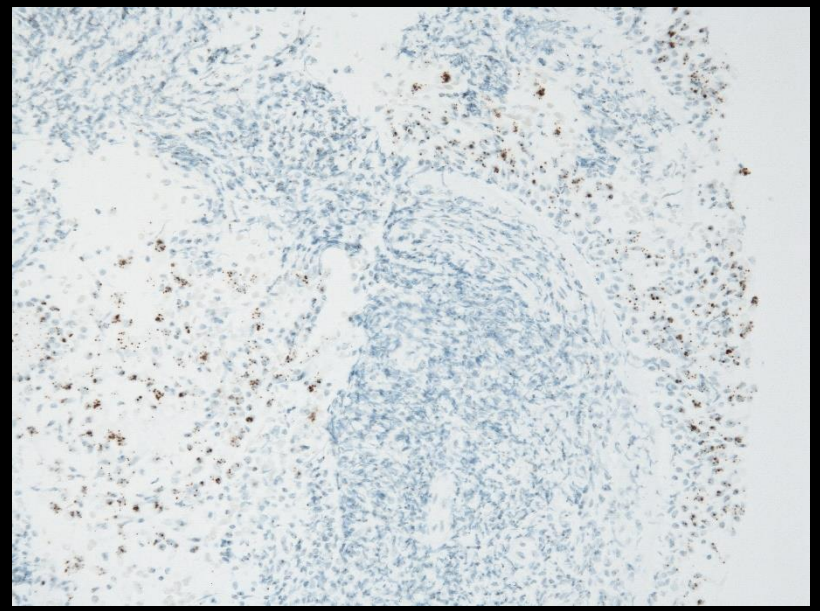
RNAscope Covid-19, TMPRSS2 and ACE2

Clinical sample (Nasal biopsy)

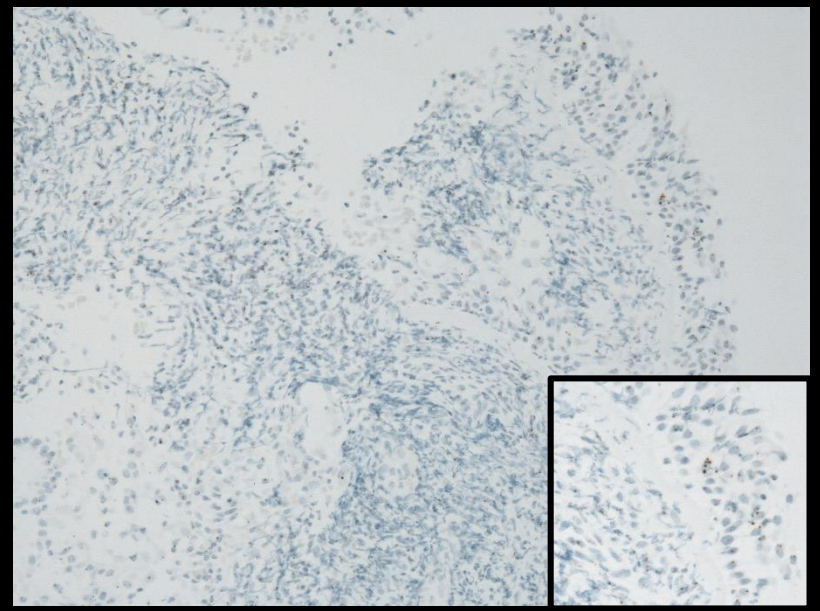
Covid-19 probe



TMPRSS2 probe



ACE2 probe



(Discovery standard protocol, CC1 16` (97°C)/ P 16` (37°C), AMP5 12 ` (37°C))

Introduction of RNAscope to the Pathology Department (Naestved/Denmark)

- Helpful in challenging diagnostic situations e.g., detection of light chain restrictions (kappa/lambda) in B-cell Lymphomas
- Confirming mRNA findings (Nanostring profiling) – which cells are positive
- Validation/verification of reaction patterns obtained with antibodies
- Lack of valid primary antibodies (research)
- BaseScope

e.g. point mutation (BRAF V600E in melanomas or colon adenocarcinomas) or gene fusion products

RESEARCH ARTICLE

Keratin 5 in Lung Cancer Specimens: Comparison of Four Antibody Clones and *KRT5* mRNA-ISH

Christian Thomsen, MD, Laura Blok-Husum, MD,* Jeanette Bæhr Georgsen, MSc,† Torben Steiniche, MD, DMSc,† and Mogens Vyberg, MD‡*

(*Appl Immunohistochem Mol Morphol* 2023;31:347–353)

TABLE 2. Positivity-rates and H-scores in Lung Cancer Subtypes for Each Keratin 5 Antibody Clone and p40

Antibody	SP27		XM26		EP1601Y		D5/16 B4		p40	
Tumor type	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	$P < 0.05$
SCC (n = 31, incl. 2 ASCs)	100	287	97	259	97	251	100	252	97	SP27 vs. all other
AC (n = 59)	25	13	0	0	0	0	0 (24*)	0	2	SP27 vs. all other
LCC (n = 17)	53	87	41	50	29	38	24	32	12	SP27 vs. all other
CS (n = 5)	60	132	40	120	40	120	40	120	40	—
LCNEC (n = 8)	50	91	38	69	25	61	25	62	25	—
SCLC (n = 10)	40	40	30	17	30	16	30	18	10	—
Overall (n = 130)	—	99	—	79	—	74	—	74	—	SP27 vs. XM26 vs. D5/16 B4 and EP1601Y

*Non-specific granular reaction, probably Mouse Ascites Golgi.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; CS, carcinosarcoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; Pos., H-score ≥ 10; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

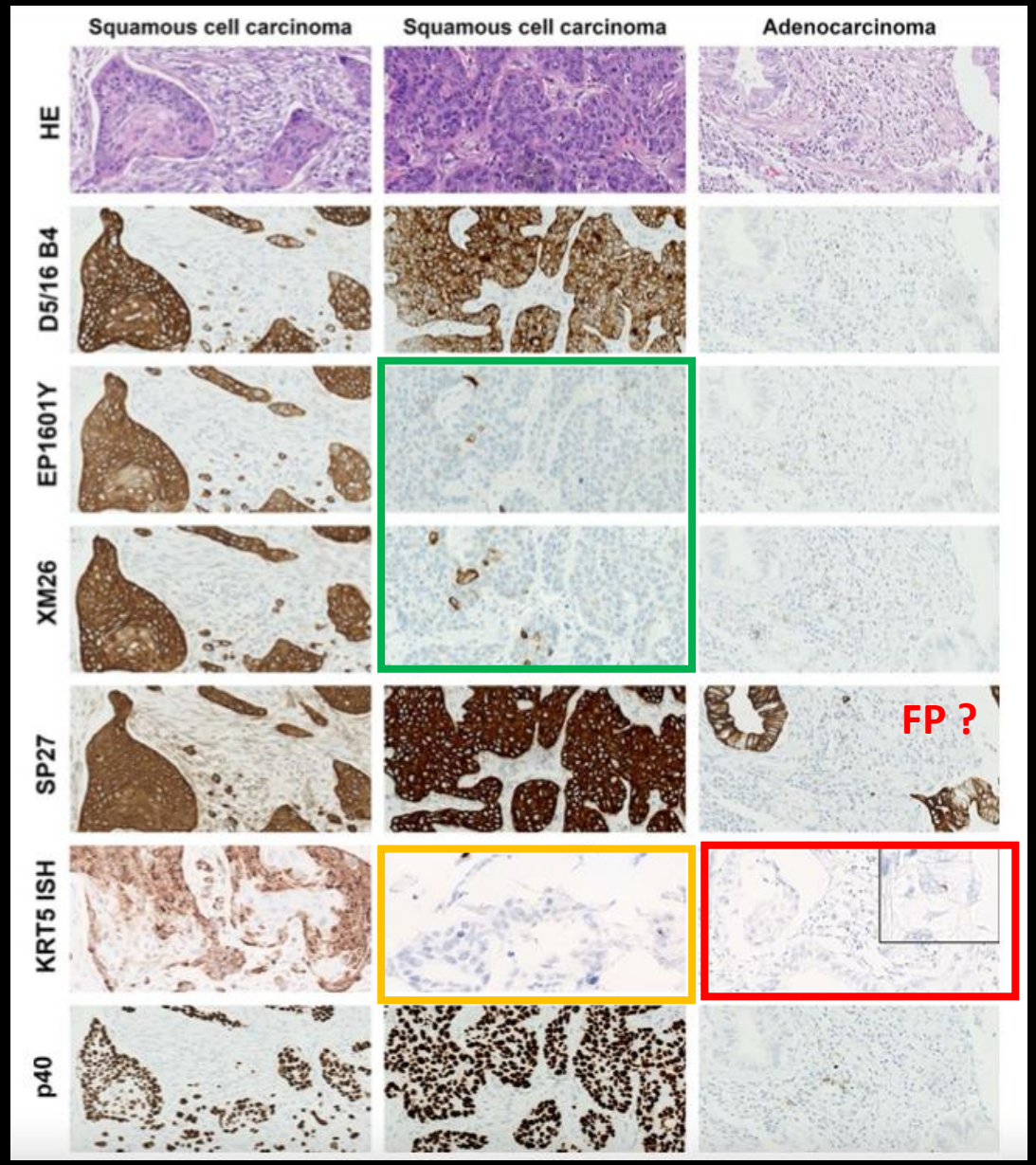


TABLE 3. KRT5 mRNA-ISH Results for Each Lung Cancer Subtype

	mRNA in situ Hybridization		
Tumor type	Sufficient quality [%], (n)	Positive* [%], (n)	ISH-score [0–300], range (median)
SCC (n = 31, incl. 2 ASCs)	77 (24)	96 (23)	0–300 (270)
AC (n = 59)	68 (40)	71 (28)	0–10 (1)
LCC (n = 17)	81 (14)	100 (14)	1–270 (4)
CS (n = 5)	60 (3)	67 (2)	0–175 (3)
LCNEC (n = 8)	NA	NA	—
SCLC (n = 10)	NA	NA	—

*Positive: Sufficient quality and ISH-score ≥ 1.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; CS, carcinosarcoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

Positive ?

ISH score very low
Interpretation/cut- off value ?

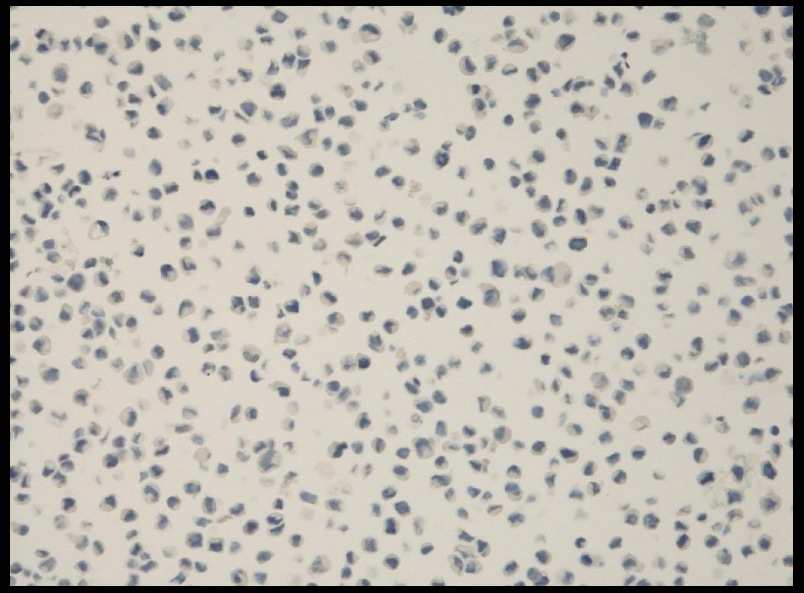
(*Appl Immunohistochem Mol Morphol* 2023;31:347–353)

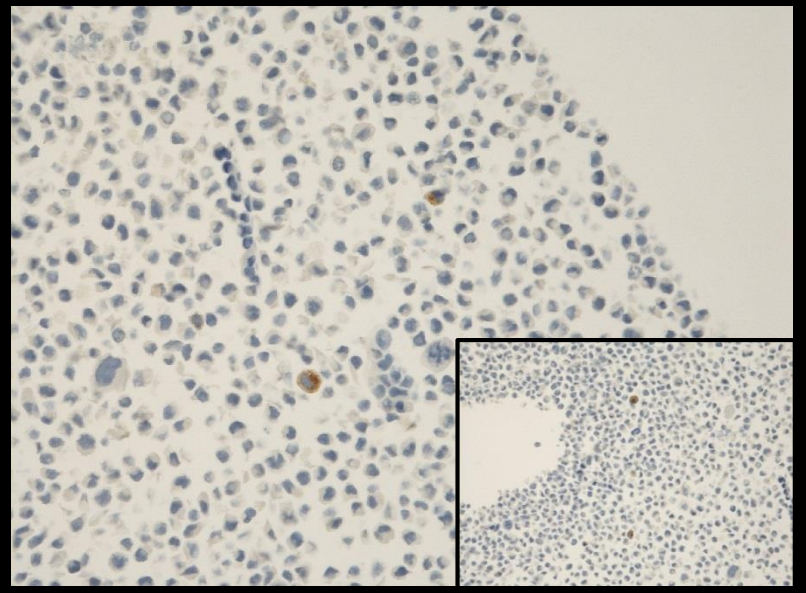
RNAscope (Duplex)

PSOR2 (IL17A-/CD3+)

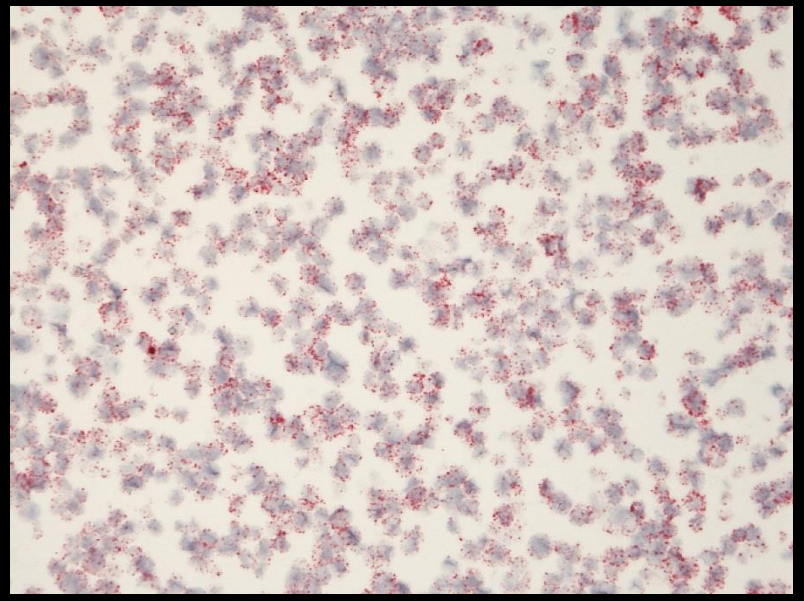
SeAX (IL17A+/CD3-)

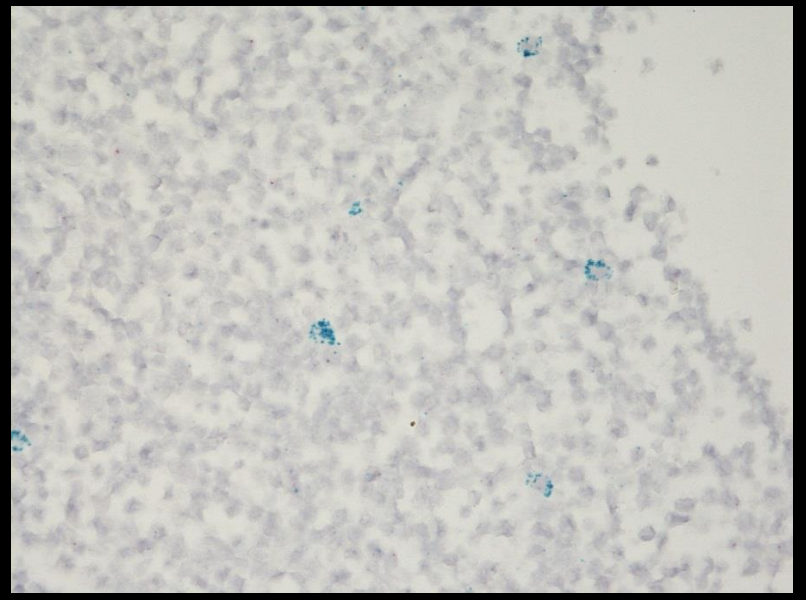
IL17a (Goat polyclonal) IHC
Single staining





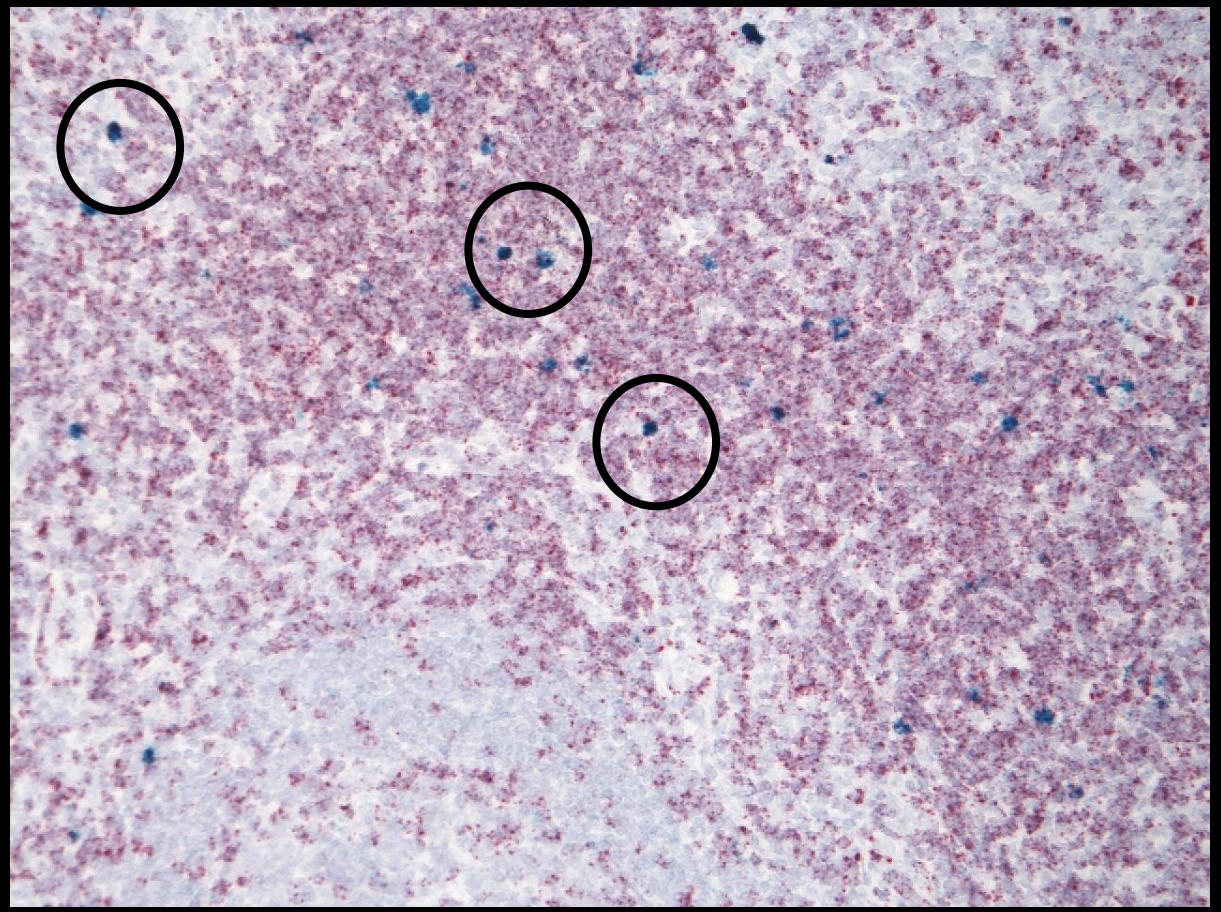
IL17a+CD3E /RNAscope



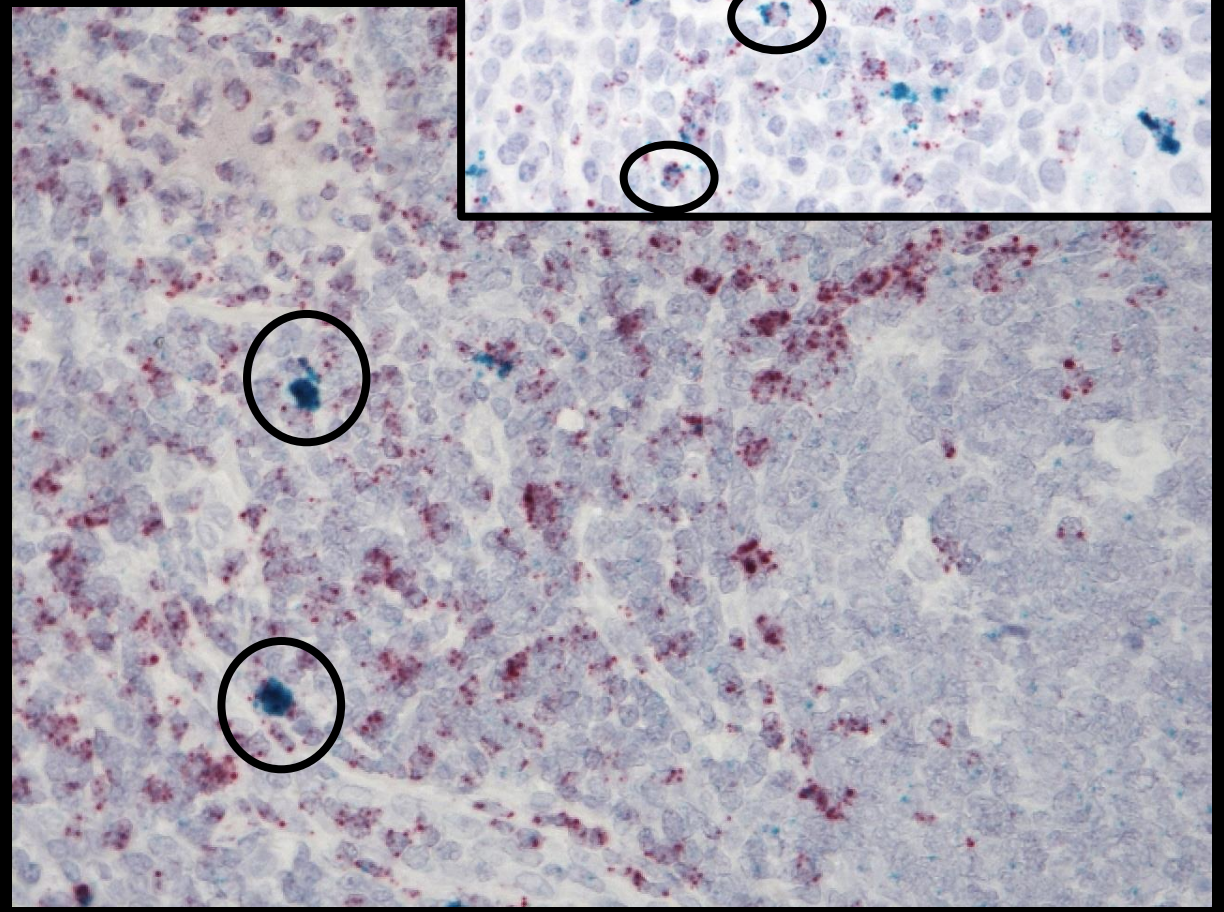


RNAScope Duplex

IL17A: Green/bluish
CD3E: Red
Tonsil



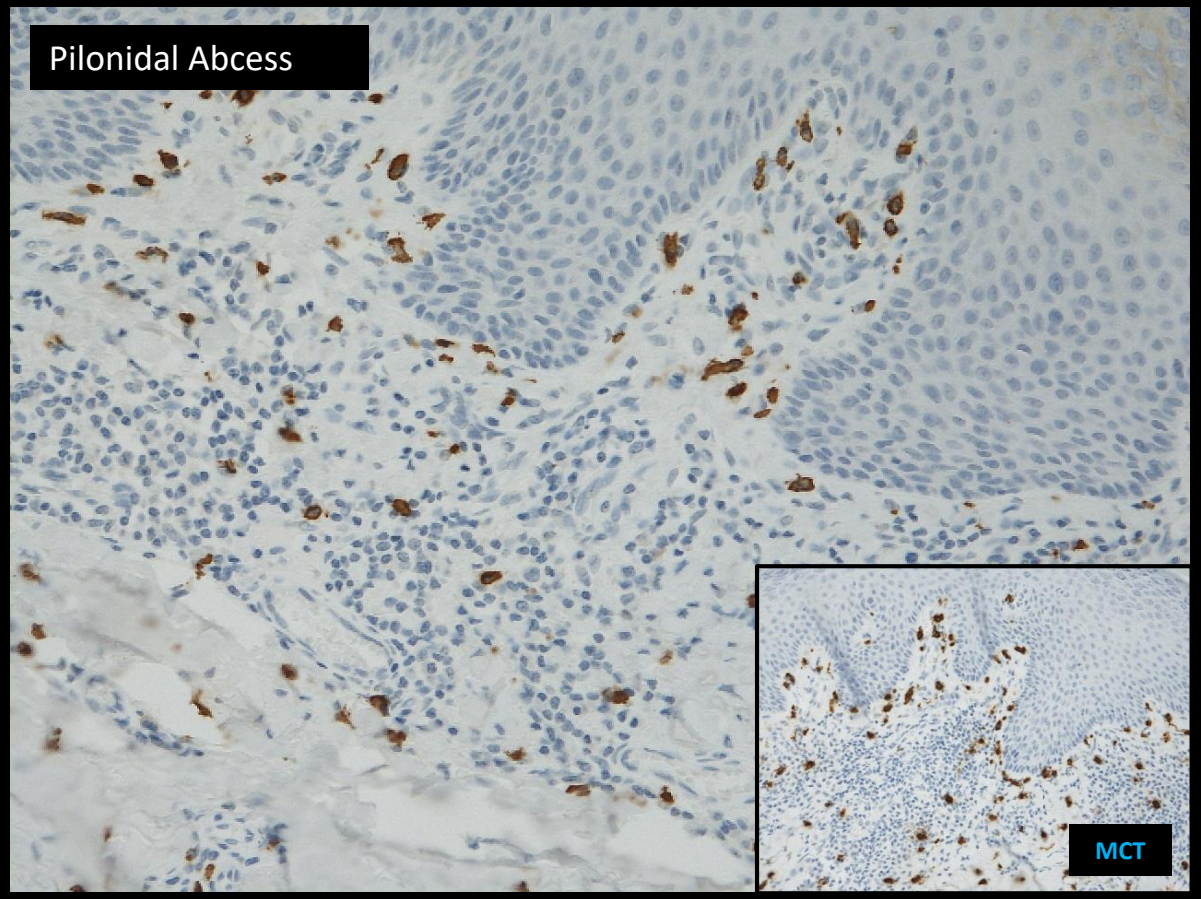
IL17A^{+}CD3E^{+} T-cells / IL17A^{-}CD3E^{+} T-cells



IL17A^{+}CD3E^{+} T-Cells ? Difficult to interpret due to very strong reaction for IL17A. The positive IL17A^{+} are large ?

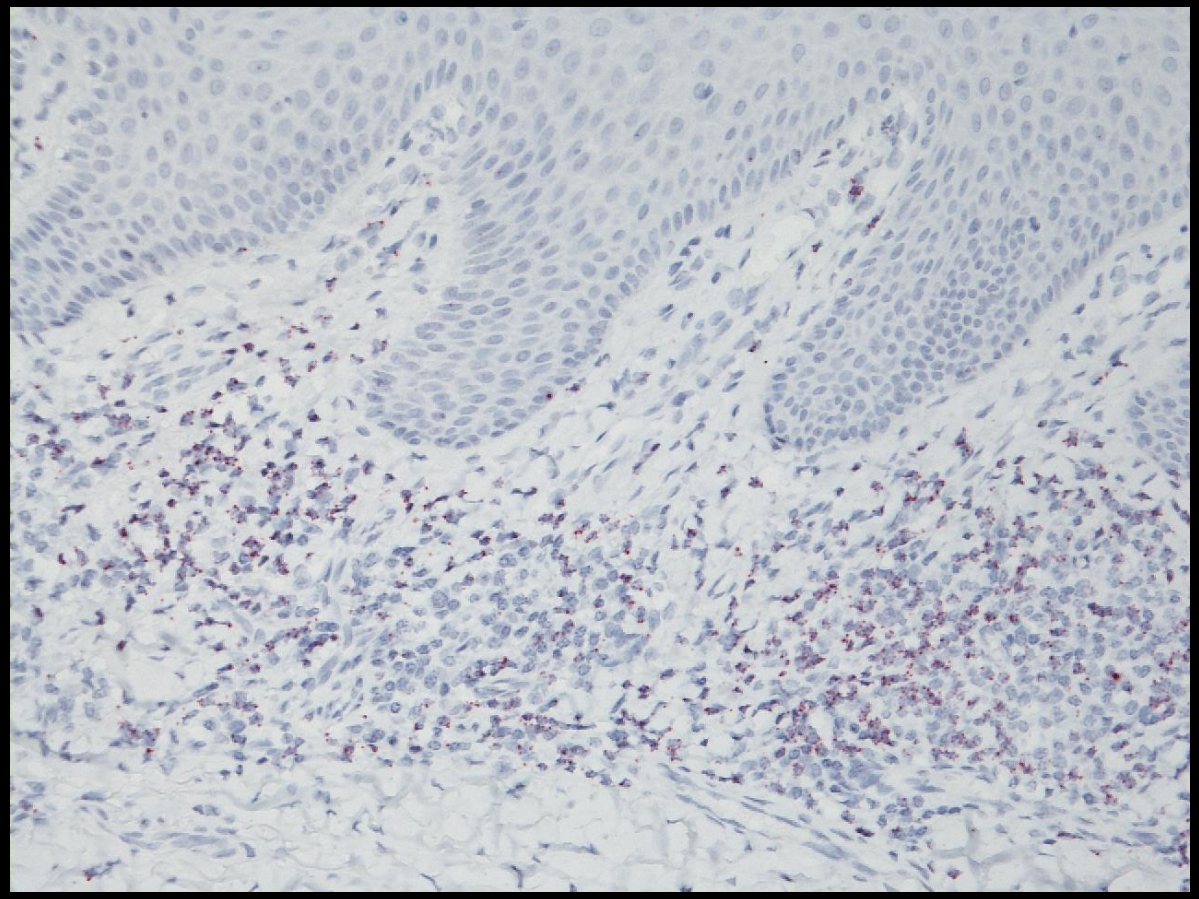
IL17A: The Big Issue ?

Immunohistochemistry: IL17A (polycloal Goat)



The mast cells/neutrophil granulocytes are positive ?

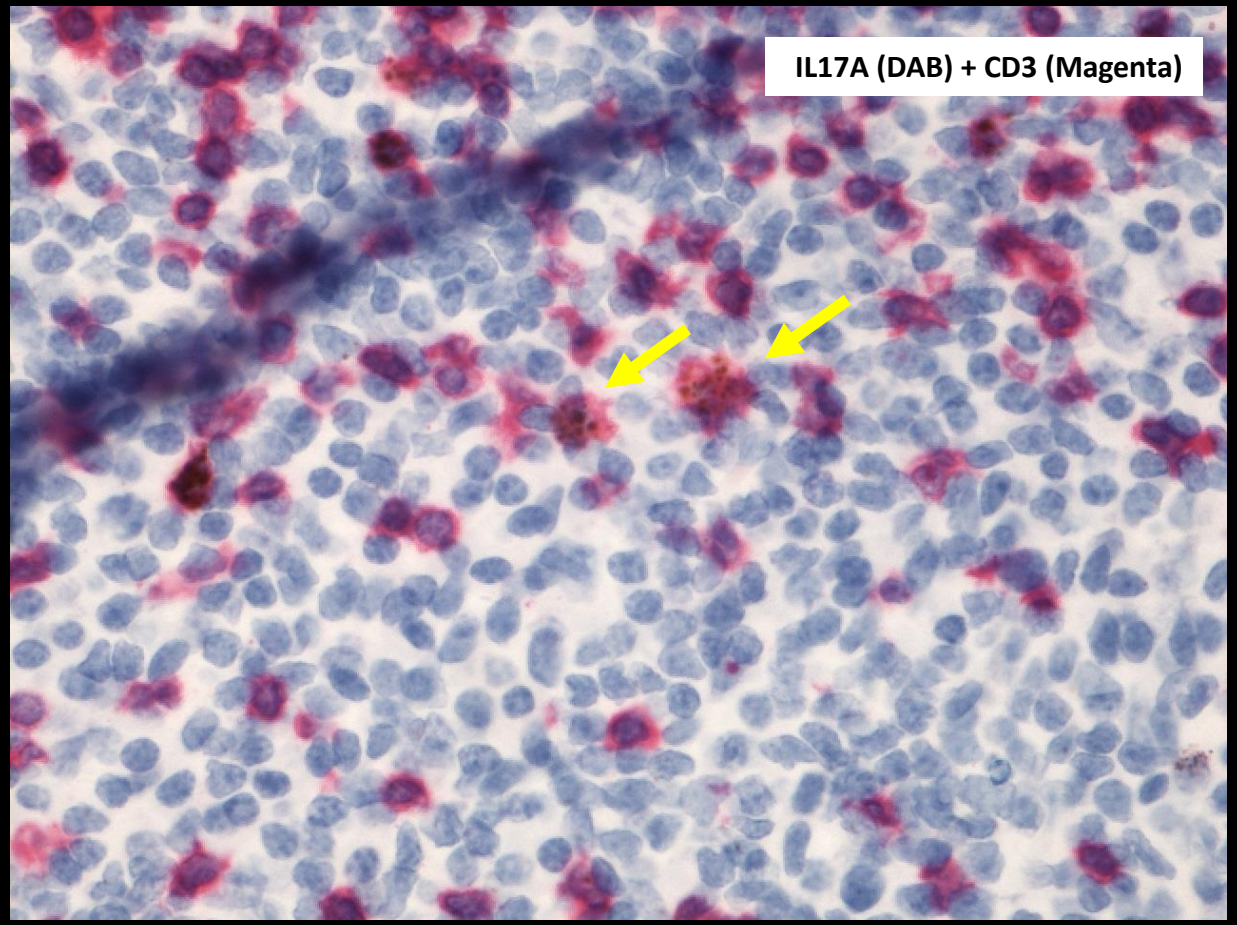
RNAScope Duplex: IL17A+CD3E

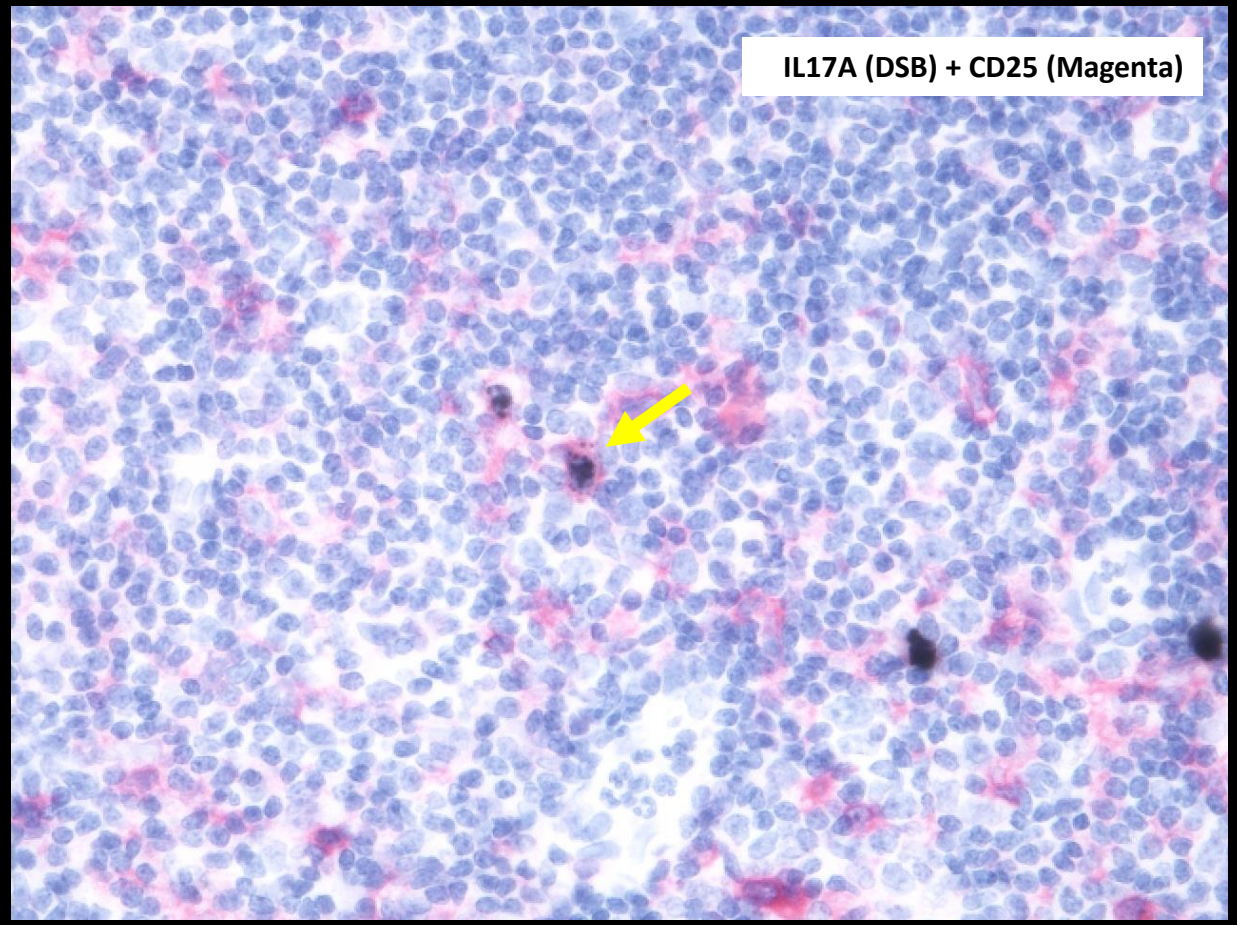


Only T-cells are demonstrated (red granular reaction) ?

Combined RNAscope + IHC ?

Manual: Dual ISH (Rnascope IL17A) + IHC (CD3 or CD25)





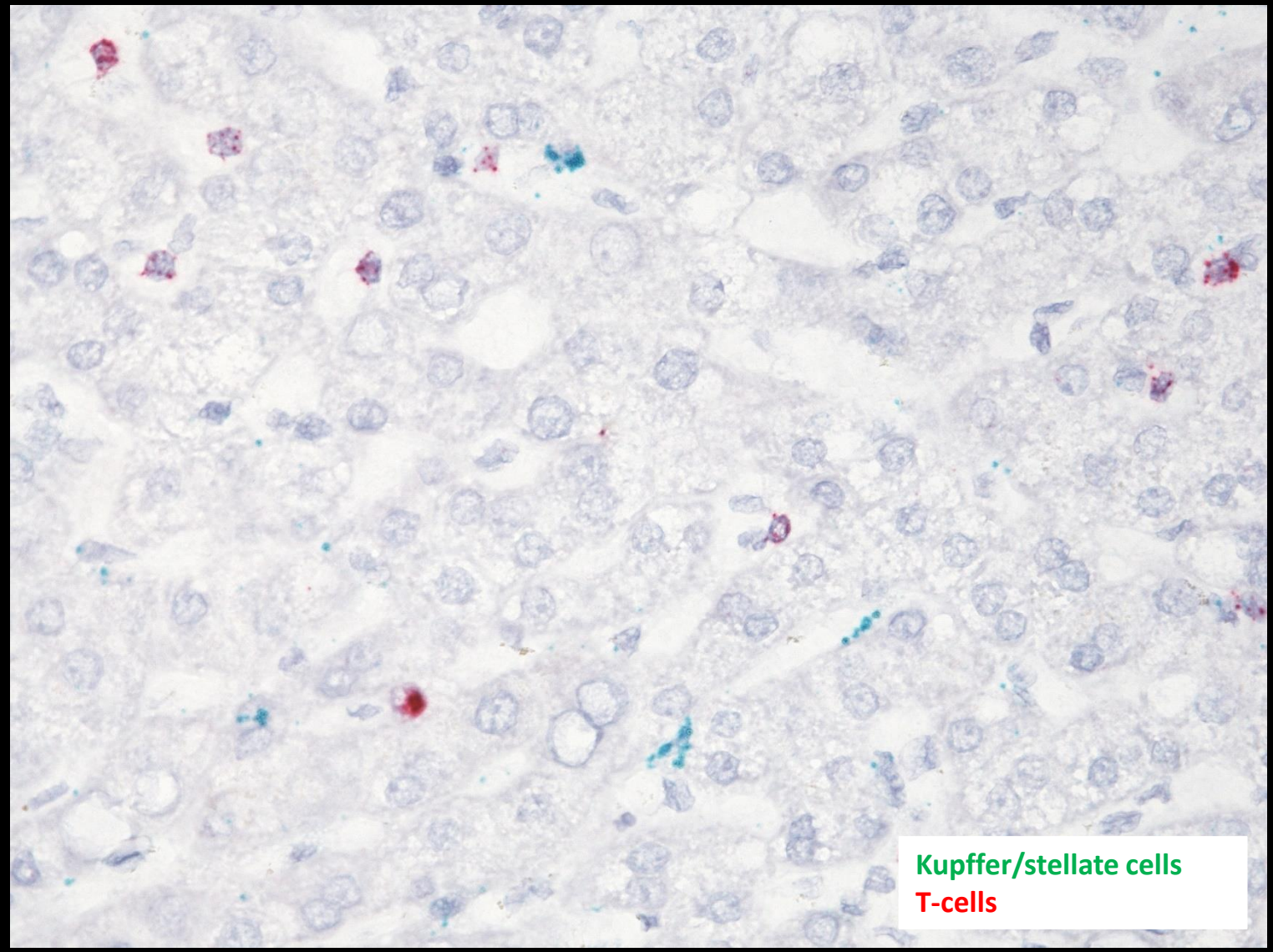
RNAscope: IL17A + H8`(TR)/P16`(Protease) + DAB or DSB/IHC:CD3, LN10 (1:50) or CD25 (1:25) + Flex+/Magenta

Manual Assay: Rnascope Duplex HGF + CD3

Manual RNAscope Duplex:

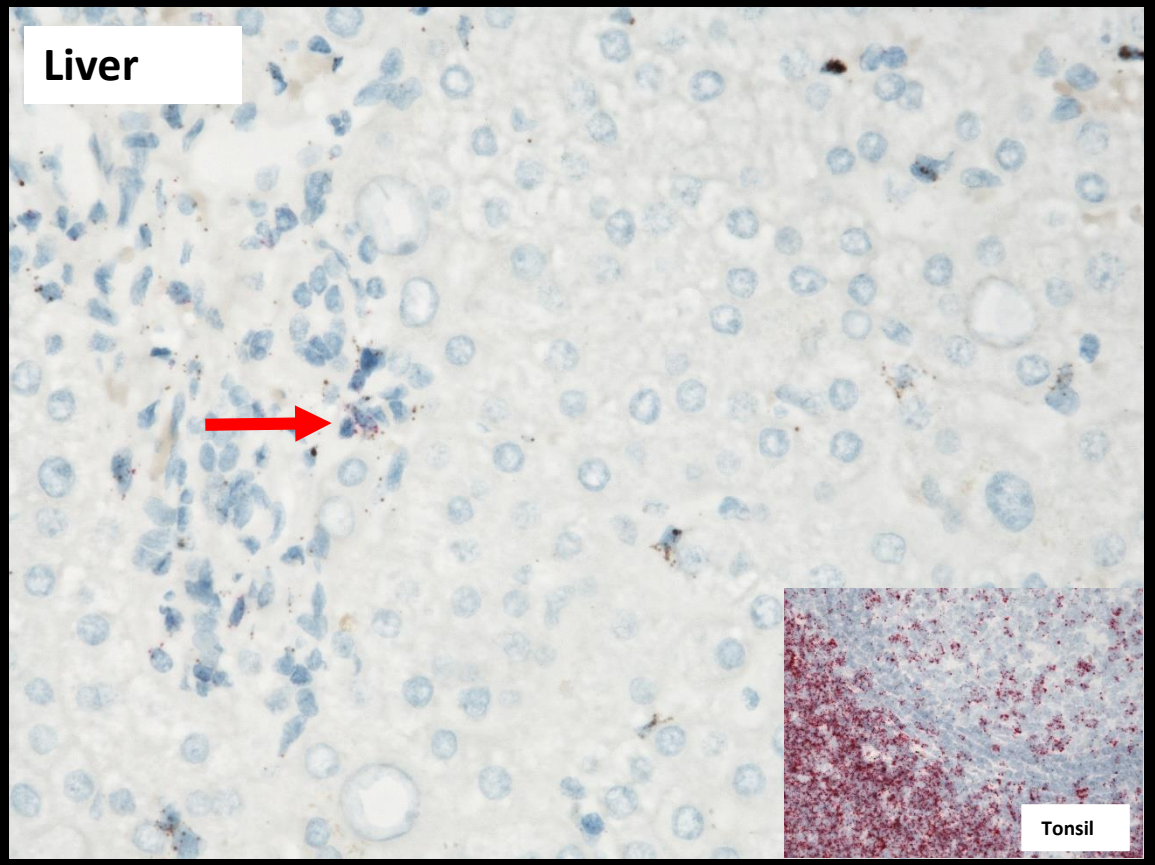
TR15/P15`(Protease)

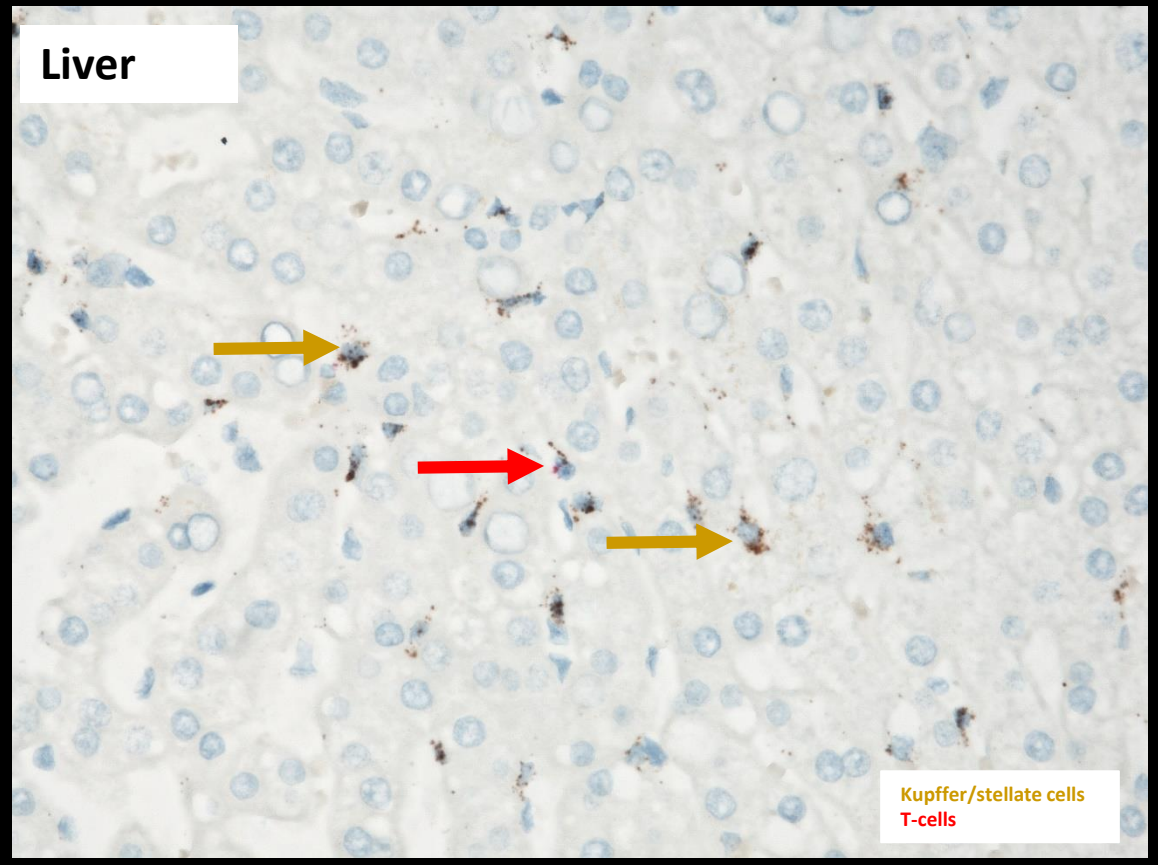
Standard detection - recommended procedure from the vendor



Ventana Discovery: Rnascope Duplex HGF + CD3

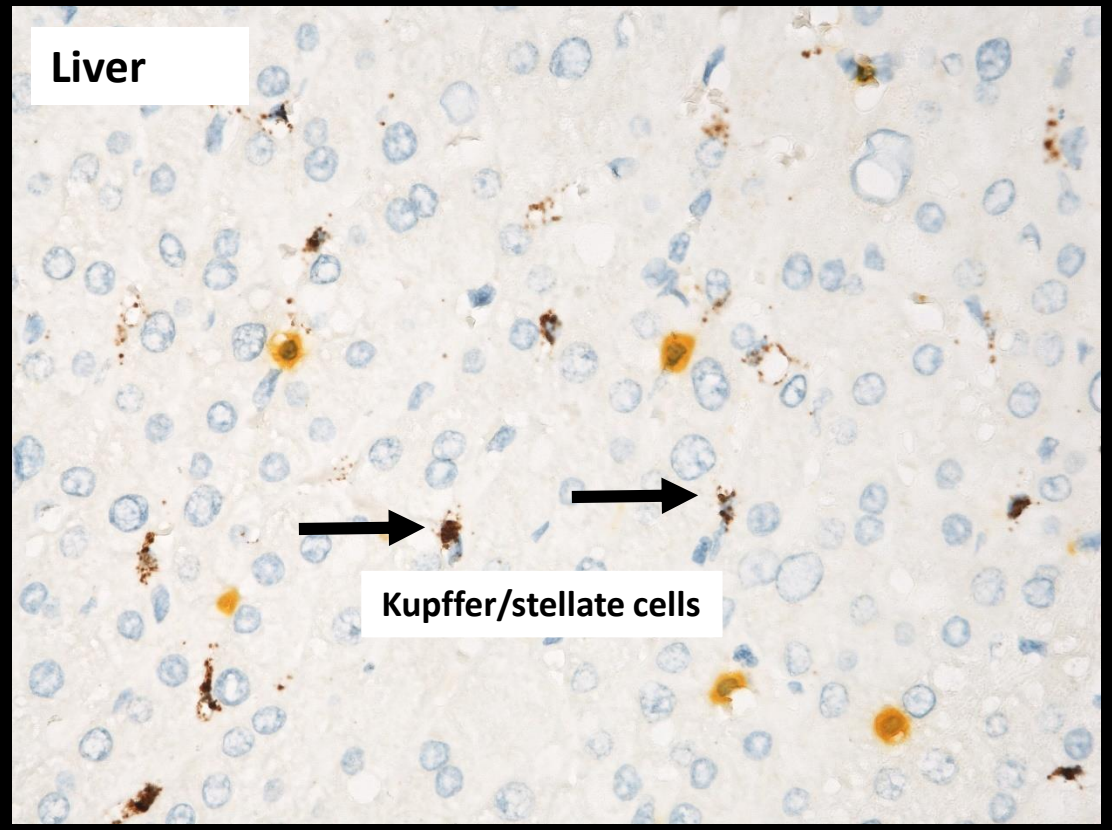
Problem: Weak red signals (T-cells) ?

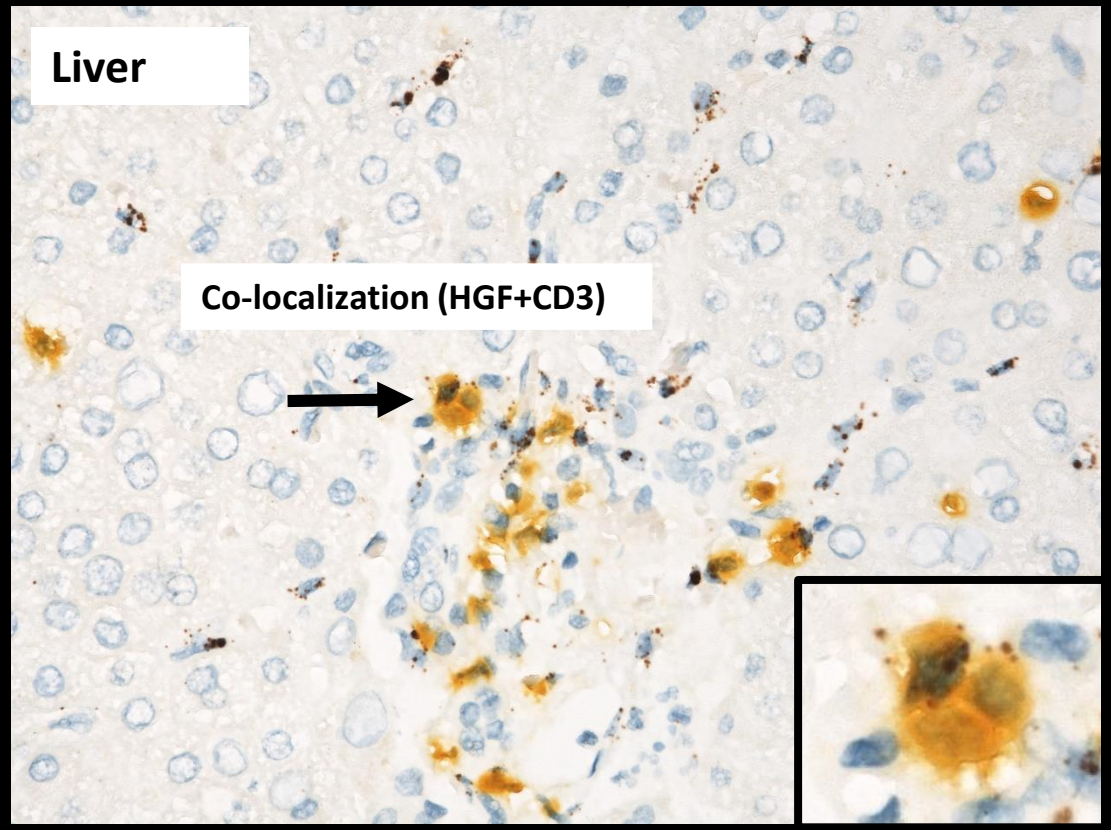




RNAscope Duplex: H16/24` (CC1)/P16`(Protease) + AMP5+8 24` + DAB /FR

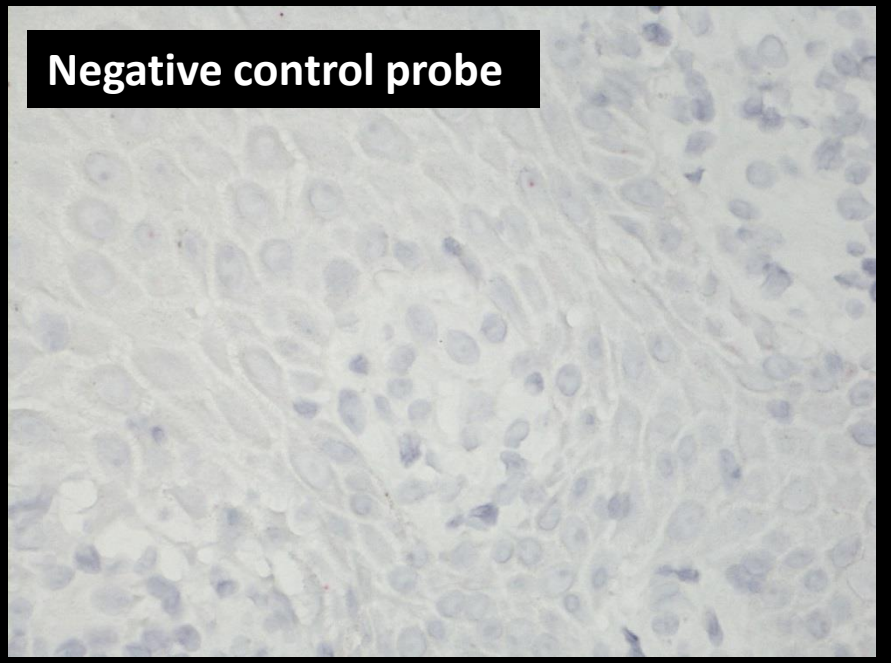
Ventana Discovery: Dual ISH (Rnascope HGF) + IHC (CD3)

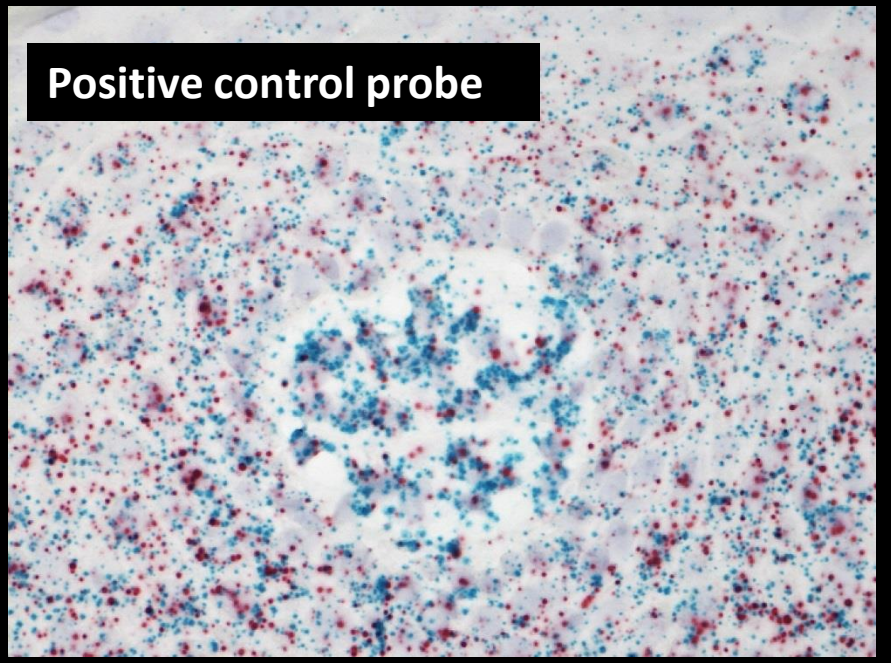




RNAscope: HGF + H24` (CC1)/P16`(Protease) + AMP5 48` + DAB /IHC:CD3, LN10 (1:50) + Anti-Mouse/NP + Anti-NP/AP + Discovery Yellow

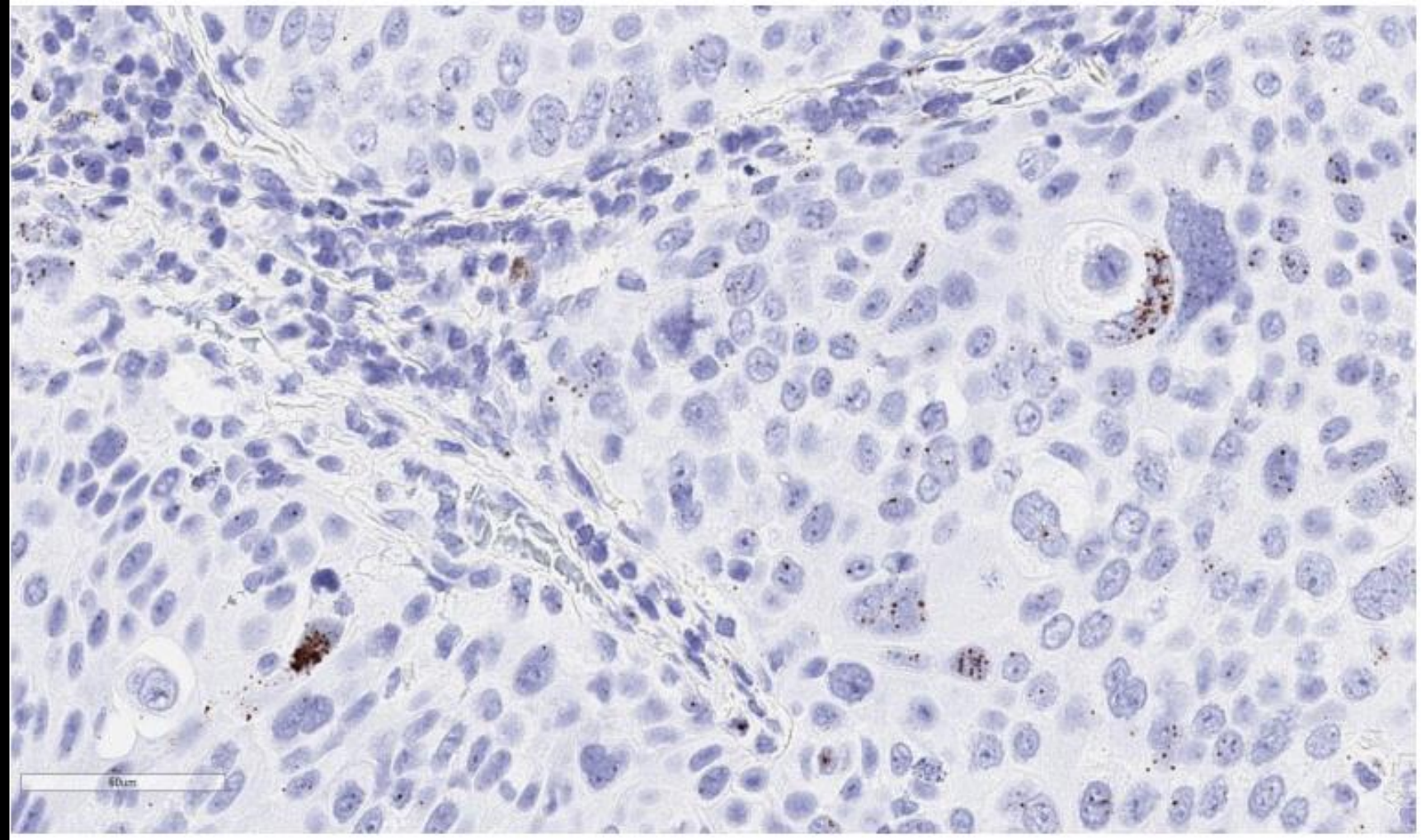
Final thoughts and remarks





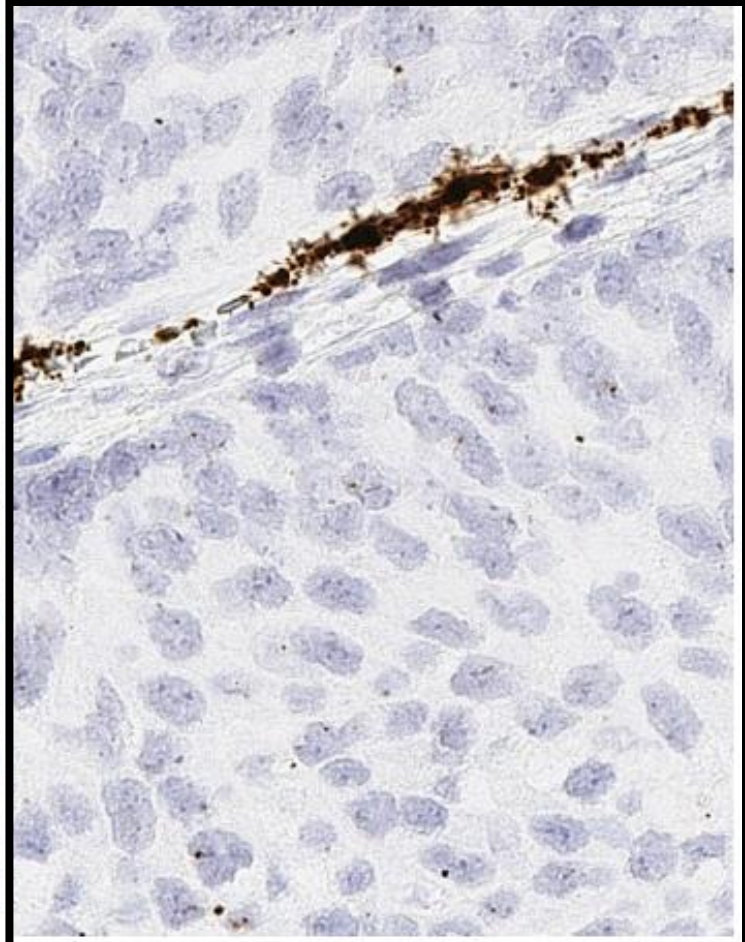
Small dot`s in nuclei`s: Detection of the DNA (genes) ?

Squamous epithelial cells should be negative for IL26/CD3E



Expression of PDL1 RNA (brown dots) in human lung cancer tissue, RNA in situ hybridization (ISH) using automated RNAscope® Leica Assay-BROWN

Dots in the nuclei`s`?



cancer tissue, RNA in situ hybridization (ISH)

Volume 49(5): 603–611, 2001
The Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Single-copy Gene Detection Using Branched DNA (bDNA) In Situ Hybridization

Audrey N. Player,[1] Lu-Ping Shen, Daryn Kenny, Vincent P. Antao, and Janice A. Kolberg

Bayer Diagnostics, Emeryville, California

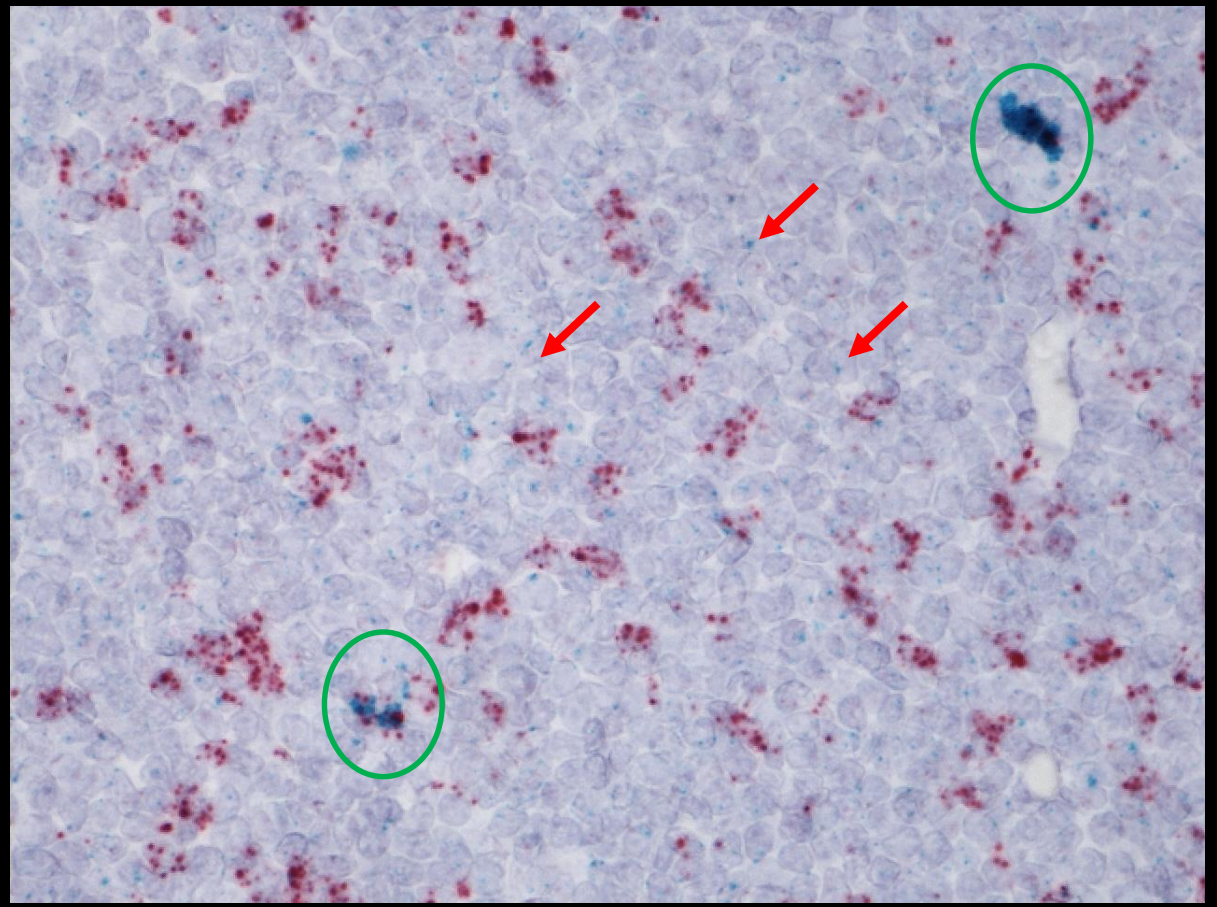
Single dots in the nuclei: Are we detecting the genes ?

SUMMARY We have developed a branched DNA in situ hybridization (bDNA ISH) method for detection of human papillomavirus (HPV) DNA in whole cells. Using human cervical cancer cell lines with known copies of HPV DNA, we show that the bDNA ISH method is highly sensitive, detecting as few as one or two copies of HPV DNA per cell. By modifying sample pretreatment, viral mRNA or DNA sequences can be detected using the same set of oligonucleotide probes. In experiments performed on mixed populations of cells, the bDNA ISH method is highly specific and can distinguish cells with HPV-16 from cells with HPV-18 DNA. Furthermore, we demonstrate that the bDNA ISH method provides precise localization, yielding positive signals retained within the subcellular compartments in which the target nucleic acid sequences are localized. As an effective and convenient means for nucleic acid detection, the bDNA ISH method is applicable to the detection of cancers and infectious agents. **(J Histochem Cytochem 49:603–611, 2001)**

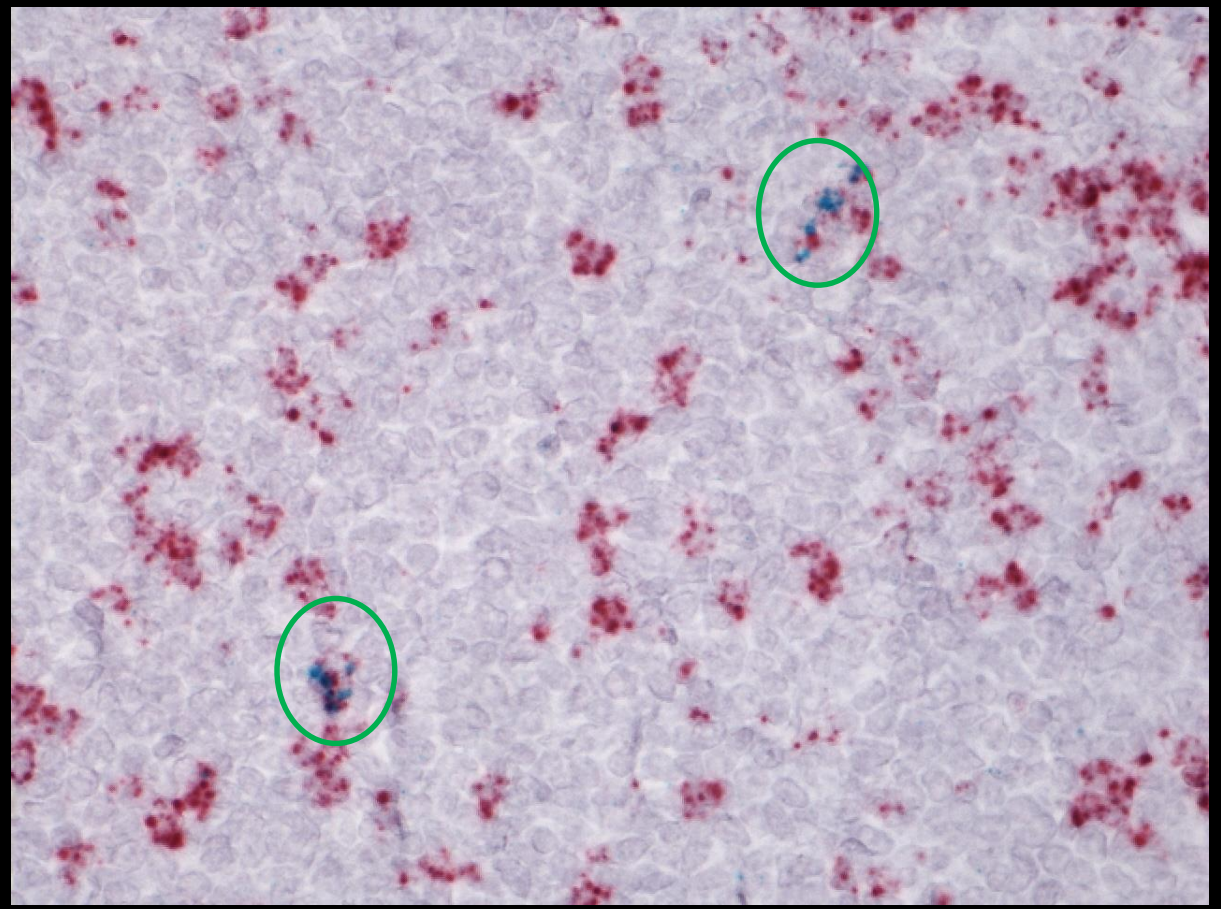
KEY WORDS
branched DNA (bDNA) signal amplification
in situ hybridization (ISH)
cervical cancer cell lines
human papillomavirus (HPV)

RNAscope IL17A/CD3E: With and without a DNAse pre-treatment step

Without DNAse



With DNAse (Qiagen 4`)

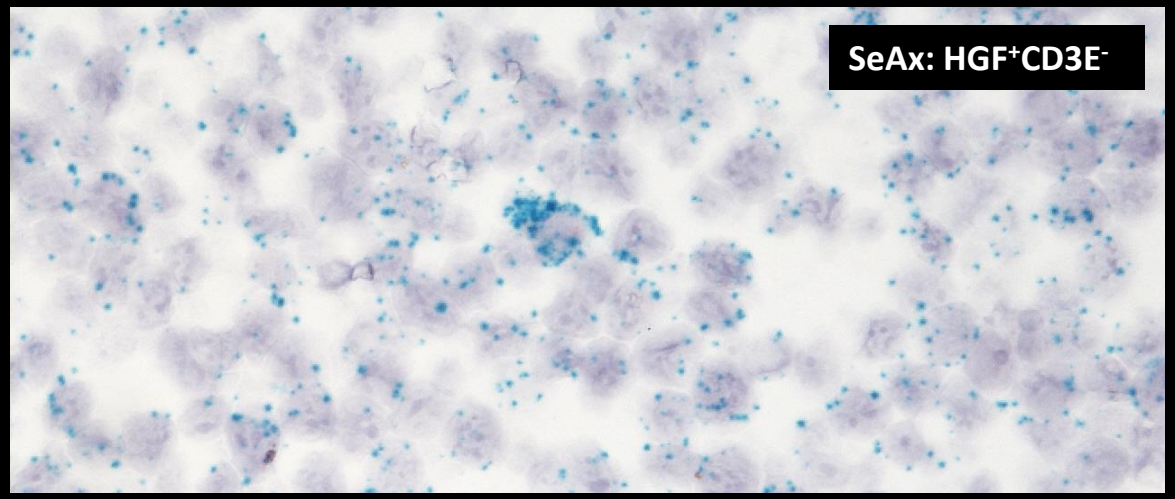


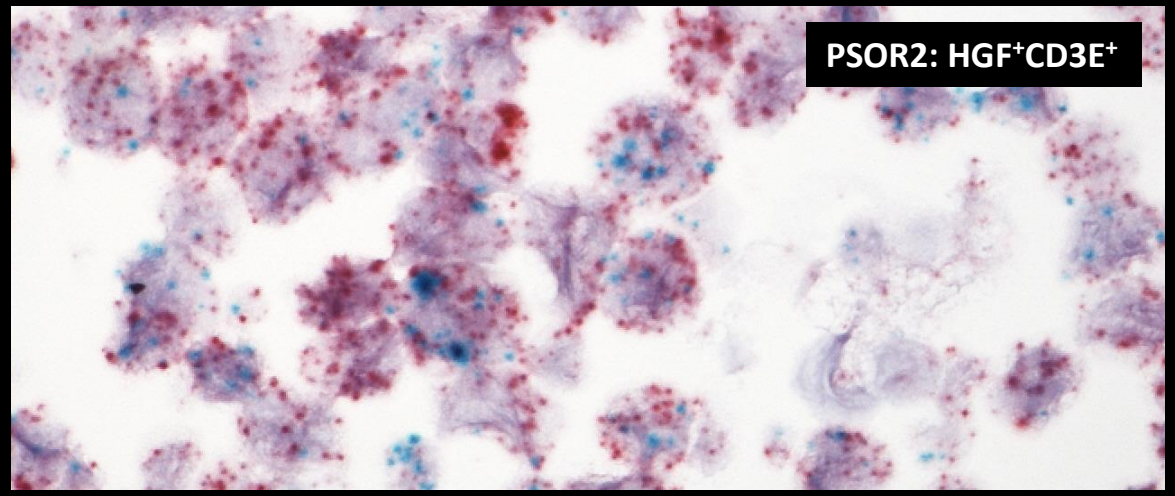
The pre-treatment with DNAse eliminated reactions related to dots in the nuclei`s. Specific signals are preserved.

bDNA In situ hybridization (RNAscope)

Summary:

- It works , especially with the SinglePlex assay
  - Based on C1 probes (DAB) and single detection reagents
- Using Duplex Kit - select the right channel of the probe
  - C1 for the most abundant expressed target mRNA (not always possible to predict)
  - Cross-reactivity and false positive result (mixed color) may be seen
- Single mRNA molecule detection - be critical ?
  - E.g., single “nuclear dots” could be the gene expression
- ViewRNA ?





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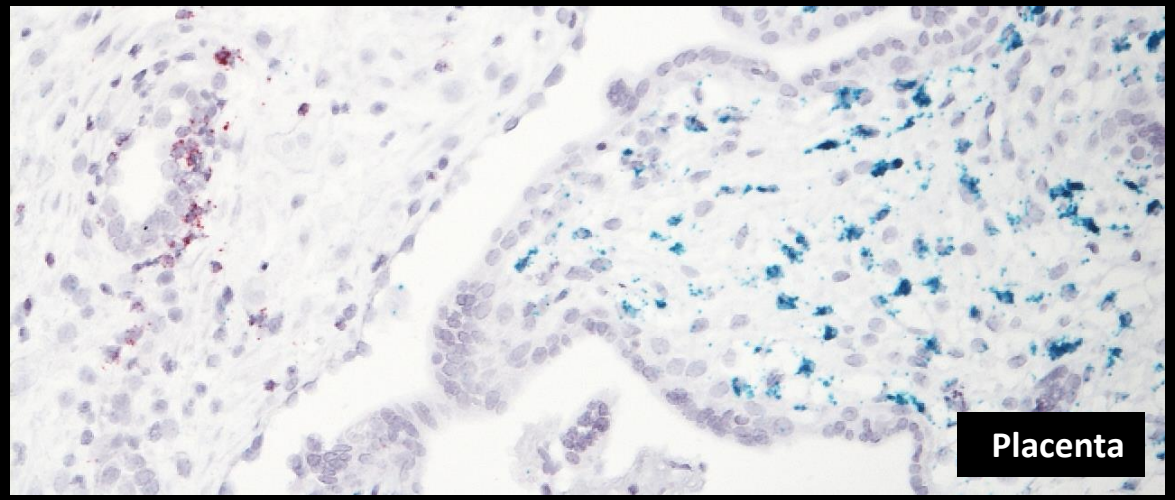
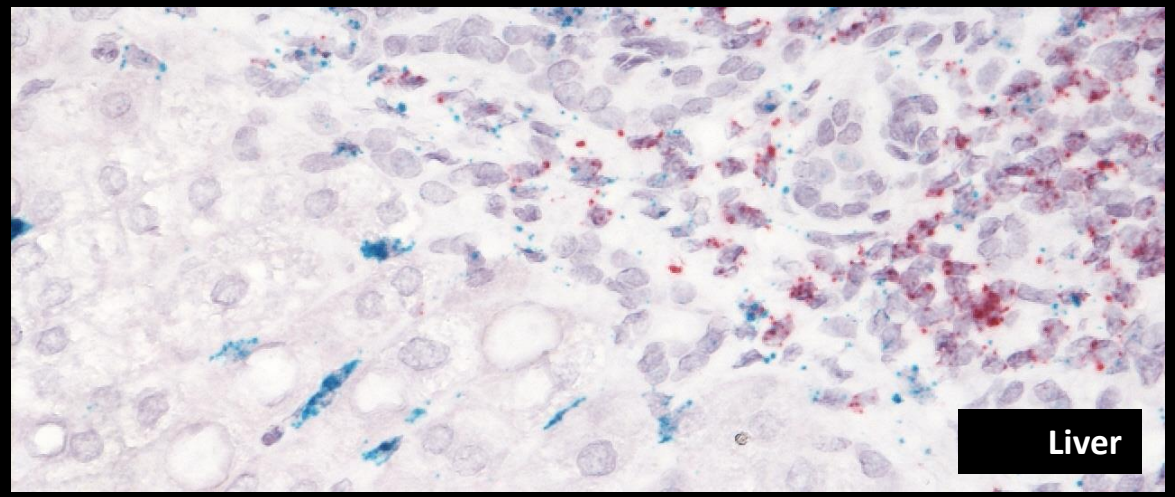




Diagnostiske metoder
Patologiafdelingen, Aalborg Universitetshospital

In Situ Hybridization (ISH)

Branched DNA ISH Technology

RNAscope/Basescope/ViewRNA

Michael Bzorek

Histotechnologist

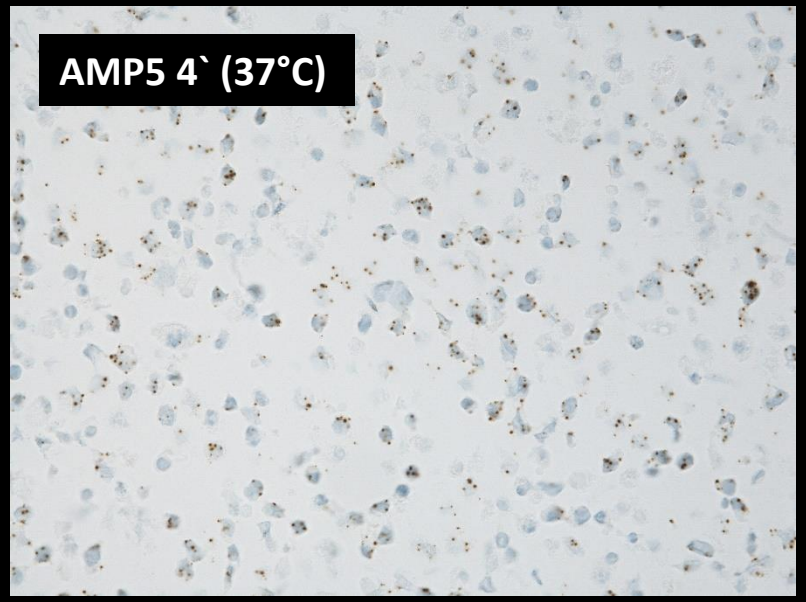
Department of Surgical Pathology

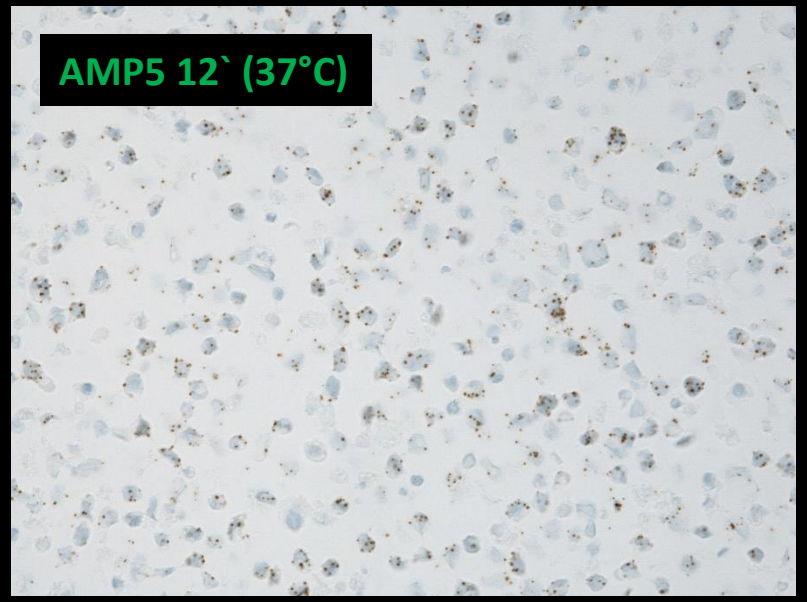
University Hospital, Region Zealand, Denmark

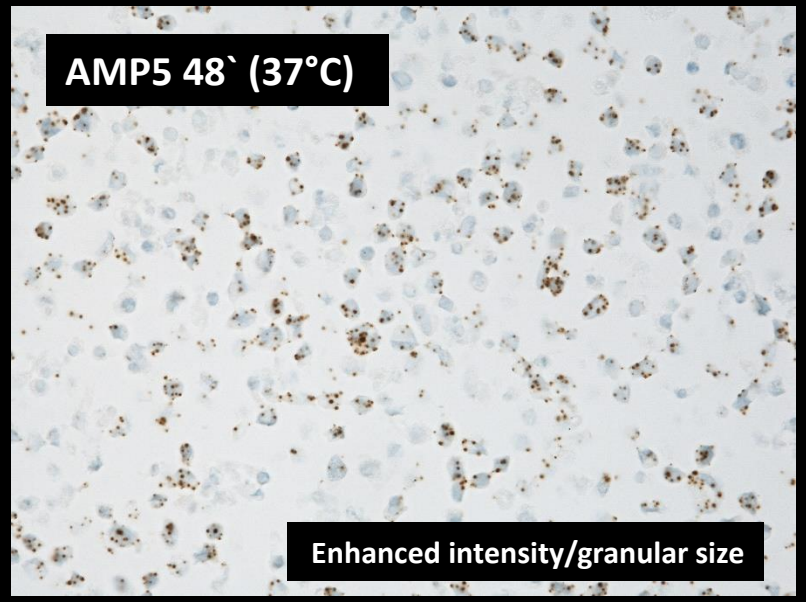
RNAscope TBP probe: Calibration of the Discovery Instrument (Ventana)

Amplifier 5 (AMP5) step – variable time

Hela Cell Line



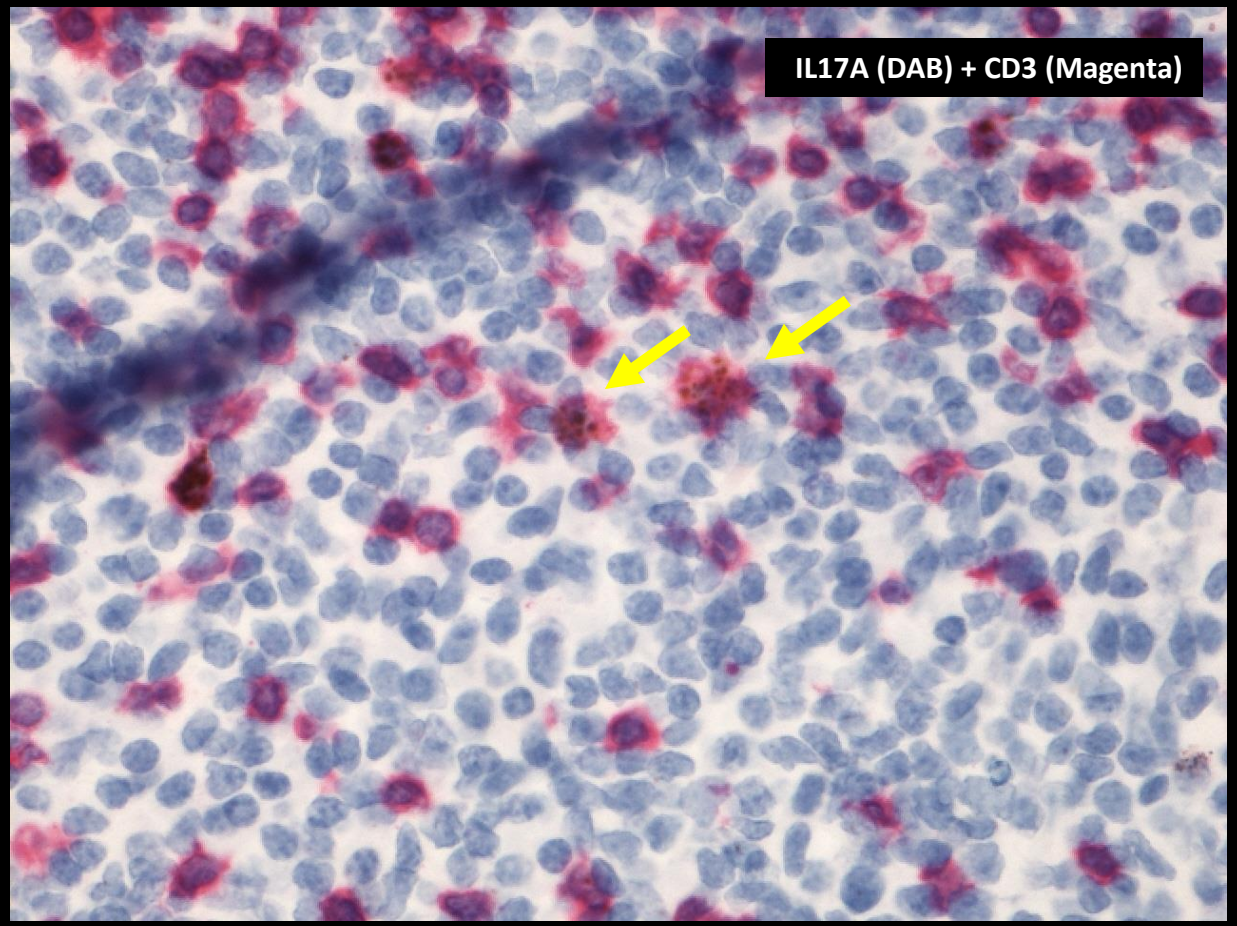


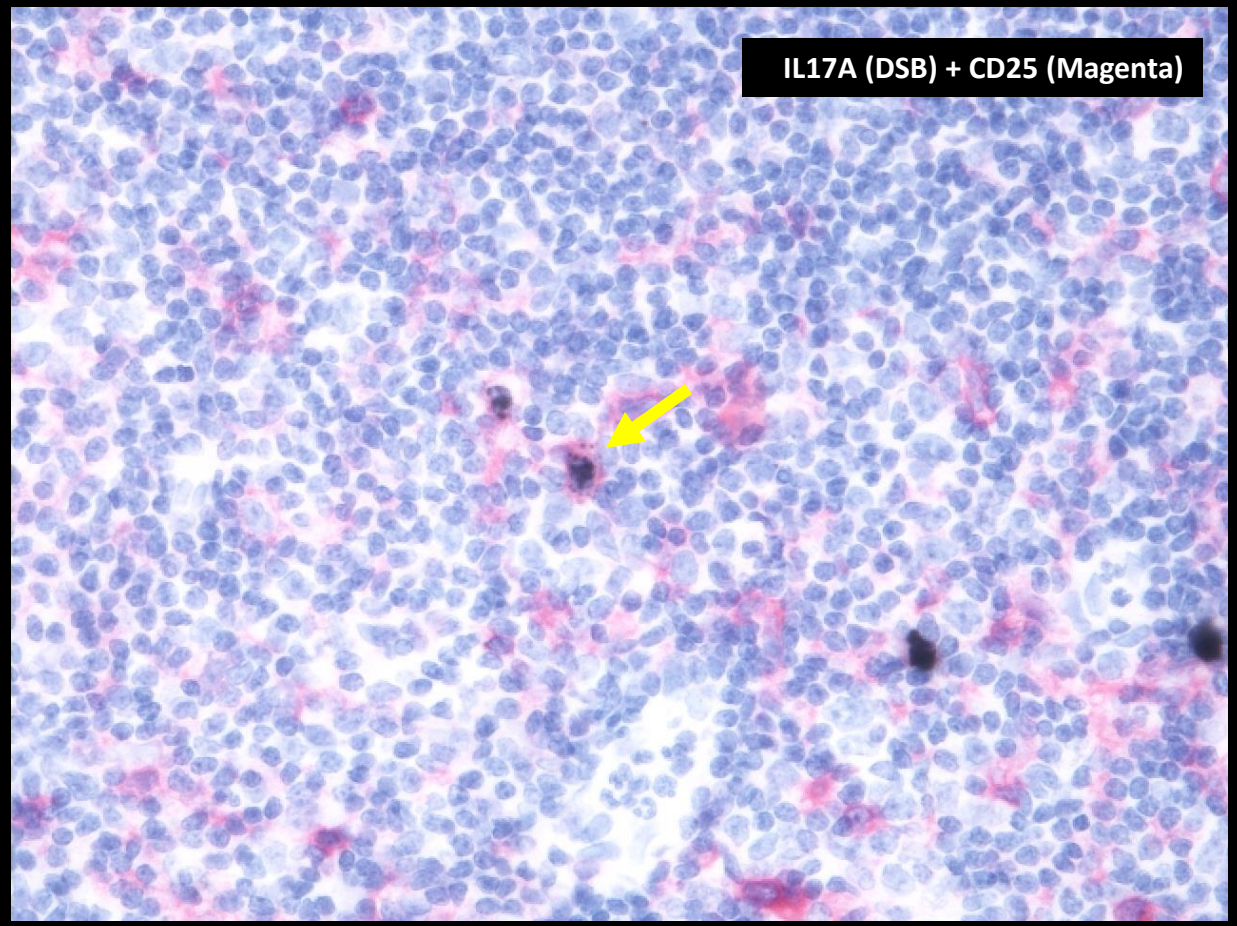


Discovery standard protocol, CC1 16` (97°C)/ P 16` (37°C), AMP5 4-48 ` (37°C)

Combined RNAscope + IHC ?

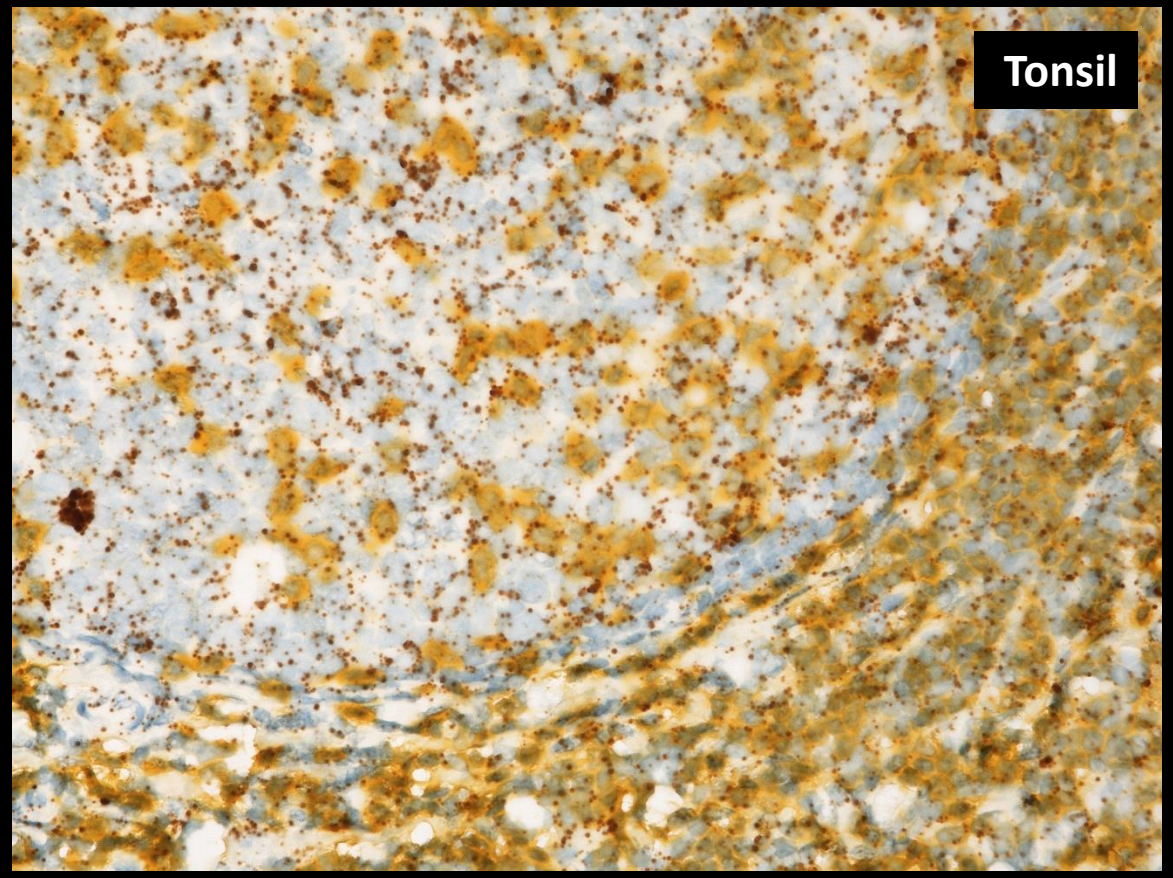
Manual: Dual ISH (Rnascope IL17A) + IHC (CD3 or CD25)

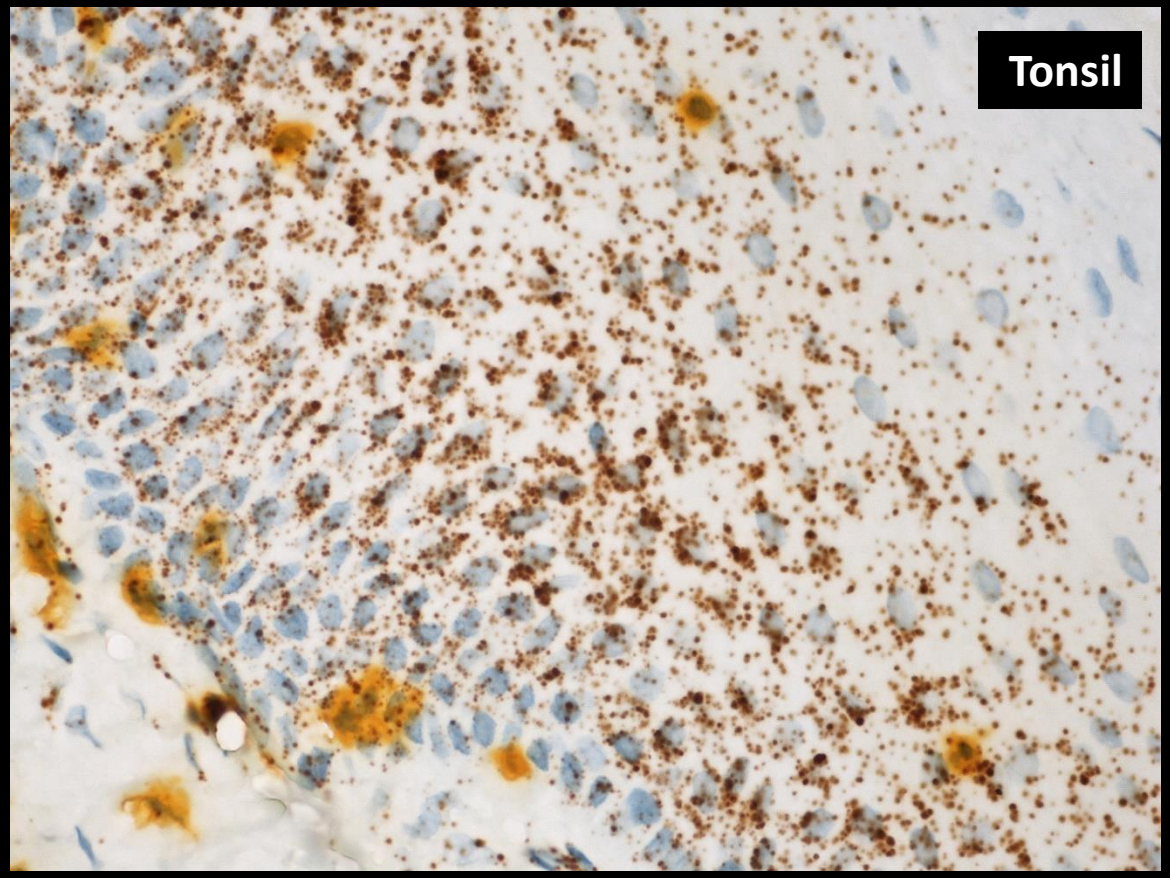




RNAscope: IL17A + H8`(TR)/P16`(Protease) + DAB or DSB/IHC:CD3, LN10 (1:50) or CD25 (1:25) + Flex+/Magenta

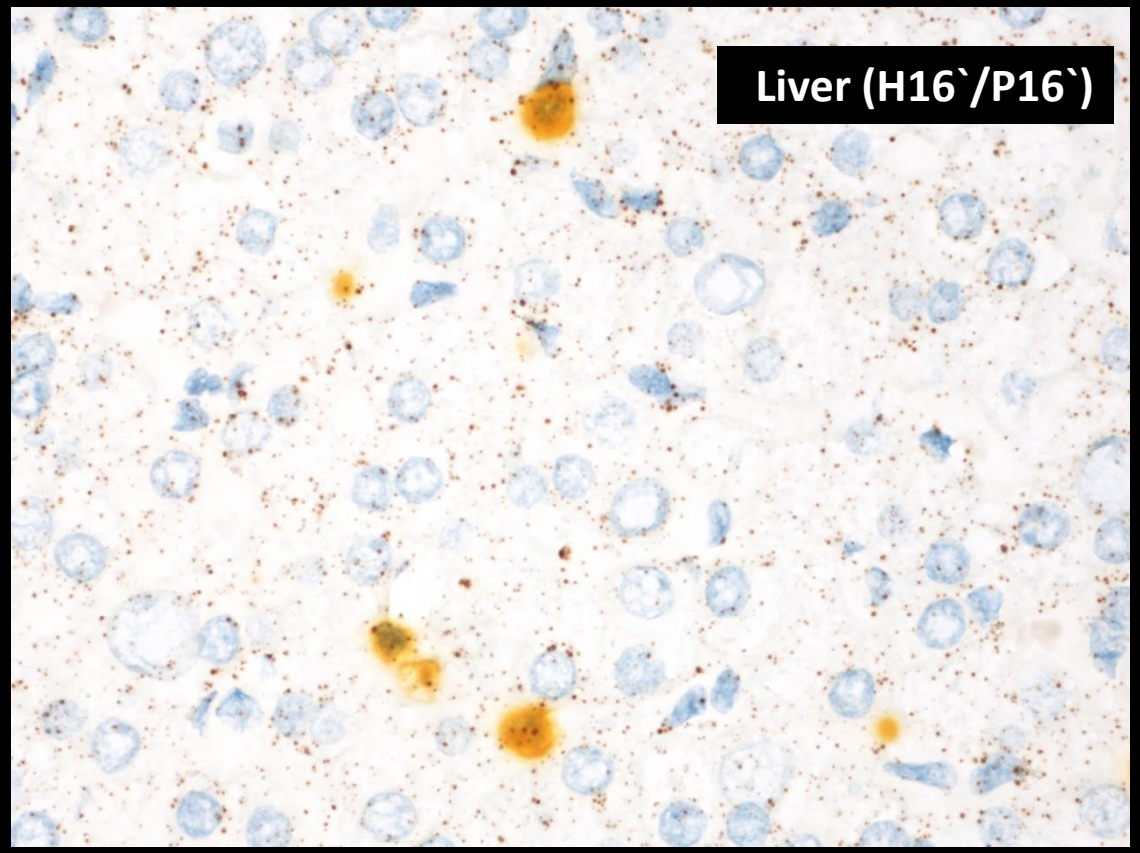
Ventana Discovery: Dual ISH (Rnascope PPIB) + IHC (CD3)

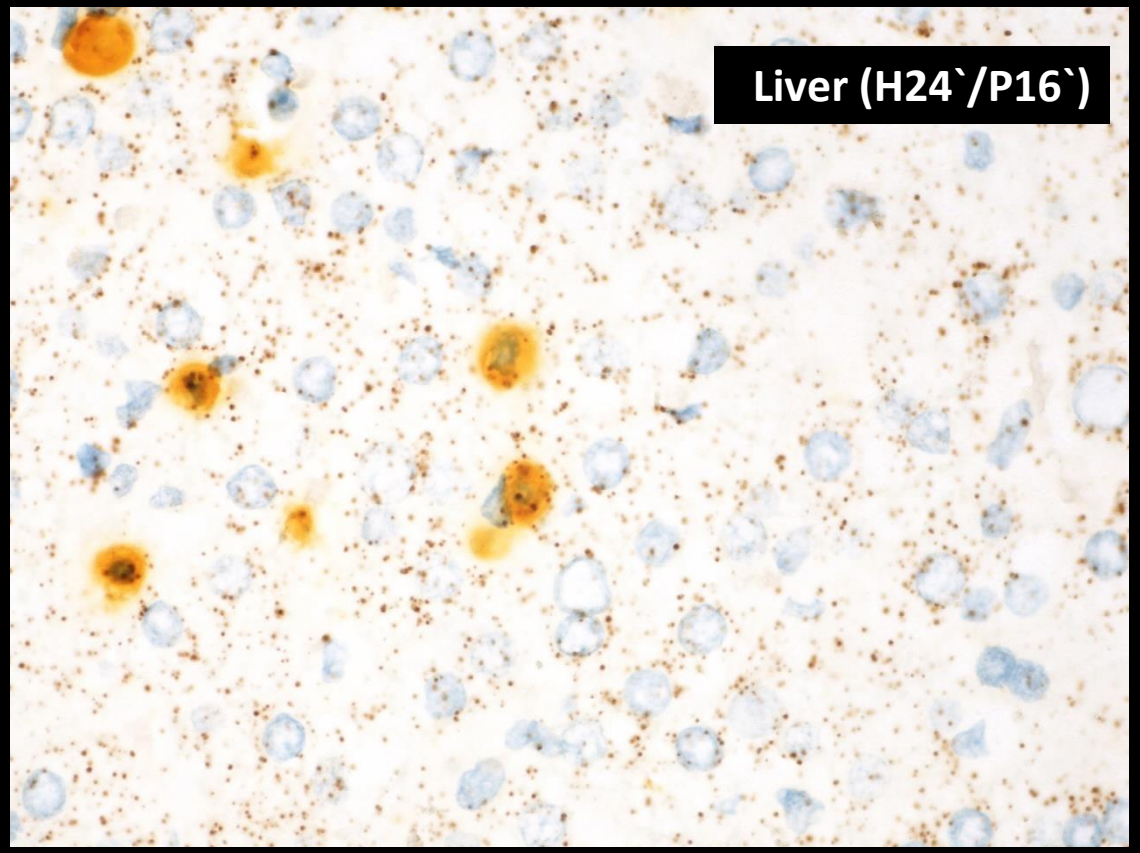




RNAscope: PPIB + H24`(CC1)/P16`(Protease) + AMP5 48` + DAB/IHC:CD3, LN10 (1:50) + Anti-Mouse/NP + Anti-NP/AP + Discovery Yellow

Ventana Discovery: Dual ISH (Rnascope PPIB) + IHC (CD3)





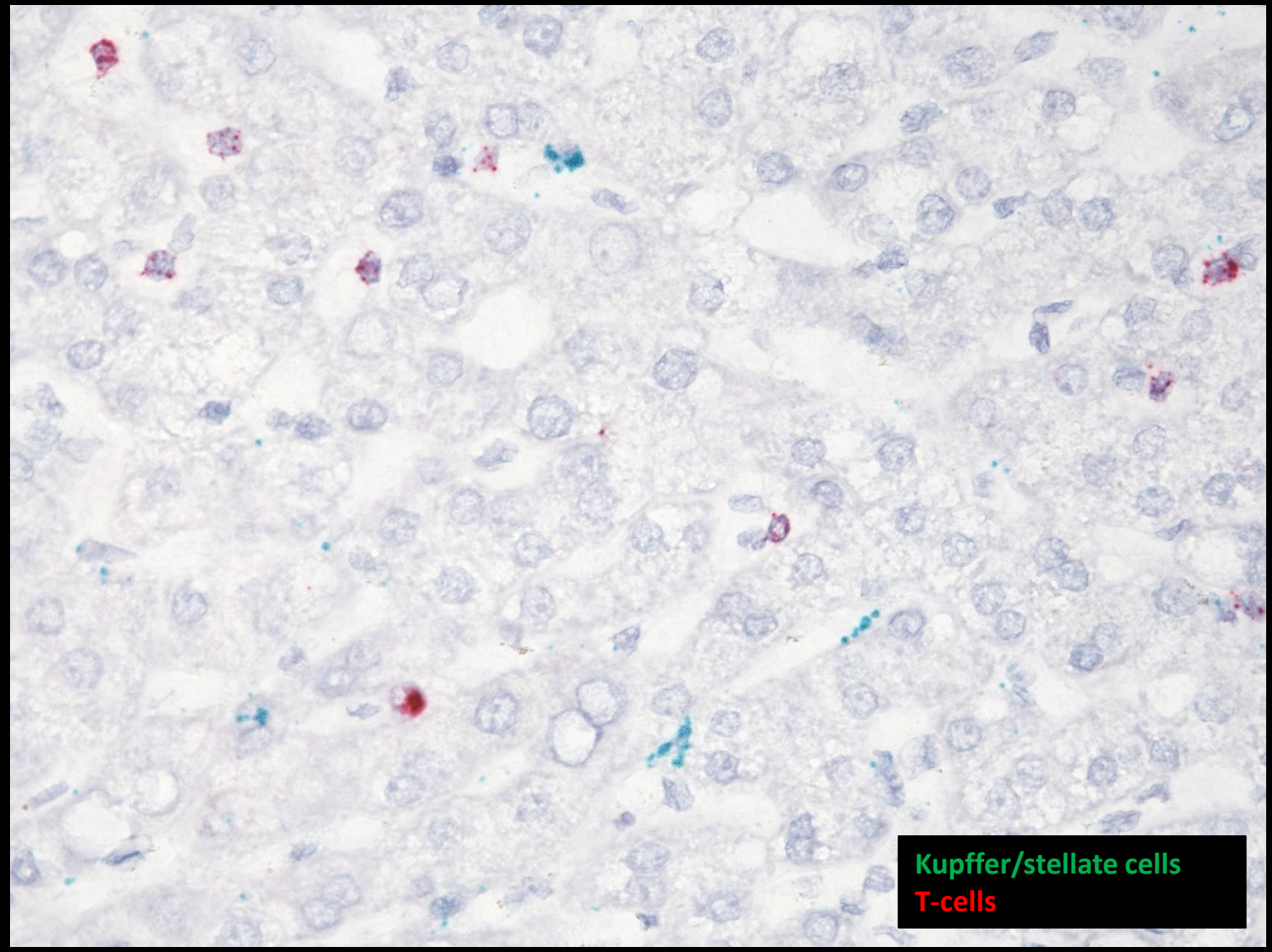
RNAscope: PPIB + H24` or H16`(CC1)/P16`(Protease) + AMP5 48` + DAB /IHC:CD3, LN10 (1:50) + Anti-Mouse/NP + Anti-NP/AP + Discovery Yellow

Manual Assay: Rnascope Duplex HGF + CD3

Manual RNAscope Duplex:

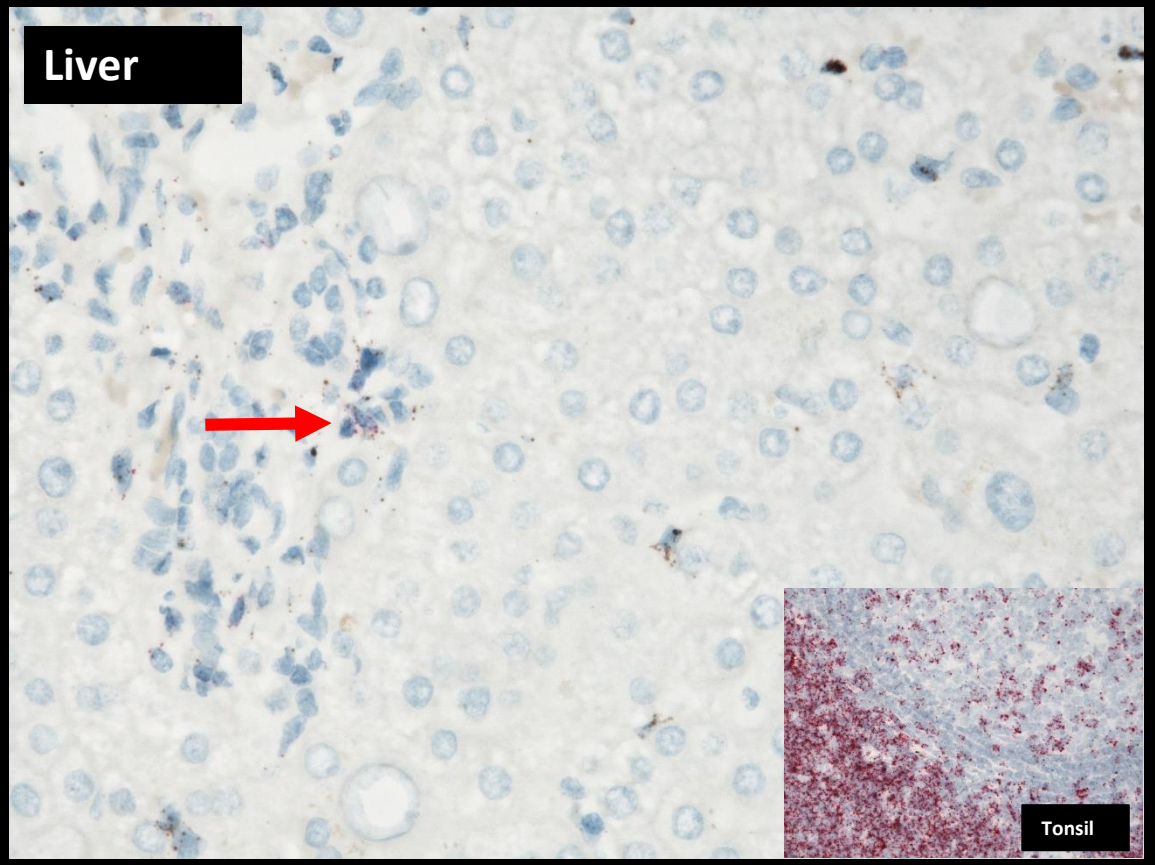
TR15/P15`(Protease)

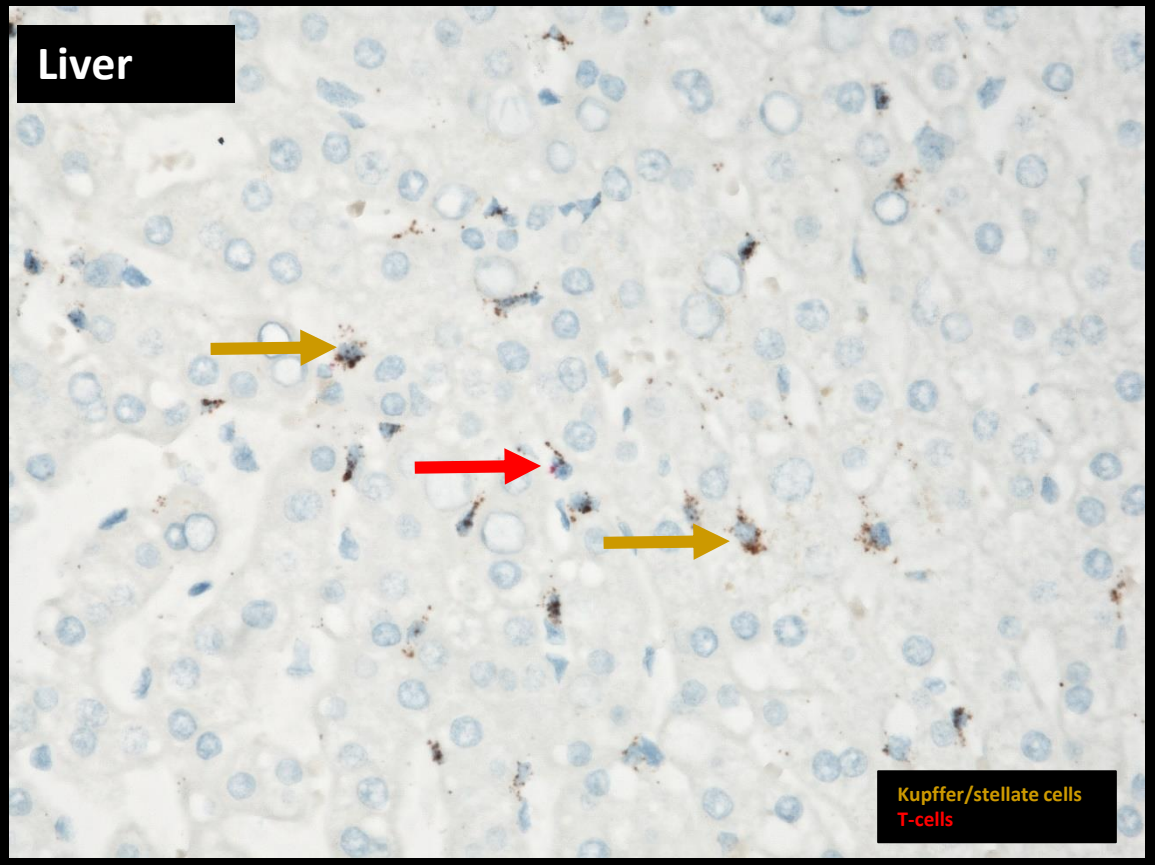
Standard detection - recommended procedure from the vendor



Ventana Discovery: Rnascope Duplex HGF + CD3

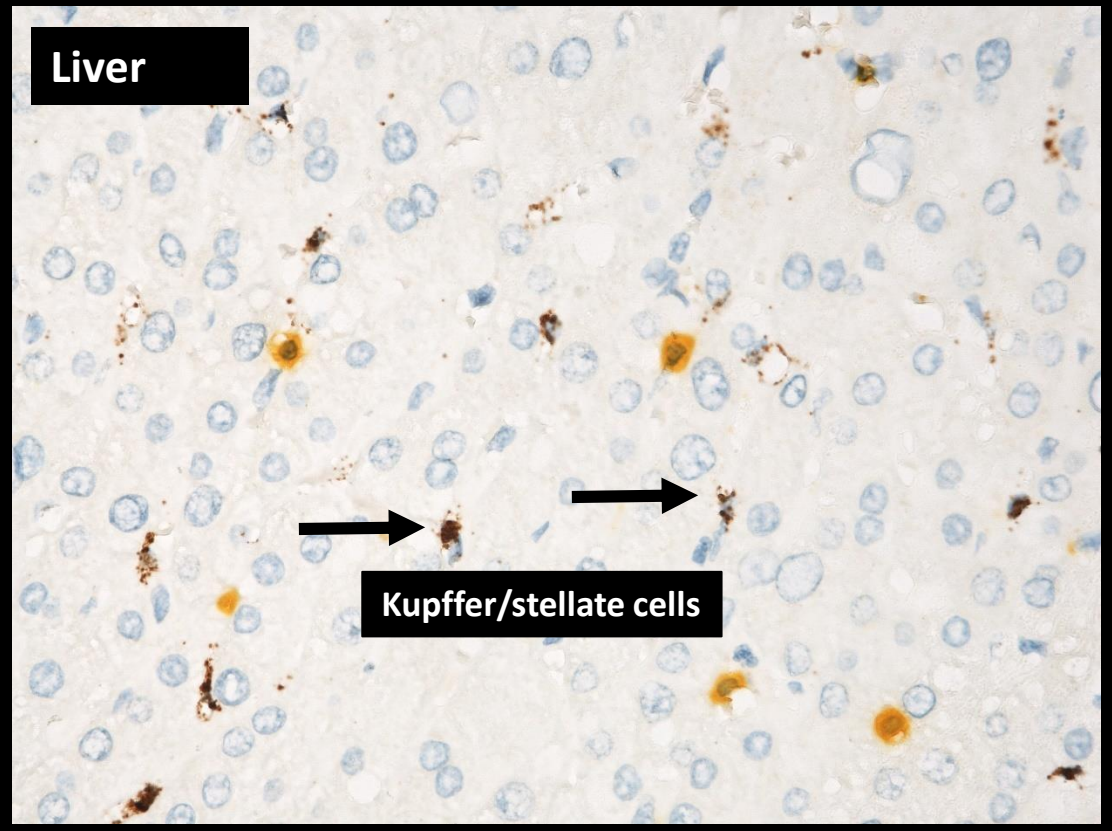
Problem: Weak red signals (T-cells) ?

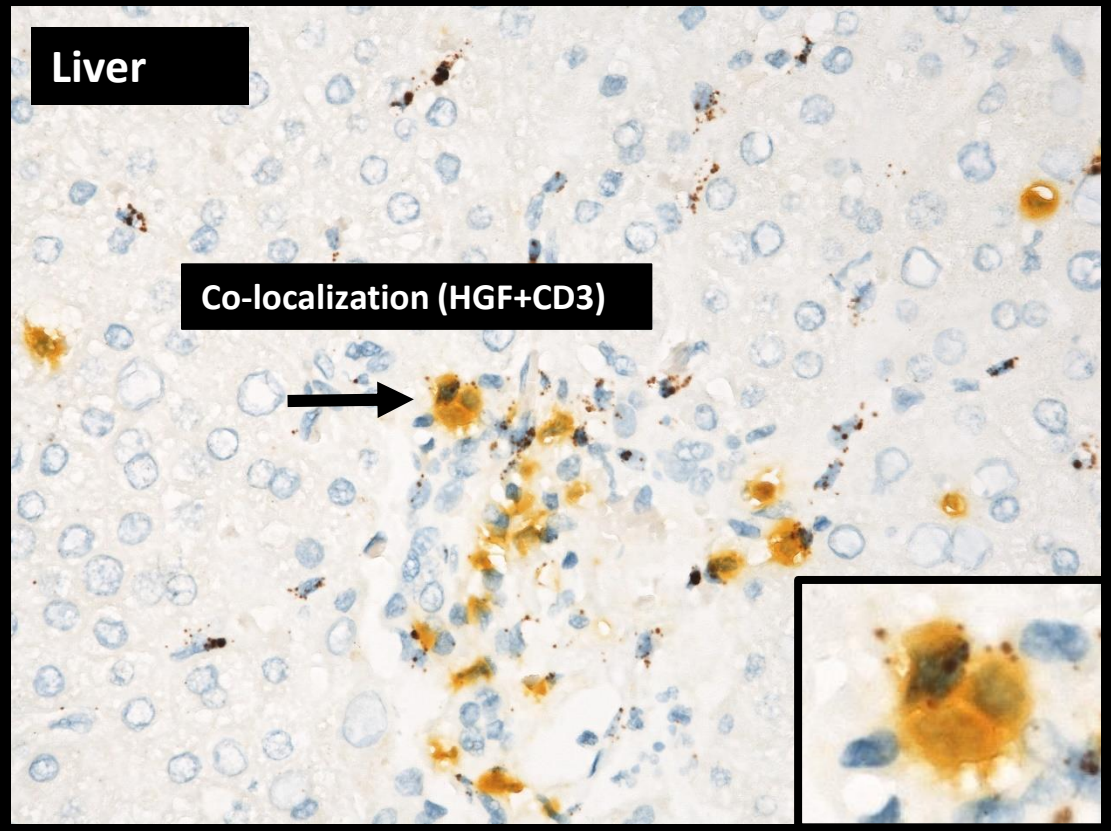




RNAscope Duplex: H16/24` (CC1)/P16`(Protease) + AMP5+8 24` + DAB /FR

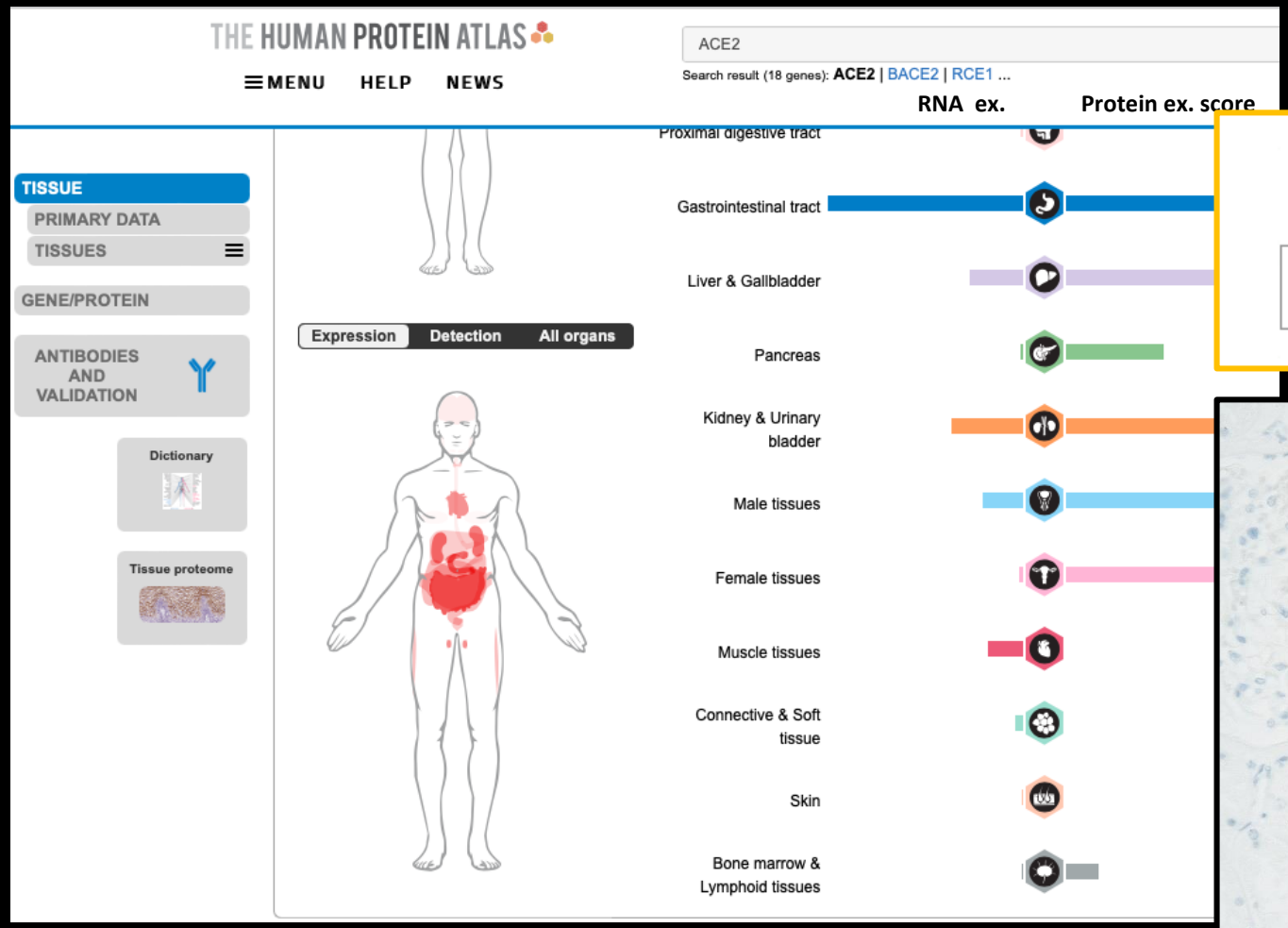
Ventana Discovery: Dual ISH (Rnascope HGF) + IHC (CD3)

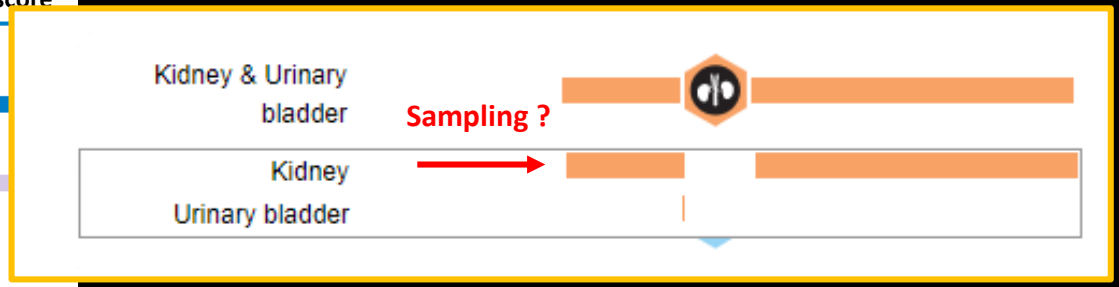


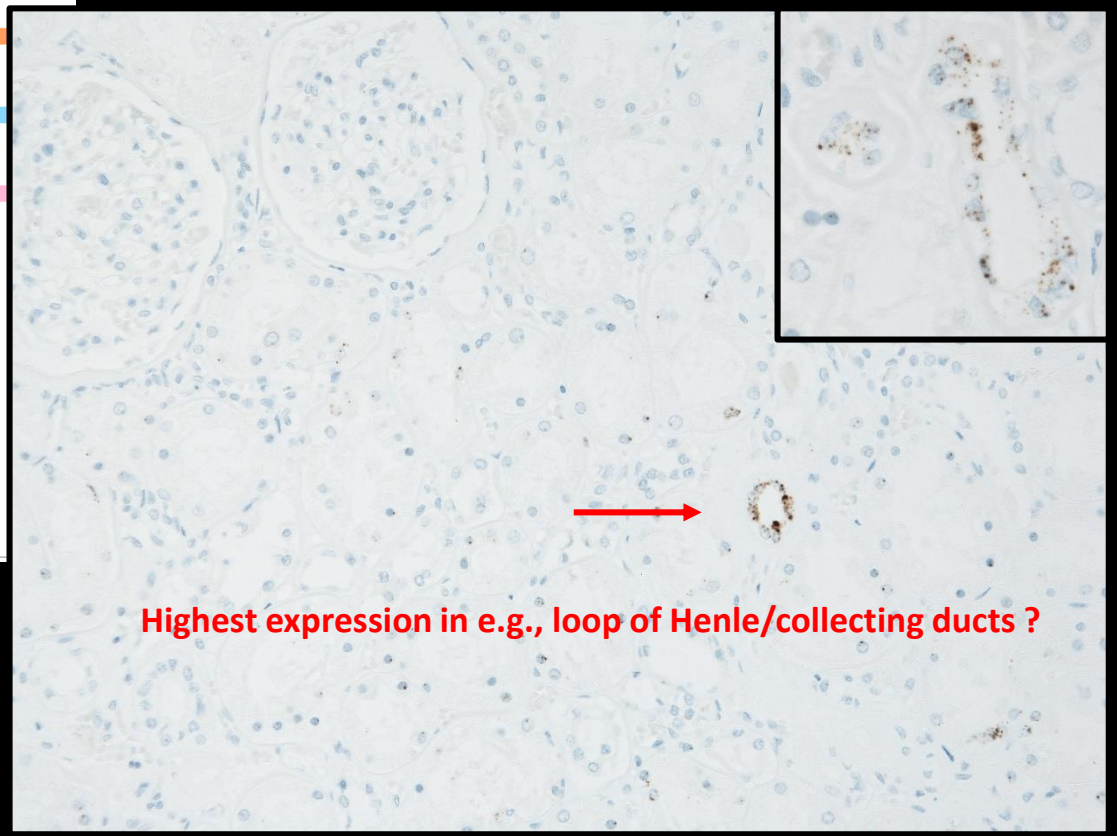


RNAscope: HGF + H24` (CC1)/P16`(Protease) + AMP5 48` + DAB /IHC:CD3, LN10 (1:50) + Anti-Mouse/NP + Anti-NP/AP + Discovery Yellow

ACE2 (RNA levels) – What to expect (normal tissue) ?







Rimsza et al. Diagnostic Pathology 2014, 9:144
http://www.diagnosticpathology.org/content/9/1/144

DIAGNOSTIC PATHOLOGY

METHODOLOGY Open Access

Kappa and lambda light chain mRNA in situ hybridization compared to flow cytometry and immunohistochemistry in B cell lymphomas

Lisa M Rimsza[1*], William A Day[2], Sarah McGinn[1], Anne Pedata[2], Yasodha Natkunam[3], Roger Warnke[3], James R Cook[4], Teresa Marafioti[5] and Thomas M Grogan[2]

mRNA Kappa or Lambda light chain

Demonstration of monoclonality in B-cell proliferations using mRNA CISH standard procedures, is most often useful in myeloma and cases with plasmacytic differentiation due to high mRNA level (Kappa or Lambda) in these disorders.

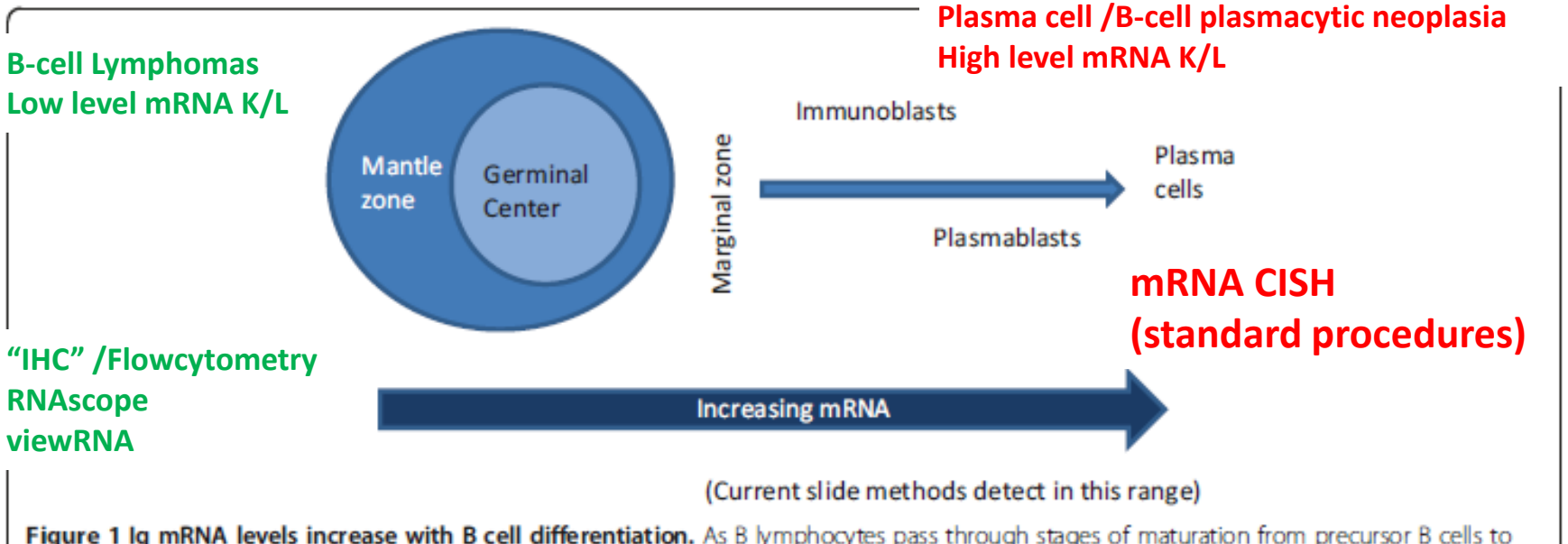


Figure 1 Ig mRNA levels increase with B cell differentiation. As B lymphocytes pass through stages of maturation from precursor B cells to naïve B cells to germinal center cells to post-germinal center cells then to plasma cells, the level of mRNA encoding immunoglobulin increases. Current slide-based methods are generally able to detect the mRNA levels found in the later stages of differentiation (generally in the post-germinal center stages).

NPY2R

Tested two NPY2R Abs on Rat tissue

Wound heling project on rat tissue

Ab should react on rat tissue

Oh Yes ☺

NPY2R Antibody (PA5-72223)

https://www.thermofisher.com/antibody/product/NPY2R-Antibody-Polyclonal/PA5-72223

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Antibody Testing Data (3)

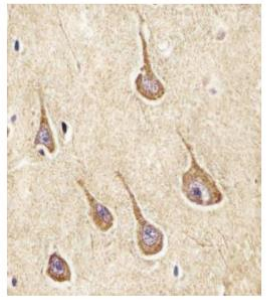


FIGURE 1/3

NPY2R Antibody (PA5-72223) in IHC

Immunohistochemical analysis of NPY2R in paraffin-embedded human brain tissue. Samples were fixed with formaldehyde and blocked with 3% BSA for 0.5 hour at room temperature. Antigen retrieval was performed by heat mediation with a citrate buffer (pH6). Samples we then incubated with NPY2R polyclonal antibody (Product # PA5-72223) using a dilution of 1:25 for 1 hours at 37°C. An undiluted biotinylated goat polyvalent antibody was used as t secondary antibody.

Give

Need help?

Let us know if we can help out.

No thanks

Start chat

Product Details

NPY2R

(Rat)

National Library of Medicine
National Center for Biotechnology Information

Gene | Gene | Advanced

Full Report | Send to:

Npy2r neuropeptide Y receptor Y2 [*Rattus norvegicus* (Norway rat)]

Download Datasets

Gene ID: 66024, updated on 22-May-2022

Summary

Symbol	Npy2r provided by RGD
Full Name	neuropeptide Y receptor Y2 provided by RGD
Primary source	RGD:620475
See related	AllianceGenome:RGD:620475
Gene type	protein coding
RefSeq status	PROVISIONAL
Organism	Rattus norvegicus
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Myomorpha; Muroidea; Muridae; Murinae; Rattus
Summary	Enables peptide YY receptor activity. Involved in several processes, including negative regulation of cell communication; regulation of nervous system process; and regulation of secretion. Located in plasma membrane. Is integral component of plasma membrane. Used to study childhood absence epilepsy and mental depression. Biomarker of obesity. Human ortholog(s) of this gene implicated in Huntington's disease; morbid obesity; and obesity. Orthologous to human NPY2R (neuropeptide Y receptor Y2). [provided by Alliance of Genome Resources, Apr 2022]
Expression	Biased expression in Brain (RPKM 13.6), Uterus (RPKM 2.4) and 1 other tissue See more
Orthologs	human mouse all

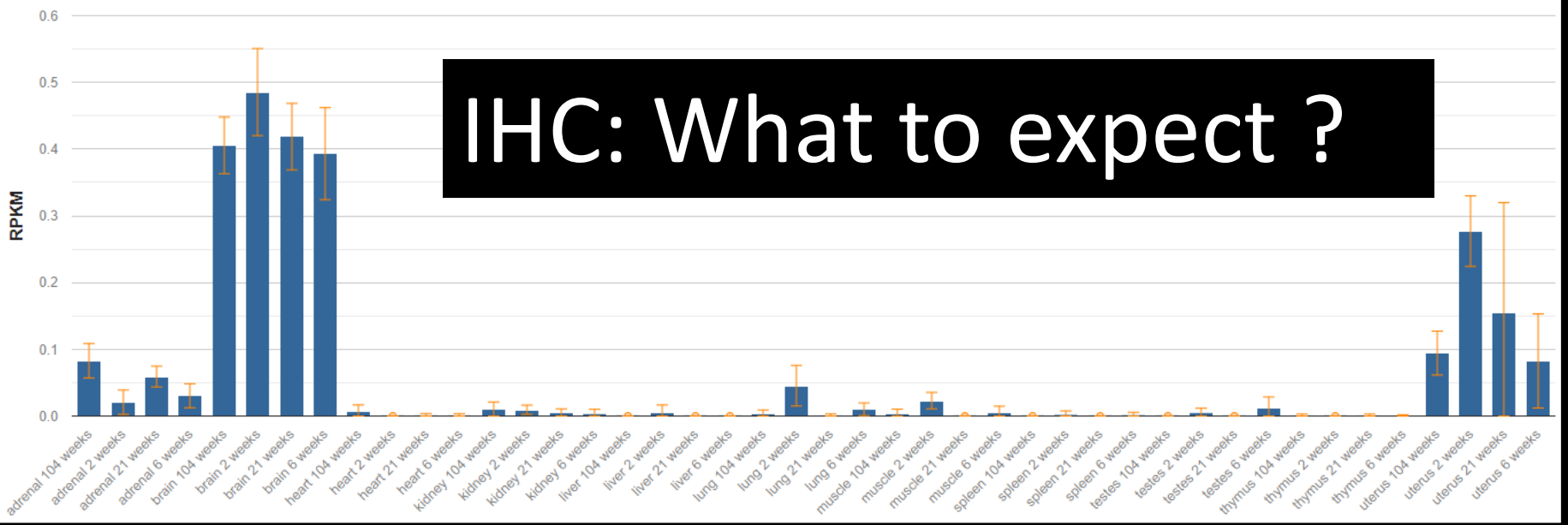
NEW Try the new Gene table
Try the new Transcript table

Expression

See details

A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages

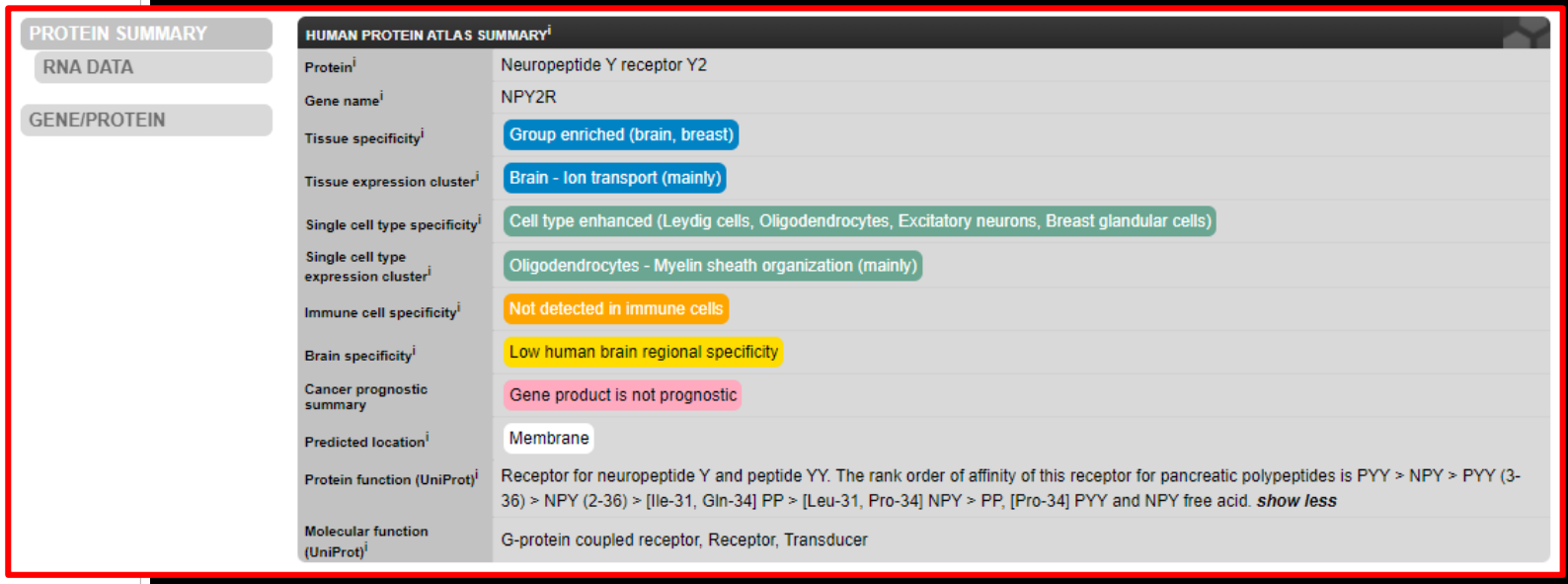
- Project title: A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages
- Description: 320 RNA samples isolated from 11 organs (adrenal gland, brain, heart, kidney, liver, lung, muscle, spleen, thymus, and testes or uterus) from both sexes of Fischer 344 rats across four developmental stages (2-, 6-, 21-, and 104-weeks-old)
- BioProject: PRJNA238328
- Publication: PMID 24510058
- Analysis date: Mon Jun 6 17:44:12 2016



IHC: What to expect ?

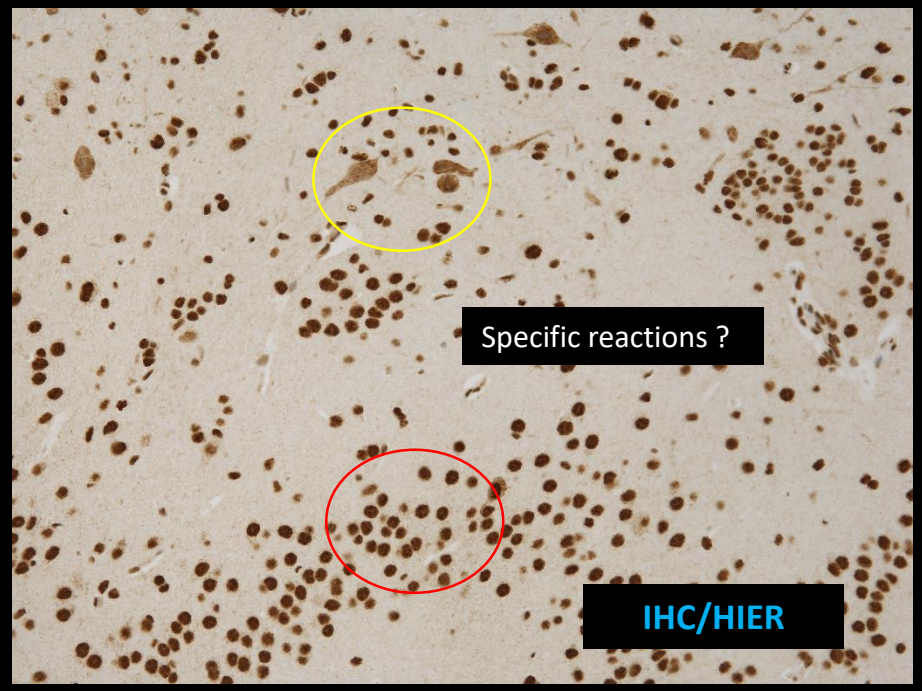
Human Protein Atlas

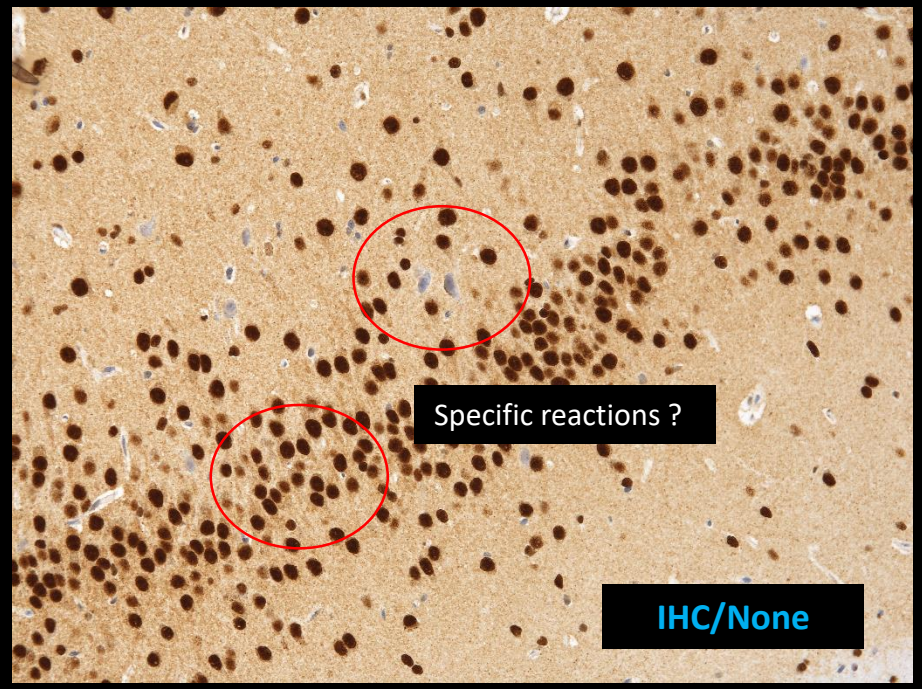
NPY2R



NPY2R, poly

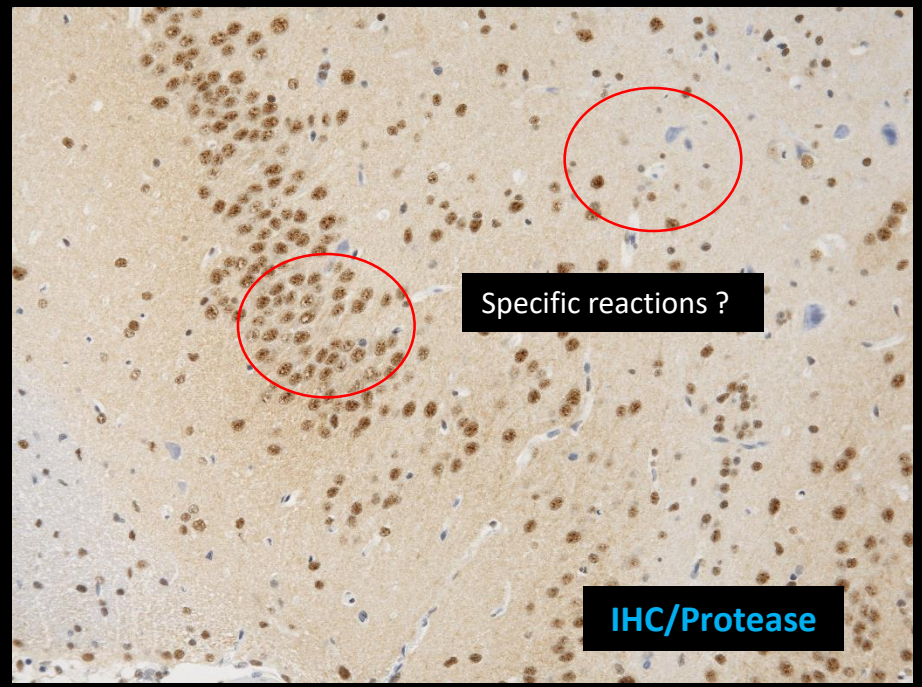
Thermo S. PA5-72223

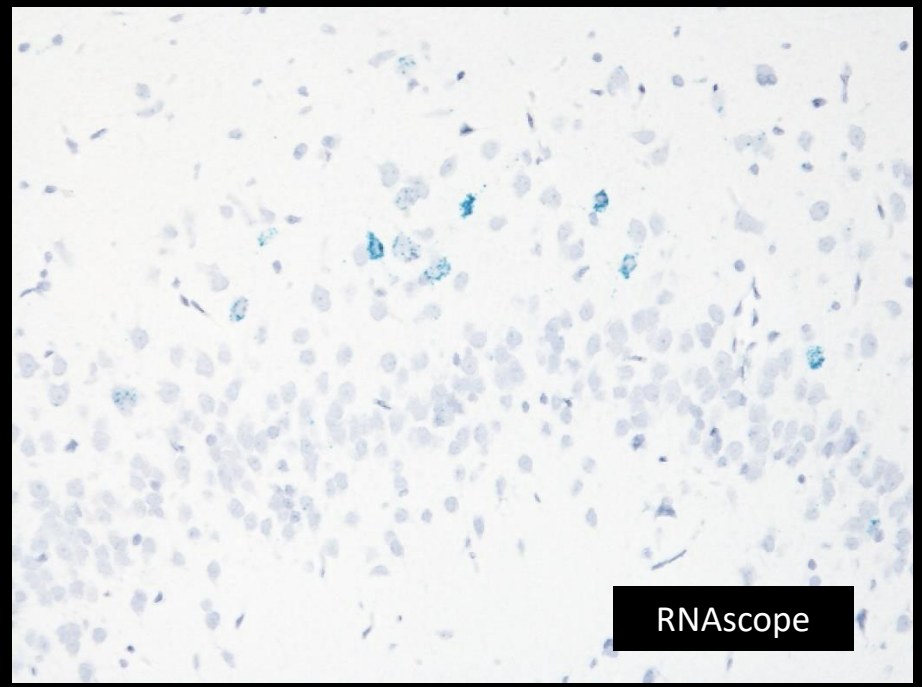




Wound-heling project on rats

Rat Brain



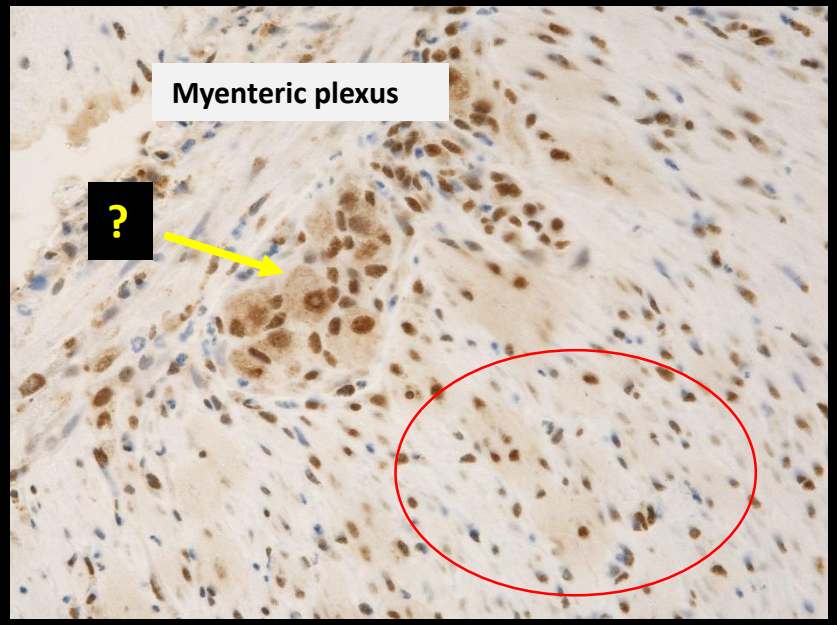


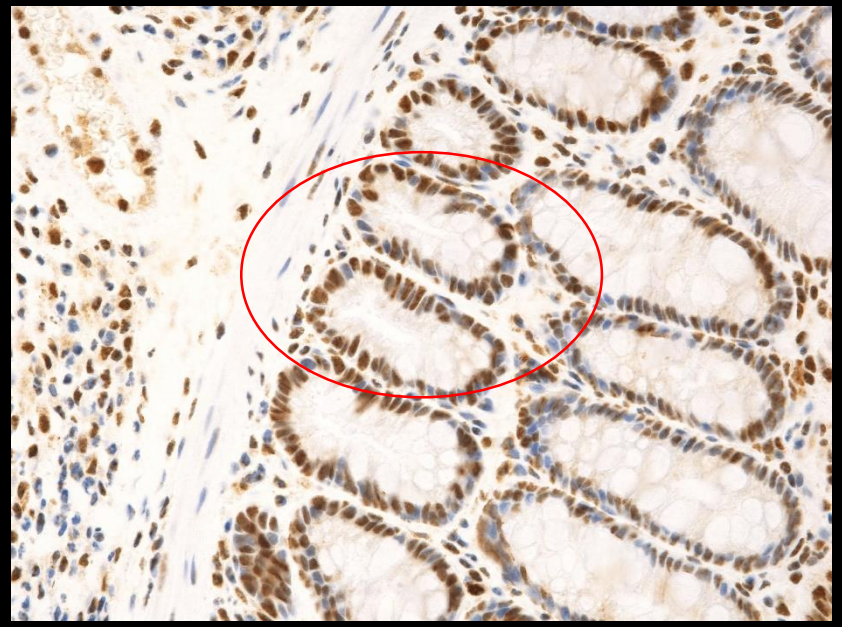
NPY2R, poly

Thermo S. PA5-72223

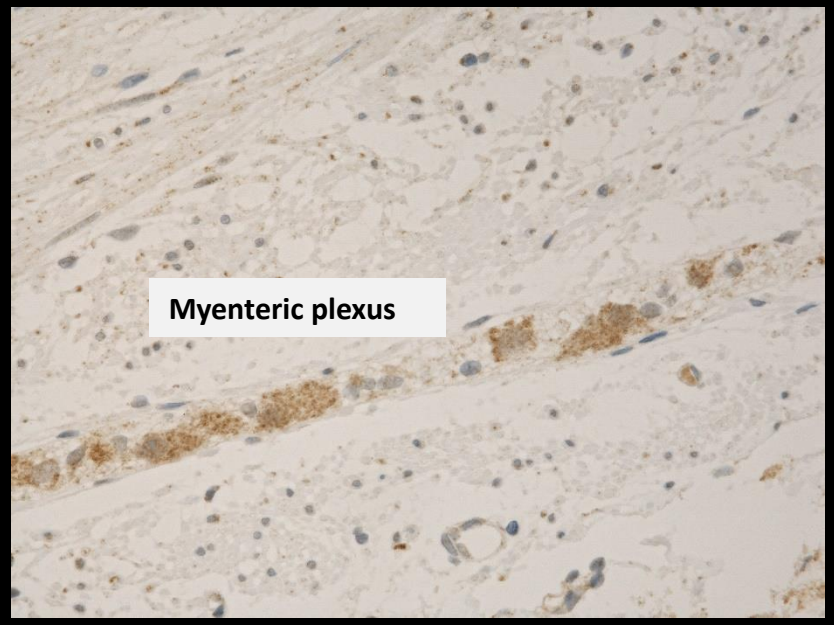
Rat intestine

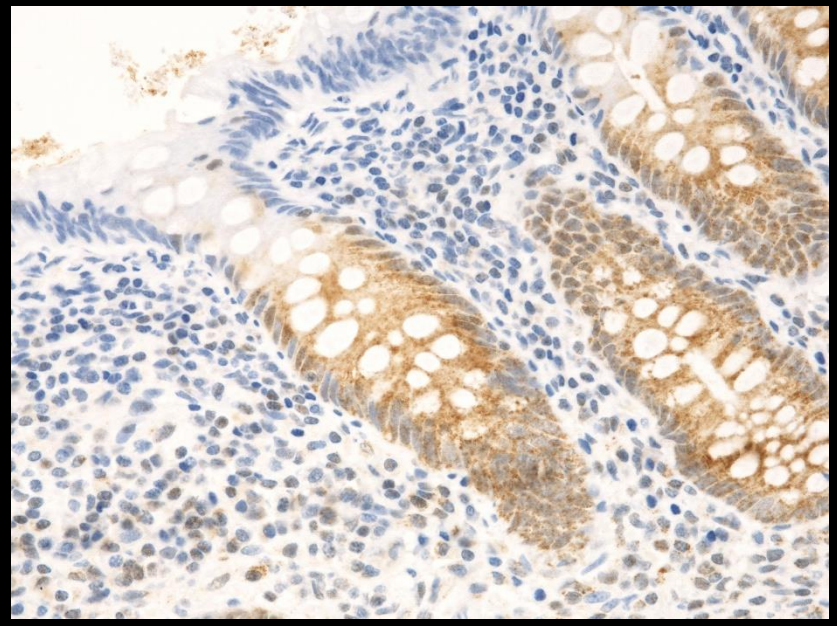
Nuclear reactions ?





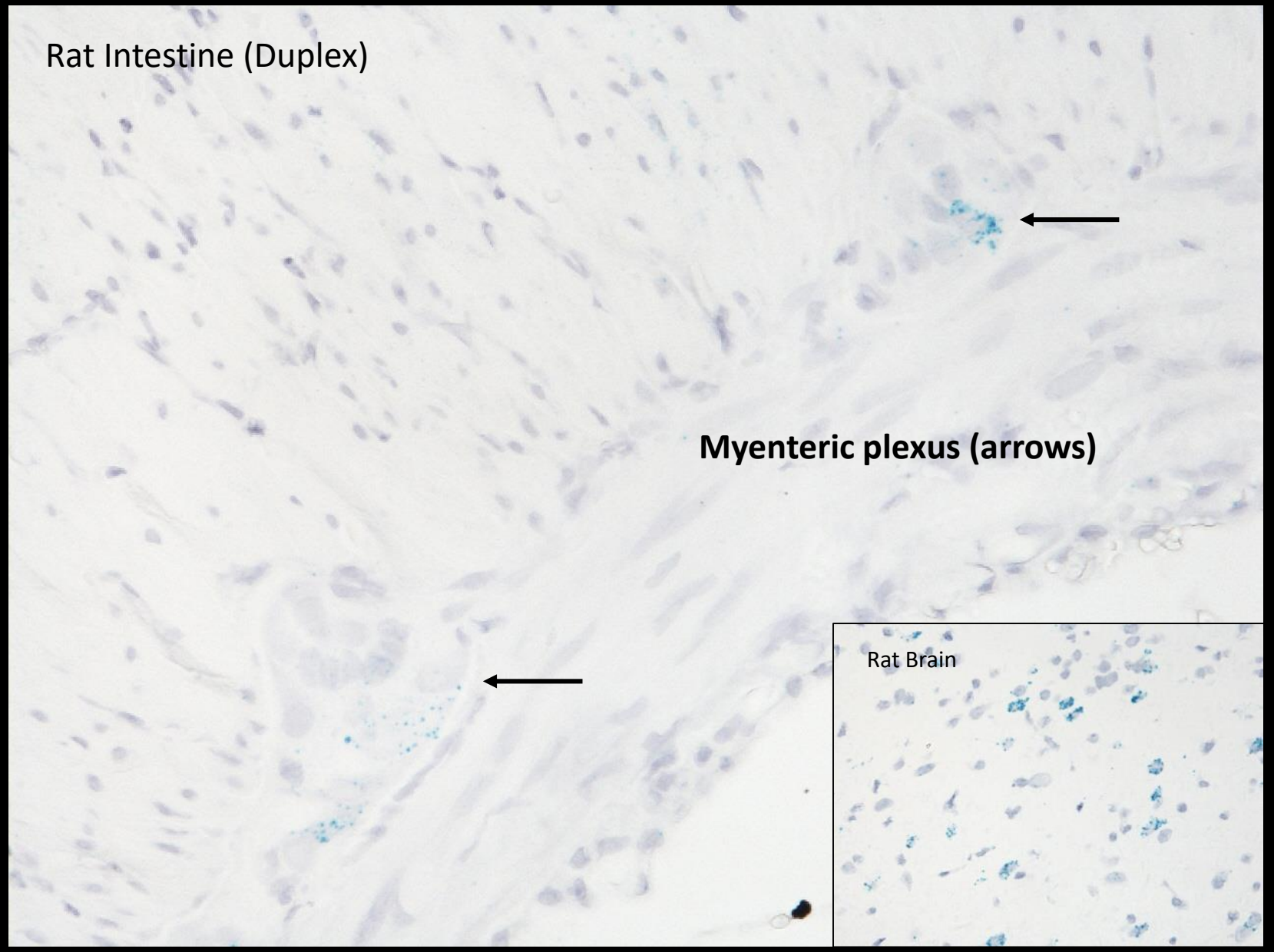
Human appendix





Compared to human tissue, rat tissue displayed a completely different expression pattern

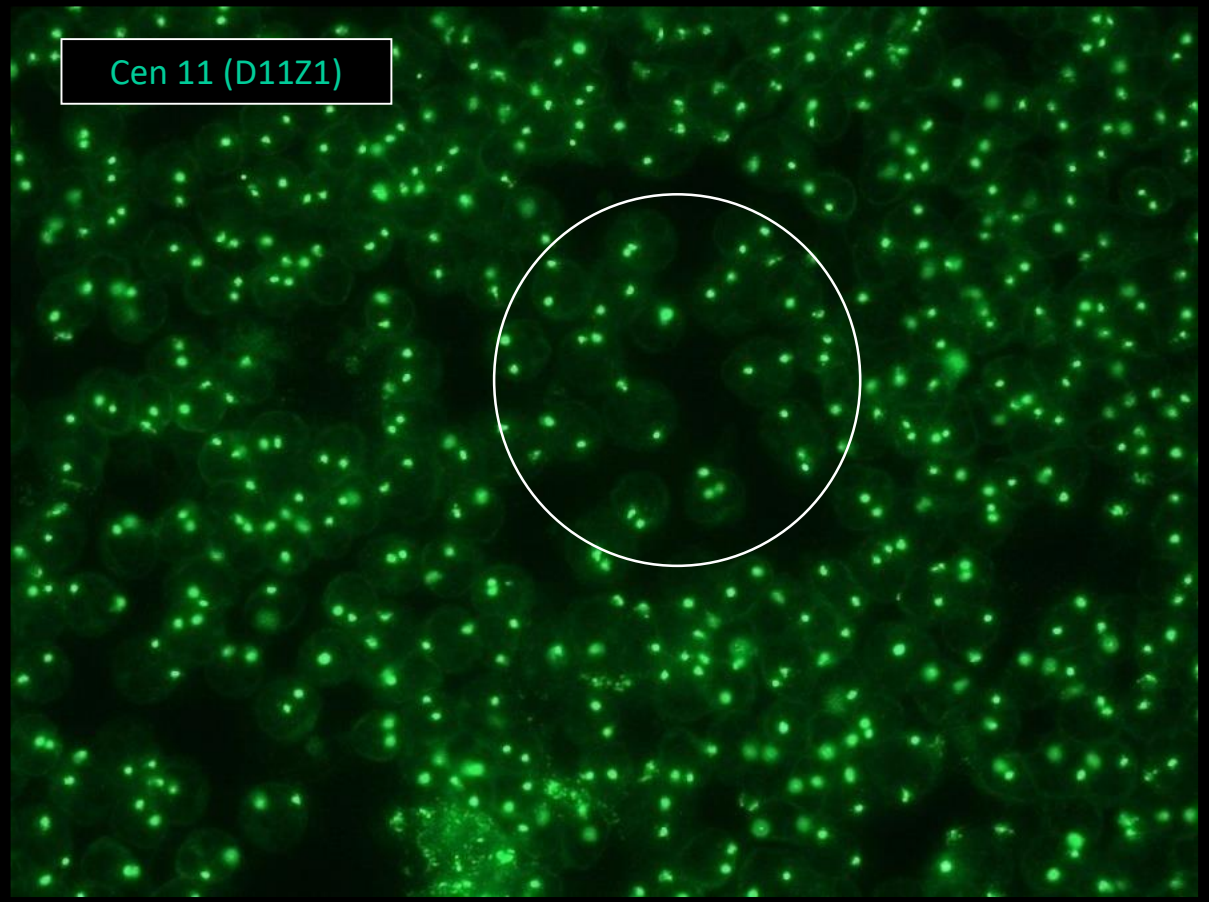
RNAscope (NPY2R)

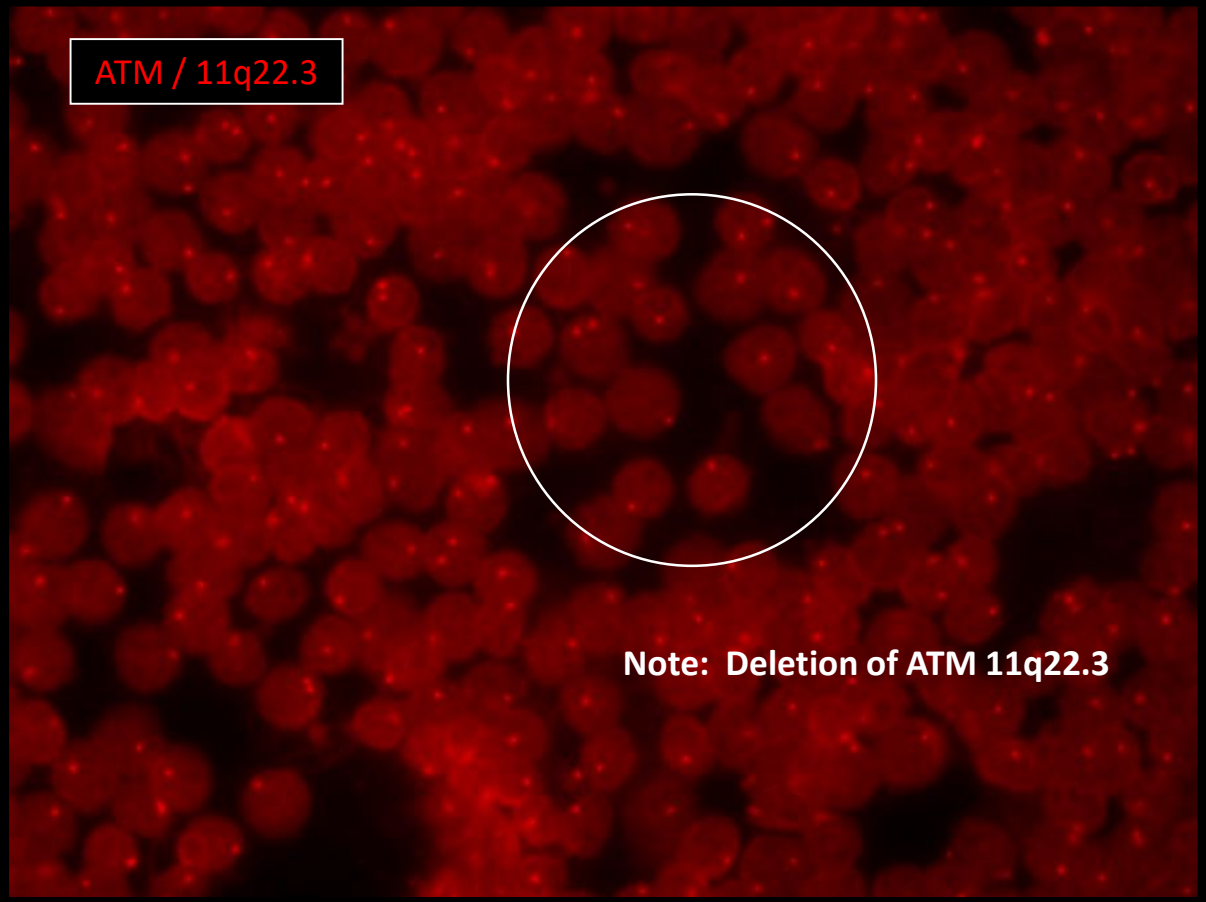


Rat intestinal epithelium was completely negative and only ganglion cells of the myenteric plexus were positive.

Patient case: CLL

B-cell enrichment using Rosette Sep





Standard FISH technology

In addition: Deletion of 13q14.3