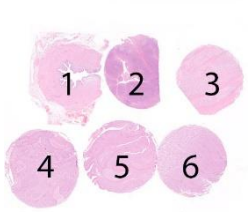
Assessment Run 68 2023

Uroplakin II/III (URO II/III)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for URO II/III, identifying and characterizing the urothelial cancer of unknown primary (CUP) origin.
Relevant clinical tissues, both normal and neoplastic, were selected to display a spectrum of antigen densities for URO II/III (see below).

Material
The slide to be stained for URO II/III comprised:



1. Urethra, 2. Tonsil, 3. Prostate adenocarcinoma, 4. Lung squamous cell carcinoma, 5-6. Urothelial carcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a URO II/III staining as optimal included:

- A moderate to strong, predominantly membranous and cytoplasmic staining reaction in virtually all umbrella cells in urethra.
- An at least weak to moderate cytoplasmic and membranous staining reaction of the majority of intermediate urothelial cells.
- An at least weak to moderate cytoplasmic and membranous staining reaction of the majority of neoplastic cells in the urothelial carcinoma tissue core no. 6.
- A moderate to strong cytoplasmic staining reaction of the majority of neoplastic cells in the urothelial carcinoma tissue core no. 5.
- No staining of other cells. Especially of central importance, the neoplastic cells of the prostate adenocarcinoma and the lung squamous cell carcinoma should be negative.

Participation

Number of laboratories registered for URO II/III, run 68	122
Number of laboratories returning slides	107 (88%)

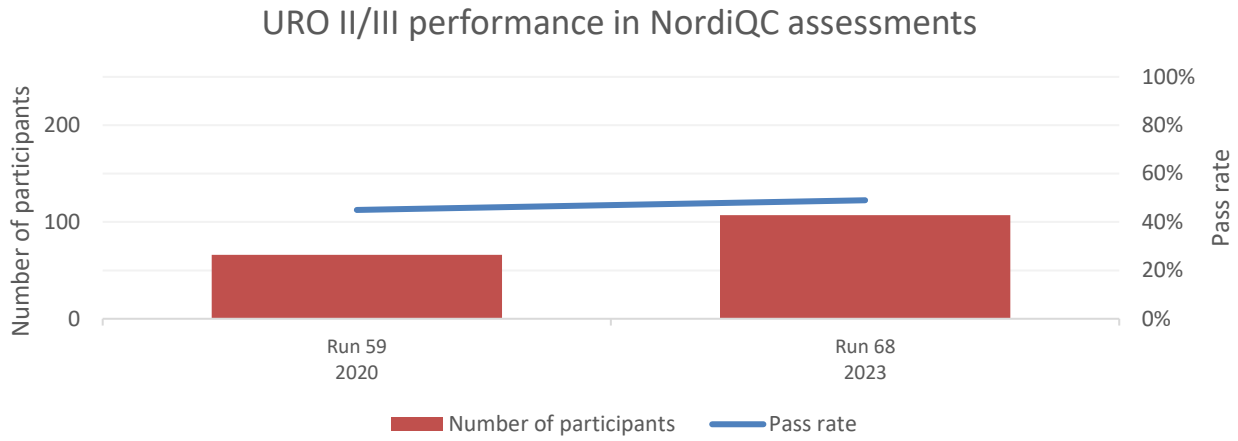
Results
At the date of assessment, 88% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

107 laboratories participated in this assessment and 49% achieved a sufficient mark (optimal or good). One laboratory used an inappropriate antibody targeting URO Ia+Ib and was not included in this assessment. Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibodies (polyclonals and clones targeted for Uroplakin III)
- Insufficient Heat Induced Epitope Retrieval (HIER) (too low temperature and/or too short efficient heating time)
- Inaccurate calibration of the titre for concentrated primary Abs (different for the platforms)

Performance history
This was the second NordiQC assessment of URO II/III. A small increase in pass rate was observed compared to the previous run (see Graph 1).

Graph 1. **Proportion of sufficient results for URO II/III in the two NordiQC runs performed**



Conclusion

Antibodies targeted for the URO II complex were most successful for the immunohistochemical demonstration of Uroplakin in this assessment with focus on identification of urothelial origin of CUP. The concentrated format of mAb clone BC21, within a laboratory developed assay, was most successful and provided the highest proportion of optimal results. The performance for e.g. mAb clone BC21 was slightly superior on Ventana BenchMark and Leica BOND compared to Dako Omnis. Of particular importance, use of an accurate calibration of the titre of the concentrated format of mAb clone BC21 tailored to the choice of IHC system was one of the most important prerequisites for a sufficient staining result. For both the concentrated format and RTU formats of mAb clone BC21 efficient HIER and a sensitive and specific 3-step detection system is recommended.

Urethra and tonsil are recommended as positive and negative tissue controls for URO II/III. In urethra, protocols must be calibrated to provide a moderate to strong, distinct predominantly membranous staining reaction in virtually all umbrella cells. A weak cytoplasmic staining reaction is seen in intermediate urothelial cells. In tonsil, no staining reaction should be seen, especially the squamous epithelial cells being negative (it has to been mentioned that internal NordiQC data has revealed that focally URO II/III can be observed in few reactive crypt epithelial cells). At present, no data is available on low-level expressing normal tissues/cells, and thus it is important to secure an "as strong as possible reaction" for URO II/III in urothelial umbrella and intermediate cells without any reaction in negative tissue controls.

Table 1. **Antibodies and assessment marks for URO II/III, run 68**

Concentrated antibodies	*Reactivity*	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb **BC21**	*URO II*	39 6 1	Biocare Medical Zytomed Systems Beijing Zhongshan	19	14	7	6	72%	41%
mAb **BC17**	*URO III*	3	Biocare Medical	-	-	1	2	-	-
mAb **AU-1**	*URO III*	4	Cell Marque	-	-	-	4	-	-
rmAb **SP73**	*URO III*	3	Cell Marque	-	-	1	2	-	-
rmAb **BP6199**	*URO III*	1	BioLynx Biotechnology	-	-	-	1	-	-
rpAb **BSB2291**	*URO III*	1	BioSB	-	-	-	1	-	-
rpAb **HPA010506**	*URO III*	1	Sigma Aldrich	-	-	-	1	-	-
Conc total		59		19	14	9	17	55%	32%
Ready-To-Use antibodies								Suff.[1]	OR.[2]
mAb **BC21** **AVI 3051 KG**	*URO II*	4	Biocare Medical	2	-	1	1	-	-
mAb **BC21** **API 3051 AA**		7	Biocare Medical	1	5	1	-	86%	14%
mAb **BC21** **MAD-000773QD**		2	MASTER DIAGNOSTICA	2	-	-	-	-	-
mAb **BC21** **MSG102**		2	Zytomed Systems	2	-	-	-	-	-
mAb **BC21** **8270-C010**		1	Sakura Finetek	1	-	-	-	-	-
mAb **BC21/6** **MSG102**		1	Zytomed Systems	-	1	-	-	-	-
MX130 **MAB-1099**		2	Fuzhou Maixin	2	-	-	-	-	-
mAb **BC21+BC17** **API 3094 AA**	*URO II/III*	3	Biocare Medical	2	1	-	-	-	-
rmAb **SP73** **760-4533**	*URO III*	16	Roche/Ventana	-	-	-	16	0%	0%
rmAb **SP73** **345R-17/18**		4	Cell Marque	-	-	-	4	-	-
mAb **AU1** **651108**		1	Progen	-	-	-	1	-	-
mAb **BC17** **BC17**		1	Master Vitro	-	-	-	1	-	-
rmAb **EP321** **CUR-0222**		1	Celnovte	-	-	1	-	-	-
715G4C3 **PA546**		1	Abcarta	-	-	1	-	-	-
pAb **PDRM002**		1	Diagnostic Biosystems	-	-	-	1	-	-
RTU total		47		12	7	4	24	41%	26%
Total		106		31	21	13	41		
Proportion				29%	20%	12%	38%	49%	

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

Detailed analysis of URO II/III Run 68

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb **BC21**: Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (CC1, Ventana/Roche) (11/25)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent), TRS pH high (5/13) (Dako/Agilent), Tris-EDTA/EGTA pH 9 (2/2) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (1/3) as retrieval buffer. The mAb was typically diluted in the range of 1:25-100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 29/38 (76%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

Table 2. **Proportion of optimal results for URO II/III for the most commonly used antibody as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark GX / XT / Ultra**		**Leica 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	ER2 pH 9.0	ER1 pH 6.0
mAb BC21	1/4**	0/1	4/9 (44%)	-	11/25 (44%)	0/1	1/4	-

* 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
Clone **MX130**, product no. **MAB-1099**, Fuzhou Maixin:
Two protocols with optimal results were stained on a Titan based on HIER using DNS buffer as retrieval buffer and 30 min. primary Ab incubation. As detection system the Titan Super Detection system with linker was used.

mAb clone **BC21**, product no. **MAD-000773QD**, Master Diagnostica:
One protocol with an optimal result was stained on a Thermo autostainer based on HIER using EDTA/EGTA pH 8 as retrieval buffer and 20 min. primary Ab incubation. As detection system the Master Plus Detection system was used without linker.

mAb clone **BC21+BC17**, product no. **API 3094 AA**, Biocare Medical:
One protocol with an optimal result was stained on an intelliPath from Biocare based on HIER in a Citrate buffer pH6 as retrieval buffer and 30 min. primary Ab incubation. As detection system ZytoChem Plus was used with linker.

mAb clone **BC21**, product no. **8270-C010**, Sakura Finetek:
One protocol with an optimal result was stained on a Tissue-Tek Genie based on HIER using Tris EDTA/EGTA pH 9 as retrieval buffer and 30 min. primary Ab incubation. As detection system the Tissue-Tek Genie Pro Detection system with linker was used.

Only limited data was available for other Ready-To-Use Abs and corresponding systems. More details can be found in comments below.

Table 3. **Proportion of sufficient and optimal results for URO II/III for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
rmAb **SP73 760-4533**	(0/7)	(0/7)	(0/9)	(0/9)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this second NordiQC assessment of URO II/III, 122 laboratories submitted a protocol, and 107 participated in the assessment. The prevalent feature of an insufficient result in this second assessment of URO II/III was characterized by reduced number of cells expected to be stained or completely false negative results and was generally caused by extended use of antibodies with low analytical sensitivity. Reduced number or false negative staining reaction was seen in 89% of the insufficient results (48/54). The majority of all laboratories were able to demonstrate Uroplakin in the apical part of superficial umbrella cells in the urethra, but only antibodies and protocols with high sensitivity managed to demonstrate Uroplakin in the intermediate cells in the urothelium (see Figs. 1a-b). Only Abs raised against Uroplakin II gave a distinct positive cytoplasmic staining reaction in the cytoplasmic compartment of intermediate urothelial cells, whereas the plaque-like' and pericellular membranous localized Uroplakin in umbrella cells was identified by both Abs against Uroplakin II and III (see Fig. 1a).

The overall low pass rate was significantly affected by use of less successful primary Abs, and in particular related to the Abs against URO III as shown in Table 1. In total, 36% (39/107) of the laboratories used an Ab for URO III either as a concentrate within a laboratory developed test (LDT) or as Ready-To-Use format, and none of these received a sufficient mark due to false negative staining reaction in one or both of the two included urothelial carcinomas.

The most popular concentrated format was the mAb clone BC21 targeting Uroplakin II being used by 43% of the laboratories (46/107). In total, 72% received a sufficient result, 41% optimal. The mAb clone BC21 was typically applied within an LDT based on HIER in an alkaline buffer and by use of a 3-step detection system. As seen in Table 2, optimal results could be obtained on all main fully automated IHC systems and especially widely applied on Dako Omnis and Ventana BenchMark with same overall performance. Borderline or poor assessment results were on the Dako Omnis mainly caused by too low dilution of the primary Ab giving excessive background reaction and on the Ventana Benchmark mainly caused by a high dilution of the primary Ab and/or too short HIER time giving a too low analytical sensitivity.

One laboratory used the ZM204 clone which is raised against URO Ia+b. This protocol was deemed as use of inappropriate Ab, as this assessment evaluate the technical performance for URO II and III.

The concentrated formats of Abs against URO III (see Table 1) were all less successful and in total used by 13 laboratories. No sufficient results were obtained despite comparable protocols settings to the mAb BC21 for URO II were applied. The insufficient results were mainly characterized by completely false negative results or in otherwise carefully calibrated protocols staining of only high expression neoplastic cells in tissue cores no 5-6 were seen, but leaving the majority of the neoplastic cells unstained (see Fig. 2b).
In addition, the mAb clone AU-1 and polyclonal Ab also provided an aberrant false positive staining result in both the tonsil and the lung squamous cell carcinoma (see Figs. 5a and 5b).

47 laboratories used Ready-To-Use (RTU) antibodies. The Ventana product 760-4533 based on the rmAb clone SP73 against URO III was most popular, but, similar to the concentrated format, no protocols were found successful in providing a sufficient result. All 16 laboratories using the SP73 clone applied it on the Ventana Benchmark platform. Different protocols were used with both 2- and 3-layer detection systems, +/- amplifier and using HIER in the range of 8-64 min. However, none of these combinations were found applicable to demonstrate Uroplakin in the neoplastic cells of the two urothelial carcinomas at the expected level.

Various RTU formats of mAb clone BC21 were used by 20 laboratories – 17 received a sufficient mark and observed on all 4 of the main IHC platforms. 6 optimal results were achieved on the Ventana Benchmark platform using CC1 in 32-64 min. and carefully calibrated for either UltraView or OptiView.
The remaining optimal results for the BC21 products were achieved on either Bond or Autostainer platforms with standard protocol settings, however the low amount of observations is not providing specific guidelines to apply the different Ab formats on the platforms.

In this NordiQC assessment run, IHC results performed on Dako Omnis in general have shown a reduced quality. Agilent Technologies has stated an issue with cross-reaction/background reaction caused by the HRP polymer product in the detection system EnVision FLEX. In cases of markers targeting cytoplasmic antigens as for the URO II/III this can give a misleading reaction that ultimately can be interpreted as false positive. 17 protocols were submitted for the Dako Omnis with a pass rate of 47% (8/17). 5 laboratories applied RTU products none with optimal results, but among the 12 laboratories using the concentrated format for BC21, 4 achieved an optimal result, while 5 had an extensive background reaction (the titre ranged from 1:25-150).
In the previous assessment Run 59 back in 2020 the recommended protocol settings for the Dako Omnis using the BC21 as concentrate was based on a dilution of 1:25 and EnVision Flex+ as detection system. The laboratories using these settings in this assessment did however produce excessive background and none received optimal results. The optimal settings in this assessment were still using the FLEX+ detection system but using the Ab in a titre range of 1:100-150. This could indicate that the Igg fraction or composition of the HRP polymer may have changed since run 59.

In this second assessment of URO II/III, including urothelial carcinomas for the TMA composition gave a clear indication that antibodies only targeting URO III seem less successful in the identification of the urothelial origin of CUP. None of the URO III Abs received a sufficient mark regardless either being a concentrated or Ready-To-Use format (see Table 1).
A study from 2014 confirms our observations showing a difference in the analytical sensitivity of 73% vs 37% for the mAb clone BC21 and mAb clone AU-1 for metastatic urothelial carcinomas[1], respectively.
In 2022, NordiQC performed a comparative study of several UP II and III antibodies and confirmed a relatively high analytical sensitivity of 69% for the mAb clone BC21, compared to only 19% and 23% for the well established clone mAb clone AU1 and the newly launched clone SP73, respectively in the identification of urothelial carcinomas[2].

The studies and NordiQC data indicate that IHC for URO II provides a superior analytical sensitivity compared to URO III.

1. Steven C Smith,* Sambit K Mohanty,* et al Uroplakin II outperforms uroplakin III in diagnostically challenging settings; Histopathology 2014, 65, 132–138.
2. Kristoffersen HL, Røge R, Nielsen S. Comparison of Antibodies to Detect Uroplakin in Urothelial Carcinomas. Appl Immunohistochem Mol Morphol. 2022;30(5):326-332.

Controls

At present and according to publications and preliminary data generated in this NordiQC assessment, urethra and tonsil are recommended as positive and negative tissue controls for URO II/III. In urethra, protocols must be calibrated to provide a moderate to strong, distinct predominantly membranous staining reaction in virtually all umbrella cells. A weak cytoplasmic staining should be seen in intermediate urothelial cells. In tonsil, no staining reaction should be seen, especially squamous epithelial cells being negative. No data are available on low-level expressing normal tissues/cells and thus it is important to secure an "as strong as possible reaction" for URO II/III in urothelial umbrella and intermediate cells without any reaction in the negative tissue controls.

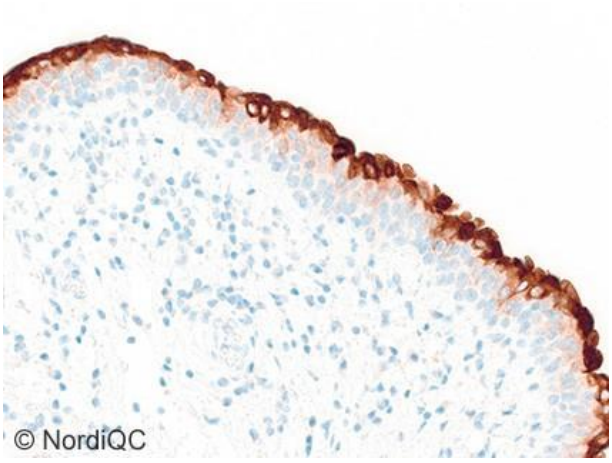


Fig. 1a (x200)
Optimal Uroplakin II/III staining of the urethra using the mAb clone BC21 - diluted, 1:25 (32 min. incubation), epitope retrieval using HIER in CC1 (48 min.) and a 3-step multimer based detection system (OptiView) performed on Benchmark Ultra (Ventana/Roche).
Virtually all umbrella cells display a strong, membranous and cytoplasmic staining reaction and the vast majority of intermediate urothelial cells show a weak to moderate cytoplasmic staining reaction. Same protocol used in Figs. 2a-4a.

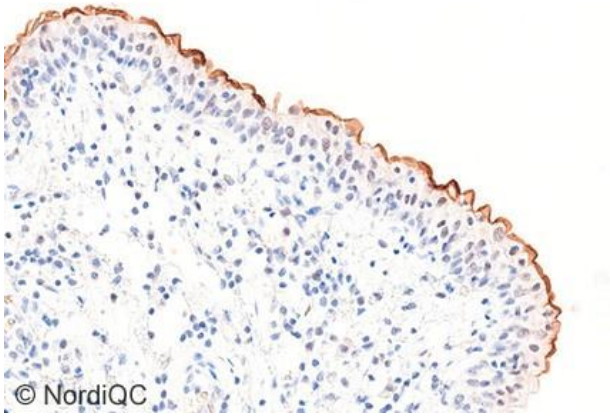


Fig. 1a (x200)
Insufficient Uroplakin II/III staining of the Urethra using the rmAb clone SP73, diluted, 1:50 (30 min. incubation), epitope retrieval using HIER in TRS High (30 min.) and a 3-step polymer based detection system (EnVision Flex+) performed on Omnis (Dako/Agilent).
The urothelium displays a moderate, plaque-like membranous, predominantly apical staining reaction in all umbrella cells but no cytoplasmic or membranous staining of the intermediate urothelial cells.
Furthermore a weak nuclear staining reaction is observed in the urothelial cells.
Same protocol used in Figs. 2b-4b.

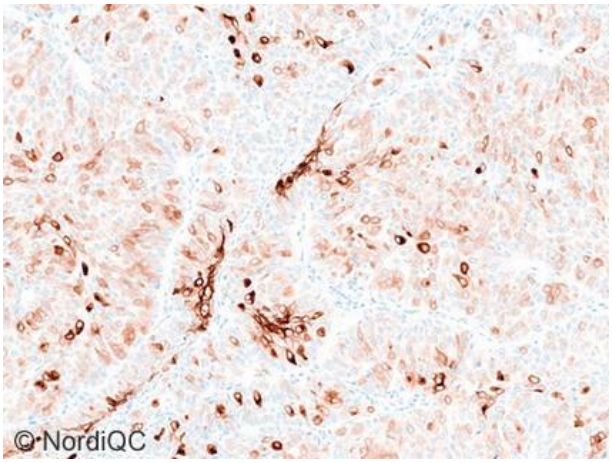


Fig. 2a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma, tissue core no 6, using same protocol as in Fig. 1a. The majority of the neoplastic cells showing a weak to moderate cytoplasmic and membranous staining reaction.

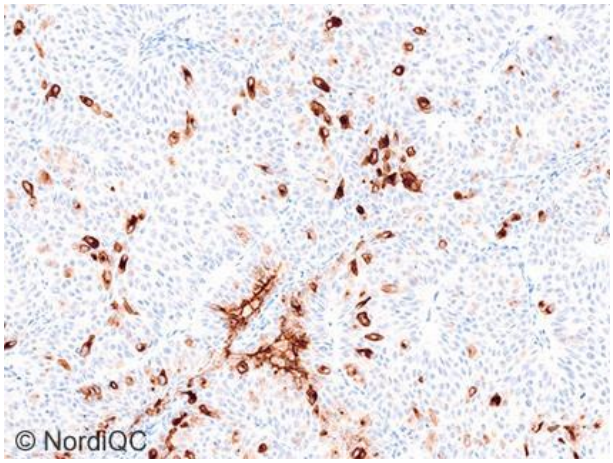


Fig. 2b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma, tissue core no 6, using same protocol as in Fig. 1b. Only a reduced number of neoplastic cells display a strong staining reaction. Also compare with Fig. 3b, same protocol.

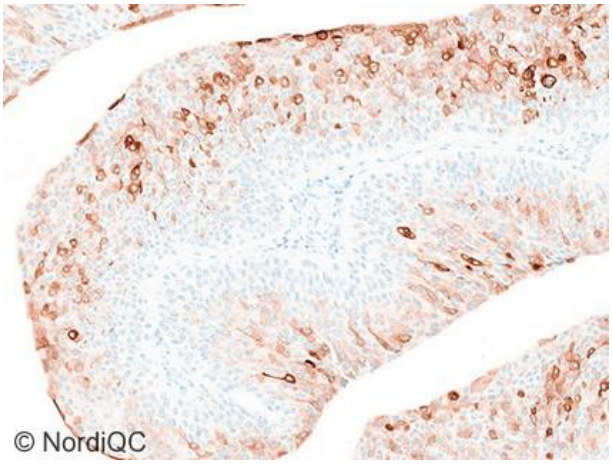


Fig. 3a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma, tissue core no 5, using same protocol as in Figs. 1a and 2a. The vast majority of the neoplastic cells display a weak to strong cytoplasmic and membranous staining reaction.

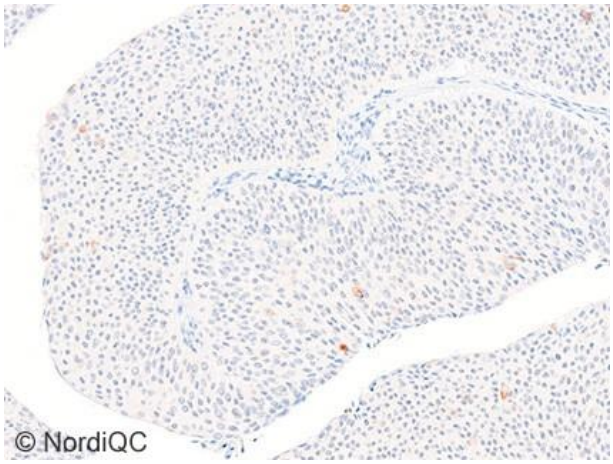


Fig. 3b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma, tissue core no 5, using same protocol as in Figs. 1b and 2b. The neoplastic cells are negative and only a dubious dot-like reaction is seen in a few cells.

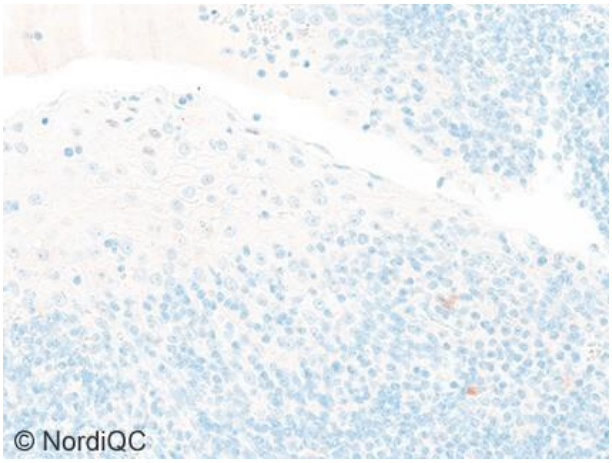


Fig. 4a (x400)
Optimal Uroplakin II/III staining of the tonsil using same protocol as in Figs. 1a – 3a. All cells are negative.

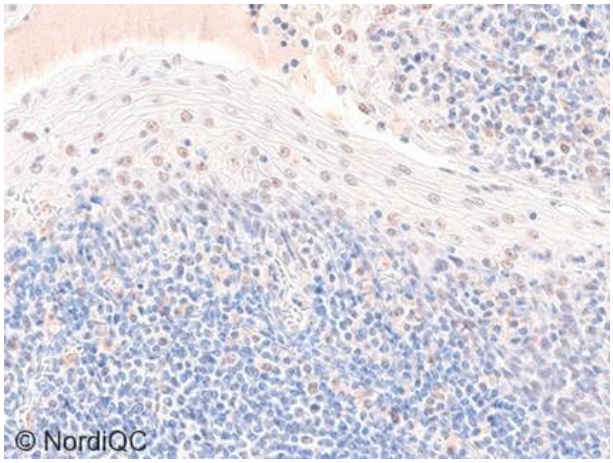


Fig. 4b (x400)
Uroplakin II/III staining of the tonsil using same insufficient protocol as in Figs. 1b – 3b. All cells are negative as expected. In addition to the reduced analytical sensitivity as seen in Figs. 1b-3b, an aberrant nuclear staining reaction is observed in the epithelial cells.

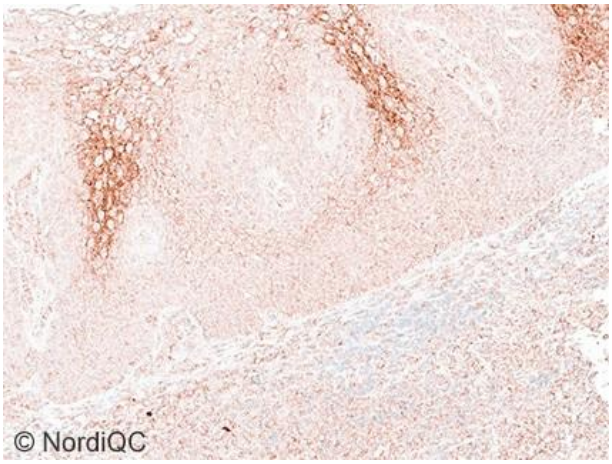


Fig. 5a (x200)
Insufficient Uroplakin II/III staining of tonsil using the mAb clone AU-1, diluted 1:25 (32 min. incubation), epitope retrieval using HIER in CC1 (64 min.) and a 2-step multimer based detection system (UltraView) performed on Benchmark Ultra (Ventana/Roche). The squamous epithelial cells and germinal centers display a moderate false positive cytoplasmic staining reaction. Same protocol used in Figs. 5b.

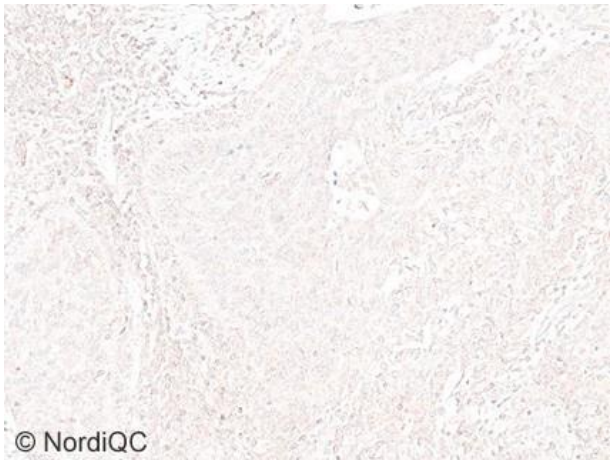


Fig. 5b (x200)
Insufficient Uroplakin II/III staining of the lung squamous cell carcinoma using same protocol as in Fig. 5a. The neoplastic cells display a false positive cytoplasmic staining reaction in the neoplastic cells. Compare with the expected level in Fig. 7a.

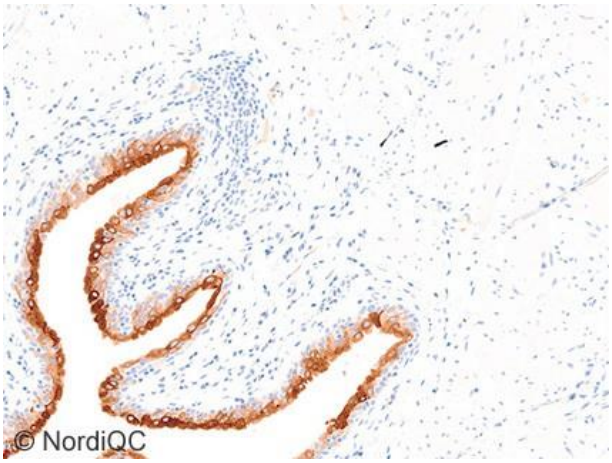


Fig. 6a (x100)
Optimal Uroplakin II/III staining of the urethra using the mAb clone BC21 - diluted, 1:100 (30 min. incubation), epitope retrieval using HIER in TRS High (24 min.) and a 3-step polymer based detection system (EnVision Flex+) performed on Omnis (Dako/Agilent). Virtually all umbrella cells display a strong, membranous and cytoplasmic staining reaction and the vast majority of intermediate urothelial cells show a weak to moderate also cytoplasmic staining reaction.
Same protocol used in Figs. 7a-8a.

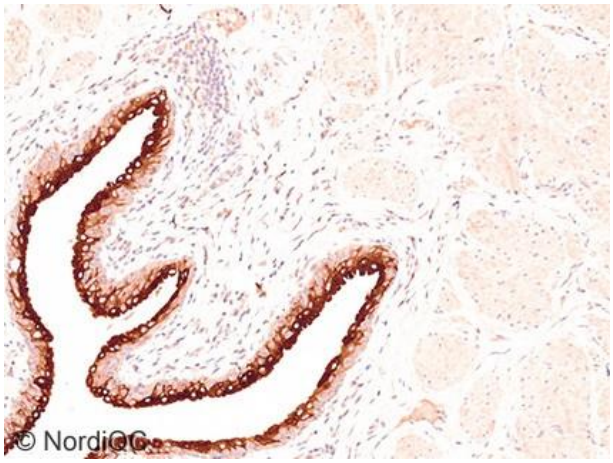


Fig. 6b (x100)
Insufficient Uroplakin II/III staining of the Urethra using the mAb clone BC21, diluted, 1:25 (30 min. incubation), epitope retrieval using HIER in TRS High (24 min.) and a 3-step polymer based detection system (EnVision Flex+) performed on Omnis (Dako/Agilent). Virtually all umbrella cells display a strong, membranous and cytoplasmic staining reaction and the vast majority of intermediate urothelial cells show a weak to moderate cytoplasmic staining reaction. However note a moderate background reaction being observed in the stroma.
Same protocol used in Figs. 7b-8b.

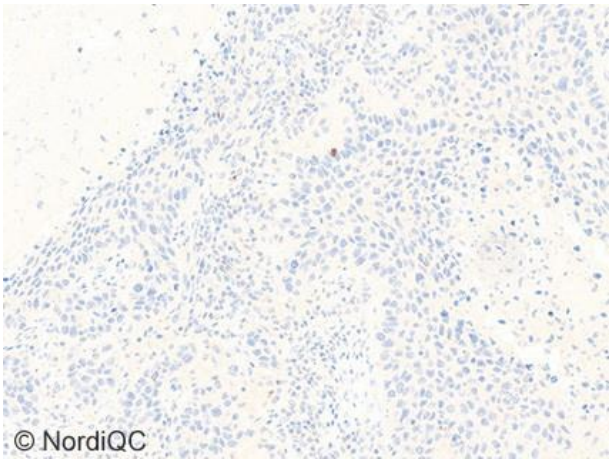


Fig. 7a (x100)
Optimal Uroplakin II/III staining of the lung squamous cell carcinoma using same protocol as in 6a.
The neoplastic cells are negative as expected.

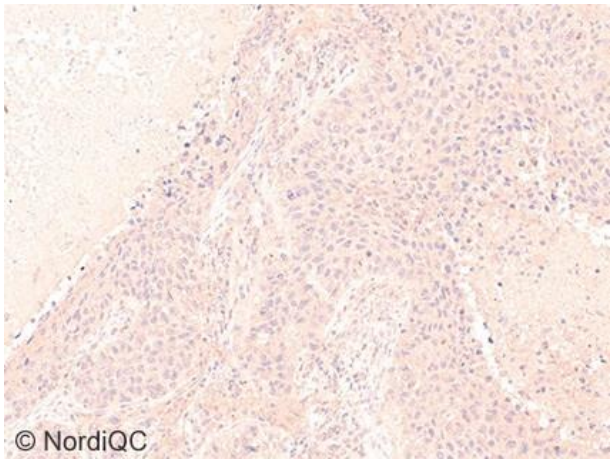


Fig. 7b (x100)
Insufficient Uroplakin II/III staining of the lung squamous cell carcinoma. All cells display a weak to moderate diffuse cytoplasmic reaction that can be interpretated as (false) positive.

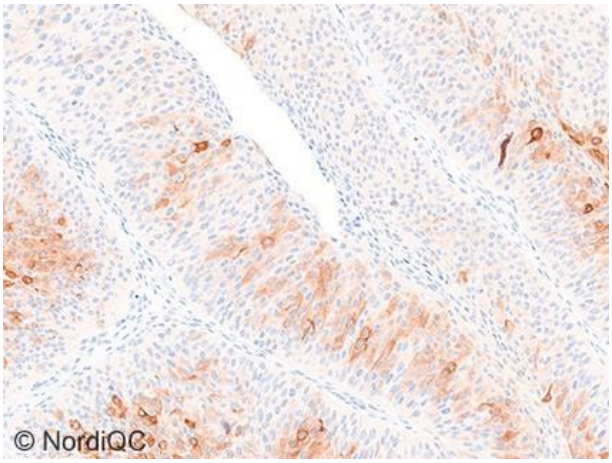


Fig 8a. (x100)
Optimal Uroplakin II/III staining of the urothelial carcinoma, tissue core no 5, using same protocol as in Figs. 6a and 7a. The majority of the neoplastic cells display a moderate to strong cytoplasmic and membranous staining reaction. A small amount of background was observed due to the HRP polymer problems with the Envision Flex detection system, but in this case accepted as the positive cells were distinctly positive.

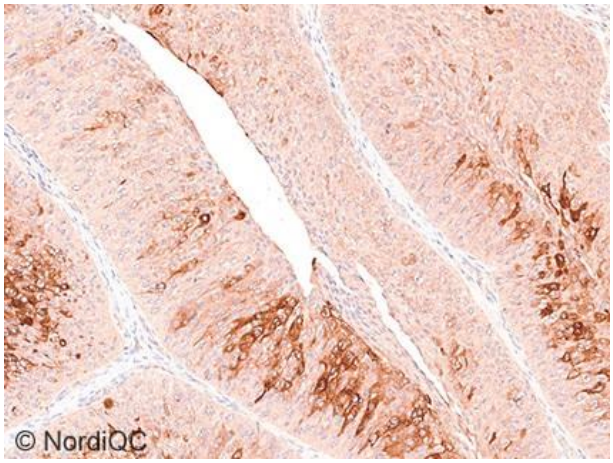


Fig 8b (x100)
Insufficient Uroplakin II/III staining of the urothelial carcinoma, tissue core no 5, using same protocol as in Figs. 6b and 7b. The majority of the cells display a distinct positive staining reaction but also a diffuse moderate background reaction that can be interpretated as (false) positive, alike the reaction observed in the lung squamous cell carcinoma Fig 7b.

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