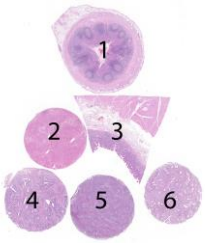
Assessment Run 47 2016

Cytokeratin 20 (CK20)

Material
The slide to be stained for CK20 comprised:



1. Appendix, 2. Liver, 3. Gastric corpus, 4. Colon adenocarcinoma, 5. Merkel cell carcinoma, 6. Urothelial carcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CK20 staining as optimal included:

- A strong, distinct cytoplasmic staining reaction of all surface epithelial cells in the appendix and an at least weak to moderate staining reaction in most crypt cells.
- An at least moderate, distinct cytoplasmic staining reaction of the vast majority of foveolar epithelial cells in the gastric mucosa.
- A moderate to strong, distinct cytoplasmic and dot-like staining reaction of virtually all neoplastic cells in the Merkel cell carcinoma.
- A weak to strong, distinct cytoplasmic staining reaction of the vast majority of neoplastic cells in the colon adenocarcinoma.
- An at least weak to moderate, distinct cytoplasmic staining reaction of the majority of neoplastic cells in the urothelial carcinoma.

Participation

Number of laboratories registered for CK20, run 47	304
Number of laboratories returning slides	284 (93%)

Results
284 laboratories participated in this assessment. 262 (92%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Insufficient HIER – too short efficient heating time and/or use of non-alkaline buffers for clone Ks20.8
- Unexplained technical issues

Performance history
This was the fourth NordiQC assessment of CK20. The pass rate increased compared to the previous runs as shown in table 2.

Table 2. **Proportion of sufficient results for CK20 in the four NordiQC runs performed**

	Run 8 2003	Run 25 2009	Run 35 2012	Run 47 2016
Participants, n=	71	130	195	284
Sufficient results	90%	64%	85%	92%

Conclusion
The mAb clone **Ks20.8** was the most widely used antibody for CK20 and provided a high pass rate and proportion of optimal results. As concentrated format within a laboratory developed (LD) assay, optimal results were obtained on all three main IHC platforms (Dako, Leica and Ventana). The newly introduced mAb clone **BS101** and rmAb clone **E19-1** also provided optimal results within LD assays. Irrespective of the clone, HIER was mandatory for an optimal result.
The Ready-To-Use systems for CK20 from Dako and Ventana, based on mAb clone **Ks20.8** and rmAb clone **SP33**, respectively, provided the highest proportion of sufficient and optimal results.
Appendix is recommended as positive tissue control for CK20. Virtually all luminal epithelial cells must show a strong cytoplasmic staining reaction, while the majority of crypt epithelial cells must show an at least weak cytoplasmic staining reaction. Liver can be used as negative tissue control in which no staining should be seen.

Table 1. **Antibodies and assessment marks for CK20, run 47**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BS101**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **Ks20.8**	97 11 5 5 2 2 1 1 1 1	Dako/Agilent Leica/Novocastra Cell Marque Thermo/Neomarkers EuroProxima Zeta Corporation Biocare DBS Euro Diagnostica PROGEN	55	58	13	0	90%	91%
rmAb clone **E19-1**	2	Immunologic	2	0	0	0	-	-
pAb **E16444**	2	Spring Bioscience	2	0	0	0	-	-
pAb **ILP 3202-C1**	1	Immunologic	1	0	0	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **Ks20.8 IR/IS777**	35	Dako/Agilent	31	4	0	0	100%	100%
mAb clone **Ks20.8 GA777**	19	Dako/Agilent	19	0	0	0	100%	100%
mAb clone **Ks20.8 PA0022**	10	Leica/Novocastra	6	3	1	0	90%	89%
mAb **Ks20.8 MAD-005105QD**	3	Master Diagnostica	2	0	1	0	-	-
mAb **Ks20.8 PM062**	1	Biocare	1	0	0	0	-	-
mAb clone **Ks20.8 E062**	1	Linaris	0	0	1	0	-	-
mAb clone **Ks20.8 Kit-0025**	1	Maixin	0	1	0	0	-	-
mAb clone **KS20.8 MON-RTU1083**	1	Monosan	0	0	1	0	-	-
mAb clone **PW31 PA0918**	1	Leica/Novocastra	0	1	0	0	-	-
rmAb clone **EPR1622Y AN557**	1	Biogenex	1	0	0	0	-	-
rmAb clone **SP33 790-4431**	78	Ventana/Roche	53	20	3	2	94%	99%
Total	284		175	87	20	2	-	
Proportion			62%	30%	7%	1%	92%	

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

Detailed analysis of CK20, Run 47

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb clone **BS101:** One protocol with an optimal result was based on heat induced epitope retrieval (HIER) using Tris-EDTA pH 9 as retrieval buffer, efficient heating time 20 min. at 98°C in PT module. The mAb was diluted 1:200, visualized by a 2-step polymer based detection system, Nordic Biosite, KDB-10007, and performed on a LabVision Autostainer.

mAb clone **Ks20.8**: Protocols with optimal results were based either on HIER, enzymatic pre-treatment or a combined pre-treatment.

With HIER, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (8/12)*, TRS pH 9 (4/9), Cell Conditioning 1 (CC1, Ventana) (16/50), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (15/18) or Tris-EDTA pH 9 (6/13) were used as retrieval buffer. The mAb was diluted in the range of 1:20-1:500. Using these protocol settings, 92 of 100 (92%) laboratories produced a sufficient staining result (optimal or good).
When using enzymatic pre-treatment, either Fast Enzyme for 5 min. at room temp. (Zytomed) (1/1) or Protease 1 for 8 min. at 36°C (Ventana) (3/11) were used. The mAb was diluted in the range of 1:50-1:400. Using these or comparable protocol settings, 8 of 10 (80%) laboratories produced a sufficient staining result.
One protocol used a combined pre-treatment with Protease 3 and CC1 (Ventana) (1/1). The mAb was diluted 1:50.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **E19-1**: Two protocols with optimal results were based on HIER using Citrate pH 6 as retrieval buffer, efficient heating time for 10-30 min. at 96-100°C. The rmAb was diluted 1:100, visualized by a 2-step polymer based detection system, Immunologic, DPVO-999HRP, and performed in a LabVision Autostainer or Tecan Freedom EVO stainer.

pAb **E16444**: Two protocols with optimal results were based on HIER using CC1 (Ventana) for 32 min. at 95°C or BORG Decloaker pH 9.5 (Biocare) for 5 min. at 110°C as retrieval buffer. The mAb was diluted in the range of 1:400-1:6,000 depending on the total sensitivity of the protocol employed.

pAb **ILP 3202-C1**: One protocol with an optimal result was based on HIER in CC1 (Ventana) for 72 min. at 95°C. The pAb was diluted 1:500, visualized by a 2-step multimer based detection system, UltraView (Ventana) and performed in a BenchMark Ultra (Ventana).

Table 3. **Proportion of optimal results for CK20 for the most commonly used antibody concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer / Omnis**		**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 **Ks20.8**	12/21** (57%)	0/2	16/48 (33%)	-	15/18 (83%)	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 **Ks20.8**, product no. **IS777/IR777**, Dako, Autostainer+/Autostainer Link:
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 95-98°C), 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 34 of 34 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **Ks20.8**, product no. **GA777**, Dako, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS pH 9 (efficient heating time 20-30 min. at 97°C), 20 min. incubation of the primary Ab and EnvisionFlex/FLEX+ (GV800/GV800+GV821) as detection system. Using these protocol settings, 19 of 19 (100%) laboratories produced a sufficient staining result.

mAb clone **Ks20.8** product no. **PA0022**, Leica/Novocastra, BOND III/MAX:
Protocols with optimal results were based on HIER using BERS2 (Bond Leica) (efficient heating time 20 min. at 99-100°C) and 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings 8 of 9 (89%) produced a sufficient staining result.

mAb clone **Ks20.8**, product no. **PM062**, Biocare, IntelliPATH
One protocol with an optimal result was based on HIER using Diva decloaker pH 6.2 in a Pressure Cooker (efficient heating time 15 min. at 110°C), 30 min. incubation of the primary Ab and MACH 4 Universal HRP-Polymer (MRH534) as detection system.

rmAb clone **SP33**, product no. **790-4431**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 32-90 min.) and 12-36 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings, 67 of 68 (99%) laboratories produced a sufficient staining result.

Comments
In this assessment and in concordance with the previous NordiQC assessments of CK20, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells and structures expected to be demonstrated. This pattern was observed in 91% of the insufficient results (20 of 22). In the remaining 9%, impaired morphology or false positive staining reaction was observed.
Virtually all laboratories were able to demonstrate CK20 in high level antigen expressing structures such as luminal epithelial cells in appendix and neoplastic cells of the colon adenocarcinoma and Merkel cell carcinoma. The demonstration of CK20 in low expressing structures as neoplastic cells of the urothelial carcinoma was more challenging and required a carefully calibrated protocol.

The mAb clone Ks20.8 was the most widely used antibody for demonstration of CK20 and provided optimal results on all three main IHC platforms from Dako, Leica and Ventana, respectively (see table 3). Used as a concentrate within a laboratory developed (LD) assay, mAb clone Ks20.8 gave an overall pass rate of 90% (113 of 126) of which 44% were optimal (see table 1). For mAb clone Ks20.8 the choice of epitope retrieval method influenced the pass rate and proportion of optimal results. HIER in an alkaline buffer was found to be more successful compared to enzymatic pre-treatment. Protocols based on HIER in alkaline buffer provided a pass rate of 92% (91 of 99) and 47% were assessed as optimal. If enzymatic pre-treatment was applied as retrieval method, the pass rate was 75% (9 of 12) and 25% optimal. Enzymatic pre-treatment seemed to result in slightly reduced sensitivity and simultaneously the morphology was frequently impaired due to excessive digestion of the cytoplasmic compartment of e.g. neoplastic cells in the Merkel cell carcinoma.

The relatively newly introduced Abs mAb clone BS101, rmAb clone E19-1 and pAb E16444 could all provide optimal results within LD assays. HIER and careful calibration of the primary Ab were the general prerequisites for the optimal results.

Ready-To-use (RTU) formats were used by 53% (151 of 284) of the laboratories.
The Ventana RTU system based on rmAb clone SP33 (**790-4431)** was the most widely used RTU system applied by 78 laboratories. Optimal results were obtained by protocol recommendations given by Ventana using HIER in CC1 for 64 min., 16 min. incubation of the primary Ab and UltraView as detection system. Laboratory modified protocol settings such as prolonged incubation time with the primary Ab and/or use of a more sensitive detection system as OptiView also provided a high proportion of optimal results. If the HIER time was reduced to 32 min. without changing other parameters to compensate for the lower sensitivity (e.g. by using OptiView as detection system) a reduced proportion of optimal results were observed.

The Dako RTU systems based on mAb clone Ks20.8 (IR/IS777 and GA777 for Autostainer and Omnis, respectively) were most successful providing an overall pass rate of 100%. Optimal results were obtained both by the Dako recommended protocol settings and by laboratory modified protocol settings adjusting incubation time with the primary Ab, HIER time and detection system.

A consistent improvement of the pass rate for CK20 has been observed in the three latest NordiQC assessments. This seems to be related to a harmonization of the protocols used within LD assays and extended use of high quality and precisely calibrated RTU systems from the main IHC system providers. For the mAb clone Ks20.8 within LD assays 19% of the protocols in run 25 were based on enzymatic pre-treatment, compared to only 9% in this run.
For the presently available RTU systems from the three main providers, Dako, Leica and Ventana grouped together a pass rate of 96% was obtained. Previously the now discontinued Ventana RTU system based on mAb Ks20.8 and the Leica RTU system based on mAb PW31 showed an inferior performance and were in this run replaced by new and improved RTU systems. In run 25, the Ventana RTU system based on Ks20.8 gave a pass rate of 50% compared to 94% for SP33 in this run. Similar observation is seen for the discontinued Leica RTU system based on PW31 giving a pass rate of 0% in run 35, compared to 90% for the newly launched system based on mAb clone Ks20.8.

Controls
It is difficult to identify a reliable and robust positive tissue control for CK20. At present, the best recommendation is still to use colon or appendix as control and to calibrate the protocol to give an intense staining reaction of virtually all the luminal epithelial cells with a high-level expression of CK20. In the crypts the majority of epithelial cells must show an at least weak to moderate cytoplasmic staining reaction. No staining reaction must be seen in non-epithelial cells in appendix or colon and can thusly serve as negative tissue control. Alternatively, liver can be used as negative tissue control for CK20. The negative tissue controls is primarily used to verify the signal-to-noise ratio of the CK20 assay.

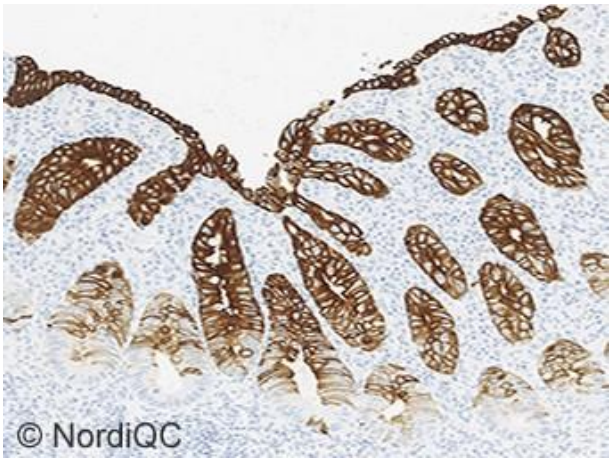


Fig. 1a
Optimal CK20 staining of the appendix using the mAb clone Ks20.8 diluted 1:100, HIER in TRS High pH 9 for 20 min., a 3-step polymer based detection kit (FLEX+) and performed on Autostainer Link, Dako.
Virtually all surface epithelial cells show a strong cytoplasmic staining reaction, while most crypt cells display an at least weak to moderate staining reaction.
No background reaction is seen.
Also compare with Figs. 2a – 5a, same protocol.

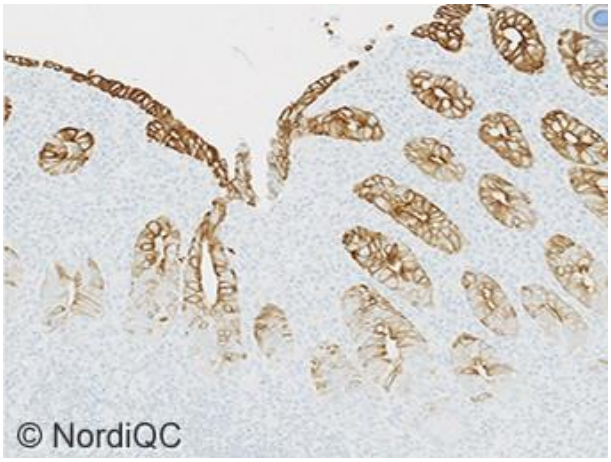


Fig. 1b
CK20 staining of the appendix using an insufficient protocol based on the mAb clone Ks20.8 diluted 1:200, HIER in CC1 pH 8.5 for 32 min., a 2-step multimer based detection kit (UltraView) and performed on BenchMark Ultra, Ventana.
The majority of epithelial cells are demonstrated but the intensity is significantly reduced. Compare with Fig. 1a – same field.
Also compare with Figs. 2b - 4b – same protocol

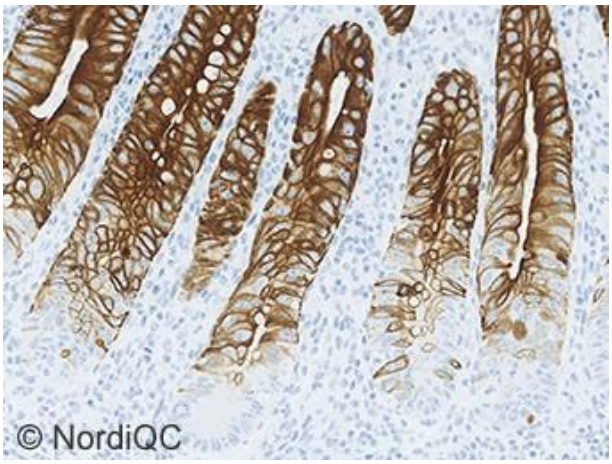


Fig. 2a
Optimal CK20 staining of the appendix using same protocol as in Fig. 1a, high power field x200.
Most crypt cells show a distinct, moderate cytoplasmic staining reaction.
No background reaction is seen.

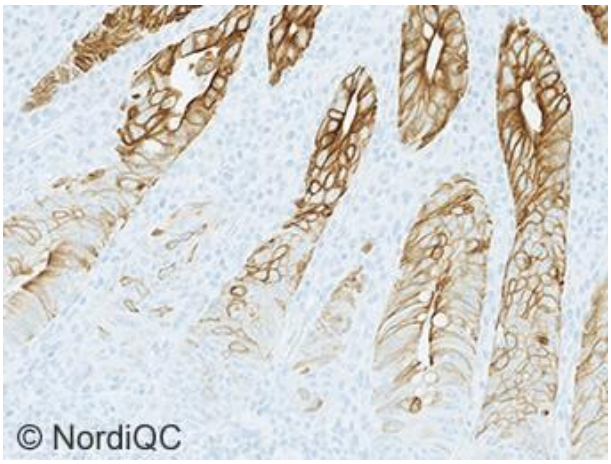


Fig. 2b
CK20 staining of the appendix using same protocol as in Fig. 1b – same field as in Fig. 2b, high power field x200.
The majority of crypt cells are demonstrated, but intensity is reduced compared to the level expected.
Also compare with Figs. 3b and 4b, same protocol.

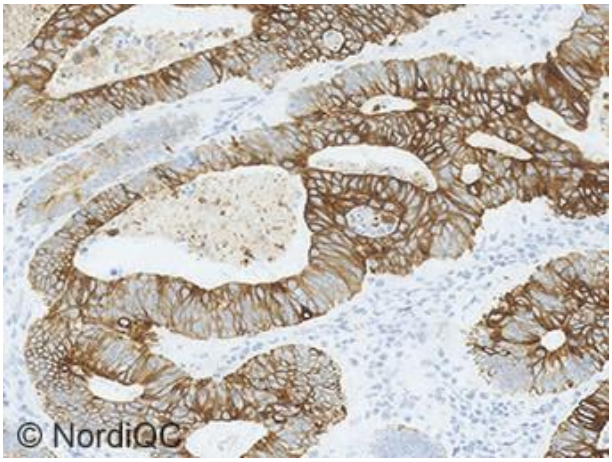


Fig. 3a
Optimal CK20 staining of the colon adenocarcinoma using same protocol as in Figs. 1a and 2a. The vast majority of neoplastic cells show a moderate cytoplasmic staining reaction.
No background reaction is seen.

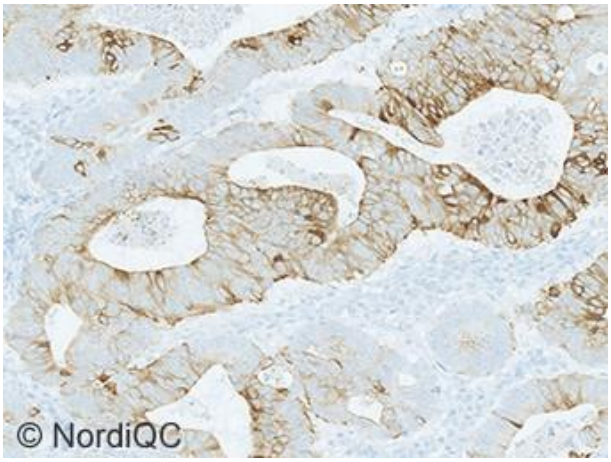


Fig. 3b
Insufficient CK20 staining of the colon adenocarcinoma using the same protocol as in Figs. 1b & 2b – same field as in Fig. 3b. The staining intensity and proportion of neoplastic cells is significantly reduced compared to the level expected and obtained in Fig. 3a.

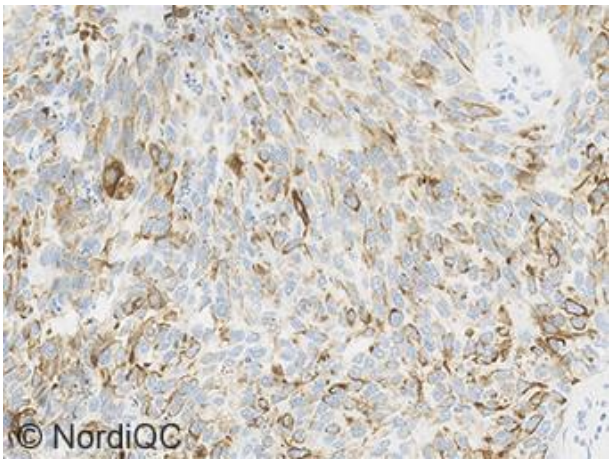


Fig. 4a
Optimal CK20 staining of the urothelial carcinoma using same protocol as in Figs. 1a - 3a. The majority of the neoplastic cells show a weak to moderate cytoplasmic staining reaction.
No background reaction is seen.

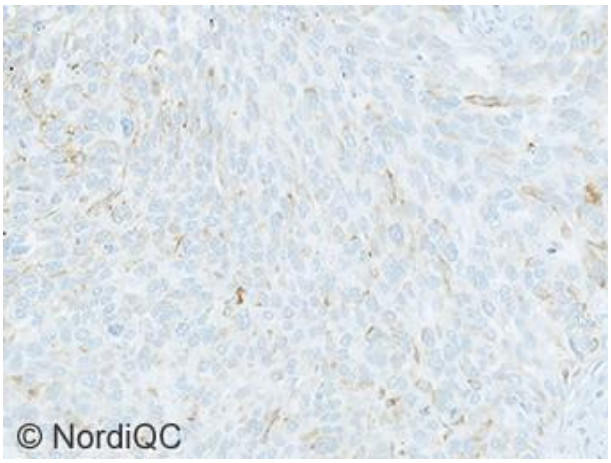


Fig. 4b
Insufficient CK20 staining of the urothelial carcionoma using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a. Only scattered neoplastic cells only show a weak and equivocal staining reaction.

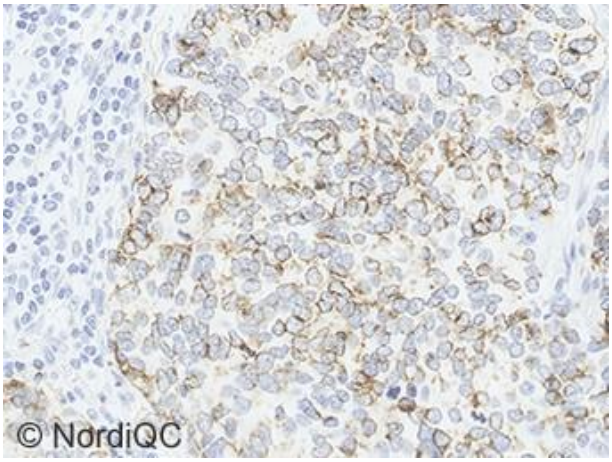


Fig. 5a
Optimal CK20 staining of the Merkel cell carcinoma using same protocol as in Figs. 1a - 4a. A moderate to strong, distinct cytoplasmic and dot-like staining reaction is seen in virtually all neoplastic cells.
The tissue architecture and morphology is preserved facilitating the interpretation. Compare with Fig. 5b and the result in same tumour using a protocol based on proteolytic pre-treatment.

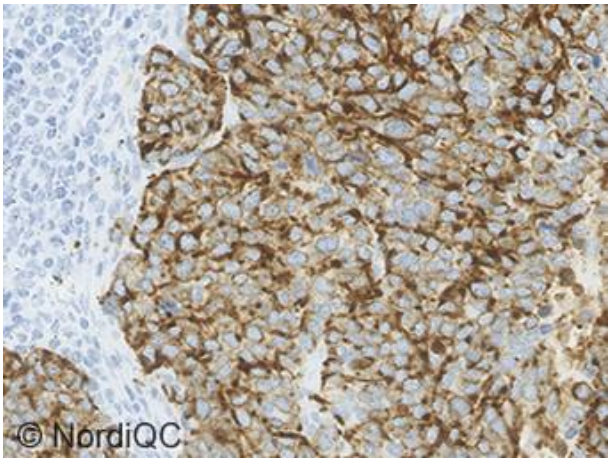


Fig. 5b
Insufficient CK20 staining of the Merkel cell carcinoma using the mAb clone Ks20.8 with a protocol based on proteolytic pre-treatment – same field as in Fig. 5a.
The morphology is heavily impaired as the cytoplasmic compartment have been digested by the enzymatic digestion. Only a partial staining reaction in few cells can be identified.
mAb clone Ks20.8 can be performed with both HIER and proteolytic pre-treatment, but HIER should be the preferred choice to secure an intact morphology and high analytical sensitivity. In this run, protocols based on HIER in an alkaline buffer provided a pass rate of 92% and 47% assessed as optimal, whereas proteolytic pre-treatment gave a pass rate of 75% and only 25% optimal marks.

SN/LE/MV/RR 11.07.2016