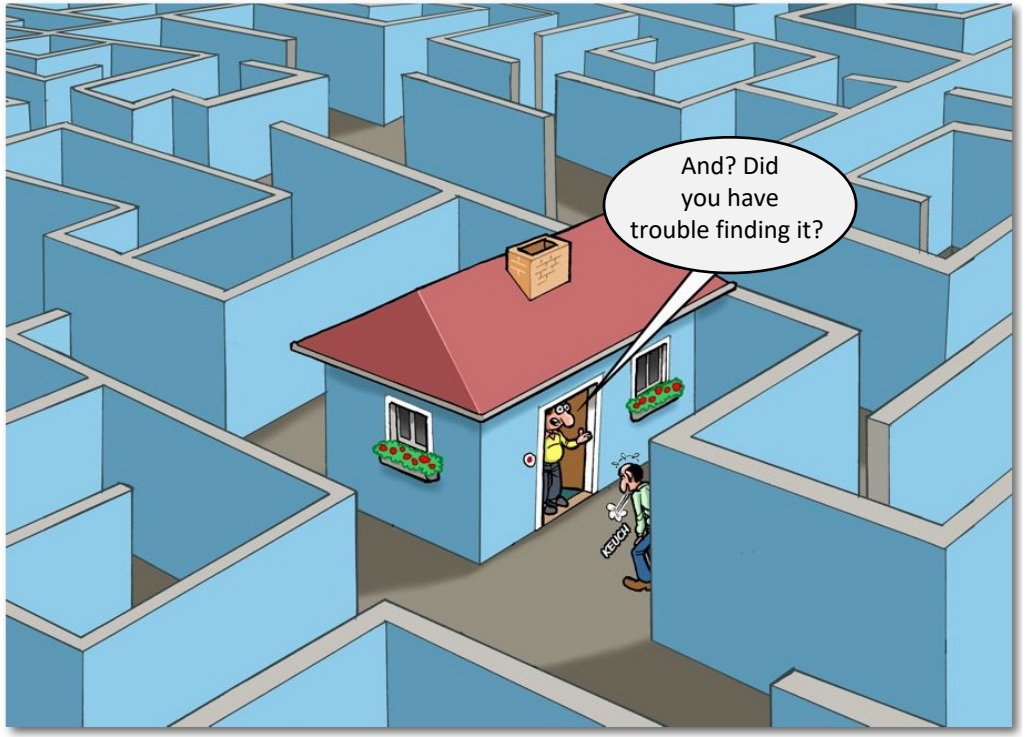
MS

Validated Antibodies

Large-scale validated antibodies for Immunohistochemistry.

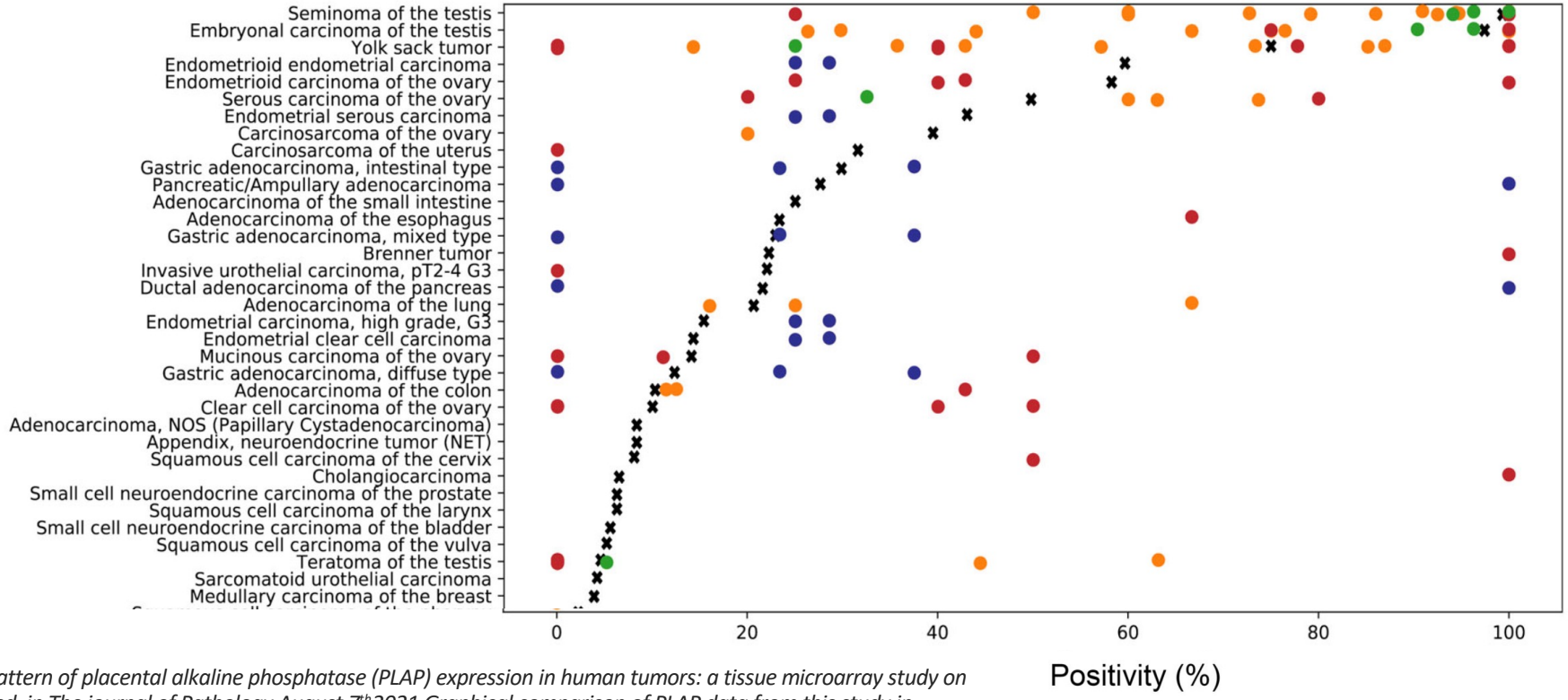
Issues in Immunohistochemistry



- Too many ”undocumented” antibodies being sold without any information on staining properties
- Neither customers nor vendors know what are the “good antibodies”
- Even in case of perfectly specific antibodies, pathologists do not know (and can not know!) in what normal tissues and which tumors all their many diagnostic antibodies should stain

More than 40% of IHC publications are completely useless because of poor antibodies

This table shows a literature summary of different research papers on PLAP staining in tumors. It demonstrates the problem pathologists and researchers are facing: depending on which paper you read, you will get completely different information about the staining intensity of an antibody – because of non-specific antibodies.

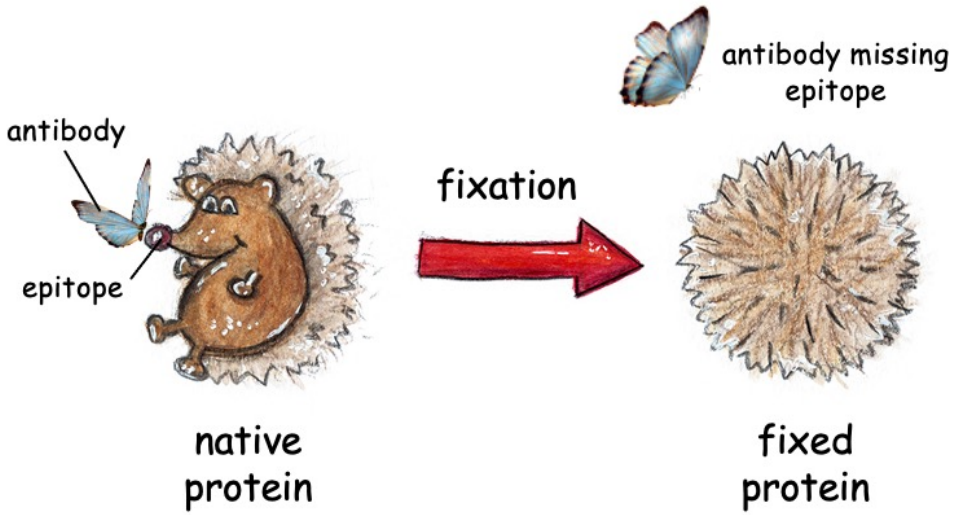


Reiswich et al. (2021): "Pattern of placental alkaline phosphatase (PLAP) expression in human tumors: a tissue microarray study on 12,381 tumors." published in The journal of Pathology August 7th 2021 Graphical comparison of PLAP data from this study in comparison with the previous literature (dots).

Why is antibody validation different for antibodies in formalin fixed immunohistochemistry?

Formalin fixation exerts a profound impact on the three-dimensional structure of proteins and the accessibility of epitopes.

Because formalin changes the proteins, traditional methods for evaluating antibodies on unfixed “perfect” proteins (Western blot, ELISA, protein microarrays) will **not** distinguish good and bad antibodies for IHC.



Epitopes that are targeted by antibodies may be masked and new antibody binding sites can evolve.

According to the *International Working Group for Antibody Validation (IWGAV)* there are only two approaches to validate antibodies for Immunohistochemistry:

- Comparison of IHC data with results from second independent method for expression measurement (i.e. RNA). **„Orthogonal strategy“**

- Comparison of IHC data with results obtained by using a second independent antibody for expression measurement. **„Independent antibody strategy“**

A proposal for validation of antibodies

Mathias Uhlen[1], Anita Bandrowski[2], Steven Carr[3], Aled Edwards[4], Jan Ellenberg[5], Emma Lundberg[1], David L Rimm[6], Henry Rodriguez[7], Tara Hiltke[7], Michael Snyder[8] & Tadashi Yamamoto[9]

We convened an *ad hoc* International Working Group for Antibody Validation in order to formulate the best approaches for validating antibodies used in common research applications and to provide guidelines that ensure antibody reproducibility. We recommend five conceptual 'pillars' for antibody validation to be used in an application-specific manner.

Antibodies are among the most frequently used tools in basic science research and in clinical assays. Despite their widespread use, as well as extensive and valuable discourse in the literature[1–6], a comprehensive scientific framework for antibody validation across research applications is lacking. As a result, the quality and consistency of data generated through the use of antibodies vary greatly. This poses an impediment to the rigor and reproducibility that are the cornerstones of the advancement of science.

The extensive discussion of antibody validation in the literature indicates a collective need for standards to validate antibody specificity and reproducibility, as well as a need for adequate reporting practices. For example, in 2010, Bourbeillon and colleagues[4] introduced the minimum information about a protein affinity reagent (MIAPAR) proposal. This proposal was meant to formalize a standard for how to report information about affinity binder reagents so that the correct reagent for a particular target could be selected for a specific application. The MIAPAR proposal is a useful guide for this purpose; however, it does not include explicit recommendations for the experimental approaches best suited to support validation of antibody specificity in particular applications.

Immunoreagents are used in a range of applications. According to the antibody reagent portal Antibodypedia (http://www.antibodypedia.com; **Supplementary Fig. 1**), their most common application is in western blot assays (immunoblotting), followed by immunohistochemistry and immunocytochemistry. In addition, the sandwich assay (e.g., ELISA), although it encompasses only a low percentage of overall antibody use, is an important application from a clinical perspective.

It is essential to note that samples are treated substantially differently in preparation for different antibody-based assays (**Supplementary Table 1**). Proteins are typically in near-native form for flow cytometry and sandwich assays, but they are wholly or partly denatured for western blot assays, immunohistochemistry, and immunocytochemistry. Because of differences in protein conformation and target accessibility, antibodies that perform well in one context may perform inadequately in others. In addition, the ratio of the target protein to other proteins in a sample may lead to significantly different levels of off-target binding. This is true even if the antibody's affinity for such proteins is much lower than its affinity for the target protein. Given this complexity it is challenging, if not impossible, to identify a simple and single benchmark for characterizing antibody performance for the full range of possible applications. Indeed, extensive characterization of antibody performance in western blotting may indicate nothing about the performance of the same antibody in an ELISA assay, where the antibody must recognize the epitope within the protein's native conformation. Likewise, an antibody may specifically recognize a cell surface protein in unfixed hematopoietic cells in flow cytometry but fail to bind the same protein in fixed liver tissue processed for immunohistochemistry. Therefore, approaches for antibody validation must be carried out in an application- and context-specific manner.

The International Working Group for Antibody Validation (IWGAV) was convened as an *ad hoc* committee of international scientists with diverse research interests but the shared goal of improving standards for antibody use and validation. Here, we propose a set of standard guidelines for validating antibodies, guidelines that may be used in an application-specific manner and that in part take advantage of technologies recently introduced by the genomics and proteomics communities. We suggest five conceptual pillars for validation of antibodies: (i) genetic strategies, (ii) orthogonal strategies, (iii) independent antibody strategies, (iv) expression of tagged proteins, and (v) immunocapture followed by mass spectrometry (MS). We suggest that at least one of these pillars should be used as a minimum criterion for claiming that a particular antibody has been adequately validated for a specific application. The use of multiple strategies

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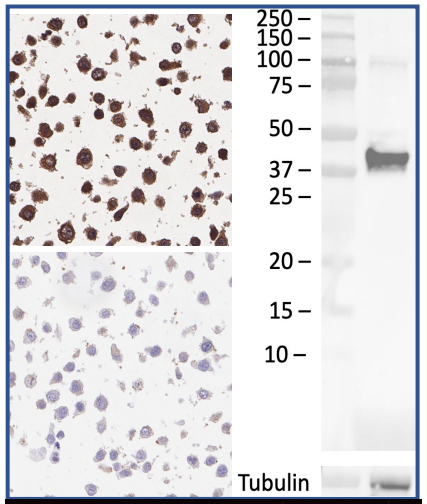
Specificity testing

Traditional approaches
not suitable for IHC



KO- Cell lines

Western Blots

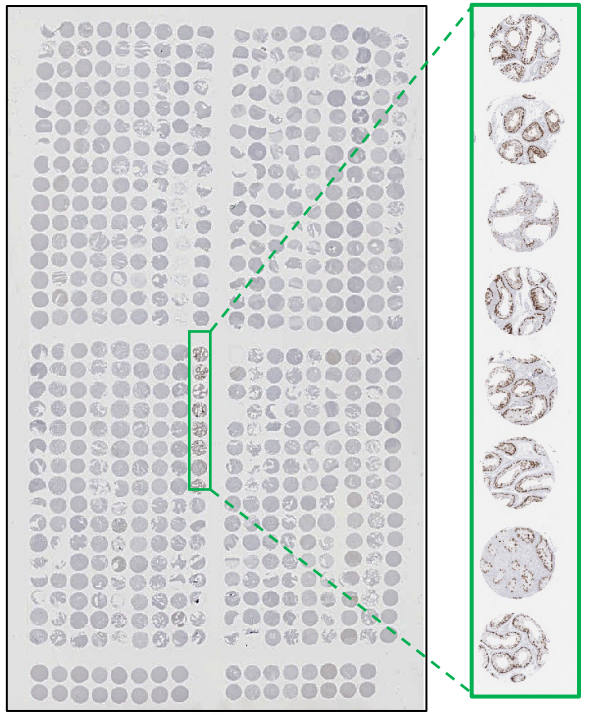
Protein Arrays

Unfixed protein

MSVA approach
recommended by IWGAV

8 tissue samples from every organ from different donors

All human proteins can be expected to be represented!



e.g. testis

Formalin fixed protein

What differentiates MSVA from the rest of the Market

MSVA is specialized in immunohistochemistry and provides a unique wealth of information for every antibody on the MSVA homepage, because

the key to successful Immunohistochemistry is:

Know your antibody!

We offer:

- Extensive validation process on tissues
- Extremely sophisticated product selection (only 130 approved products out of 4000 candidates)
- All of our antibodies work exactly as described and visualized in our normal and cancer tissue galleries on our website
- Only 1 antibody per target to avoid confusion on what clone to select
- Protocol recommendations (Manual Protocol, Dako, Leica & Ventana autostainers)
- Performance characteristics for studies with MSVA antibodies on >10,000 tumors summarized on our website under “Compatibility of Antibodies”
  (already available for: PLAP, CPA1, Mesothelin, CK19, CK6, MUC5AC, Arginase-1, DOG-1)

MS Validated Antibodies

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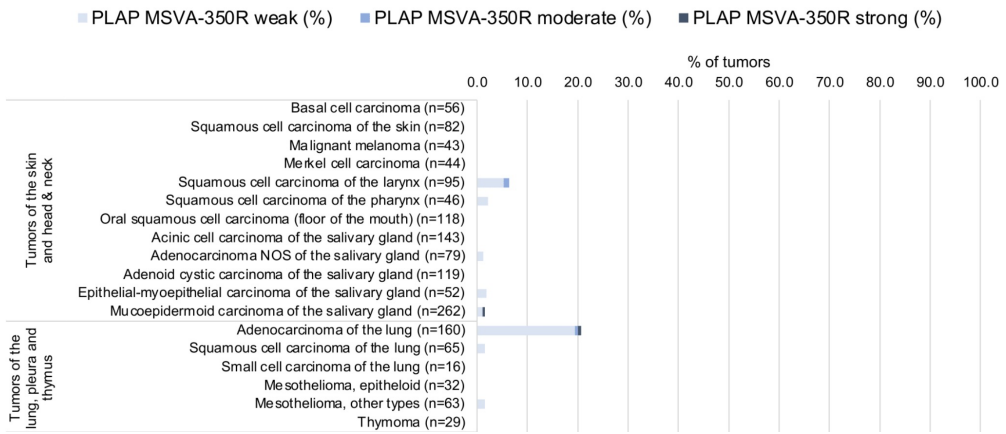
MSVA homepage – a highly useful guide for our antibody users.

Compatibility of Antibodies

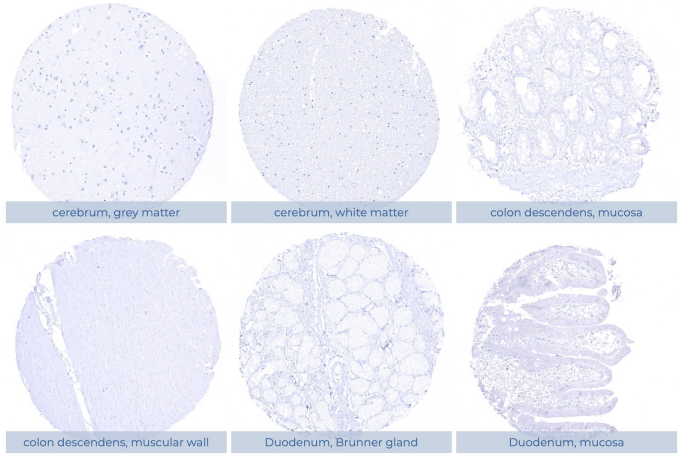
Data compilation from studies using recommended protocols

Data from the publication: "Pattern of placental alkaline phosphatase (PLAP) expression in human tumors: a tissue microarray study on 12,381 tumors". Published by Reiswich et al. in the journal of Pathology: Clinical Research, August 7th 2021.
Summarized in own graphics:

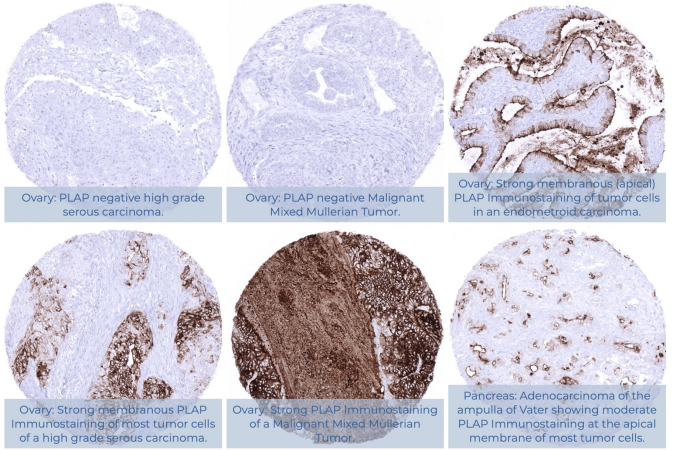
1. PLAP staining in tumors "organ-specific" with antibody MSVA-350R



Data shown on >60 different Normal Tissues



Normal tissue gallery | **Cancer tissue gallery**



Comprehensive and visualized information on staining patterns in tumors

And links to other relevant sources (HPA, TCGA Database)

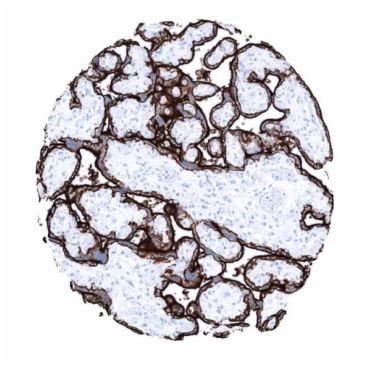
MSVA homepage – a highly useful guide for our antibody users.

Protocol Recommendations

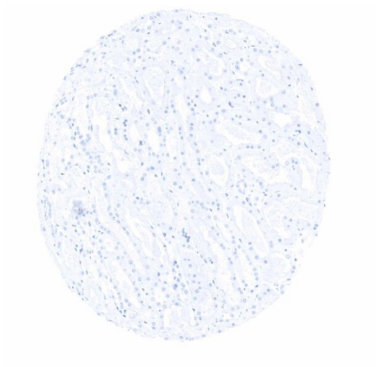
We provide protocol recommendations for Manual Protocol, Dako, Leica & Ventana autostainers to reproduce the results shown in our gallery

Agilent / Dako – Autostainer Link 48

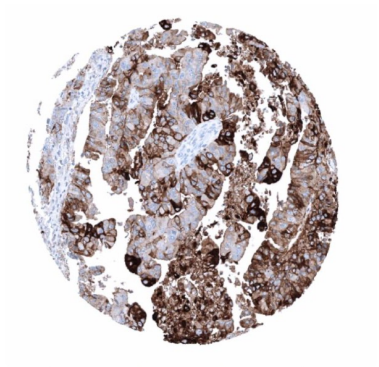
Pretreatment in PT-Link for 30 minutes at 95°C (pH high); FLEX peroxidase blocking for 5 minutes (room temperature), MSVA-350R **1:150** for 20 minutes (room temperature), FLEX+ mouse/rabbit (LINKER) for 15 minutes (room temperature), horseradish peroxidase (HRP) for 20 minutes (room temperature), FLEX DAB+Sub-Chromo for 10 minutes (room temperature), FLEX hematoxylin for 5 minutes (room temperature).



Dako – Placenta



Dako – Kidney



Dako – Esophagus Adenocarcinoma

Staining Pattern in Normal Tissues

PLAP – Staining pattern in normal tissues (MSVA-350R)

Brain	Cerebrum	Negative.
	Cerebellum	Negative.
Endocrine Tissues	Thyroid	Negative.
	Parathyroid	Negative.
	Adrenal gland	Negative.
	Pituitary gland	Negative.
Respiratory system	Respiratory epithelium	Negative.
	Lung	Negative.
Gastrointestinal Tract	Salivary glands	Negative.
	Esophagus	Negative.
	Stomach	Negative.
	Colon	Negative.
	Duodenum	Negative.
	Rectum	Negative.
	Small intestine	Negative.
	Liver	Negative.
	Gallbladder	Negative.
	Pancreas	Negative.
Genitourinary	Kidney	Negative.
	Urothelium	Negative.
Male genital	Prostate	Negative.
	Seminal vesicles	Negative.
	Testis	Negative.
	Epididymis	Negative.
Female genital	Breast	Negative.
	Uterus, ectocervix	Negative.
	Uterus endocervix	A weak PLAP staining can be seen at the surface apical membrane of epithelial cells (not in all samples).
	Uterus, endometrium	A weak PLAP staining can be seen at the surface apical membrane of epithelial cells (not in all samples).

Practical approach of MSVA

Look up 1	Look up RNA data on the Human Protein Atlas (HPA)
Stain 2	Stain the antibody on a Tissue Microarray containing 20 different Normal Tissues and 18 different tumor types. See if staining matches HPA
Compare 3	Compare with independent clones for the same target to confirm or disconfirm staining

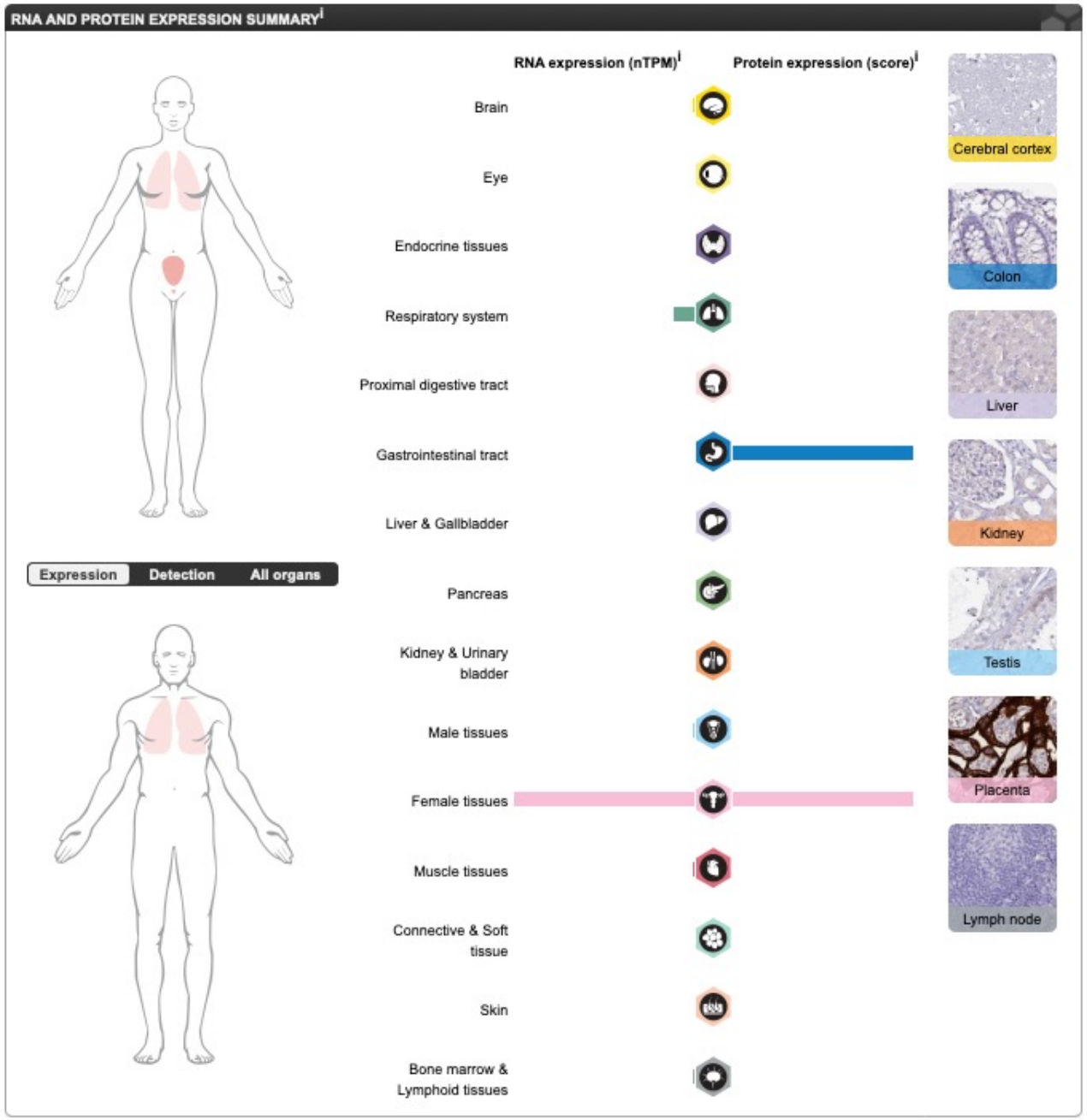
Look up

1

We look up the protein on the Human Protein Atlas to see in what tissues we expect staining.

For the example of PLAP we expect an antibody that almost exclusively stains in female tissues.

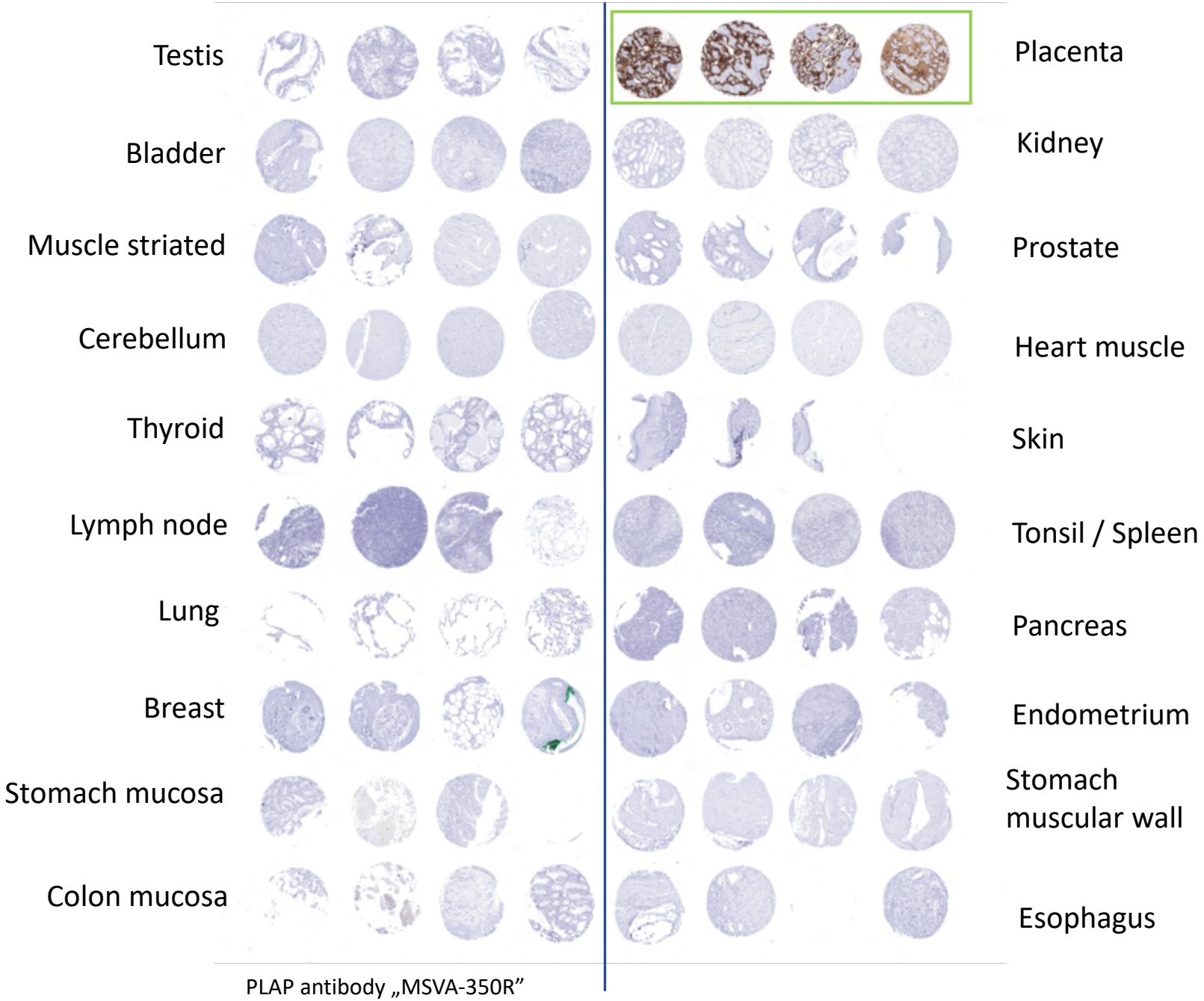
RNA expression PLAP – Source: Human Protein Atlas



Stain 2

We stain the TMA with our antibody. Here with PLAP clone MSVA-350R.

It shows staining in the placenta. The results are as expected based on the RNA data from the HPA.

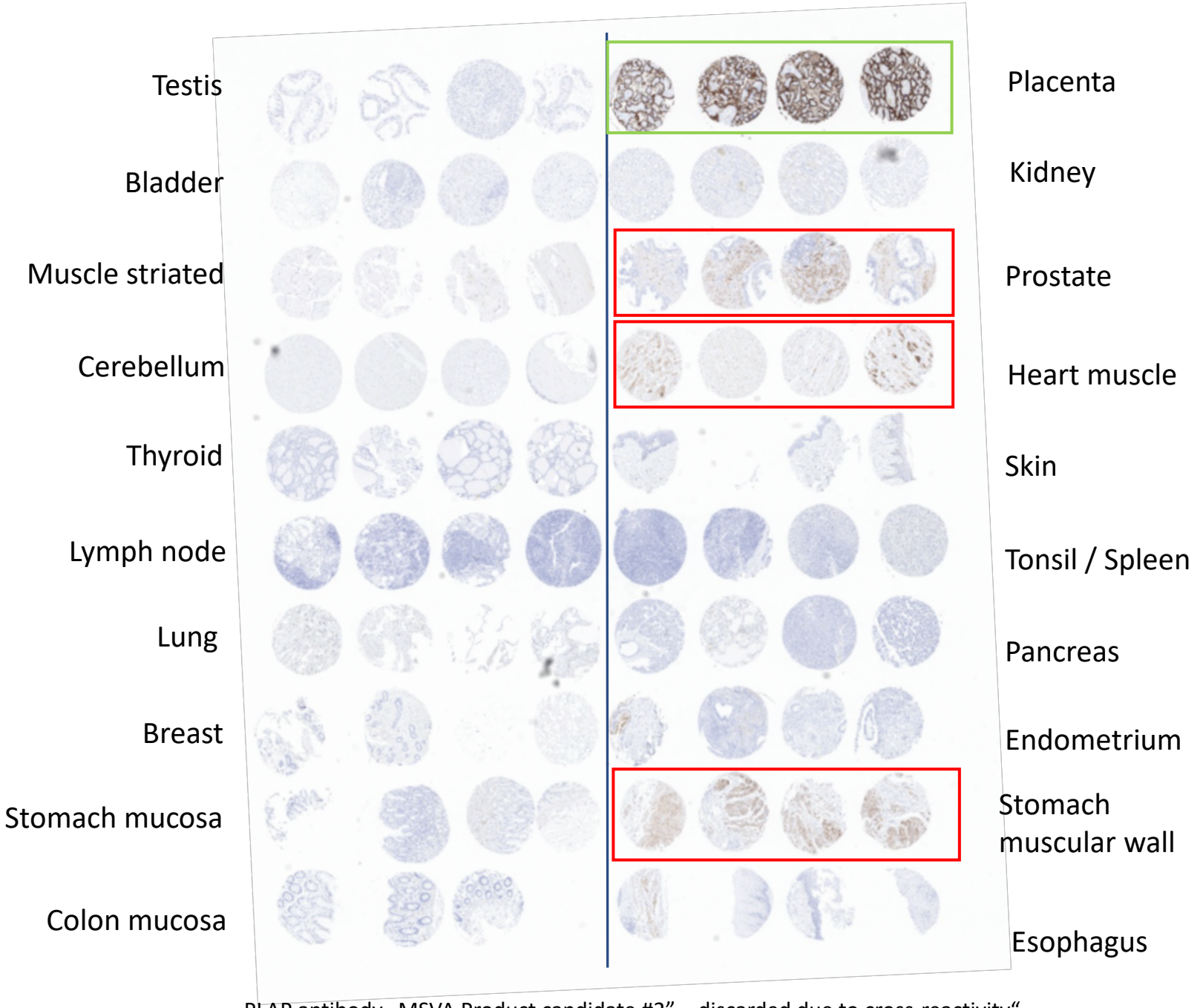


PLAP antibody „MSVA-350R"

Compare 3

We compare with independent clones to confirm the staining. Here the placenta staining is confirmed.

The other positive stainings are obviously non specific. As the other antibody does not show them and RNA expression does not suggest these stainings.

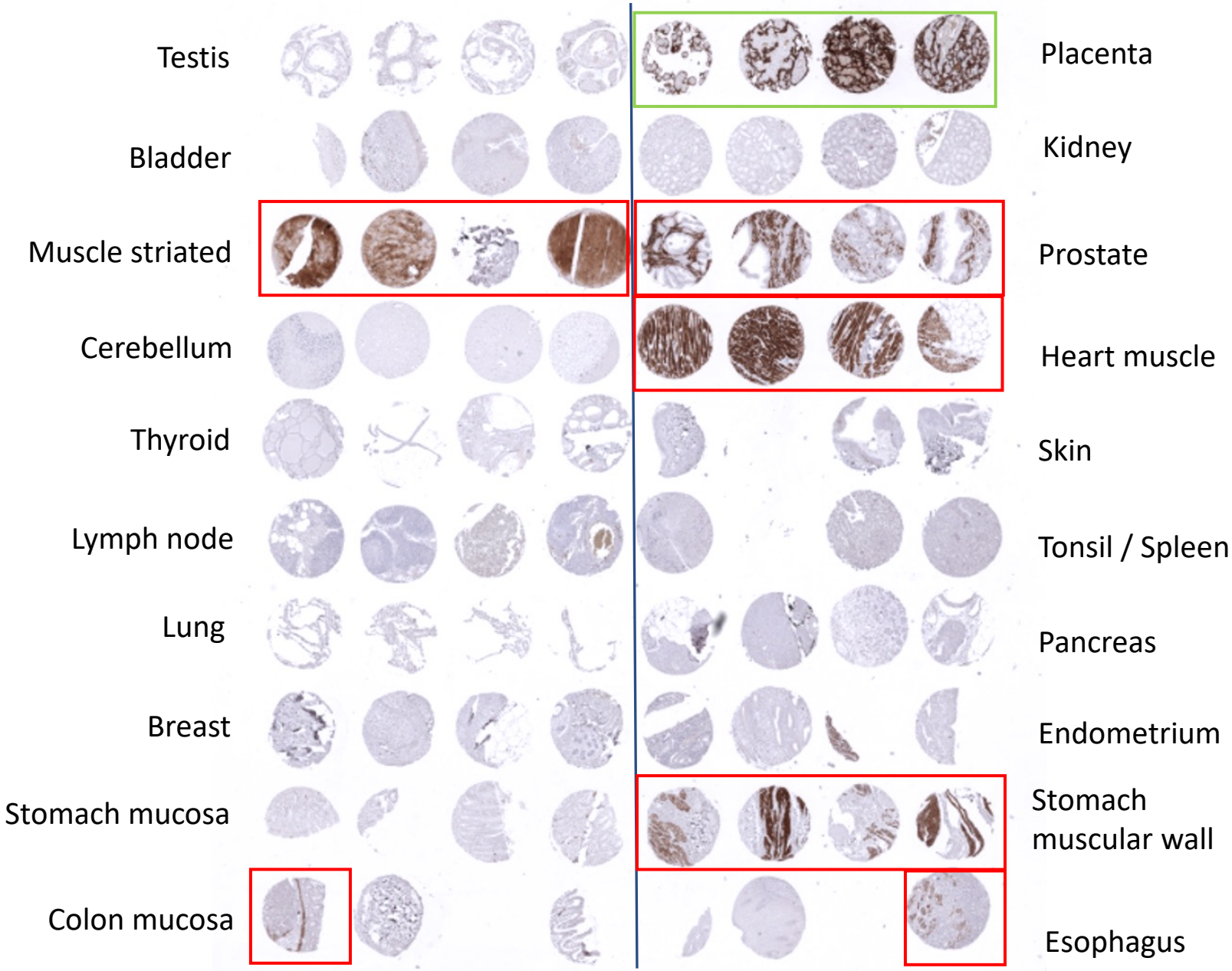


PLAP antibody „MSVA Product candidate #2" – discarded due to cross-reactivity"

Compare 3

We compare with an established clone (8A9) to confirm the results. Again: Placenta staining is confirmed.

And even more non-specificity for this PLAP antibody!

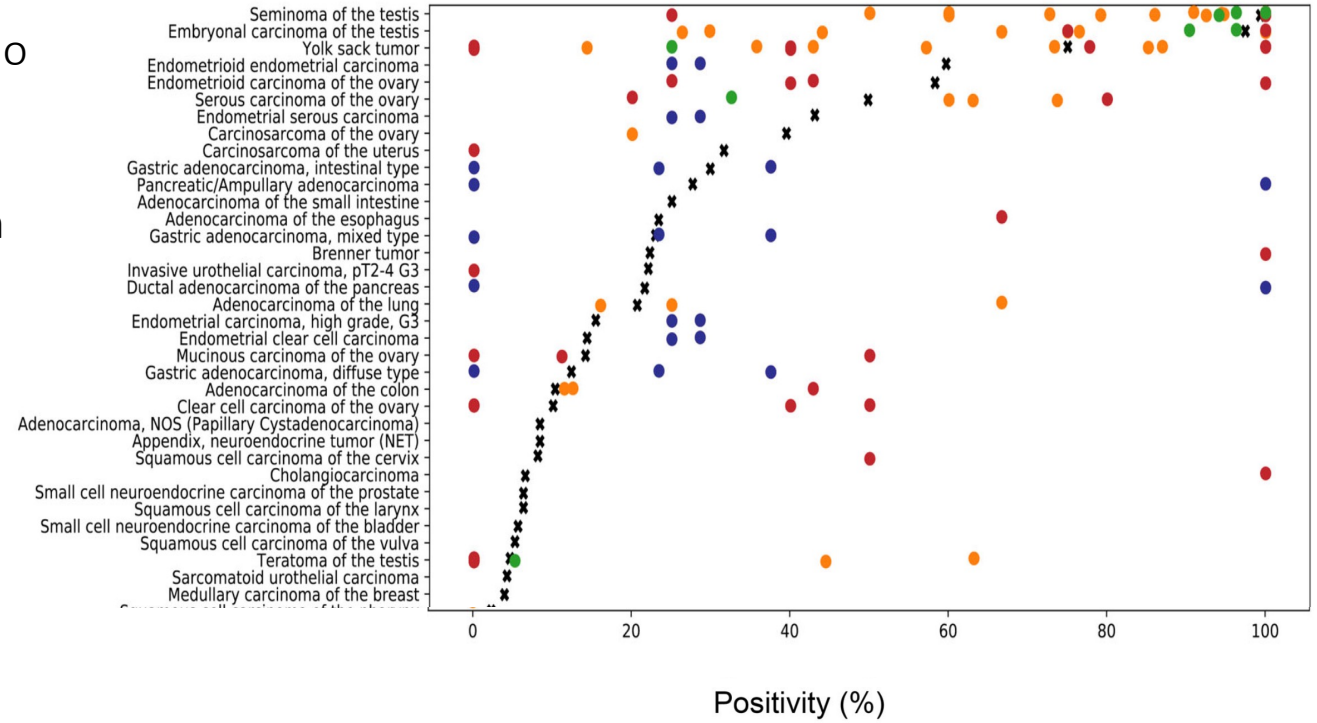


PLAP antibody clone 8A9

Practical problems with unspecific antibodies

1) Inconvenience for the pathologist but not necessarily dangerous for patients.

2) **Disaster for researchers!!** Most of the time they do not have the capacities to fully validate an antibody. They trust the vendor and just use some control tissues to validate the antibody –> Disaster gets even bigger when multiplex IHC is used



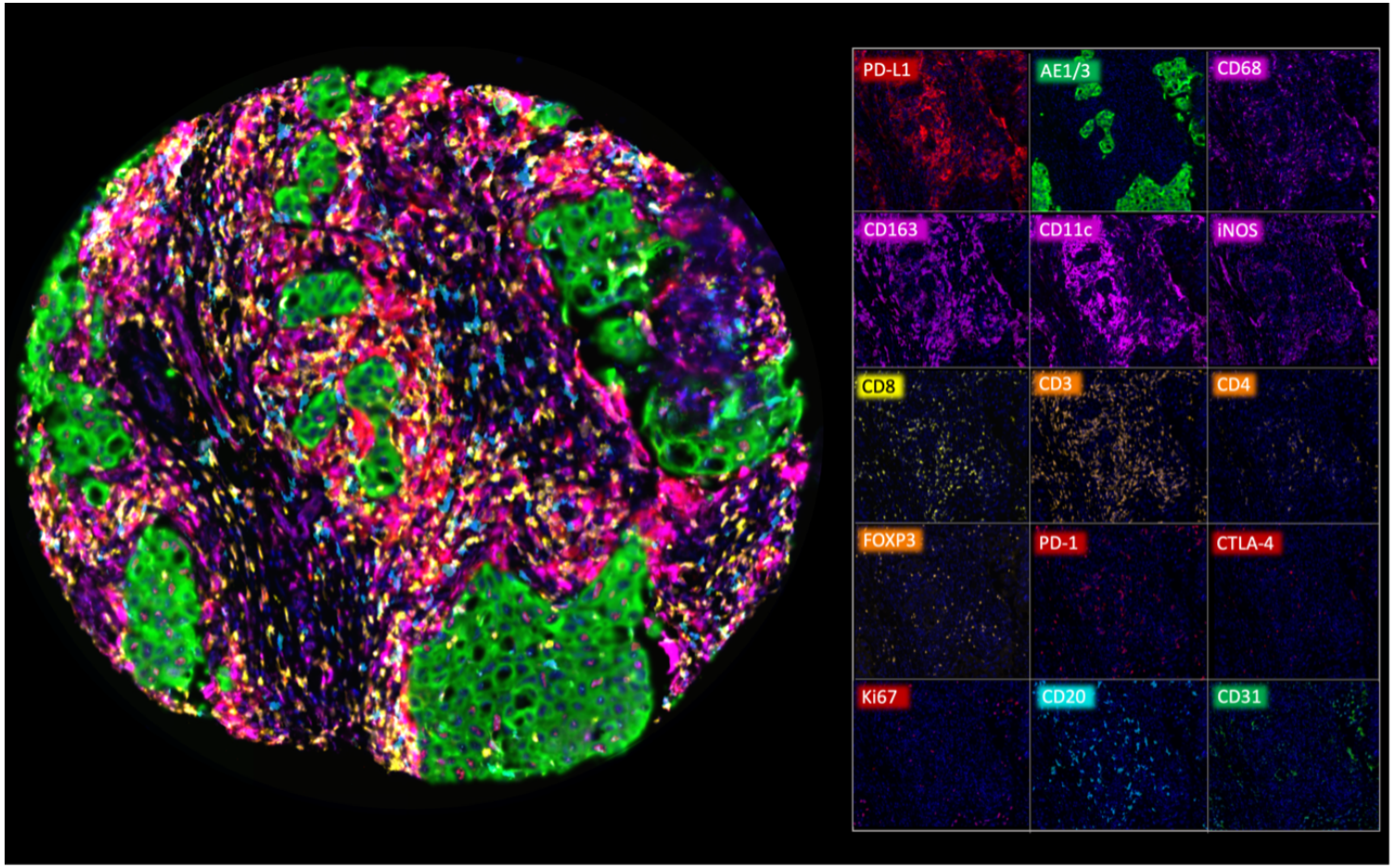
The future of immunohistochemistry

Multiplex fluorescence immunohistochemistry (mfIHC) allows the application of multiple antibodies to one tissue slide. Here a panel with 15 Antibodies

In the future even more antibodies will be used simultaneously with mfIHC.

Non-specific or cross-reactive antibody staining of one or several antibodies used in a multicolour panel can severely contaminate the data and conclusions drawn from experiments.

Therefore, high-quality antibodies with good documentation is pivotal for experimental success!



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