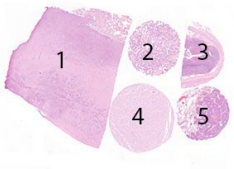
## Assessment Run 74 2025

# Claudin 4 (CLDN4)

**Purpose**
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CLDN4, identifying malignant mesothelioma. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for CLDN4 (see below).

**Material**
The slide to be stained for CLDN4 comprised:




1. Mesothelioma, 2. Placenta, 3. Appendix, 4. Clear cell renal cell carcinoma (ccRCC)
5. Lung adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CLDN4 staining as optimal included:

- A moderate to strong membranous staining reaction of all epithelial cells in the appendix.
- An at least weak to moderate membranous staining reaction of virtually all cytotrophoblasts in placenta.
- An at least weak membranous staining reaction of the vast majority of neoplastic cells in the ccRCC.
- A moderate membranous staining reaction of virtually all neoplastic cells in lung adenocarcinoma.
- No staining reaction of the neoplastic cells in the mesothelioma. Lymphocytes and muscle cells in appendix are also expected to be negative.

**KEY POINTS FOR CLDN4 IMMUNOASSAYS**
- The mAb clone **3E2C1** and rmAb clone **EP417** are recommendable both as a concentrated Abs and as RTUs.
- The widely used mAb clone **3E2C1** seems less reproducible on Ventana BenchMark platforms.
- Placenta and appendix are recommendable as positive and negative tissue controls.

**Participation**

| | |
|---|---|
| Number of laboratories registered for CLDN4, run 74 | 107 |
| Number of laboratories returning slides | 98 (92%) |

All slides returned after the assessment were assessed and received advice if the result being insufficient, but the data were not included in this report.

**Results**
98 laboratories participated in this assessment. One laboratory submitted a protocol for CLDN18.2 and was excluded from this CLDN4 assessment. 70 (72%) achieved a sufficient mark (optimal or good), see Table 1a (see page 2). Tables 1b and 1c summarizes the antibodies (Abs) used and assessment marks (see page 2 and 3).

The most frequent cause of insufficient staining reactions was:
- Less successful primary Ab
- Less successful performance of mAb clone 3E2C1 on Ventana BenchMark platforms
- Too low concentration of the primary antibody
- Use of a less sensitive detection system

**Performance history**
This was the first NordiQC assessment of CLDN4 and the overall pass rate was 72%.

**Controls**
In appendix, virtually all epithelial cells must show a moderate to strong membranous staining reaction. Lymphocytes and smooth muscle cells should be negative. In placenta, an at least weak to moderate membranous staining reaction should be seen in virtually all cytotrophoblasts.

**Conclusion**
The rmAb clone **EP417** was the most successful Ab for the demonstration of CLDN4. As concentrated (conc.) format within a laboratory developed assay, optimal results were obtained on the main stainer platforms from Ventana/Roche and Dako/Agilent. Efficient HIER in an alkaline buffer and carefully calibrated primary Ab together with a sensitive detection system were the most important prerequisites for a sufficient staining. The widely used mAb clone 3E2C1 could also give optimal results but gave an inferior and less reproducible performance on Ventana BenchMark platforms.

Table 1a. **Overall results for CLDN4, run 74**

| | n | Optimal | Good | Borderline | Poor | Suff.[1] | OR[2] |
|---|---|---|---|---|---|---|---|
| Concentrated antibodies | 68 | 16 | 30 | 13 | 9 | 68% | 24% |
| Ready-To-Use antibodies | 29 | 12 | 12 | 2 | 3 | 82% | 41% |
| Total | 97 | 28 | 42 | 15 | 12 | | |
| Proportion | | 29% | 43% | 16% | 12% | 72% | |

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1b. **Concentrated antibodies and assessment marks for CLDN4, run 74**

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | OR[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **3E2C1** | 15 | Biocare Medical | 3 | 7 | 4 | 1 | 67% | 20% |
| | 24 | Invitrogen | 3 | 15 | 3 | 3 | 75% | 13% |
| mAb clone **A-12** | 2 | Gennova | 0 | 0 | 0 | 2 | - | - |
| rmAb clone **EP417** | 8 | BioSB | 1 | 4 | 2 | 1 | 63% | 13% |
| | 8 | Cell Marque | 6 | 2 | 0 | 0 | 100% | 75% |
| rmAb clone **EPRR17575** | 4 | Abcam | 1 | 1 | 2 | 0 | - | - |
| rmAb clone **QR137** | 1 | Quartett | 1 | 0 | 0 | 0 | - | - |
| pAb, **ab15104** | 4 | Abcam | 1 | 1 | 2 | 0 | - | - |
| pAb, **PA580481** | 1 | ThermoFisher | 0 | 0 | 0 | 1 | - | - |
| pAb, **16195-1-AP** | 1 | Proteintech | 0 | 0 | 0 | 1 | - | - |
| Total | 68 | | 16 | 30 | 13 | 9 | | |
| Proportion | | | 24% | 44% | 19% | 13% | 68% | |

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).

Table 1c. **Ready-To-Use antibodies and assessment marks for CLDN4, run 74**

| Ready-To-Use antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | OR[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **3E2C1** **MAB-1106** | 1 | Fuzhou Maixin | 0 | 1 | 0 | 0 | - | - |
| mAb clone **3E2C1** **API 3121 AA** | 8 | Biocare Medical | 5 | 2 | 0 | 1 | 88% | 63% |
| mAb clone **A-12** **AMB08-5M** | 1 | BioGenex | 0 | 0 | 0 | 1 | - | - |
| mAb clone **AA11** **MFM-0188** | 1 | Bioin Biotechnology | 0 | 1 | 0 | 0 | - | - |
| mAb clone **GM104** **GT2339** | 1 | Gene Tech | 1 | 0 | 0 | 0 | - | - |
| rmAb clone **EP417** **BSB 3792-3/7/15** | 7 | Bio SB | 0 | 5 | 1 | 1 | 71% | 0% |
| rmAb clone **EP417** **468-17/18** | 9 | Cell Marque | 5 | 3 | 1 | 0 | 89% | 56% |
| Ab clone **172D5F1** **PA616** | 1 | Abcarta | 1 | 0 | 0 | 0 | - | - |
| Total | 29 | | 12 | 12 | 2 | 3 | | |
| Proportion | | | 41% | 41% | 7% | 11% | 82% | |

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).

## Detailed analysis of CLDN4, Run 74

The following protocol parameters were central to obtain optimal staining:

### Concentrated antibodies

mAb clone **3E2C1**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Target Retrieval Solution (TRS, Dako/Agilent) High pH (5/13)* or Cell Conditioning 1 (CC1, Ventana/Roche) (1/20) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:250 and a 3-step detection system was applied. Using these protocol settings, 20 of 23 (87%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP417**: Protocols with optimal results were based on HIER using TRS High pH (2/5) (Dako/Agilent), TRS Low pH (1/1) (Dako/Agilent) or CC1 (Ventana/Roche) (4/10) as retrieval buffer. The rmAb was diluted in the range of 1:25-1:100. Using these protocol settings, 13 of 15 (87%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for CLDN4 for the most commonly used antibody concentrates on the four main IHC systems***

| Concentrated antibodies | **Dako/Agilent Autostainer[1]** | | **Dako/Agilent Omnis** | | **Ventana/Roche BenchMark[2]** | | **Leica Biosystems Bond[3]** | |
|---|---|---|---|---|---|---|---|---|
| | TRS pH 9.0 | TRS pH 6.1 | TRS pH 9.0 | TRS pH 6.1 | CC1 pH 8.5 | CC2 pH 6.0 | BERS2 pH 9.0 | BERS1 pH 6.0 |
| mAb clone **3E2C1** | 0/1** | - | 5/10 (50%) | - | 1/18 (6%) | 0/1 | 0/3 | - |
| rmAb clone **EP417** | 2/2 | - | 0/2 | 1/1 | 4/10 (40%) | - | - | - |

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** Number of optimal results/number of laboratories using this buffer.
1) Autostainer Link 48.
2) BenchMark Ultra, Ultra plus
3) Bond III

### Ready-To-Use antibodies and corresponding systems

No Ready-To-Use Abs with a corresponding system (≥5 assessed protocols) were used in this assessment.

**Comments**
In this assessment, the prevalent feature of an insufficient result was a too weak or completely false negative staining result in structures expected to be positive. This pattern was observed in 93% of the insufficient results (25 of 27). In addition, four of the false negative staining results also gave a false positive staining reaction of cells and structures expected to be negative. Two insufficient results (7%) were caused by a poor signal-to-noise ratio or excessive background staining. Virtually all laboratories were able to demonstrate CLDN4 in high-level antigen expressing structures such as neoplastic cells of the ccRCC and normal epithelial cells in the appendix. Demonstration of CLDN4 in low-level expressing structures as neoplastic cells of the lung adenocarcinoma and cytotrophoblasts in placenta were more challenging and required a carefully calibrated protocol.

70% (68 of 97) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for CLDN4. The most successful **rmAb clone EP417** was used by 16 participants, giving an overall pass rate of 81%, 44% optimal. Optimal results could be obtained on the main platforms from Dako/Agilent and Ventana/Roche (see Table 2). 10 participants applied the Ab on a Ventana BenchMark platform. All used HIER in CC1 and nine applied OptiView as detection system with a pass rate of 89% (8 of 9). On Dako Omnis, one optimal result was obtained by HIER in TRS pH 6.1 in combination with a 3-step detection system.

The **mAb clone 3E2C1** was the most widely used antibody for demonstration of CLDN4 and as a concentrate, gave an overall pass rate of 72%, only 15% optimal. The main prerequisites for sufficient staining were use of HIER in an alkaline buffer, careful calibration of the titre of the primary Ab and preferably a 3-step detection system. If using a 3-step detection system, a pass rate of 81% (25 of 31), 19% optimal (n=6) was seen compared to 38% observed for 2-step detection systems, none optimal. As seen in Table 2, the clone was found challenging on the Ventana BenchMark platform with only 6% optimal in total. If using OptiView or UltraView with amplification, a pass rate of 76% was seen (13 of 17). Similar protocol settings were applied, but the reasons for downgrading from optimal to good was either related to a generally weak staining reaction or excessive background, complicating identification of a recommendable and reproducible protocol for mAb clone 3E2C1 on Ventana BenchMark platforms. Irrespective of protocol and platform applied, it was observed that the mAb clone 3E2C1 gave an aberrant granular dot-like staining reaction primarily seen in the mesothelioma being otherwise negative for CLDN4. The granular staining was seen in the vicinity of the neoplastic cells but also stromal compartment. This pattern is described by Ohta et al. *Claudin-4 as a marker for distinguishing malignant mesothelioma from lung carcinoma and serous adenocarcinoma. Int J Surg Pathol*. 2013 Oct;21(5):493-501

In total, six laboratories applied a **polyclonal** Ab as a concentrate. In concordance with other NordiQC assessments, polyclonal Abs in general gave an inferior performance, and cannot be recommended when monoclonal Abs with the expected reaction profiles are accessible.

At present no RTU IHC assays are available from the main IHC system providers as Dako/Agilent, Ventana/Roche and Leica Biosystems and consequently commercially available RTU formats for CLDN4 without any intended IHC platform have to be evaluated and tested by the laboratories concerning best protocol settings.

The RTU format based on mAb clone 3E2C1 **API 3121 AA** (Biocare Medical) gave high proportions of sufficient and optimal results as shown in Table 1c. Optimal and sufficient results were seen on the fully automated platforms from Dako/Agilent and Leica Biosystems, with similar protocol settings as for the corresponding concentrated format.

The RTU **468-17/18** (Cell Marque) based on rmAb clone EP417 also gave high proportions of sufficient and optimal results as shown in Table 1c. Optimal and sufficient results were seen on the fully automated platforms from Dako/Agilent, Ventana/Roche and Leica Biosystems, with similar protocol settings as for the corresponding concentrated format.

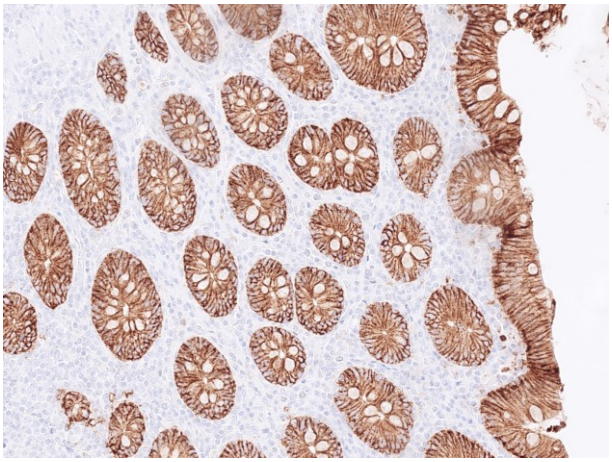


Fig. 1a
Optimal CLDN4 staining reaction of the appendix mucosa using the rmAb clone EP417 in a concentrated format (1:100), using HIER in CC1, OptiView as detection system and performed on BenchMark Ultra. The epithelial cells show a moderate to strong staining reaction.
Also compare with Figs. 2a - 6a – same protocol.

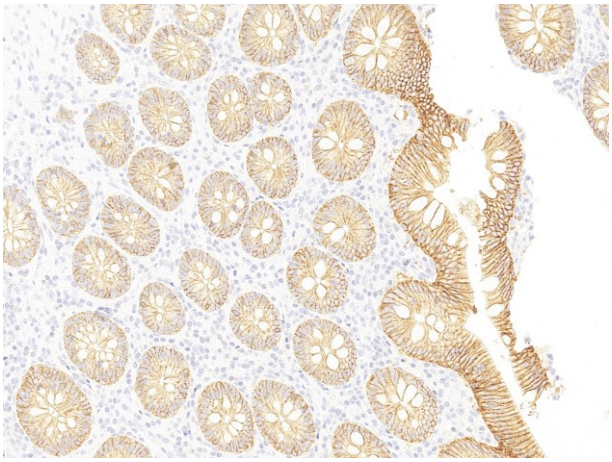


Fig. 1b
Insufficient CLDN4 staining reaction of the appendix mucosa using mAb clone 3EC21 as a concentrated format (1:100) using HIER in CC1 and UltraView as detection system, performed on BenchMark Ultra. A weaker and less distinct membranous staining reaction was seen, compared to optimal result in Fig. 1a – same area.
Also compare with Figs. 2b - 4b – same protocol.

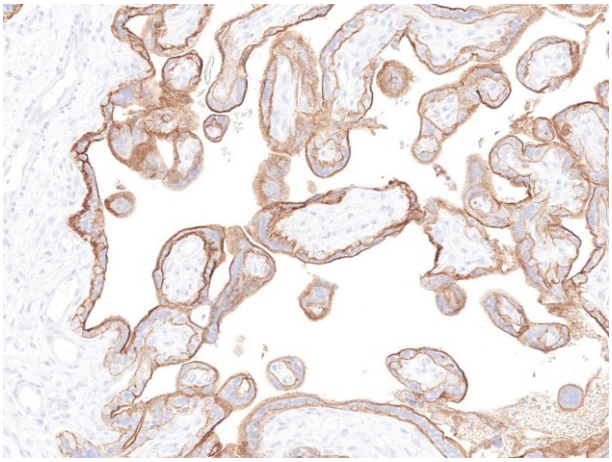


Fig. 2a
Optimal CLDN4 staining reaction of the placenta using same protocol as in Fig. 1a. A weak to moderate, predominantly membranous staining reaction of cytotrophoblasts was seen.
Also compare with Figs. 3a - 6a – same protocol.

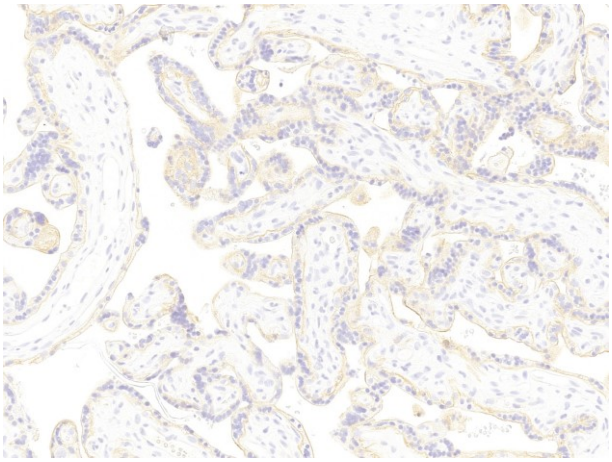


Fig. 2b
Insufficient CLDN4 staining reaction of the placenta using same protocol as in Fig. 1b – same area as in Fig. 2a. The cytothrophoblasts show a too weak and diffuse membranous staining reaction. Also compare with Figs. 3b - 4b – same protocol.

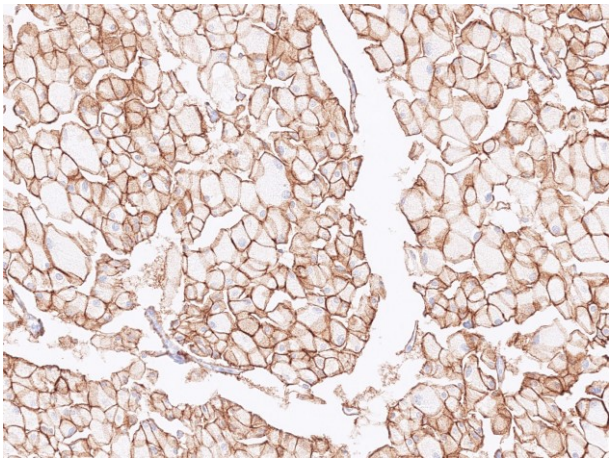


Fig. 3a
Optimal CLDN4 staining reaction of the ccRCC using same protocol as in Figs. 1a and 2a.
Virtually all the neoplastic cells show a moderate and distinct membranous staining reaction.

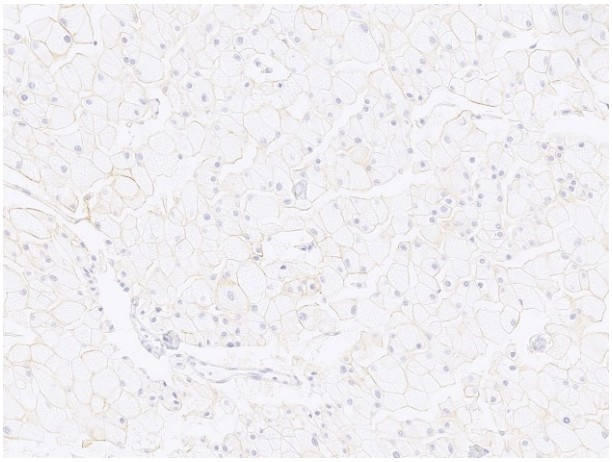


Fig. 3b
Insufficient CLDN4 staining reaction of the ccRCC using the same insufficient protocol as in Figs. 1b and 2b – same field as in Fig. 3a. In general, a significantly weaker staining reaction is seen.
Also compare with Fig. 4b and 5b – same protocol.

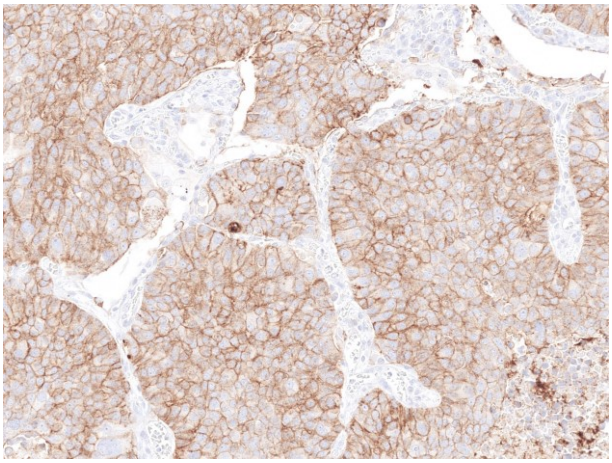


Fig. 4a
Optimal CLDN4 staining reaction of the lung adenocarcinoma using same protocol as in Figs. 1a – 3a.
Virtually all neoplastic cells show a weak to moderate membranous staining reaction.

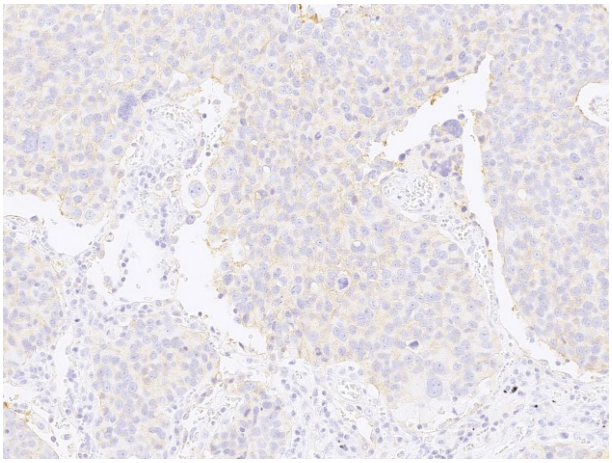


Fig. 4b
Insufficient CLDN4 staining reaction of the lung adenocarcinoma, using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. The neoplastic cells are virtually all false negative.

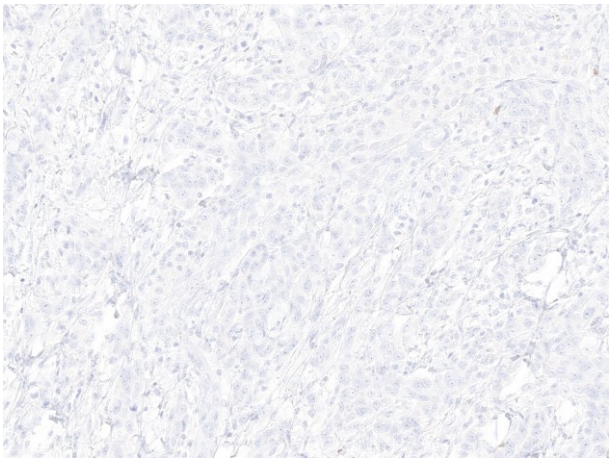


Fig. 5a
Optimal CLDN4 staining reaction of the mesothelioma using same protocol as in Figs. 1a – 4a.
No staining reaction is observed.

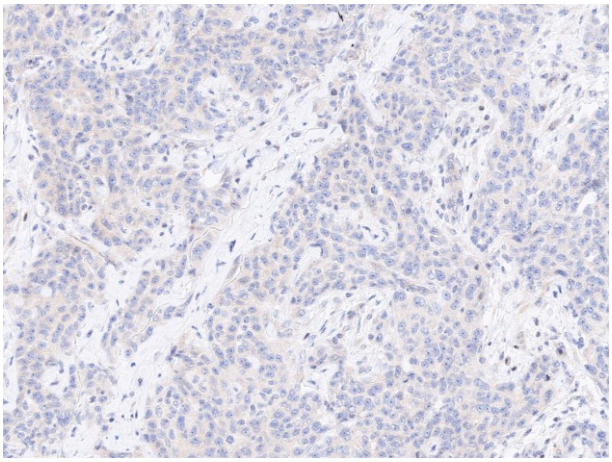


Fig. 5b
CLDN4 staining reaction of the mesothelioma using same protocol as in Figs. 1b - 4b but stained in a different laboratory.
A faint cytoplasmic staining reaction is observed in the neoplastic cells.
The mAb clone 3EC21 was not found to give reproducible results on the Ventana BenchMark platforms giving both too weak reaction and/or an excessive background as shown in Figs. 5b, 6b and 7b.

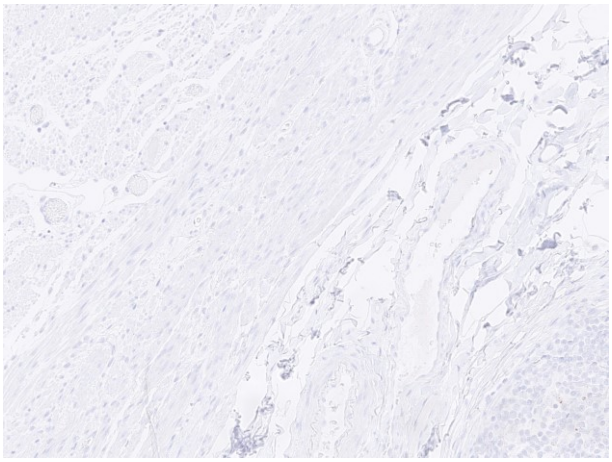


Fig. 6a
Optimal CLDN4 staining reaction of the smooth muscle cells and nerves of lamina muscularis mucosae using same protocol as in Figs. 1b – 5b.
No staining reaction is observed.

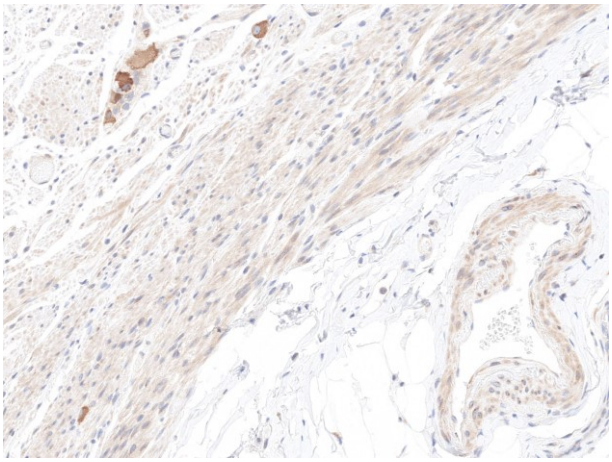


Fig. 6b
Insufficient CLDN4 staining of the appendix in which smooth muscle cells and nerves of lamina muscularis mucosae show an aberrant cytoplasmic staining reaction.
The same protocol as in Figs. 1b – 5b, performed in same lab as Fig. 5b.

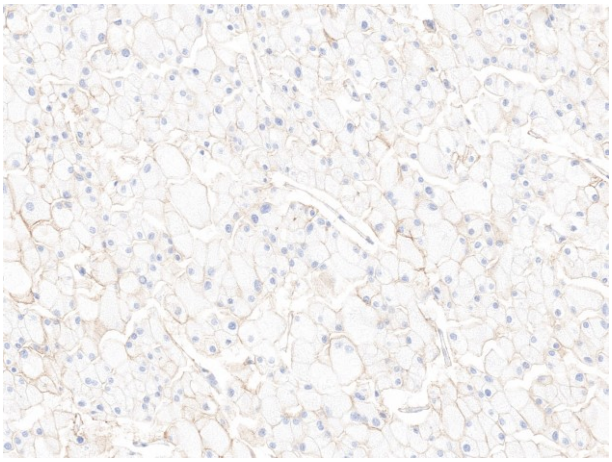


Fig. 7a
CLDN4 staining reaction of the ccRCC using the same protocol as in Figs. 1b – 6b, performed in same lab as Figs. 5b – 6b.
In general, a significantly weaker staining reaction is seen. Compare with optimal result in Fig. 3a.
The protocol based on mAb clone 3EC21 in this laboratory gave both a too weak staining reaction as seen in this Fig. but also an excessive background reaction as shown in Figs. 5b, 6b and 7b.

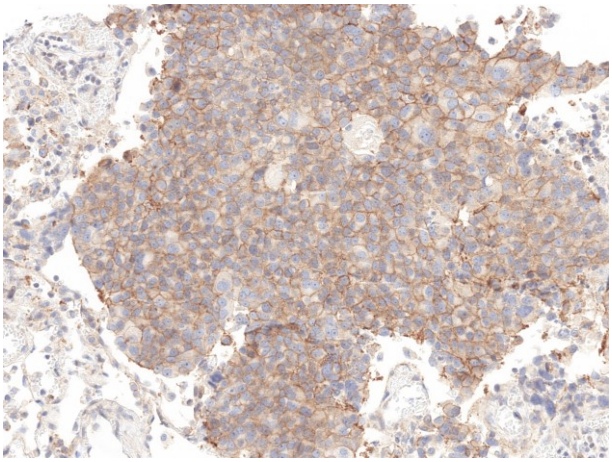


Fig. 7b
Insufficient CLDN4 staining reaction of the lung adenocarcinoma using the same protocol as in Figs. 1b – 6b and 7a, performed in same lab as Figs. 5b – 6b and 7a.
In addition to a diffuse membranous staining reaction, a weak to moderate cytoplasmic staining reaction is observed in the neoplastic cells complicating the read out.

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