Assessment Run 62 2021
Cytokeratin 20 (CK20)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for cytokeratin 20 (CK20) used to identify carcinoma origin in the diagnostic work-up of cancer of unknown primary (CUP) origin. Relevant clinical tissues, both normal and neoplastic, were selected for a broad spectrum of antigen densities for CK20 (see below).

Material
The slide to be stained for CK20 comprised:

1. Tonsil, 2. Appendix, 3. Urothelial carcinoma, 4. Breast carcinoma, 5. Colon adenocarcinoma.

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.
- 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.
- No staining of the neoplastic cells of the breast carcinoma.
- No staining of the tonsil.

Participation

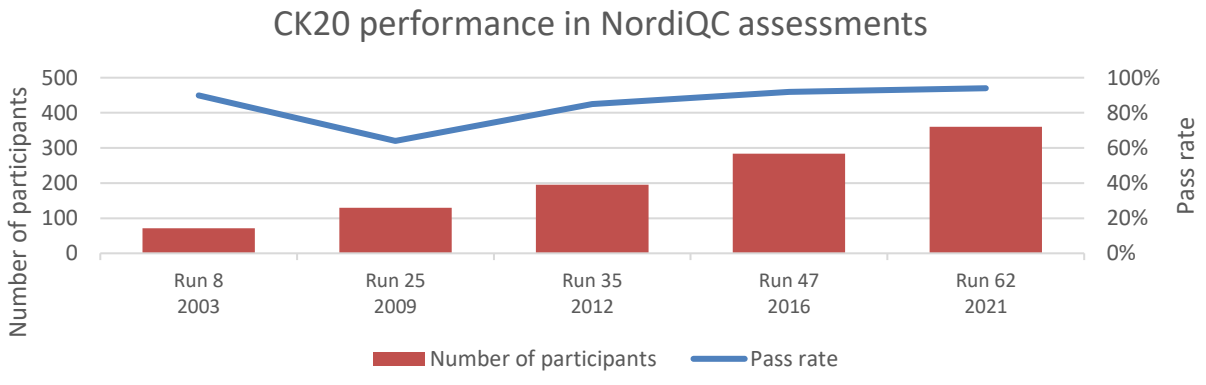
Number of laboratories registered for CK20, run 62	380
Number of laboratories returning slides	360 (95%)

Results
360 laboratories participated in this assessment. 338 (94%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 3). All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Use of proteolytic pretreatment
- Insufficient Heat Induced Epitope Retrieval (HIER) – too short efficient heating time and/or use of non-alkaline buffers for clone Ks20.8

Performance history
This was the fifth NordiQC assessment of CK20. The pass rate increased slightly compared to the previous runs as shown in Graph 1.

Graph 1. **Proportion of sufficient results for CK20 in the five NordiQC runs performed**



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 four main IHC platforms (Dako/Agilent, Leica Biosystems and Ventana/Roche). The mAb clones **BS101**, **ZM42** and pAb clone **E16444** also provided optimal results within LD assays. The Ready-To-Use (RTU) systems for CK20 from Dako/Agilent, Leica Biosystems and Ventana/Roche, 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. Tonsil can be used as negative tissue control in which no staining reaction should be seen.

Table 1. **Antibodies and assessment marks for CK20, run 62**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **BS101**	1	Nordic Biosite	1	-	-	-	-	-
mAb clone **Ks20.8**	69 18 10 2 1 1 1 1 1 2 2	Dako/Agilent Leica Biosystems Cell Marque Thermo/Neomarkers Zeta Corporation Biocare BIO SB DBS Euro Diagnostica PROGEN Quartett	65	29	13	1	87%	60%
mAb clone **ZM42**	1	Zeta Corporation	1	-	-	-	-	-
rmAb clone **EP23**	1	Epitomics	-	-	1	-	-	-
pAb clone **E16440**	1	Spring Bioscience	1	-	-	-	-	-
Ready-To-Use (RTU) antibodies							Suff.[1]	OR.[2]
rmAb clone **SP33 790-4431**[3]	19	Ventana/Roche	16	3	-	-	100%	84%
rmAb clone **SP33 790-4431**[4]	105	Ventana/Roche	89	14	2	-	98%	85%
mAb clone **Ks20.8 IR/IS777**[3]	18	Dako/Agilent	14	4	-	-	100%	78%
mAb clone **Ks20.8 IR/IS777**[4]	16	Dako/Agilent	12	3	1	-	94%	75%
mAb clone **Ks20.8 GA777**[3]	33	Dako/Agilent	31	2	-	-	100%	94%
mAb clone **Ks20.8 GA777**[4]	27	Dako/Agilent	19	7	1	-	96%	70%
mAb clone **Ks20.8 PA0022**[3]	5	Leica Biosystems	4	1	-	-	100%	80%
mAb clone **Ks20.8 PA0022**[4]	10	Leica Biosystems	7	3	-	-	100%	70%
mAb clone **Ks20.8 8304-C010**	4	Sakura Finetek	1	3	-	-	-	-
mAb **Ks20.8 MAD-005105QD**	2	Master Diagnostica	1	1	-	-	-	-
mAb **Ks20.8 PM062/IP062G10**	2	Biocare	-	1	1	-	-	-
mAb **Ks20.8 320M-10**	1	Cell Marque	1	-	-	-	-	-
mAb clone **Ks20.8 E062**	1	Linaris	-	-	1	-	-	-
clone **MX059 MAB-0834**	2	Fuzhou Maixin	2	-	-	-	-	-
mAb clone **KS20.8 MAD-005105**	1	Vitro SA	-	1	-	-	-	-
rmAb clone **KS20.8 CCM-1113**	1	Celnovte	1	-	-	-	-	-
rmAb clone **IHC220**	1	GenomeMe	-	-	1	-	-	-
Total	360		266	72	21	1		
Proportion			74%	20%	6%	-	94%	

1) Proportion of sufficient stains (optimal or good). (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).

Detailed analysis of CK20, Run 62
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **Ks20.8**: Protocols with optimal results were based either on HIER, enzymatic pre-treatment or a combined pre-treatment.
Using HIER, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (2/5)*, TRS pH 6,1 (3-in-1) (Dako/Agilent) (1/1), Cell Conditioning 1 (CC1, Ventana/Roche) (33/44), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (17/21), Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/7) or Tris-EDTA pH 9 (1/3) were used as retrieval buffer. The mAb was diluted in the range of 1:50-1:800. Using these protocol settings, 72 of 85 (85%) laboratories produced a sufficient staining result (optimal or good).
Enzymatic pre-treatment was used for 4-8 min at 36°C (3/12) with Protease 1 (Ventana/Roche). The mAb was diluted in the range of 1:50-1:200. Using these or comparable protocol settings, 11 of 12 (92%) laboratories produced a sufficient staining result.
One protocol used a combined pre-treatment with Protease 3 and CC1 (Ventana/Roche). The mAb was diluted 1:50.
* (number of optimal results/number of laboratories using this HIER buffer)

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

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark GX / XT / Ultra**			**Leica Biosystems Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	Enzyme P1	ER2 pH 9.0	ER1 pH 6.0
mAb clone **Ks20.8**	2/5 (40%)	1/1	5/7 (71%)	-	33/44 (75%)	-	3/12 (25%)	17/21 (81%)	1/7 (14%)

* 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 Tris-EDTA/EGTA pH 9 (efficient heating time 10-40 min. at 90-99°C), 18-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 29 of 30 (97%) 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), 12-30 min. incubation of the primary Ab and Envision FLEX/FLEX+ (GV800/GV800+GV821) as detection system. Using these protocol settings, 59 of 59 (100%) laboratories produced a sufficient staining result.

mAb clone **Ks20.8** product no. **PA0022**, Leica Biosystems, BOND III/MAX:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 10-30 min. at 97-100°C), 8-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings 13 of 13 (100%) produced a sufficient staining result.

mAb clone **Ks20.8** product no. **8304-C010**, Sakura Finetek, Sakura Genie:
One protocol with an optimal result was based on HIER in Sakura Finetek High pH Antigen Retrieval solution (efficient heating time 45 min. at 98°C) and 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro kit 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 CC1 (efficient heating time 16-90 min.) and 8-60 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit (760-080) or OptiView (760-700) were used as detection systems. Using these protocol settings, 117 of 118 (99%) laboratories produced a sufficient staining result.

Table 4. **Proportion of sufficient and optimal results for CK20 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb Ks20.8 **IR/IS777**	100% (18/18)	78% (14/18)	94% (15/16)	75% (12/16)
Dako Omnis mAb Ks20.8 **GA777**	100% (33/33)	94% (31/33)	96% (26/27)	70% (19/27)
Leica BOND mAb Ks20.8 **PA0022**	100% (5/5)	80% (4/5)	100% (10/10)	70% (7/10)
VMS Ultra/XT/GX rmAb SP33 **790-4431**	100% (19/19)	84% (16/19)	98% (103/105)	85% (89/105)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer were included.

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 86% of the insufficient results (19 of 22). In the remaining 14%, 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. 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 four IHC platforms from Dako/Agilent, Leica Biosystems and Ventana/Roche, respectively (see Table 3). Used as a concentrate within a laboratory developed (LD) assay, the Ab. gave an overall pass rate of 87% (94 of 108) of which 60% were optimal (see table 1). 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 90% (74 of 82) and 73% were assessed as optimal. If enzymatic pre-treatment was applied as retrieval method, the pass rate was 82% (14 of 17) and 23% optimal. Enzymatic pre-treatment seemed in some cases to increase the intensity of the neoplastic cells of the urothelial carcinoma. However it resulted overall in a slightly reduced analytical sensitivity and simultaneously the morphology was frequently impaired due to excessive digestion of the cytoplasmic compartment of e.g. neoplastic cells in the urothelial carcinoma.
In addition, choice and level of sensitivity for the detection systems also impacted the proportion of optimal results.
This was e.g. seen for the participants using the Ventana Benchmark IHC platform, where 81% (22/27) of protocols based on OptiView as detection system gave an optimal result, compared to 65% (11/17) if UltraView was applied.

The Abs mAb clones BS101, clone ZM42 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.

RTU formats were used by 69% (248 of 360) of the laboratories.
The Ventana/Roche RTU system based on rmAb clone SP33, **790-4431** was the most widely used RTU system applied by 124 laboratories. Optimal results were obtained by protocol recommendations given by Ventana/Roche using HIER in CC1 for 64 min., 16 min. incubation of the primary Ab and UltraView as detection system. Only 19 laboratories used the recommended protocol settings giving a pass-rate of 100%, 84% optimal.
105 laboratories modified the protocol settings such as prolonged incubation time of the primary Ab and/or use of a more sensitive detection system as OptiView. This gave a very similar result with a pass rate of 98%, 85% optimal (see Figs. 1a-3a).

The Dako/Agilent RTU system based on mAb clone Ks20.8, **GA777** for Dako Omnis was the second most popular RTU system being applied by a total of 60 participants. 33 laboratories used the RTU format with the vendor recommended protocol using HIER in TRS high for 30 min., 20 min. incubation of the primary antibody and EnVision FLEX+ with mouse linker for 10 min. as detection system. With the vendor recommended protocol 100% of the laboratories received a sufficient result, 94% optimal. 27 laboratories

modified the protocol primarily by omission of the mouse linker and/or changing the incubation time of the primary Ab. These protocol modifications still gave a very high pass rate of 96% but reduced the proportion of optimal results to 70%. The modifications typically reduced the total analytical sensitivity of the protocol providing a weaker intensity and proportion of cells demonstrated. This was especially seen in the neoplastic cells of the urothelial carcinoma and epithelial cells of crypts in the appendix (Figs 1b-3b).

34 Laboratories used the Dako/Agilent RTU system of mAb clone Ks20.8, **IR/IS777** for the Dako Autostainer with very similar results compared to the corresponding GA777 format. Optimal results could be obtained by using both vendor recommended, and laboratory modified protocols. The typical modification being a change of the primary Ab incubation time but without any significant impact on the pass rate.

In total 15 laboratories used the Leica Biosystems RTU system **PA0022** based on mAb clone Ks20.8. Overall a pass rate of 100% was obtained and as shown in both Table 1 and 4 virtually same performance was observed for protocols based on the vendor recommended and laboratory modified settings. It was observed that HIER in low pH, BERS 1, being used by 2 participants, provided a reduced analytical sensitivity compared to the level obtained by HIER in high pH, BERS2 as recommended by Leica Biosystems. Data however to be interpreted with caution due to few observations.

A consistent improvement of the pass rate for CK20 has been observed in the past two 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. Concerning harmonization of protocols for CK20, the increased use of HIER on the expense of proteolysis as pre-treatment has contributed positively to the improved pass rate. In run 25, 19% of the protocols within LD assays were based on enzymatic pre-treatment compared to only 4% (16 of 360) in this run for the mAb clone Ks20.8. In the previous assessments for CK20 run 25 and 47 a pass rate of 19% and 75%, respectively was obtained for protocols based on enzymatic pre-treatment compared to 76% and 92% if HIER was used.
The mAb clone Ks20.8 was found to be very robust and provided optimal results on all four main IHC platforms within a LD assay used by a relatively wide spectrum concerning HIER time, Ab titre and incubation time.
For the presently available RTU systems from the three main providers, Dako/Agilent, Leica Biosystems and Ventana/Roche grouped together a pass rate of 97% was obtained and the general access to accurate RTU systems have in the last two runs been a significant pillar for the improved pass rate compared to previous runs.

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 thus it can also serve as negative tissue control. Alternatively, tonsil 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.

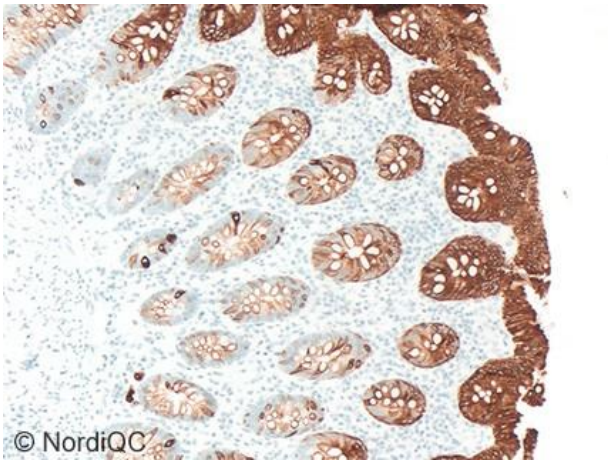


Fig. 1a
Optimal CK20 staining of the appendix using the Ventana/Roche RTU format of rmAb clone SP33 (790-4431) by incubation of 16 min. in primary Ab, HIER in CC1 pH 8.5 for 64 min., a 3-step multimer based detection kit (OptiView) and performed on Benchmark Ultra, Ventana. Virtually all surface epithelial 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 – 3a, same protocol.

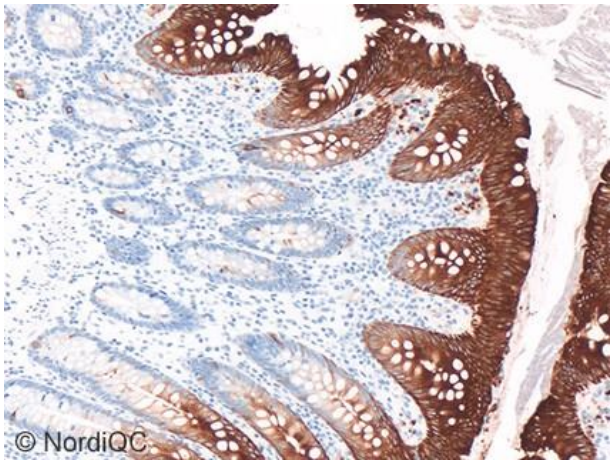


Fig. 1b
Insufficient CK20 staining of the appendix using the Dako/Agilent RTU format of Ks20.8 (GA777) by incubation of 10 min. in primary Ab, HIER in TRS High pH 9 for 30 min., a 2-step polymer based detection kit (EnVision FLEX) and performed on Omnis, Dako. The majority of surface epithelial cells are demonstrated but the intensity and number of stained cells are significantly reduced in the base of the crypts. Compare with Fig. 1a. Also compare with Figs. 2b - 3b – same protocol

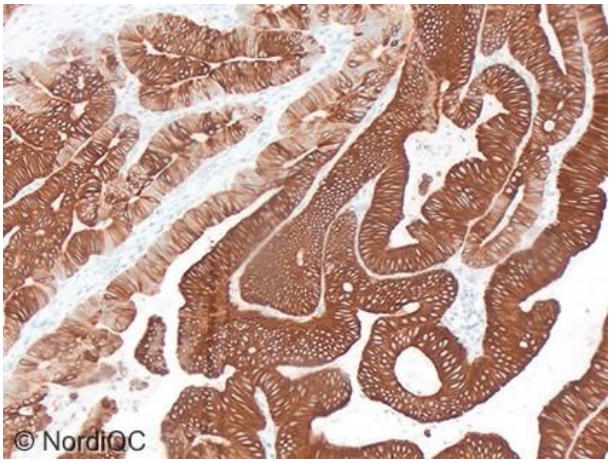


Fig. 2a
Optimal CK20 staining of the colon adenocarcinoma using same protocol as in Figs. 1a and 3a. The vast majority of neoplastic cells show a strong cytoplasmic staining reaction. No background reaction is seen.

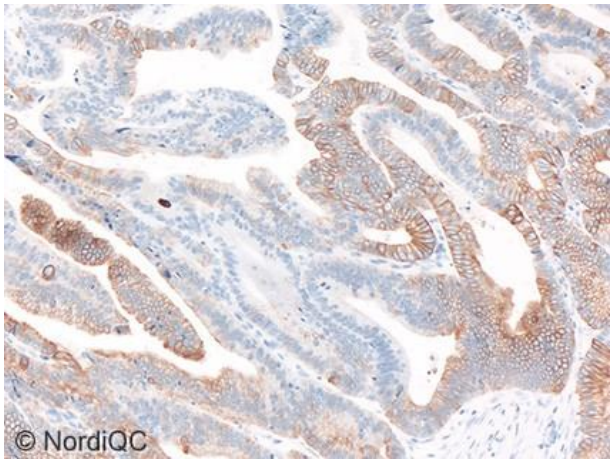


Fig. 2b
Insufficient CK20 staining of the colon adenocarcinoma using the same protocol as in Figs. 1b and 3b. The staining intensity and proportion of neoplastic cells is significantly reduced compared to the level expected and obtained in Fig. 2a.

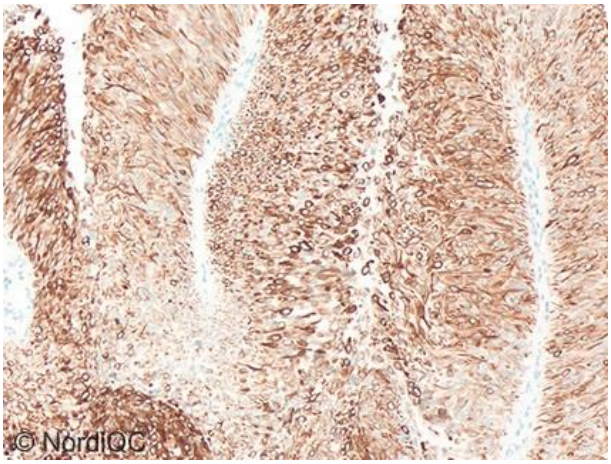


Fig. 3a
Optimal CK20 staining of the urothelial carcinoma using same protocol as in Figs. 1a - 2a. The majority of the neoplastic cells show a weak to moderate cytoplasmic staining reaction. No background reaction is seen.

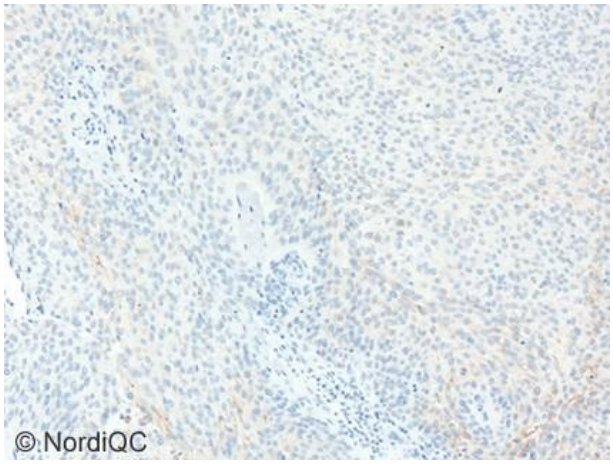


Fig. 3b
Insufficient CK20 staining of the urothelial carcionoma using same protocol as in Figs. 1b - 2b Only scattered neoplastic cells show a weak and vague staining reaction.

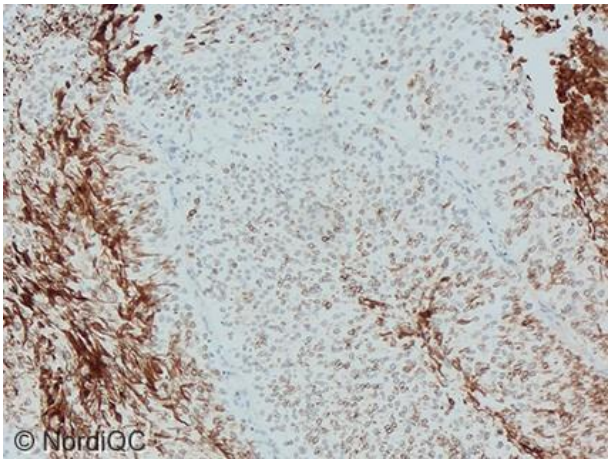


Fig. 4a
Insufficient result for CK20 in the urothelial carcinoma using mAb clone Ks20.8 with enzymatic pretreatment. The proteolytic pre-treatment enhances the intensity in some areas but the excessive digestion of the cytoplasmic compartment of the neoplastic cells hampers the morphology and many cells being negative with only nuclear contours left. Same protocol as in Fig. 4b.

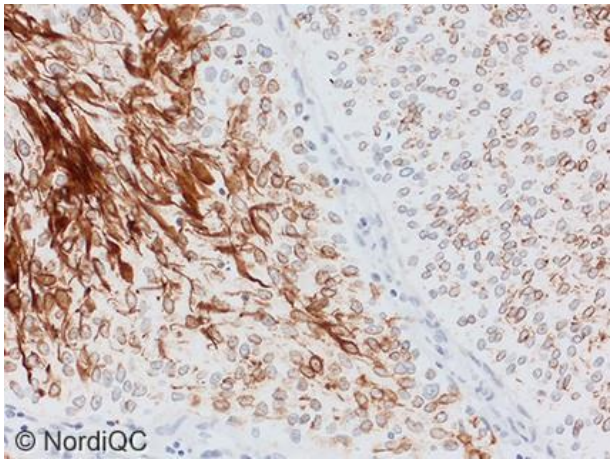


Fig. 4b
Same protocol and area as in Fig 4a but with higher magnification. The demonstration of CK20 in the cytoplasmic compartment is compromised due to the excessive digestion and the impaired morphology characterized by many nuclei without cytoplasm or membrane.

TJ/LE/SN 23.06.2021