

Material

The slide to be stained for CK-LMW comprised:

1. Appendix, 2. Liver, 3. Esophagus, 4. Renal cell carcinoma,
5. Breast ductal carcinoma., 6. Colon neuroendocrine carcinoma

All tissues were fixed in 10% neutral buffered formalin.



Criteria for assessing a CK-LMW staining as optimal included:

- A strong, distinct cytoplasmic staining reaction of virtually all appendiceal columnar epithelial cells, bile duct epithelial cells and an at least weak predominantly membranous staining reaction of the large majority of the hepatocytes.
- A moderate to strong, distinct cytoplasmic staining reaction of the majority of the neoplastic cells of the breast ductal carcinoma and the renal cell carcinoma.
- An at least weak to moderate cytoplasmic staining reaction in the majority of the neoplastic cells of the colon neuroendocrine carcinoma. A weak to moderate cytoplasmic staining reaction in smooth muscle cells and basal squamous epithelial cells of the esophagus was frequently seen using an antibody towards CK 8.

193 laboratories participated in this assessment. 32 participants used an inappropriate antibody like CK-PAN, CK7, CK19 and CK20. Of the remaining 161 laboratories 77 % achieved a sufficient mark (optimal or good). Antibodies (Abs) used and marks are summarized in table 1.

Table 1. Antibodies and assessment marks for CK-LMW, run 38

| Concentrated Antibodies | Reactivity | n | Vendor | Optimal | Good | Borderline | Poor | Suff. ¹ | Suff. OPS ² |
|---------------------------------------|------------|----|--|---------|------|------------|------|--------------------|------------------------|
| mAb clone 5D3 | CK 8/18 | 20 | Leica/Novocastra 4 Thermo/Neomarkers 3 Monosan 2 Biocare 2 Biogenex 1 Vector | 10 | 11 | 9 | 2 | 66 % | 95 % |
| mAb clones B22.1&B23.1 | CK 8/18 | 1 | Cell Marque | 0 | 1 | 0 | 0 | - | - |
| mAb clone BS83 | CK 8/18 | 1 | Nordic Biosite | 0 | 1 | 0 | 0 | - | - |
| mAb clone C51 | CK 18* | 4 | Invitrogen/Zymed | 3 | 1 | 0 | 0 | - | - |
| mAb clone CAM5.2 | CK 8 (7) | 26 | Becton Dickenson 2 Immunologic 1 Zytomed | 2 | 10 | 13 | 4 | 41 % | 100 % |
| mAb clone DC10 | CK 18 | 18 | Dako 9 Leica/Novocastra 2 Thermo/Neomarkers 1 Biogenex 1 ID Labs 1 Invitrogen/Zymed | 17 | 14 | 1 | 0 | 97 % | 97 % |
| mAb clone K8.8+DC10 | CK 8/18 | 1 | Thermo/Neomarkers | 0 | 0 | 1 | 0 | - | - |
| mAb clone TS1 | CK 8 | 3 | Leica/Novocastra 2 Thermo/Neomarkers 1 Gene Tech | 4 | 1 | 1 | 0 | 83 % | 100 % |
| mAb clone TS1 + mAb clone DC10 | CK 8/18 | 1 | Homemade cocktail: Thermo/Neomarkers | 0 | 1 | 0 | 0 | - | - |
| rmAb clone EP17 | CK 8 | 3 | Epitomics | 3 | 0 | 0 | 0 | - | - |

| | | | | | | | | | |
|---|----------|-----|---------------------|------|------|------|-----|-------|-------|
| mAb clone EP1628Y | CK 8 | 1 | Epitomics | 1 | 0 | 0 | 0 | - | - |
| Ready-To-Use Antibodies | | | | | | | | | |
| mAb clone 5D3 PM056 | CK 8/18 | 1 | Biocare | 0 | 1 | 0 | 0 | - | - |
| mAb clone 5D3 PA0067 | CK 8/18 | 6 | Leica/Novocastra | 5 | 1 | 0 | 0 | 100 % | 100 % |
| mAb clone 5D3 RTU-5D3 | CK 8/18 | 2 | Leica/Novocastra | 2 | 0 | 0 | 0 | - | - |
| mAb clone 35betaH11 760-2637 | CK 8 | 5 | Ventana/Cell Marque | 0 | 0 | 2 | 3 | - | - |
| mAb clone 35betaH11 MON-RTU1075 | CK 8 | 1 | Monosan | 0 | 0 | 1 | 0 | - | - |
| mAb clones B22.1&B23.1 760-4344 | CK 8/18 | 17 | Ventana/Cell Marque | 15 | 2 | 0 | 0 | 100 % | 100 % |
| mAb clones B22.1&B23.1 MAD-001005QD | CK 8/18 | 1 | Master Diagnostica | 1 | 0 | 0 | 0 | - | - |
| mAb clone CAM5.2 790-4555 | CK 8 (7) | 2 | Ventana | 1 | 1 | 0 | 0 | - | - |
| mAb clone DC10 IR618 | CK 18 | 15 | Dako | 14 | 1 | 0 | 0 | 100 % | 100 % |
| Total | | 161 | | 78 | 46 | 28 | 9 | - | |
| Proportion | | | | 48 % | 29 % | 17 % | 6 % | 77 % | |

1) Proportion of sufficient stains (optimal or good),

2) Proportion of sufficient stains with optimal protocol settings only, see below.

* Claimed by Invitrogen/Zymed to be CK8

The following protocol parameters were central to obtain optimal staining:

Concentrated Antibodies

mAb clone **5D3**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/8)*, TRS pH 9 (Dako) (3/6), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (2/3), Tris-EDTA/EGTA pH 9 (2/3) or Citrate pH 6 (1/2) as retrieval buffer. The mAb was typically diluted in the range of 1:40-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings 18 of 19 (95 %) laboratories produced a sufficient staining (optimal or good).

* (number of optimal results/number of laboratories using this buffer)

mAb clone **C51**: Protocols with optimal results were based on HIER using TRS pH 9, 3-in-1 (Dako) (2/2) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50-1:100. Using these protocol settings 3 of 3 (100 %) laboratories produced an optimal staining (optimal or good).

mAb clone **CAM5.2**: The two protocols with optimal results were based on either proteolytic pre-treatment using Protease 1(Ventana) or a combined pre-treatment using proteolysis (Protease 3, Ventana) followed by HIER in Cell Conditioning 1 (CC1;BenchMark, Ventana). Using proteolysis as single pre-treatment, the Ab was used concentrated (1:1), whereas the Ab was used at a titre of 1:25 for the protocol based on the combined pre-treatment.

mAb clone **DC10**: Protocols with optimal results were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (1/2), TRS pH 9 (Dako) (1/3), CC1 (BenchMark, Ventana) (7/12), BERS 2 (Leica) (3/6), Diva Decloaker pH 6.2 (Biocare) (1/1) or Tris-EDTA/EGTA pH 9 (4/6) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 28 of 29 (97 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **TS1**: Protocols with optimal results were based on either HIER using BERS 2 (Leica) (3/3) or a combined pre-treatment based on proteolysis in Protease 3 (Ventana) and HIER using CC1 (BenchMark,

Ventana) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50-1:1.000 depending on the total sensitivity of the protocol employed. Using these protocol settings 4 of 4 (100 %) produced an optimal staining.

rmAb clone **EP17**: Protocols with optimal results were based on HIER using CC1 (BenchMark, Ventana) (3/3) as the retrieval buffer. The mAb was diluted 1:100.

rmAb clone **EP1628Y**: The protocol with an optimal result was based on HIER using TRS pH 9 (3-in-1) (Dako) (1/1) as the retrieval buffer. The mAb was diluted 1:600.

Table 2 summarizes the overall proportion of optimal staining results using the two most frequently used concentrated Abs and IHC stainer platforms.

Table 2. **Optimal results for CK-LMW using concentrated antibodies on the 3 main IHC systems***

| Concentrated antibodies | Dako | | Ventana | | Leica | |
|-------------------------|----------------------------|------------|----------------------|------------|----------------|------------|
| | Autostainer Link / Classic | | BenchMark XT / Ultra | | Bond III / Max | |
| | TRS pH 9.0 | TRS pH 6.1 | CC1 pH 8.5 | CC2 pH 6.0 | ER2 pH 9.0 | ER1 pH 6.0 |
| mAb clone 5D3 | 36 % 4/11** | - | 0 % 0/5 | - | 67 % 2/3 | - |
| mAb clone DC10 | 67 % 2/3 | - | 64 % 7/11 | - | 50 % 3/6 | - |

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms

** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use Antibodies

mAb clone **5D3** (prod. no. PA0067, Leica): Protocols with optimal results were based on HIER using BERS 1 or BERS 2 (Bond, Leica), 15-20 min incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 5 of 5 (100 %) laboratories produced an optimal staining.

mAb clone **B22.1&B23.1** (prod. no. 760-4344, Ventana/Cell Marque): Protocols with optimal results were based either on HIER using mild or standard CC1 or a combined pre-treatment with Protease 2 or 3 for 4 – 8 min (Ventana) followed by HIER in CC1 (mild), 16-32 min incubation of the primary Ab and iView (760-091), UltraView (760-500) or OptiView(760-700) as detection system. Using these protocol settings 16 of 16 (100 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **CAM5.2** (prod. no. 790-4555, Ventana): The protocol with an optimal result was based on proteolysis in Protease 1 (Ventana) for 8 min, 32 min incubation of the primary Ab and UltraView (760-500) as detection system. Using these protocol settings 1 of 1 (100 %) laboratory produced an optimal staining.

mAb clone **DC10** (prod. no. IR618, Dako): Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min at 95-99°C) and 20-30 min incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 15 of 15 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient staining were:

- Less successful antibodies (all 6 protocols based on the mAb clone 35BH11 gave an insufficient result)
- Less successful performance of the mAb clone 5D3 on the BenchMark platform (Ventana)
- Inappropriate epitope retrieval (e.g. enzymatic pre-treatment for the mAb clones 5D3)
- Too low concentration of the primary Ab.

In this assessment and in concordance with the previous CK-LMW assessments in NordiQC, the prevalent feature of an insufficient staining was a too weak or false negative reaction of the cells expected to be demonstrated. The majority of the laboratories were able to demonstrate CK-LMW in structures with high-level antigen expression as epithelial cells of the appendix and the bile ducts of the liver, whereas the demonstration of CK-LMW in structures with a reduced and low-level antigen expression such as the hepatocytes and the neoplastic cells of the neuroendocrine carcinoma was more difficult and only seen with appropriate protocol settings.

The choice of the primary Ab had a great impact on the pass rate, as the proportion of sufficient results varied between 0% (mAb clone 35BH11, n=6) and 98 % (mAb clone DC10, n= 47).

It was also observed that the choice of epitope retrieval method has to be tailored to the individual primary Ab. For the mAb clone 5D3 applied as a concentrate, all 6 of 6 protocols based on proteolytic pre-treatment gave an insufficient result. If HIER was performed as the epitope retrieval method and

otherwise similar protocol settings were applied, a pass rate of 95 % was seen, out of which 45 % was optimal. For unexplained reasons, the mAb clone 5D3 gave an insufficient result (5/5) on the BenchMark IHC platforms (Ventana) despite the protocols were based on HIER in CC1.

Otherwise identical protocols for the mAb clone 5D3 gave sufficient and optimal results on the corresponding IHC platforms from Leica and Dako.

In this context the vendors' data sheets for the mAb clone 5D3 give misleading guidelines concerning the epitope retrieval: Thermo Scientific / NeoMarkers and Biocare both recommend proteolysis as pre-treatment for the mAb clone 5D3, while Leica / Novocastra recommends HIER for the clone when sold as a Ready-To-Use format prod. no. PA0067, but proteolysis for the concentrated format!

The impact of the choice of primary Ab and epitope retrieval is illustrated in table 3, where the cumulated data for the 4 most widely used clones in the last five assessments for CK-LMW is listed. Note, e.g., the over-all pass rate of 62 % for the mAb clone 5D3, compared to 81 % when HIER was applied and 11 % when protease was used.

Table 3. **Pass rates for four CK-LMW clones using different epitope retrieval methods**

| Pass rate for run 16, 20, 25, 33 & 38 | | | | | | | | |
|---------------------------------------|-----------|------------|-----------|------------|-------------------|------------|--------------------|------------|
| | Total | | HIER | | Prot. pre-treatm. | | HIER + proteolysis | |
| | Protocols | Sufficient | Protocols | Sufficient | Protocols | Sufficient | Protocols | Sufficient |
| mAb clone CAM 5.2 | 126 | 56 (44 %) | 41 | 14 (34 %) | 66 | 39 (59 %) | 9 | 3 (33 %) |
| mAb clone DC10 | 159 | 151 (95 %) | 158 | 149 (95 %) | 0 | 0 | 2 | 2 (100 %) |
| mAb clone 5D3 | 107 | 66 (62 %) | 80 | 65 (81 %) | 27 | 3 (11 %) | 0 | 0 |
| mAb clone 35BH11 | 54 | 6 (11 %) | 32 | 4 (13 %) | 22 | 2 (11 %) | 0 | 0 |

Controls

In this assessment and as observed in the previous NordiQC assessments, liver is a recommendable positive control for CK-LMW. Virtually all the hepatocytes must show an at least moderate predominantly membranous staining reaction, while the epithelial cells lining the bile ducts must show a strong cytoplasmic staining reaction. No staining should be seen in the connective tissue and lymphocytes in the portal rooms. Appendix cannot be recommended as positive control as the epithelial cells express a high concentration of CK-LMW and thus cannot be used to calibrate the protocol for CK-LMW to demonstrate CK-LMW in low antigen expressing cells and neoplasias.

Basal squamous cells in the esophagus will display a weak to moderate cytoplasmic staining reaction if using Abs towards CK8 as e.g. the mAb clone EP17.

Performance history

This was the 6th assessment of CK-LMW in NordiQC. A small increase in the pass rates was seen compared to previous runs. Many factors may contribute to the improved proportion of sufficient results. The tailored recommendations given to the laboratories obtaining an insufficient result seem to have a positive impact. In run 33, 40 laboratories were given a tailored recommendation and subsequently submitted a staining in this run. 19 laboratories followed the recommendation of which 16 (84 %) improved to a sufficient result, while 21 laboratories did not change their protocol and only 5 of these obtained a sufficient result (24 %). Recommendations given to laboratories using the mAb clone 5D3 on the Ventana BenchMark platform were less successful.

The improved pass rate was also related to the high quality and extended use of the Ready-To-Use (RTU) systems for CK-LMW from the three main providers Ventana, Dako and Leica as the RTU systems from these companies in this assessment showed a pass-rate of 100 % thus being superior to in-house validated protocols for CK-LMW based on the same clones.

Table 4. **Proportion of sufficient results for CK-LMW in six NordiQC runs**

| | Run 9 2003 | Run 16 2006 | Run 20 2007 | Run 25 2009 | Run 33 2011 | Run 38 2013 |
|--------------------|------------|-------------|-------------|-------------|-------------|-------------|
| Participants, n= | 54 | 66 | 74 | 99 | 141 | 161 |
| Sufficient results | 57 % | 45 % | 67 % | 66 % | 64 % | 77 % |

Conclusion

The mAb clones **5D3**, **C51**, **DC10**, **B22.1&B23.1** and the mAb clones **EP17** and **EP1628Y** could all be used to obtain an optimal staining result for CK-LMW. The epitope retrieval and protocol settings have to be specifically tailored to each of the clones/cocktails. The mAb clone 5D3 gave an inferior performance on the BenchMark IHC platform (Ventana) and misleading data-sheets are still provided by the vendors for the concentrated format of this clone.

The Ready-To-Use formats for the mAb clones B22.1&B23.1 (Ventana), DC10 (Dako) and 5D3 (Leica) gave a pass rate of 100 %.

Liver is an appropriate positive control for CK-LMW: The majority of hepatocytes must show an at least

moderate staining with an enhancement along the cell membranes.



Fig. 1a
Optimal staining for CK LMW of the appendix using the mAb clone 5D3 for CK 8/18 optimally calibrated, HIER in an alkaline buffer and performed on the Autostainer Link stainer, Dako.
Virtually all the columnar epithelial cells show a strong cytoplasmic staining reaction, while no background staining is seen.
Also compare with Figs. 2a - 3a, same protocol.

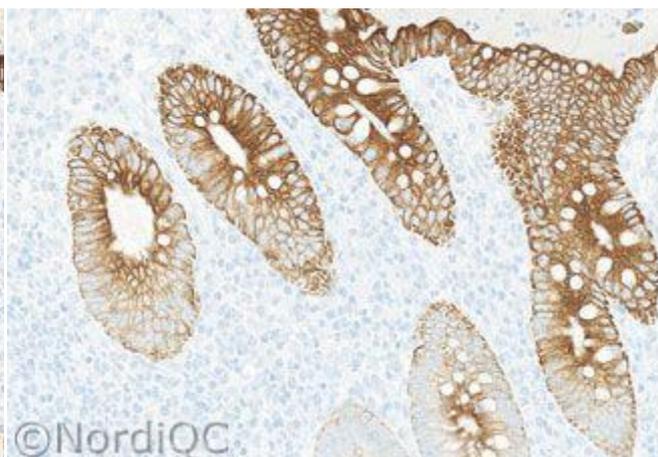


Fig. 1b
Insufficient staining for CK LMW of the appendix using the mAb clone 5D3 for CK 8/18, HIER in an alkaline buffer and performed on the BenchMark ULTRA stainer, Ventana – same field as in Fig. 1a. The mAb clone 5D3 gave same insufficient staining result by all protocol settings used on the BenchMark stainers.
Only the luminal columnar epithelial cells show a moderate to strong cytoplasmic staining, while virtually no staining is seen in the basal part of the crypts.
Also compare with Figs. 2b - 3b, same protocol.

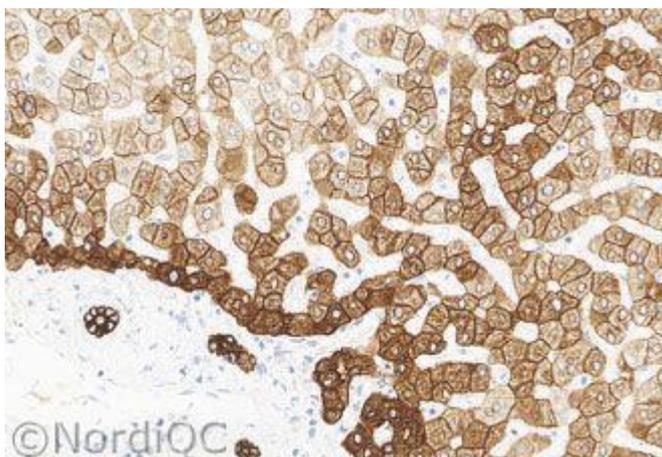


Fig. 2a
Optimal staining for CK LMW of the liver using the same protocol as in Fig. 1a.
The majority of the hepatocytes show a distinct, moderate staining reaction with a membrane enhancement, while the columnar epithelial cells of the bile ducts show a strong cytoplasmic staining reaction.
Same protocol used in Figs. 1a - 3a.

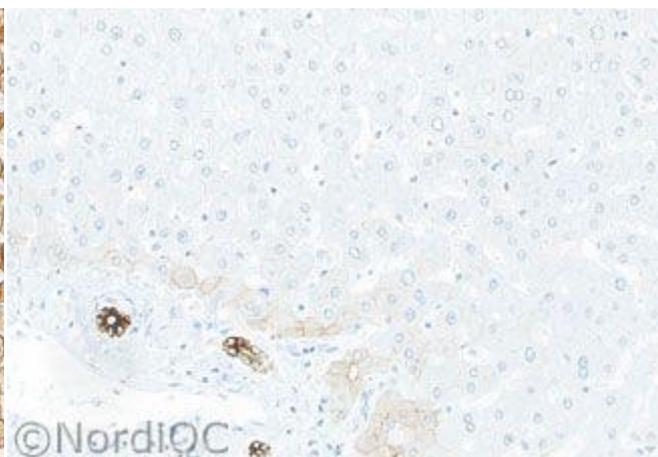


Fig. 2b
Insufficient staining for CK LMW of the liver using the same protocol as in Fig. 1b - same field as in Fig. 2a.
Only the bile duct epithelial cells are demonstrated, while the hepatocytes are almost negative.
Same protocol used in Figs. 1b - 3b.

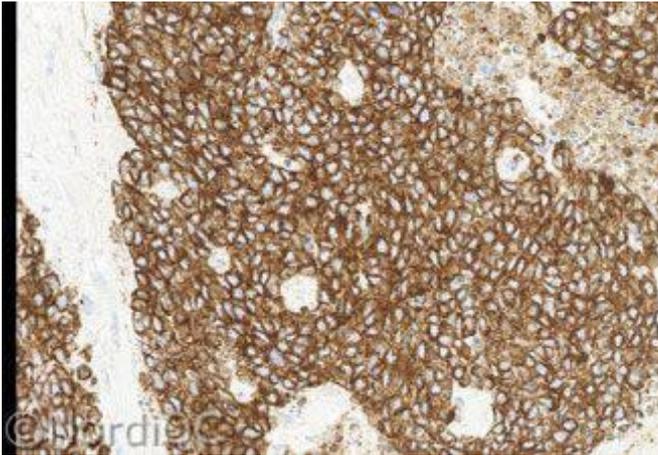


Fig. 3a
 Optimal staining for CK-LMW of the colon neuroendocrine carcinoma. Virtually all the neoplastic cells show a distinct, moderate to strong cytoplasmic staining reaction. No background staining is seen – necrotic cells are stained.
 Same protocol used in Figs. 1a - 3a.

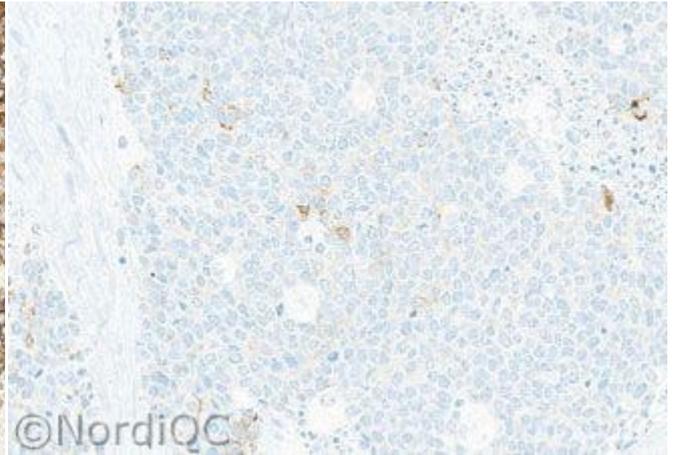


Fig. 3b
 Insufficient staining for CK LMW of the colon neuroendocrine carcinoma - same field as in Fig. 3a. Only scattered neoplastic cells show a weak and diffuse staining reaction.
 Same protocol used in Figs. 1b - 3b.

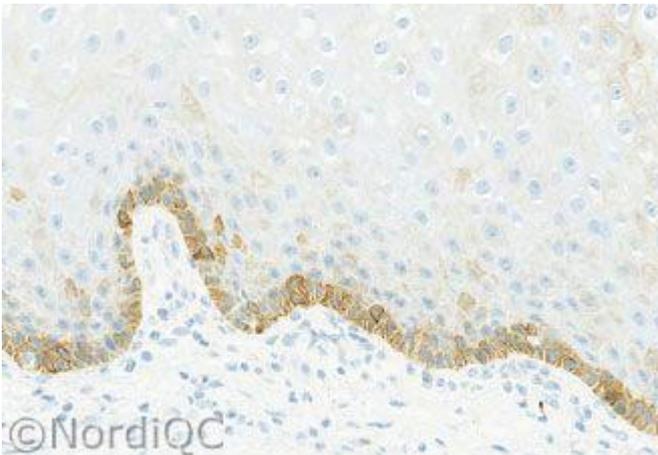


Fig. 4a
 Optimal staining for CK-LMW of the esophagus using the mAb clone EP17 for CK8. Virtually all the basal squamous epithelial cells show a moderate cytoplasmic staining reaction, while only dispersed intermediate squamous epithelial cells show a faint staining reaction.
 Compare with Fig. 4b using the mAb clone DC10 for CK18.

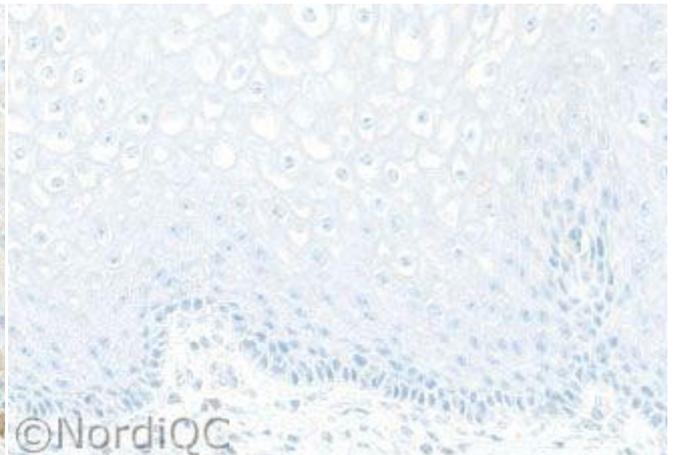


Fig. 4b
 Optimal staining for CK-LMW of the esophagus using the mAb clone DC10 for CK18. No staining reaction is seen in the basal squamous epithelial cells.

SN/RR/LE 13-6-2013