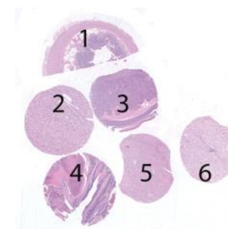
Assessment Run 45 2015

Epithelial cell-cell adhesion molecule (Ep-CAM)

Material

The slide to be stained for Ep-CAM comprised:



1. Appendix, 2. Kidney, 3. Basal cell carcinoma, 4. Colon adenocarcinoma,
5-6. Renal cell carcinoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing Ep-CAM staining as optimal included:

- A strong and distinct, predominantly membranous, staining reaction of virtually all columnar epithelial cells in the appendix.
- A moderate to strong, predominantly membranous, staining reaction of virtually all epithelial cells in the renal collecting tubules.
- An at least weak, predominantly basolateral, staining reaction of epithelial cells in the proximal tubules and membranous staining of epithelial cells lining the Bowman capsule in the kidney.
- A moderate to strong and distinct, predominantly membranous, staining of virtually all the neoplastic cells in the basal cell carcinoma and colon adenocarcinoma.
- An at least weak but distinct, predominantly membranous, staining reaction of dispersed (>10%) neoplastic cells in the two renal cell carcinomas.

Participation

Number of laboratories registered for Ep-CAM, run 45	213
Number of laboratories returning slides	195 (92%)

Results

195 laboratories participated in this assessment. Three participants used an inappropriate Ab such as mAb clone CAM5.2 for low molecular weight cytokeratin. Of the remaining 192 laboratories, 43% achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:

- Less successful performance of mAb clone Ber-EP4 on BenchMark and BOND IHC platforms.
- Proteolytic pre-treatment
- Too low concentration of the primary Ab
- Use of low sensitive detection systems

Performance history

This was the fourth NordiQC assessment of Ep-CAM and, as shown in table 2, the pass rates of the two latest runs were at a virtual identical low level.

Table 2: **Proportion of sufficient results for Ep-CAM in the four NordiQC runs performed**

	Run 17 2006	Run 23 2008	Run 32 2011	Run 45 2015
Participants, n=	74	78	141	192
Sufficient results	54%	63%	45%	43%

Conclusion

The mAb clones **BS14**, **Ber-EP4**, **MOC31** and **VU-1D9** could all be used to obtain an optimal staining result. For the most widely used Ab for Ep-CAM mAb clone Ber-EP4, HIER in special formulated buffers as TRS low pH 6.1 (Dako) and Diva pH 6 (Biocare) provided the highest proportion of sufficient and optimal results. The mAb clones BS14, MOC31 and VU-1D9 could provide an optimal result using HIER in standard HIER buffers. Use of sensitive 3-step polymer/multimer detection systems were for all clones within laboratory developed assay superior to 2-step systems. The Dako RTU systems based on mAb clone Ber-EP4 were the most successful assays for Ep-CAM. Kidney and tonsil are recommendable as positive and negative tissue controls for Ep-CAM. In kidney, virtually all epithelial cells lining the collecting tubules must

show a moderate to strong, predominantly membranous, staining reaction, whereas an at least weak, predominantly basolateral, staining reaction must be seen in the majority of epithelial cells in the proximal tubules and also in scattered epithelial cells lining the Bowman capsule. In tonsil, no staining reaction should be seen in lymphocytes or smooth muscle cells in vessels and only dispersed squamous epithelial cells should be demonstrated.

Table 1. **Antibodies and assessment marks for Ep-CAM, run 45**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **9C4**	1	BioLegend	0	0	0	1	-	-
mAb clone **BS14**	2	Nordic Biosite	2	0	0	0	-	-
mAb clone **C-10**	1	Santa Cruz Biotech	0	0	1	0	-	-
mAb clone **Ber-Ep4**	77 2 2	Dako Diagnostic BioSystems Thermo/NeoMarkers	9	16	38	18	31%	89%
mAb clone **MOC-31**	19 3 1 1	Dako Leica/Novocastra Cell Marque Monosan	9	6	6	3	63%	100%
mAb clone **VU-1D9**	3 3 1 1	Novocastra Thermo/LabVision Merck Millipore Thermo/Pierce	3	3	2	0	75%	75%
rmAb clone **E144**	1	Abcam	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **Ber-Ep4 760-4383**	36	Ventana/Cell Marque	0	6	21	9	17%	-
mAb clone **Ber-Ep4 IR/IS637**	19	Dako	4	12	1	2	84%	100%
mAb clone **Ber-Ep4 GA637**	9	Dako	7	1	1	0	89%	100%
mAb **Ber-Ep4 PM107**	1	Biocare	0	0	0	1	-	-
mAb **Ber-Ep4 MAD-001709QD**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone **Ber-Ep4 MON-RTU1096**	1	Monosan	0	0	1	0	-	-
mAb clone **MOC-31 790-4561**	3	Ventana	0	1	2	0	-	-
mAb clone **MOC-31 248M-18**	1	Cell Marque	0	0	1	0	-	-
mAb clone **MOC-31 PA0797**	1	Leica/Novocastra	0	1	0	0		
mAb clone **MOC-31 MAB-0280**	1	Maixin	0	1	0	0	-	-
mAb clone **VU-1D9**	1	Unknown	0	0	1	0		
Total	192		34	47	76	35	-	
Proportion			18%	25%	39%	18%	43%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of Ep-CAM, Run 45
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **BS14**: Two protocols provided optimal results.
One protocol was based on HIER using Tris-EDTA pH 9 as retrieval buffer, dilution of 1:100 of the primary Ab and a 3-step polymer based detection kit (FLEX+, Dako). The other protocol was based on HIER in Cell Conditioning 1 (CC1, Ventana) followed by enzymatic pre-treatment with Protease 3 (Ventana), dilution of

1:100 of the primary Ab and a 3-step multimer based detection kit (OptiView, Ventana). Using these protocol settings 2 of 2 (100%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **Ber-Ep4**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/2)*, TRS pH 6.1 (Dako) (6/17) or DIVA Decloaker pH 6 (Biocare) (1/3) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 16 of 18 (89%) laboratories produced a sufficient staining result.

mAb clone **MOC-31**: Protocols with optimal results were typically based on HIER using TRS pH 6.1 (Dako) (7/9), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/1) or Tris-EDTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:100. Using these protocol settings 11 of 11 (100%) laboratories produced a sufficient staining result.

mAb clone **VU-1D9**: Two protocols with optimal results were both based on HIER using Cell Conditioning 1 (CC1, Ventana) (3/5) as retrieval buffer. The mAb was diluted in the range of 1:80-500. Using these protocol settings 3 of 4 (75%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for Ep-CAM for the most commonly used antibody as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica 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 **Ber-EP4**	2/2	6/13** (43%)	0/21 (0%)	0/2	0/1	0/2

* 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)

Ready-To-Use antibodies and corresponding systems

mAb clone **Ber-Ep4**, product no. **IS637/IR637**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 6.1 (efficient heating time 10-20 min. at 96-99°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings 8 of 8 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **Ber-Ep4**, product no. **GA637**, Dako, Dako Omnis:
Protocols with optimal results were based on HIER using TRS pH 6.1 (efficient heating time 30 min. at 97°C), 30 min. incubation of the primary Ab and EnVision FLEX+ (GV800+GV821) as detection system. Using these protocol settings 8 of 8 (100%) laboratories produced a sufficient staining result (optimal or good).

Comments

In concordance with the previous NordiQC assessments for Ep-CAM, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells and structures expected to be demonstrated. Virtually all participating laboratories were able to stain Ep-CAM in high-level antigen expressing cells as columnar epithelial cells of appendix, neoplastic cells in the basal cell carcinoma and colon adenocarcinoma, whereas demonstration of Ep-CAM in neoplastic cells of the two renal clear cell carcinomas was more challenging and only seen when appropriate protocol settings were applied. Too weak or false negative staining result was seen in 98% of the insufficient results (109 of 111).

mAb clone Ber-EP4 was the most widely used antibody for the demonstration of Ep-CAM. Using this Ab within a laboratory developed (LD) assay, it was observed that the proportion of sufficient results was highly influenced by the pre-treatment conditions and the IHC platform used. First of all, the pass rate was significantly lower if proteolytic pre-treatment was used compared to HIER. If proteolytic pre-treatment was used, only 2 of 22 protocols (9%) were assessed as sufficient and none of these were optimal. If HIER was applied, 22 of 56 protocols (39%) were assessed as sufficient, of which 9 (16%) were optimal. A significant difference in the overall performance for the mAb clone Ber-EP4 was also related to the specific HIER buffer used and thus indirectly to the IHC platform applied. When the protocol was performed on a Dako IHC platform as Autostainer Link 48 or Omnis using HIER in TRS low pH 6.1 (Dako), 10 of 13 protocols (77%) provided a sufficient staining result, of which 6 (46%) were optimal. Previous assessments have indicated that the performance and level of the chosen assays sensitivity for mAb clone Ber-EP4 is significantly improved if HIER is based on the special formulated buffers, TRS pH 6.1 (Dako)

and Diva pH 6.2 (Biocare) compared to HIER using other buffers. This inevitable has an impact on the performance for the demonstration of Ep-CAM on IHC platforms lacking the possibility to perform HIER in these special formulated buffers. Consequently, only 2 of 21 protocols (10%) based on HIER in CC1 on the fully automated platforms BenchMark XT or Ultra (Ventana) provided a sufficient result of which none were optimal, although applying similar protocol settings as titre range and a sensitive detection system comparable to other assays e.g. Dako systems.

The inferior performance of the mAb clone Ber-EP4 on IHC platforms without access to the special HIER buffers as listed above, makes it crucial to identify Abs providing the expected reaction pattern using standard HIER buffers available for these platforms. In this assessment it was observed that the mAb clones BS14, MOC31 and VU-1D9 might be alternatives to Ber-EP4, as all three Abs could provide an optimal staining result using protocols based on standard HIER buffers for the respective IHC platforms from BenchMark and BOND. Especially the newly launched mAb clone BS14 might be an option for the demonstration of Ep-CAM on BenchMark platforms. An optimal staining result was obtained on this platform using HIER in CC1 followed by proteolysis in P3 and OptiView as detection system. In support of this observation, the other protocol assessed as optimal was based on HIER in TRIS-EDTA pH 9 and thus may reflect that BS14 is an antibody reacting with an antigenic epitope being less critical to use of the special formulated HIER buffers. However, more studies are required to evaluate on the robustness and consistency of the performance of the mAb clone BS14.

Irrespective of the clone applied within a LD assay, the use of 3-step polymer/multimer based detection systems gave an increased number of sufficient results compared to 2-step systems. Using a 3-step system as EnVision FLEX+ (Dako) a pass rate of 50% (22 of 44 protocols) was seen compared to 31% (21 of 68 protocols) for 2-step systems as e.g. EnVision FLEX (Dako).

The Dako Ready-To-Use (RTU) systems IR/IS637 and GA637 for Autostainer and Omnis, respectively, were the most successful assays in this assessment, see table 1. For the RTU system, GA637, a pass rate of 100% was seen if the protocol was applied in concordance to the recommendations given in the official package insert, which in brief is based on HIER in TRS low pH 6.1 and a 3-step polymer based detection system. The IR/IS637 RTU system also provided a high proportion of sufficient results, but only a few were evaluated as optimal. Both the official protocol recommendations and laboratory modified protocols, typically adjusting HIER and/or Ab times, could be used to obtain sufficient and optimal results for the two RTU systems.

The pass rate for the Ventana RTU system based on mAb clone Ber-EP4 was 17% (6 of 36) and none were assessed as optimal. As for the LD assays, inaccessibility and use of the special formulated HIER buffers consequently seemed to influence the performance of the RTU system. No sufficient results were obtained using the recommended protocol settings as listed in the official package insert (Ab incubation for 16 min., HIER in CC1 32 min. and UltraView as detection system). Using a laboratory modified protocol based on HIER in CC1 for 16-32 min. followed by enzymatic pre-treatment in Protease 3 for 4 min., Ab incubation for 16-60 min. and OptiView as detection system, 3 of 3 protocols provided a sufficient result.

Controls

Kidney and tonsil are recommendable as positive and negative tissue controls for Ep-CAM. In kidney virtually all epithelial cells lining the collecting tubules must show a moderate to strong predominantly membranous staining reaction, whereas an at least weak predominantly basolateral staining reaction must be seen in the majority of epithelial cells in the proximal tubules and also in scattered epithelial cells lining the Bowman capsule. In tonsil, no staining reaction should be seen in lymphocytes or smooth muscle cells in vessels and only dispersed squamous epithelial cells should be demonstrated.

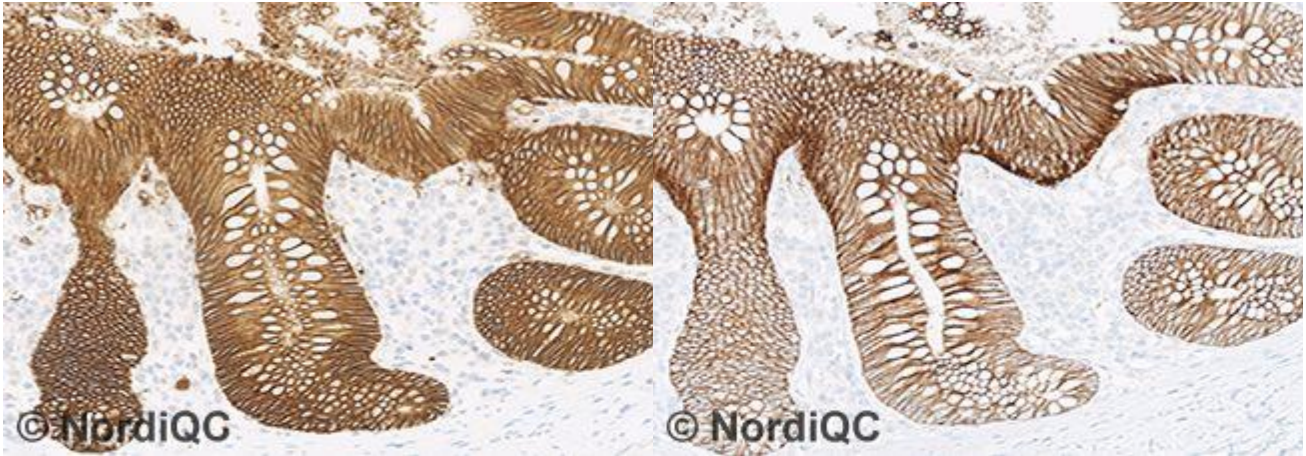


Fig. 1a
Optimal Ep-CAM staining of appendix using mAb clone Ber-EP4 diluted 1:50, HIER in TRS Low pH 6.1 (Dako) for 20 min. in PT-link, a 3-step polymer based detection kit and performed on Autostainer Link 48, Dako.
Virtually all columnar epithelial cells show a strong distinct predominantly membranous staining reaction. No background staining is observed. Few macrophages in lamina propria show an intracytoplasmic staining most likely due to uptake of epithelial cells.
Also compare with Figs. 2a – 4a, same protocol.

Fig. 1b
Ep-CAM staining of appendix using mAb clone Ber-EP4 with an insufficient protocol – same field as in Fig. 1a. The primary Ab was used at a titre of 1:50, HIER in CC1 pH 8.5 (Ventana) and a 2-step multimer based detection system providing a too low sensitivity.
Virtually all epithelial cells are demonstrated, though with a slightly reduced intensity.
However also compare with Figs. 2b - 4b – same protocol, indicating appendix cannot be recommended as positive tissue control for Ep-CAM due to a too high level of the antigen expression compared to the level seen in many carcinomas.

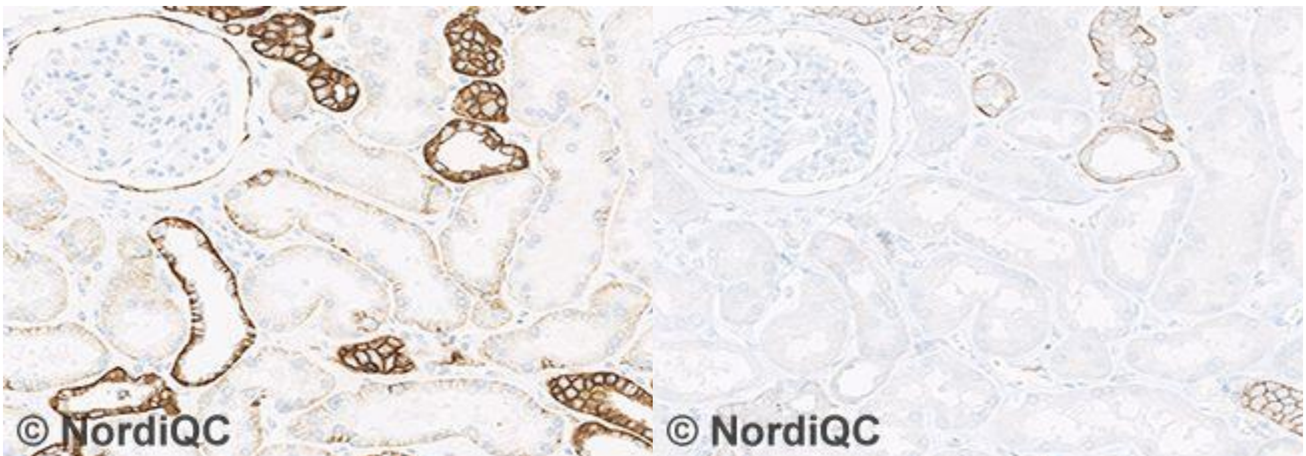


Fig. 2a
Optimal Ep-CAM staining of kidney using same protocol as in Fig. 1a.
The epithelial cells of the renal collecting tubules show a moderate to strong membranous staining reaction, while the epithelial cells of the Bowman capsule and proximal tubules only show a weak predominantly basolateral reaction.

Fig. 2b
Insufficient Ep-CAM staining of kidney using same protocol as in Fig. 1b - same field as in Fig. 2a.
Only the epithelial cells of the collecting tubules are demonstrated, whereas cells with reduced Ep-CAM expression are unstained.
Also compare with Figs. 3b and 4b – same protocol.

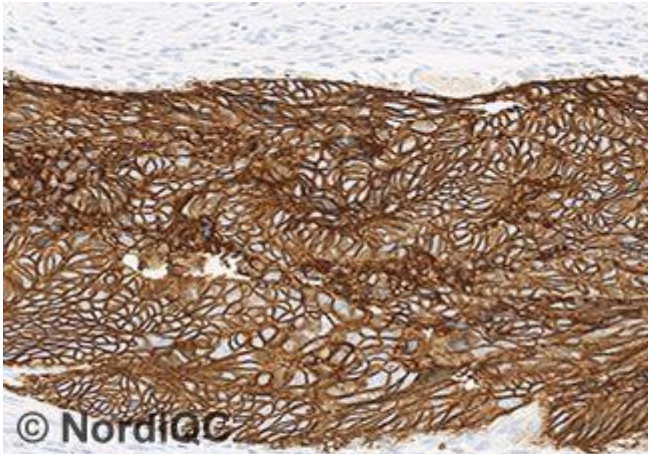


Fig. 3a
Optimal Ep-CAM staining of the colon adenocarcinoma using same protocol as in Figs. 1a and 2a.
Virtually all neoplastic cells show a strong predominantly membranous staining reaction.
No background staining is seen.

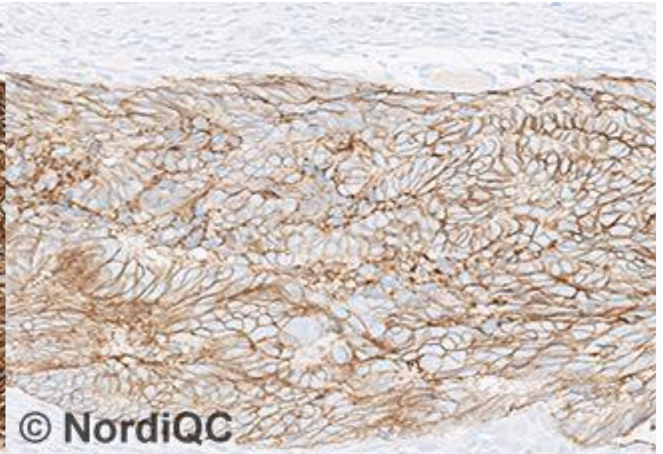


Fig. 3b
Ep-CAM staining of the colon adenocarcinoma using the same protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
Virtually all neoplastic cells are demonstrated, though with a reduced intensity.
However also compare with Fig 4b – same protocol.

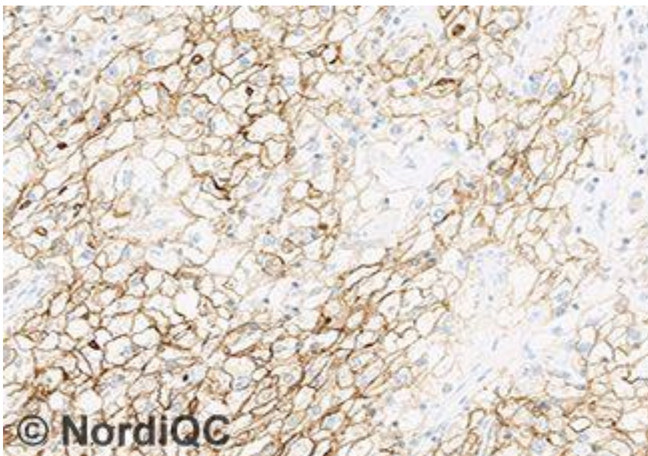


Fig. 4a
Optimal Ep-CAM staining of the renal clear cell carcinoma, tissue core no. 6 using same protocol as in Figs. 1a - 3a.
The majority of neoplastic cells show a moderate, distinct membranous staining reaction.

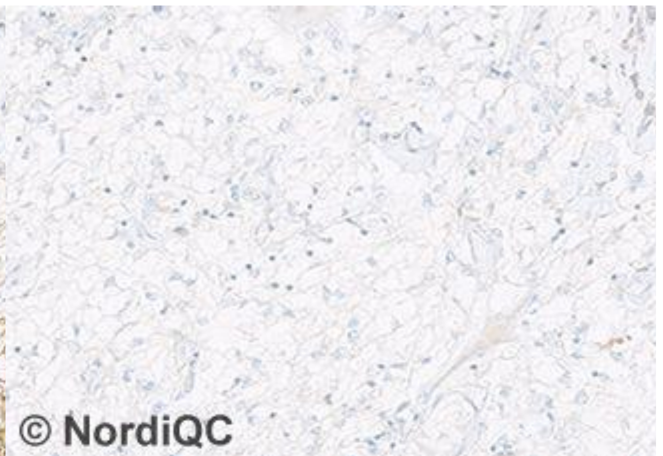


Fig. 4b
Insufficient Ep-CAM staining of the renal clear cell carcinoma, tissue core no. 6 using same protocol as in Figs. 1b – 3b – same field as in Fig. 4a.
All neoplastic cells are false negative. The protocol applied in Figs. 1b – 4b has only been calibrated to demonstrate Ep-CAM in cells/tissues with high level expression as columnar epithelial cells in appendix but not in cells with reduced expression, which in particular is seen in renal carcinomas.

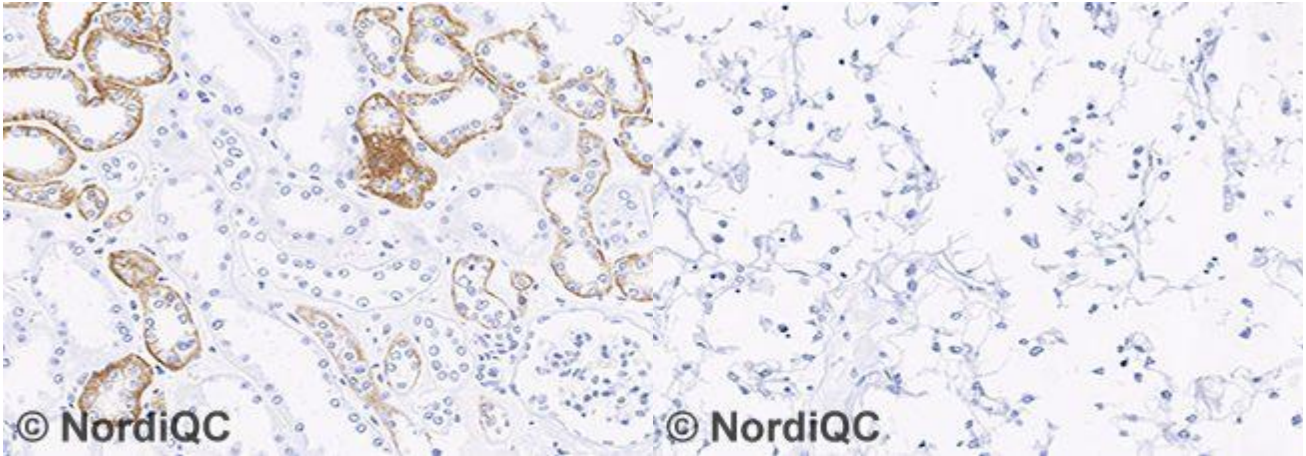


Fig. 5a
Staining for Ep-CAM of the normal kidney using the mAb clone Ber-EP4 with an insufficient protocol based on proteolytic pre-treatment.
Only epithelial cells of the collecting tubules are demonstrated while epithelial cells lining the proximal tubules and Bowman capsule are negative.
Also compare with Fig. 5b – same protocol.

Fig. 5b
Insufficient Ep-CAM staining of the renal clear cell carcinoma tissue core no. 6 using same protocol as in Fig. 5a.
The neoplastic cells are all false negative as the fragile membranes have been digested by the proteolytic pre-treatment.
HIER in TRS low pH 6.1 (Dako) and Diva pH 6 (Biocare) provided an increased sensitivity and at the same time an improved preservation of morphology compared to proteolysis – see Figs. 2a and 4a.

SN/LE/MV/RR 03.12.2015