Enhanced validation data

Anti-Claudin 18.2 recombinant antibody - ab222512

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Enhanced validation designed for your needs

We understand the challenge of finding the right antibody clone – highly specific and sensitive to your intended target – at early selection stages of your development program. To de-risk this clone selection process for you, we generated enhanced validation data for our best recombinant antibody clones to some of the most promising targets.

Our enhanced validation gives you an extra level of confidence in an antibody clone

— Provides additional data on the specificity and sensitivity of our recombinant antibodies in immunohistochemistry (IHC) and other relevant techniques

— Carried out in a custom manner, specific both to the target and the relevant research & clinical settings

— Builds upon our high-quality standard validation

Our framework for enhanced validation

— Our enhanced validation focuses on generating detailed IHC expression profiles for promising immuno-oncology targets in selected formalin-fixed paraffin-embedded (FFPE) human normal tissues and cancer tissue microarrays (TMAs).

— In this study, we demonstrate the sensitivity and specificity of anti-Claudin 18.2 recombinant monoclonal antibody (ab222512) in IHC in selected tissues and TMAs using a BOND™ RX Research Stainer (Leica®).

— We also show biophysical characterization (BPQC) data evaluating antibody identity by mass spectrometry, antibody aggregation by dynamic light scattering, and antibody purity by size exclusion chromatography.

Target overview

Function

- Plays a major role in tight junction-specific obliteration of the intercellular space, through calcium in dependent cell-adhesion activity.

Tissue specificity

- Concentrated at the cell-cell borders of epithelial cells.

Sequence similarities

- Belongs to the Claudin family.

Isoforms

- The human gene CLDN18 has two protein isoforms, Claudin 18.1 and Claudin 18.2. The two isoforms differ within the N-terminal 69 amino acids. Claudin 18.1 expression is restricted to the lung. In contrast, Claudin 18.2 expression is restricted to the stomach.[1]

Figure 1. Peptide COBALT alignment of Claudin 18.1 and Claudin 18.2. Sequence differences occur at the N-terminal (1-69aa) region of the protein. Homologs amino acids are indicated in red; mismatch amino acids are indicated in blue. The analysis was performed using an NCBI online tool, COBALT2.

Cellular localization

Cell junction, tight junction. Cell membrane.

Normal tissue expression

Expressed in differentiated gastric mucosa epithelial cells[1] and absent from gastric stem zone and lung[1].

Cancer expression

Primary gastric cancers and metastases[1,3,4]
Ectopic activation in pancreatic, esophageal (adenocarcinoma), ovarian and lung tumors[1,3,5]

Target information from: UniProt accession P56856
The UniProt Consortium
The Universal Protein Resource (UniProt) in 2010
Nucleic Acids Res. 38:D142-D148 (2010).

Materials and methods
IHC staining protocol: BOND™ RX Research Stainer (Leica®)

Prestaining protocol		
Step	**Reagents**	**Pre-programmed protocol**
Dewax	Bond™ dewax solution (#AR922), alcohol, BOND wash solution (#AR9590)	Prestaining protocol: Dewax
Antigen retrival	Bond™ epitope retrieval ER2 solution (#AR9640)	Prestaining protocol: HIER with ER2, 20 min, 98°C

Staining protocol				
Step	**Reagents**	**Number of washes**	**Incubation time, min**	**Temperature**
Peroxide block	3-4% (v/v) Hydrogen peroxide	-	5	
Wash	Bond™ wash solution	3x	0	
Primary antibody	Anti-Claudin 18.2 rabbit monoclonal antibody [EPR19202] - ab222512 Diluted in Bond™ primary antibody diluent (#AR93523) to final concentration of 2µg/mL (FFPE Tissue)	-	15	
Wash	Bond™ wash solution	3x	0	
Secondary detection	Refine detection kit - polymer (#DS0900)	-	8	
Wash	Bond™ wash solution	2x	0	Ambient
	Deionized water	1x	0	
Visualization	Refine detection kit - mixed DAB refine (#DS0900)	1x	10	
Wash	Deionized water	3x	0	
Counterstain	Refine detection kit - hematoxylin (#DS0900)	-	5	
	Deionized water	1x	0	
Wash	Bond™ wash solution	1x	0	
	Deionized water	1x	0	

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Table 1. IHC staining protocol on BOND™ RX Research Stainer (Leica®). The protocol used is the same as the default IHC protocol F on BOND™ RX Research Stainer (Leica®), apart from the standard post-primary step, which has been excluded from our protocol. All steps were performed at room temperature.
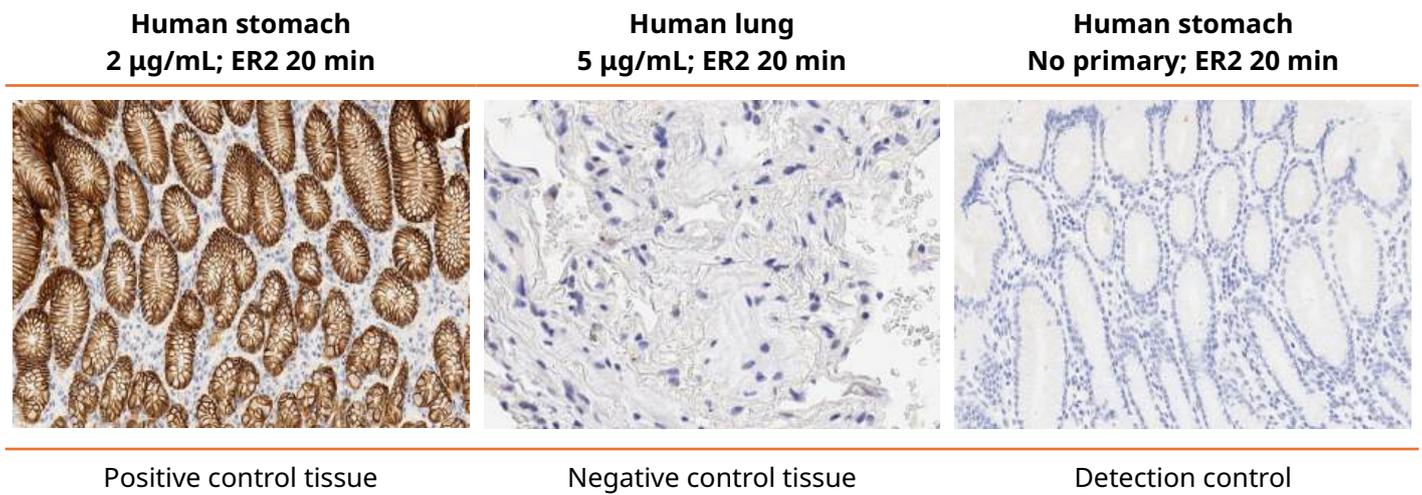
Human cancer tissue					
Tissue micro array	**Cores**	**Cases**	**Normal/ Benign cases**	**Cancer cases**	**Source (#catalog number)**
Colorectal cancer	102	102	5	97	Pantomics (#REC1021)
Stomach cancer	150	150	5	70	Pantomics (#STC1501)
Pancreatic cancer	102	102	16	86	Pantomics (#PAC1021)
Esophagus cancer	102	102	5	97	Pantomics (#ESC1021)

Table 2. List of human cancer TMAs used in the enhanced validation.

Claudin 18.2 expression in normal human tissues

Claudin 18.2 expression was detected in human stomach mucosa epithelial cells but was absent from the lung tissue.

Human stomach **2 µg/mL; ER2 20 min**	**Human lung** **5 µg/mL; ER2 20 min**	**Human stomach** **No primary; ER2 20 min**
Positive control tissue	Negative control tissue	Detection control

Figure 2. Representative IHC images show that Claudin 18.2 expression was detected in the stomach mucosa epithelial cells (positive control tissue) and absent from the lung tissue (negative control tissue). IHC staining of human normal TMA samples was performed using Anti-Claudin18.2 (ab22512) at a final concentration of 2 µg/mL or 5 µg/mL. The omission of primary antibody demonstrates no background staining from the secondary detection kit. Positive staining in brown; hematoxylin counterstain in blue. Magnification, x20.

Indirect ELISA

Figure 3 shows that our anti-Claudin 18.2 antibody only binds to the Claudin 18.2 synthetic peptide and doesn't bind to the Claudin 18.1 isoform.
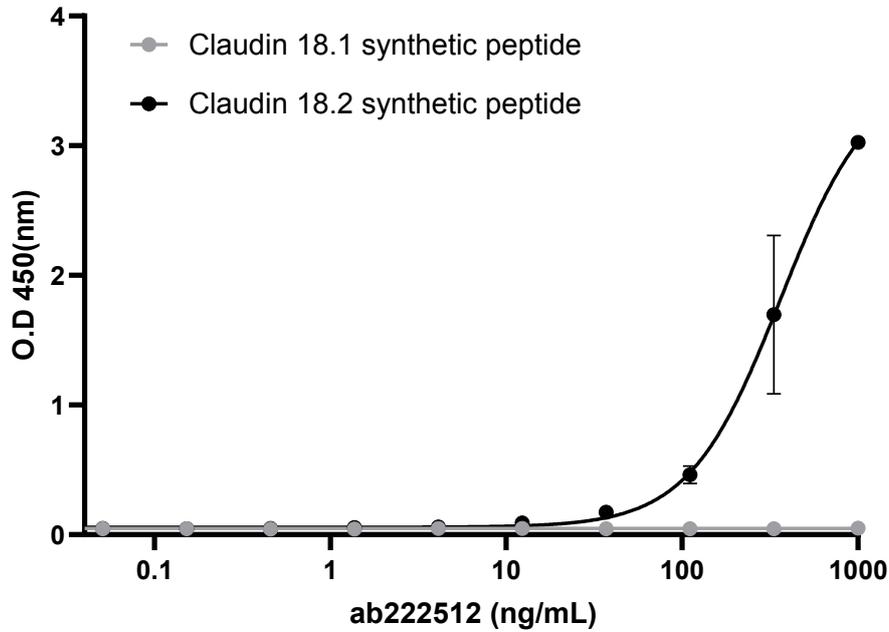
Figure 3. Indirect ELISA showing anti-Claudin18.2 (ab222512) binding to Claudin 18.2 synthetic peptide. The graph shows that anti-Claudin18.2 (ab222512) only binds to the Claudin18.2 synthetic peptide. Plates were coated with Claudin 18.1 and Claudin 18.2 synthetic peptides at 1000 ng/mL. The anti-Claudin18.2 (ab22512) binding was assessed in a serial dilution range of 0.016–1000 ng/mL (3-fold serial dilution). Claudin 18.2 synthetic peptide represents the Claudin18.2 [EPR19202] epitope and Claudin 18.1 synthetic peptide represents the equivalent region but is Claudin 18.1 isoform-specific. Binding was detected using pre-adsorbed secondary antibody, goat anti-rabbit IgG H&L (HRP, ab97080) at 1/2000 dilution.

IHC peptide blocking assay

Figure 4 further confirms that our anti-Claudin 18.2 antibody specifically binds to Claudin 18.2 isoform.
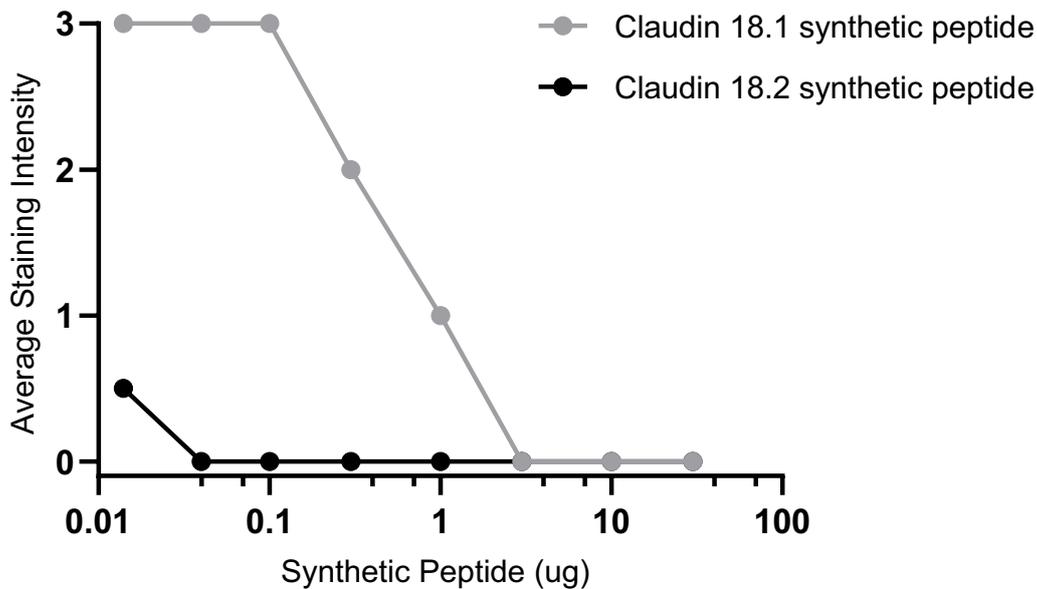
Figure 4. A graphical representation of Claudin 18.2 specific staining intensity in human stomach, following the pre-absorption of the primary antibody (Claudin18.2, ab22512) with either Claudin 18.1 or Claudin 18.2 synthetic peptides (synthetic peptide concentration range 0.014–30 µg, 3-fold dilution series). Claudin 18.2 specific staining was blocked by Claudin 18.2 synthetic peptide. Claudin 18.1 synthetic peptide failed to block Claudin 18.2 specific staining, except for when synthetic peptide levels reached saturation (>3µg), which is expected given the 80% sequence similarity between synthetic peptides.

Claudin 18.2 synthetic peptide represents the Claudin18.2 [EPR19202] epitope; Claudin 18.1 synthetic peptide represents the equivalent region but is Claudin 18.1 isoform-specific. IHC staining of human normal stomach samples was performed using anti-Claudin18.2 (ab22512) at a final concentration of 2 µg/mL. Semi-quantitative scoring of the average tumor expression intensity (0-3+, with a 0.5 interval) was performed by eye.

Representative images of IHC peptide blocking assay

Claudin 18.2 expression was still detected in the human stomach tissue following the antibody's pre-absorption with Claudin 18.1 synthetic peptide, but it was completely eliminated following the pre-absorption with Claudin 18.2 peptide (Figure 5).

No peptide	**Negative Control**

Claudin 18.1 – Synthetic Peptide	**Claudin 18.2 – Synthetic Peptide**

Peptide (1µg)	Peptide (1µg)

Peptide (0.3µg)	Peptide (0.3µg)

Peptide (0.1µg)	Peptide (0.1µg)

Peptide (0.04µg)	Peptide (0.04µg)

Peptide (0.014µg)	Peptide (0.014µg)
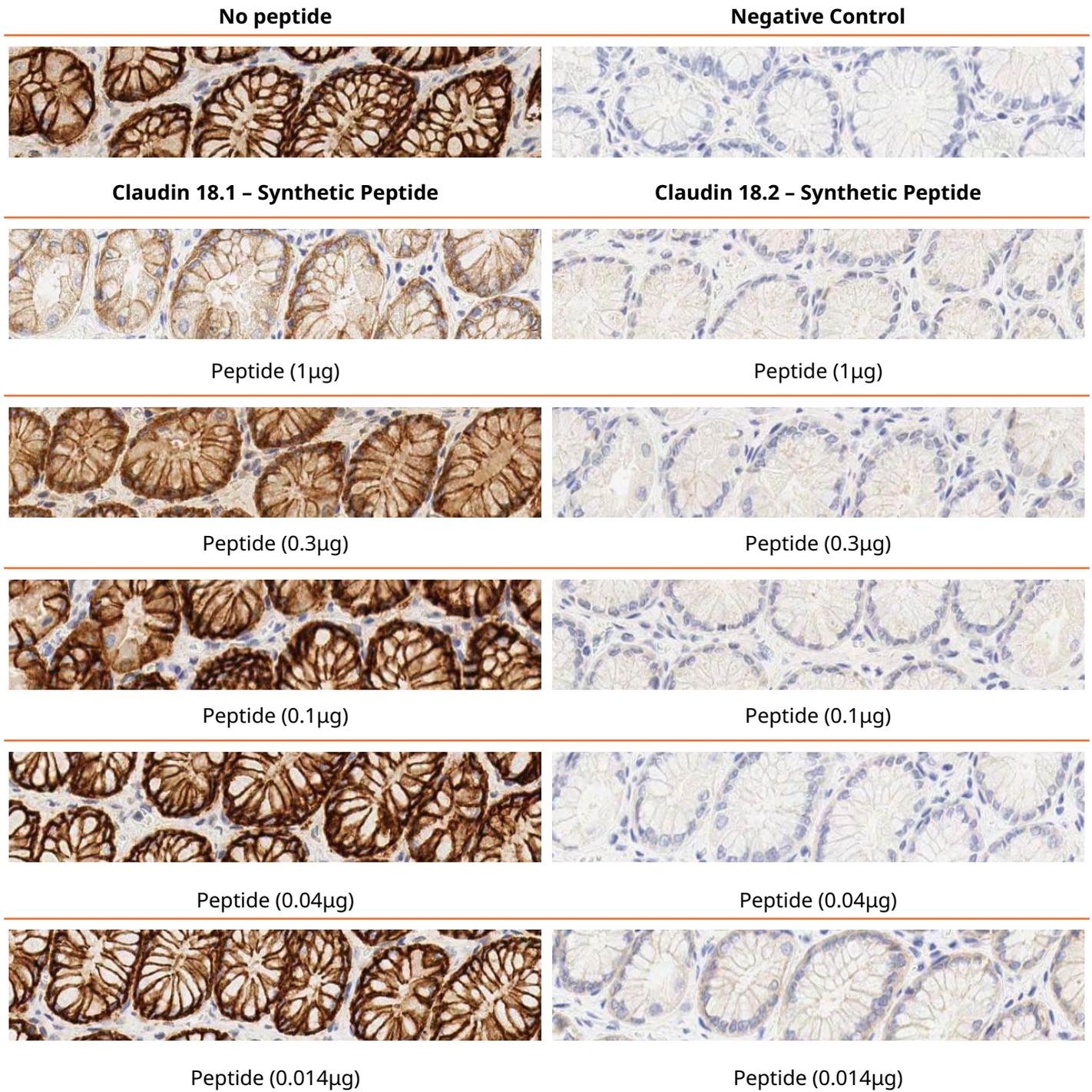
Figure 5. Claudin 18.2 staining in human stomach, following the pre-absorption of the primary antibody (anti-Claudin18.2, ab22512) with either Claudin 18.1 or Claudin 18.2 synthetic peptides (synthetic peptide concentration range 1 µg - 0.014 µg, 3-fold dilution series). Claudin 18.2 synthetic peptide represents the Claudin18.2 [EPR19202] epitope; Claudin 18.1 synthetic peptide represents the equivalent region but is Claudin 18.1 isoform-specific. IHC staining of human stomach samples was performed using anti-Claudin18.2 (ab22512) at a final concentration of 2 µg/ml. Positive staining in brown; hematoxylin counterstain in blue. Magnification, x20.

Claudin 18.2 expression in stomach cancer TMA

Below are the representative images of Claudin 18.2 expression in stomach cancer TMA (Figure 6).
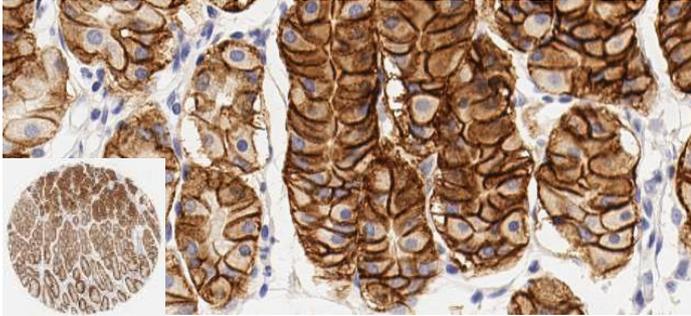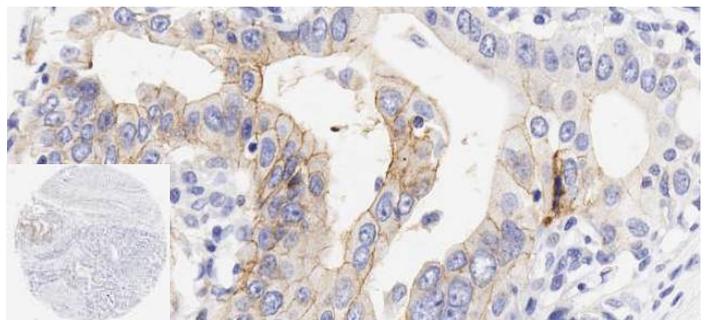
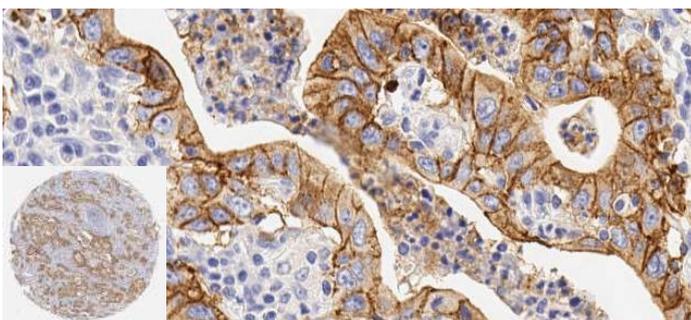| **Normal stomach (3+)**	**Chronic gastritis (2+)**
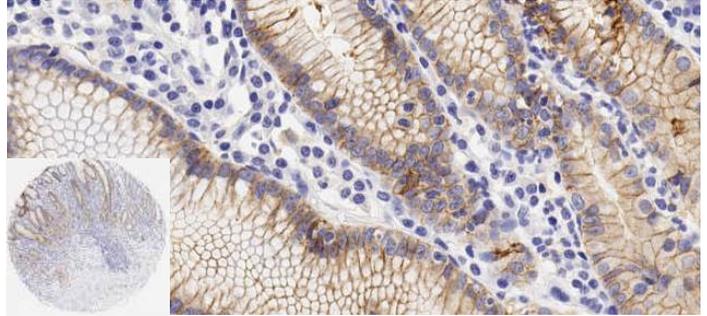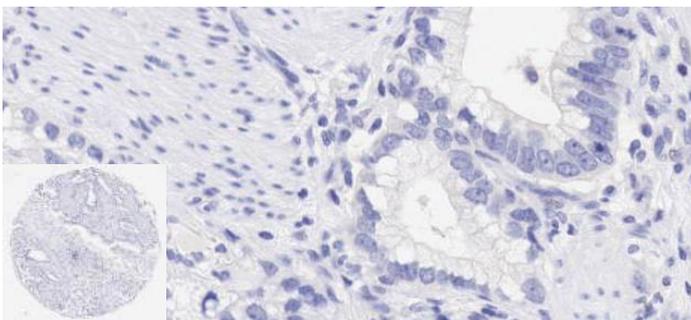
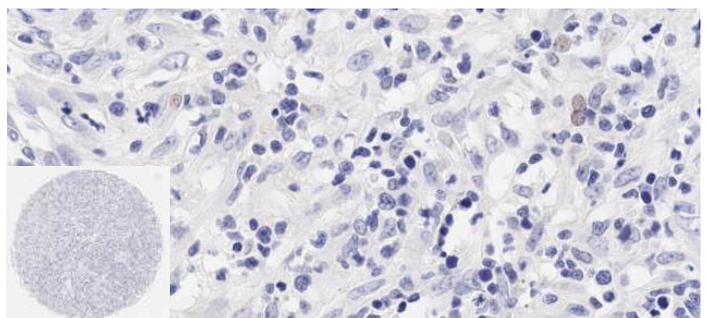| **Adenocarcinoma (0)**	**Adenocarcinoma (1+)**

Adenocarcinoma (2+)	**Adenocarcinoma (3+)**
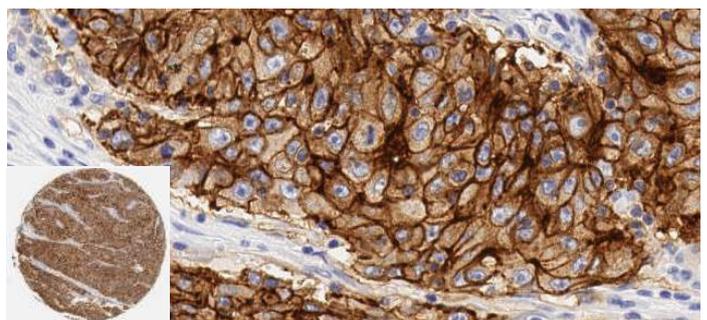
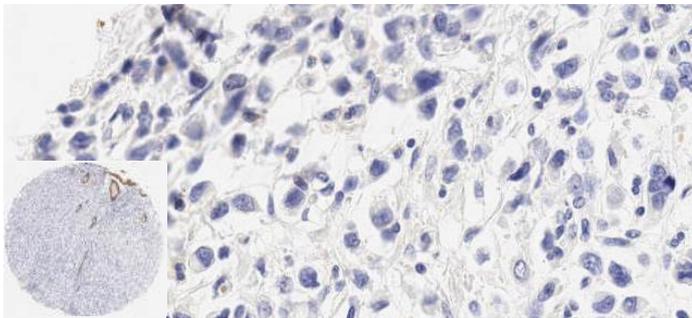| **Gastrointestinal stromal tumor, GIST (0)**	**Lymphoma, MALT-B cell lymphoma (0)**
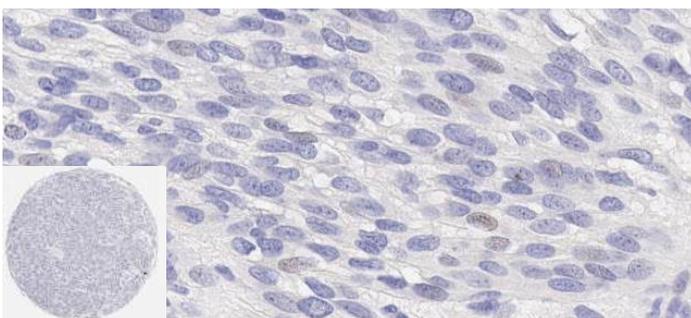
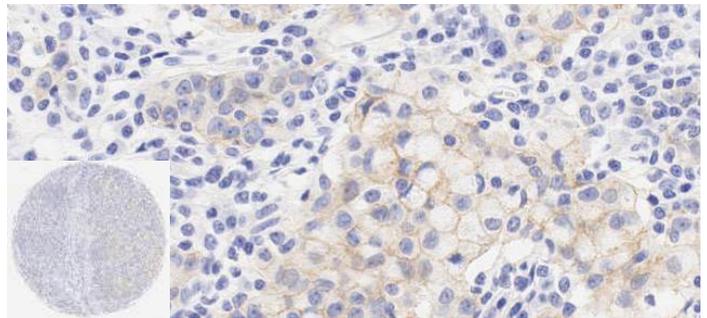**Lymphoma, non-Hodgkin T-cell lymphoma (0)**
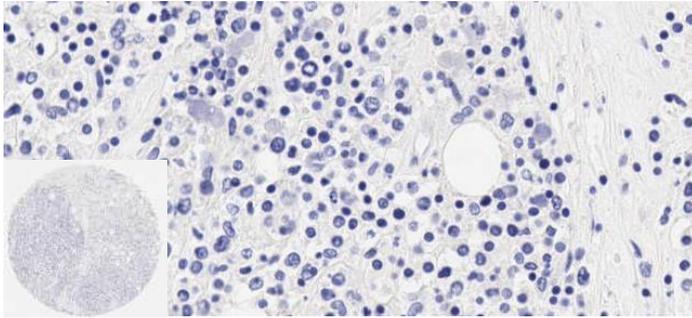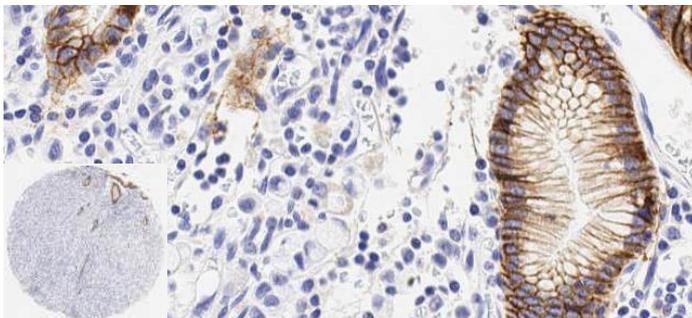
Mucinous adenocarcinoma (1)
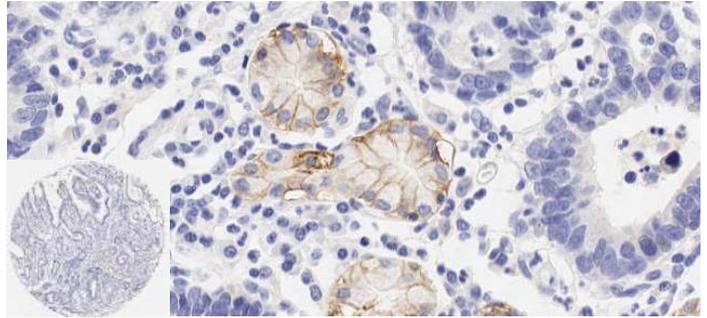
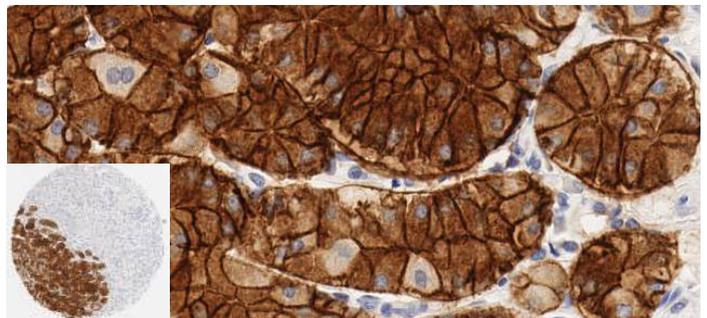**Signet ring cell carcinoma (0)**
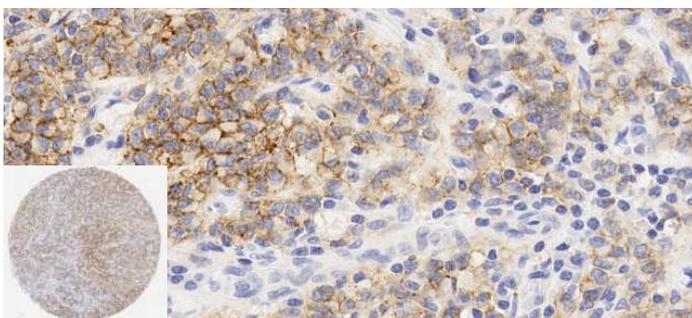
Signet ring cell carcinoma (1+)

Signet ring cell carcinoma (2+)
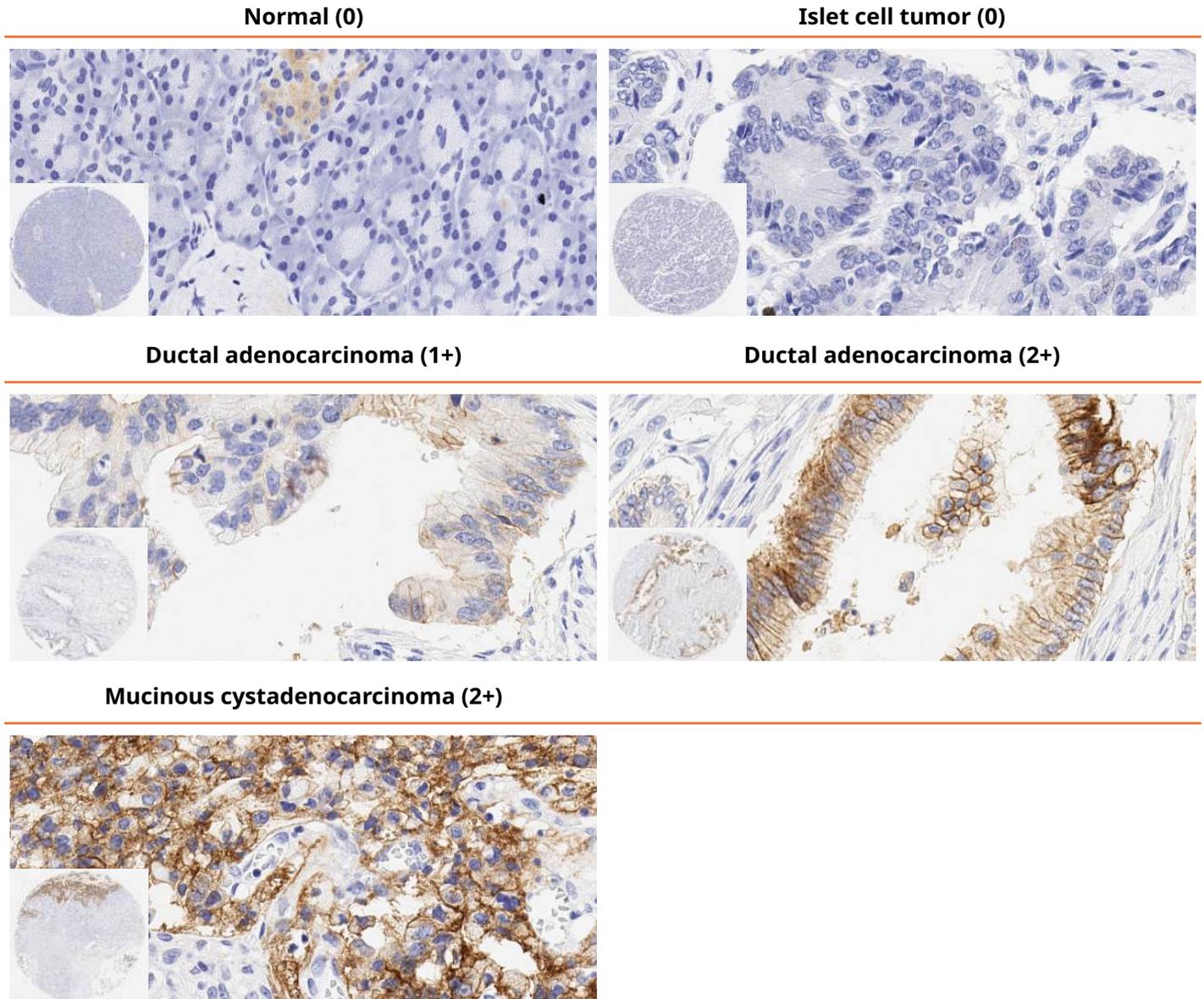
Signet ring cell carcinoma (3+)

Undifferentiated carcinoma (2+)

Figure 6. Claudin 18.2 expression intensity in stomach cancer. Semi-quantitative scoring of the average tumor expression intensity (0-3+,with a 0.5 interval) was performed by eye. Representative images show Claudin 18.2 expression intensity observed in each pathological cancer subtype. Positive staining in brown. Hematoxylin counterstain in blue. Magnification, x5 and x20.
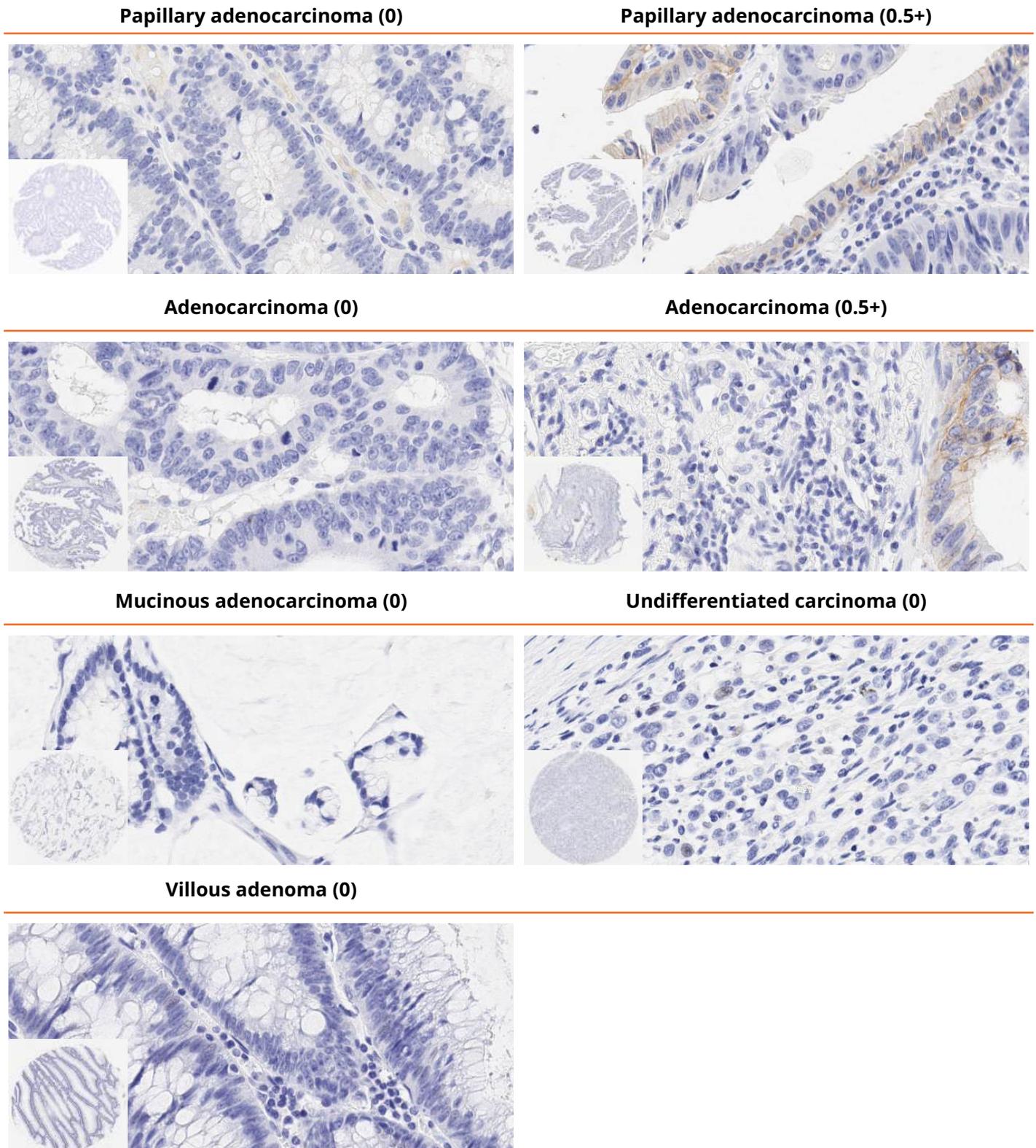
Claudin 18.2 expression in pancreatic cancer TMA

Below are the representative images of Claudin 18.2 expression in pancreatic cancer TMA (Figure 7).

Normal (0)   **Islet cell tumor (0)**

Ductal adenocarcinoma (1+)   **Ductal adenocarcinoma (2+)**

Mucinous cystadenocarcinoma (2+)

Figure 7. Claudin 18.2 expression intensity in pancreatic cancer. Semi-quantitative scoring of the average tumor expression intensity (0-3+, with a 0.5 interval) was performed by eye. Representative images show Claudin 18.2 expression intensity observed in each pathological cancer subtype. Positive staining in brown. Hematoxylin counterstain in blue. Magnification, x5 and x20.

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Claudin 18.2 expression in colorectal cancer TMA

Below are the representative images of Claudin 18.2 expression in colorectal cancer TMA (Figure 8).

Papillary adenocarcinoma (0)

Papillary adenocarcinoma (0.5+)

Adenocarcinoma (0)

Adenocarcinoma (0.5+)

Mucinous adenocarcinoma (0)

Undifferentiated carcinoma (0)

Villous adenoma (0)

Figure 8. Claudin 18.2 expression intensity in colorectal cancer. Semiquantitative scoring of the average tumor expression intensity (0-3+, with a 0.5 interval) was performed by eye. Representative images show Claudin 18.2 expression intensity observed in each pathological cancer subtype. Positive staining in brown. Hematoxylin counterstain in blue. Magnification, x5 and x20.
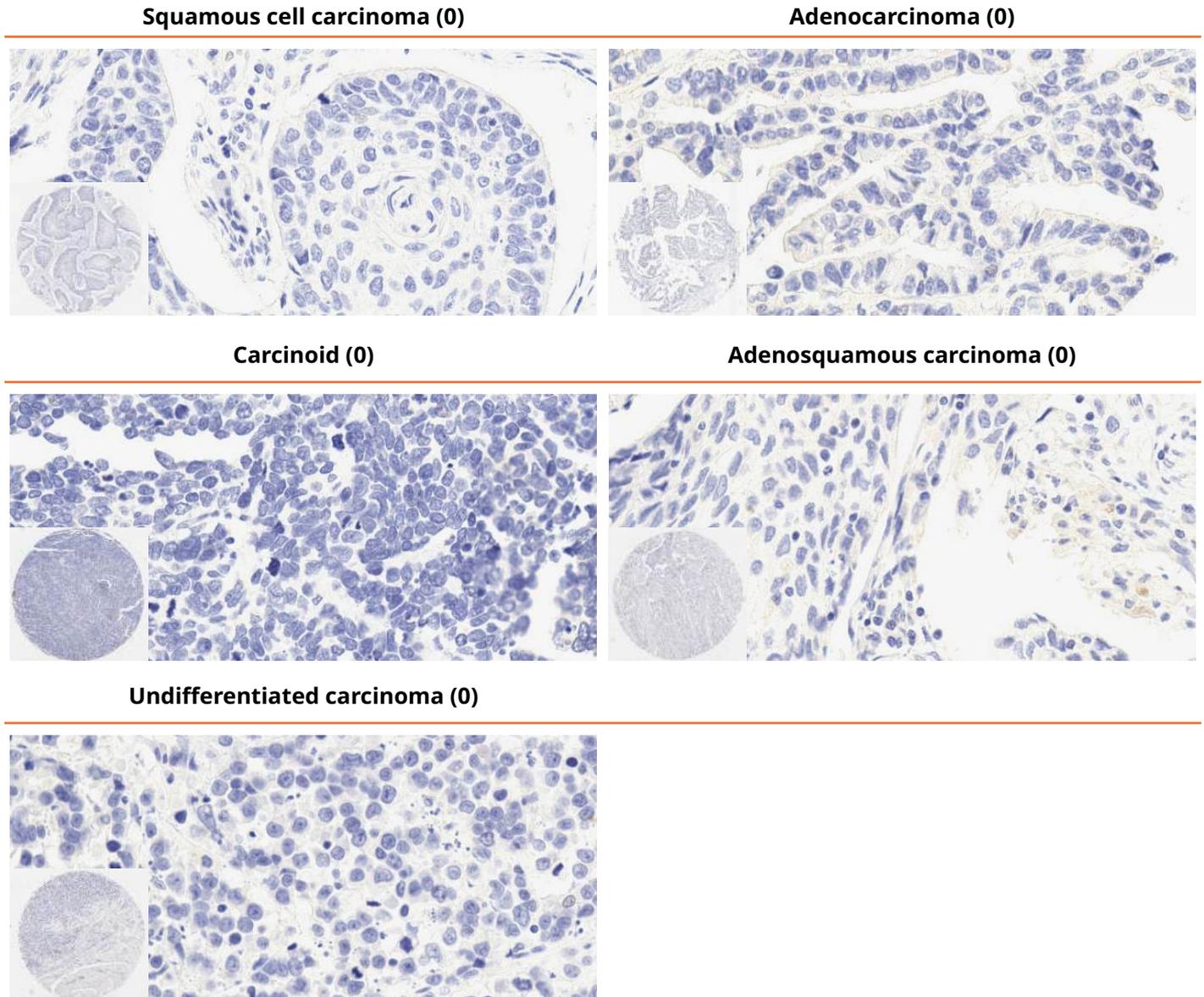
Claudin 18.2 expression in esophagus cancer TMA

Below are the representative images of Claudin 18.2 expression in esophagus cancer TMA (Figure 9).

Squamous cell carcinoma (0)

Adenocarcinoma (0)

Carcinoid (0)

Adenosquamous carcinoma (0)

Undifferentiated carcinoma (0)

Figure 9. Claudin 18.2 expression intensity in esophagus cancer. Semi-quantitative scoring of the average tumor expression intensity (0-3+ with a 0.5 interval) was performed by eye. Representative images of each expression intensity observed in each pathological cancer subtype. Positive staining in brown. Hematoxylin counterstain in blue. Magnification, x5 and x20.

Cancer TMA whole core image analysis

Claudin 18.2 expression varied in the analyzed cancer TMAs (Figure 10), with stomach and pancreatic cancer TMAs having higher H-scores compared to colorectal and esophagus cancer TMAs.
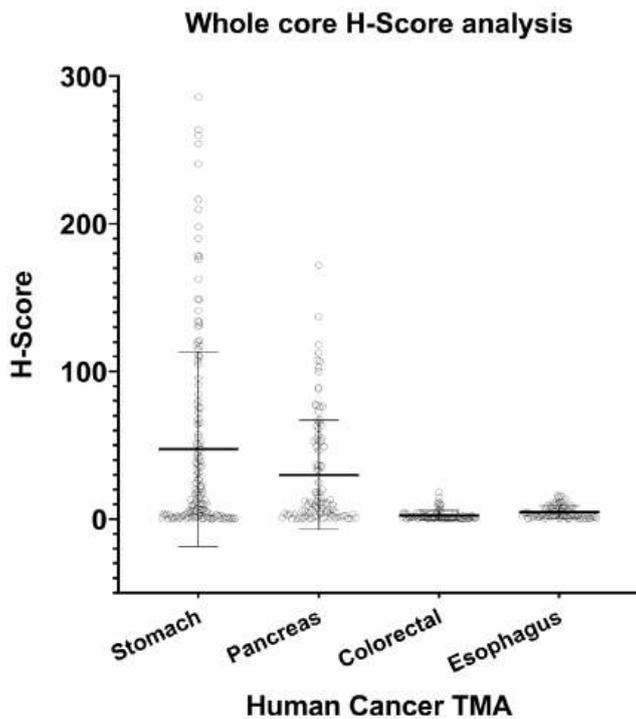
Figure 10. Cancer TMA whole core H-score analysis. Image analysis was conducted with QuPath v0.2.3 software using in-built analysis algorithms. Whole core analysis was performed counting all negative and positive cells and separating positivity into 1+, 2+ and 3+ positivity. A combined H-score was created for each core. Colorectal (15/102) and Esophagus (15/102) cores were excluded due to technical artefact.

# Mass spectrometry to evaluate antibody identity

Mass spectrometry analysis confirmed the identity of anti-Claudin 18.2 antibody, with the observed molecular weights of the heavy and light chains matching the theoretical molecular weights obtained from the plasmid sequence (Figure 11).
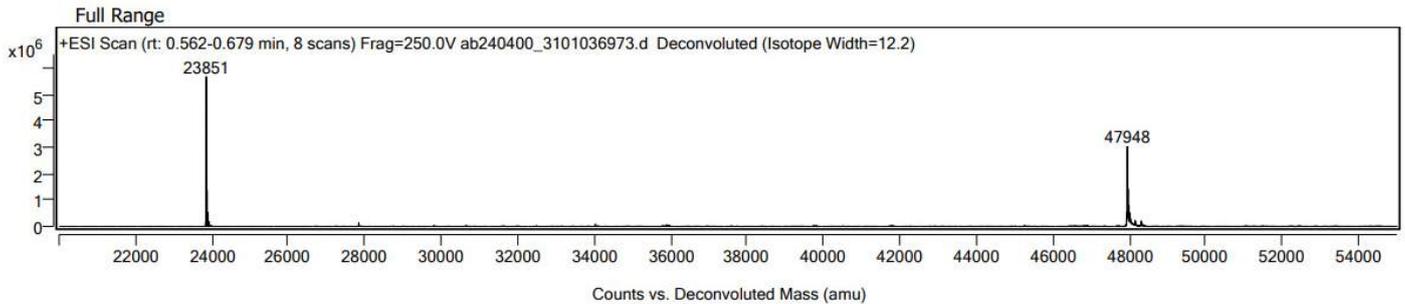
Figure 11. Deconvoluted mass spectrum (MS) of denatured and de-glycosylated antibody. Anti-Claudin 18.2 antibody [EPR19202] was denatured into heavy and light chains at 75°C for 10 minutes and de-glycosylated with Protein Deglycosylation Mix II at 37°C for 16 hours to remove N-linked and common O-linked glycans. The MS spectrum was acquired using a TOF mass spectrometer and deconvolute by Bioconfirm software.

# Dynamic light scattering to evaluate antibody aggregation

Dynamic light scattering analysis detected a limited level of antibody aggregates (Figure 12).
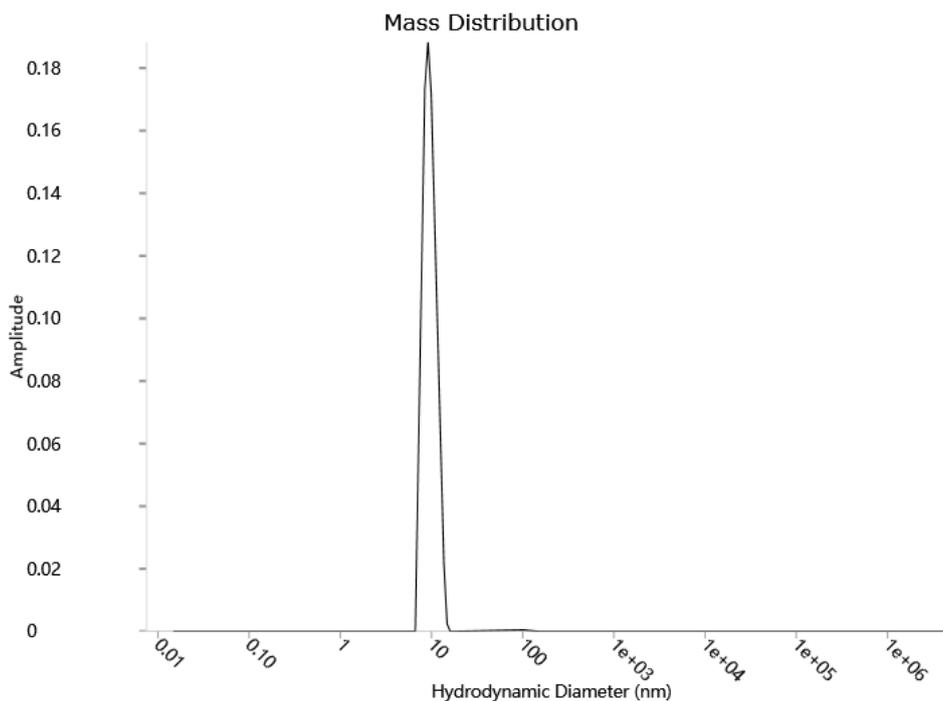
Figure 12. Size and size distribution of intact antibody. Data was acquired by a biologics stability screening platform. The hydrodynamic diameter of the anti-Claudin 18.2 antibody [EPR19202] is 10.34 nm, which accounts for 99.8% of the mass, demonstrating a limited level of aggregates.

Size exclusion chromatography to evaluate antibody purity

Size exclusion chromatography analysis showed that the purity of the anti-Claudin 18.2 antibody is at 99.21% (Figure 13).
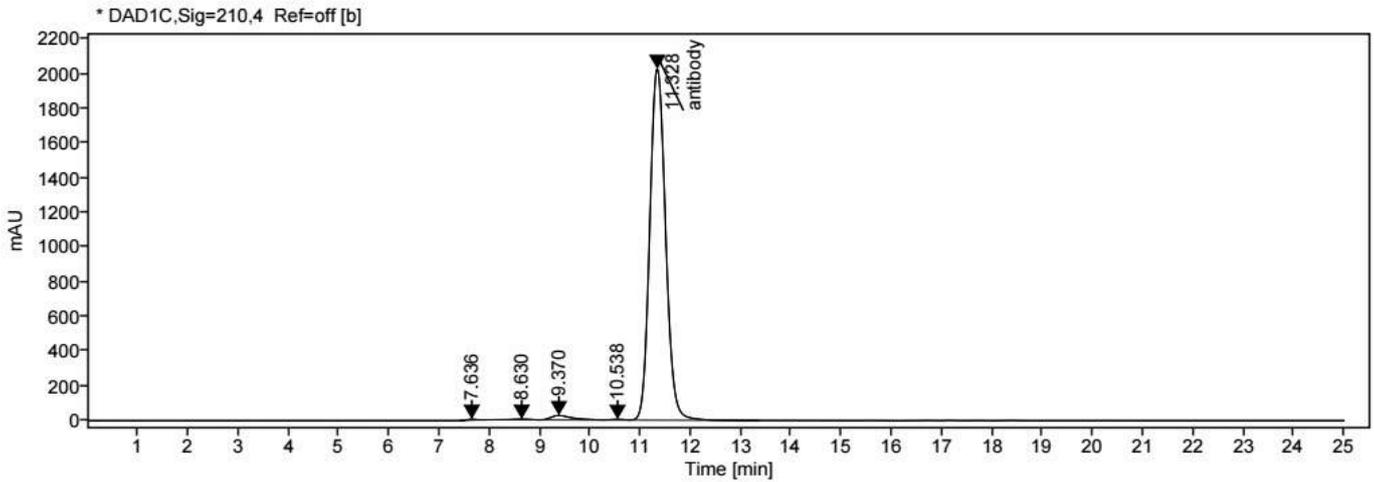
Figure 13. Size exclusion chromatography of intact antibody. Data was acquired by high-performance liquid chromatography with AdvanceBio SEC column. Anti-Claudin 18.2 antibody [EPR19202] is a predominantly monomeric peak at 11.328 min with a peak area percent of 99.21%.

References

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2. Papadopoulos JS and Agarwala R. COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics* **23**:1073-79 (2007). Available at: https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt. cgi?CMD=Doc.

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5. Moentenich V, Gebauer F, Comut E, et al. Claudin 18.2 expression in esophageal adenocarcinoma and its potential impact on future treatment strategies. *Oncol Lett.* **19**(6):3665-3670 (2020).

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