

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 origin of 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, 2-3. Urothelial carcinoma, 4. Prostate adenocarcinoma, 5. Tonsil,
6. Lung squamous cell 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.
- A weak to moderate cytoplasmic and membranous staining reaction of the neoplastic cells in the urothelial carcinoma tissue core no. 2.
- A moderate to strong cytoplasmic staining reaction of the neoplastic cells in the urothelial carcinoma tissue core no. 3.
- 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 59	101
Number of laboratories returning slides	66 (65%)

The number of laboratories returning slides has decreased in this run 59 compared to previous assessments, due to the COVID-19 pandemic. All slides returned after the assessment will be assessed and receive advice if the result is insufficient but will not be included in this report.

Results

66 laboratories participated in this assessment. 30 (45%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and the assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:

- Less successful primary antibodies (clones targeted for Uroplakin III)
- Too low concentration of the primary antibody
- Insufficient HIER (too low temperature and/or too short efficient heating time)

Performance history

This was the first NordiQC assessment of URO II/III and the overall pass rate was very low (see Table 2).

Table 2. Proportion of sufficient results for URO II/III in the first NordiQC run performed

	Run 59 2020
Participants, n=	66
Sufficient results	45%

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 Nordic Immunohistochemical Quality Control, Uroplakin II/III run 59 2020

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 inferior on Ventana BenchMark and Leica BOND compared to Dako Omnis. Irrespective of the primary Ab applied, efficient HIER, use of appropriate titer and incubation time tailored to the choice of IHC system were the most important prerequisites for a sufficient staining result.

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. 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 59**

Concentrated antibodies	Reactivity	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	OR ²
mAb BC21	<i>URO II</i>	24	BioCare Medical	13	10	3	1	85%	37%
		3	Zytomed Systems						
mAb AU-1	<i>URO III</i>	2	Cell Marque	-	-	-	2	-	-
rmAb SP73	<i>URO III</i>	7	Cell Marque	-	-	1	7	0%	0%
		1	Immunologic						
rmAb ERP18799	<i>URO II</i>	1	ABCAM	-	1	-	-	-	-
rmAb EP321	<i>URO III</i>	1	Bio SB	-	-	1	-	-	-
rpAb AB82173	<i>URO III</i>	2	ABCAM	-	-	-	2	-	-
Ready-To-Use antibodies								Suff. ¹	OR. ²
mAb BC21 AVI 3051 KG	<i>URO II</i>	1	Biocare Medical	-	1	-	-	-	-
mAb BC21 API 3051 AA		1	Biocare Medical	-	1	-	-	-	-
mAb BC21 MAD-000773QD		1	Vitro SA	1	-	-	-	-	-
mAb BC21 MSG102		1	Zytomed Systems	-	1	-	-	-	-
mAb BC21+BC17 API 3094 AA	<i>URO II/III</i>	3	Biocare Medical	-	2	1	-	-	-
rmAb SP73 760-4533 (VRPS)³	<i>URO III</i>	1	Roche/Ventana	-	-	1	-	-	-
rmAb SP73 760-4533 (LMPS)⁴		16	Roche/Ventana	-	-	2	14	0%	0%
rmAb SP73 345R-17/18		1	Cell Marque	-	-	-	1	-	-
Total		66		14	16	9	27		
Proportion				21%	24%	14%	41%	45%	

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 URO II/III Run 59

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb **BC21**: Protocols with optimal results were typically based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana) (6/16)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) or TRS pH high (3/3), Tris-EDTA/EGTA pH 9 (2/2), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/4), Citrate pH 6 (1/1) 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, 21/22 (96%) laboratories produced a sufficient staining result (optimal or good).

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

Table 3. **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	1/1	-	2/2	-	5/15 (33%)	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 mAb clone BC21, product no. MAD-000773QD, Vitro SA:

The one protocol with an optimal result were stained on a Thermo autostainer based on HIER using EDTA/EGTA pH 8 as retrieval buffer and 20 min. Ab incubation. As detection system the Master Plus Detection system was used without linker.

No protocols for other Ready-To-Use Abs and corresponding systems provided an optimal result. More details can be found in comments below.

Comments

In this first NordiQC assessment of URO II/III, 101 laboratories submitted a protocol, but only 66 participated in the assessment. The main reason for this low percentage was due to the COVID-19 pandemic, that made it difficult for the laboratories to both receive and return the slides within the deadline for this report.

The prevalent feature of an insufficient result in this first assessment of URO II/III was characterized by too weak or completely false negative results and was generally caused by extended use of antibodies with low analytical sensitivity. Too weak or false negative staining reaction was seen in 55% of the insufficient results (36/66). 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, 47% (31/66) 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 41% of the laboratories (27/66). In total, 85% received a sufficient result, 48% 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 3, optimal results could be obtained on all main fully automated IHC systems. However, the proportion of optimal results was slightly lower on both Ventana BenchMark and Leica BOND compared to Dako Omnis, but this might be related to the titres selected for the primary Ab and the low number of observations. Borderline or poor assessment results (4/27) were mainly caused by a too low titre of the primary Ab or HIER at low pH.

The concentrated formats of Abs against URO III mAb AU-1, rmAb SP73, rmAb EP321 and pAb AB82173 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 especially in the urothelial carcinoma, tissue core 3 and/or reduced number of cells demonstrated in the urothelial carcinoma, tissue core no 2.

In addition, the rmAb clone SP73 and pAb AB82173 also provided an aberrant false positive staining result (primarily observed on e.g. Dako Autostainer platform) (see Figs. 5a and 5b).

Twelve laboratories used the SP73, AU-1 and AB82173 clones, and with carefully calibrated protocols were able to stain single neoplastic cells in tissue core no 2 but leaving the majority of the neoplastic cells unstained (see Fig. 2b). This also applied for tissue core no. 3, where optimal protocols based on URO II targeted antibodies provided a moderate to strong cytoplasmic staining reaction in the neoplastic cells, while protocols based on the three mentioned Abs for URO III did not demonstrate neoplastic cells.

Two laboratories applied the SP73 as concentrate on an Autostainer platform with similar protocols as for the Ventana platform, and both protocols provided a false positive result. Both protocols based on HIER in high pH gave a cytoplasmic staining reaction in muscle cells, and a weak to moderate staining reaction of the cytoplasm and nuclear compartment in the neoplastic cells of the squamous cell lung carcinoma. A nuclear staining reaction was also seen in both epithelial cells of the tonsil and the neoplastic cells of the urothelial carcinoma (see Figs. 5-6).

The pAb AB82173 stained on either Autostainer or Omnis platform using HIER in high pH also showed false positive staining reaction of the muscle cells in all tissue cores but did not react with the nuclei like the SP73 clone.

One protocol was based on the rmAb clone EP321 for URO III and was slightly more successful compared to the other URO III Abs. The protocol based on rmAb clone EP321 demonstrated some of the intermediate cells in the urothelium and a focal reaction of the urothelial carcinoma tissue core no. 3 but a large amount of the neoplastic cells still remained unstained and same observation was seen in the urothelial carcinoma tissue core no. 2 (see Fig. 7).

In summary, the following patterns was observed:

- mAb clone AU-1; false negative,
- rmAb clone EP321; false negative,
- rmAb clone SP73; false negative and false positive (smooth muscle cells and nuclei of several cell types – see Fig. 5-6),
- pAb AB82173; false negative and false positive (smooth muscle cells).

25 laboratories used Ready-to-use antibodies. The Ventana product 760-4533 based on the rmAb clone SP73 clone against URO III was most popular, but, similar to the concentrated format, no protocols were found successful in providing a sufficient result. All 18 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 32-76 min. However, none of these combinations were found applicable to demonstrate Uroplakin in the neoplastic cells of the two urothelial carcinomas.

RTU formats of mAb clone BC21 were used by 4 laboratories – only 1 received an optimal mark using the MAD-000773QD product from Vitro SA on a Thermo Autostainer.

Three laboratories used the Biocare RTU product API 3094 AA based on a cocktail of mAb clone BC21 and BC17 for Uroplakin II and III, respectively. No optimal marks were obtained, and due to too low number of observations, no reliable information of the potential and performance of this cocktail with inclusion of a "Uroplakin III" Ab can be extracted. Two protocols provided a result assessed as sufficient.

In this first assessment of UROII/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 2013 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 carcinomas¹, respectively. The study and NordiQC data indicate that IHC for URO II provides a superior analytical sensitivity compared to URO III. However, the conclusions must be drawn with some caution due to the low number of participants included in this assessment and limited access to publications comparing the overall level of analytical sensitivity and specificity of the commercially available Abs for URO II/III.

In order to elucidate on the performance of Uroplakin Abs, NordiQC will perform an extended study to optimize Abs for URO II/III and using these optimized protocols on a large cohort of urothelial carcinomas and other carcinomas to characterize the analytical sensitivity and specificity. In addition to this, repeating the assessment of URO II/III will provide more data on any potential platform dependencies for the Abs applied. In this assessment, the performance for e.g. mAb clone BC21 was slightly inferior on Ventana BenchMark and Leica BOND compared to Dako Omnis.

1. Steven C Smith,* Sambit K Mohanty,* et al Uroplakin II outperforms uroplakin III in diagnostically challenging settings; *Histopathology* 2014, 65, 132–138.

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.

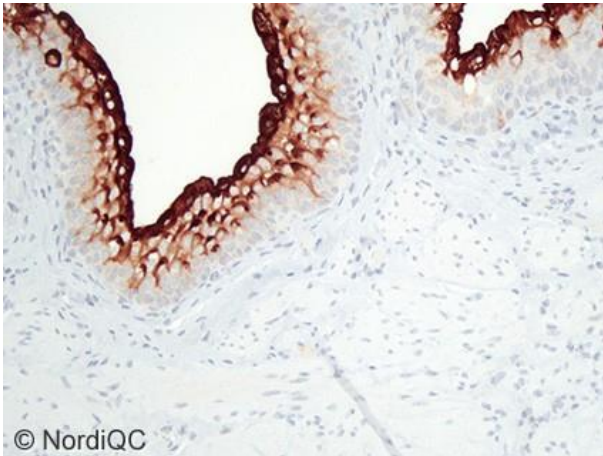


Fig. 1a (x200)
Optimal Uroplakin II/III staining of the urethra using the mAb clone BC21 - diluted, 1:25 (30 min. incubation), epitope retrieval using HIER in TRS High (25 min.) and a 3-step multimer based detection system (EnVision Flex+) performed on Autostainer (Dako). Virtually all umbrella cells display a strong, membranous and cytoplasmic staining reaction and the vast majority of intermediate urothelial cells show a weak to moderate predominantly apical but also cytoplasmic staining reaction. Same protocol used in Figs. 2a-4a.

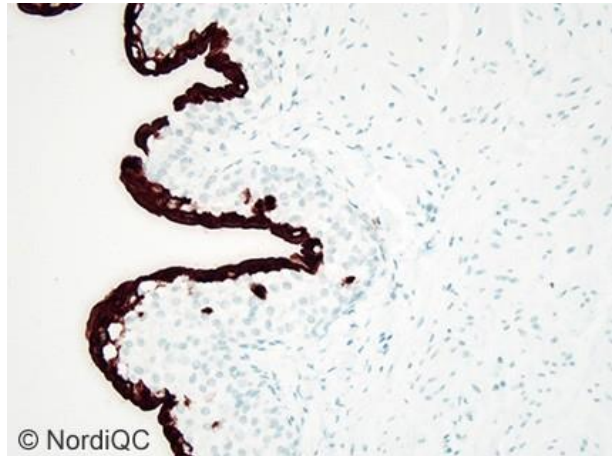


Fig. 1a (x200)
Insufficient Uroplakin II/III staining of the Urethra using the rmAb clone SP73, diluted, 1:100 (32 min. incubation), epitope retrieval using HIER in CC1 (32 min.) and a 3-step multimer based detection system (OptiView with amplification) performed on BenchMark (Ventana). The urothelium displays a strong, plaque-like membranous, predominantly apical staining reaction in all umbrella cells but no cytoplasmic or membranous staining of the intermediate urothelial cells. Same protocol used in Figs. 2b-4b.

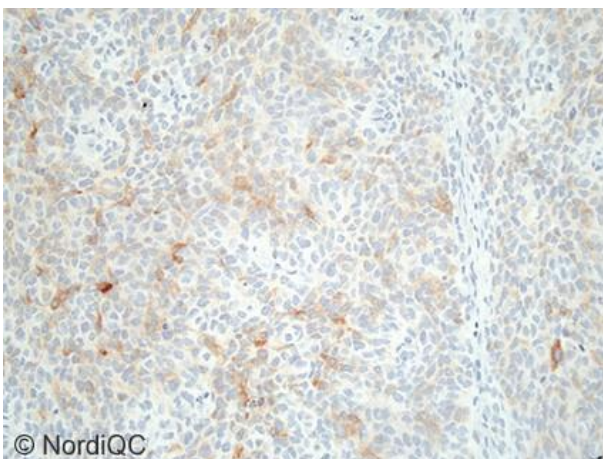


Fig. 2a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma tissue core no 2 using same protocol as in Fig. 1a. The majority of the neoplastic cells showing a weak to moderate cytoplasmic and membranous staining reaction.

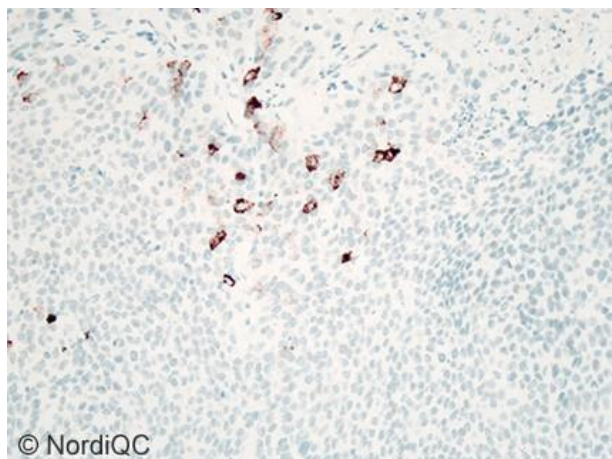


Fig. 2b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma tissue core no 2 using same protocol as in Fig. 1b. The vast majority of neoplastic cells are almost completely negative and only a few scattered cells display a strong staining reaction.

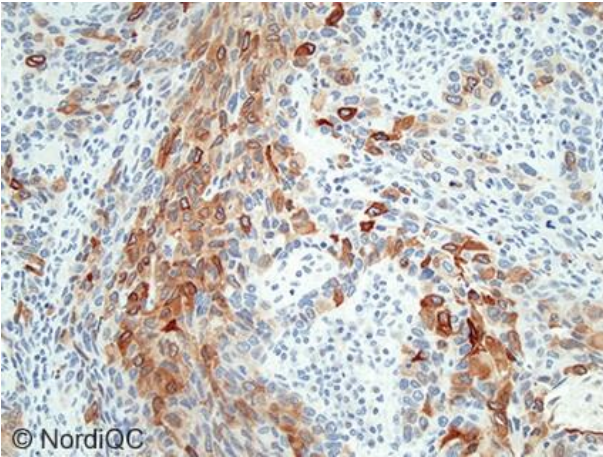


Fig. 3a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma tissue core no 3 using same protocol as in Figs. 1a and 2a. The vast majority of the neoplastic cells display a moderate to strong cytoplasmic and membranous staining reaction.

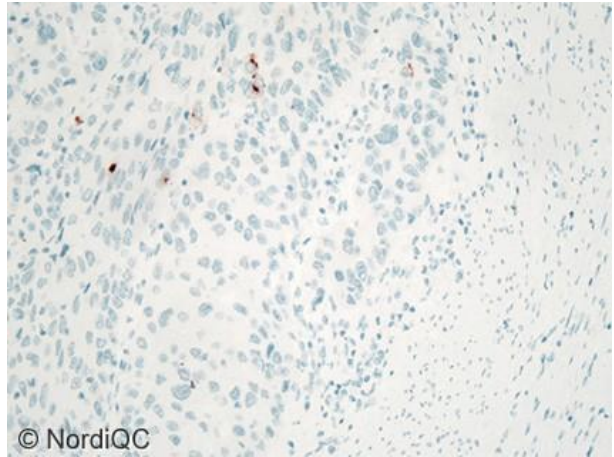


Fig. 3b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma tissue core no 3 using same protocol as in Figs. 1b and 2b. The neoplastic cells are negative and only a dubious dot-like reaction are seen in a few cells.

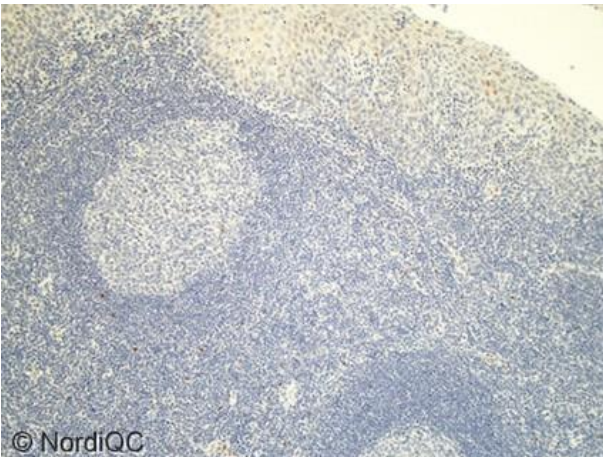


Fig. 4a (x100)
Optimal Uroplakin II/III staining of the tonsil using same protocol as in Figs. 1a - 3a. All cells are negative.

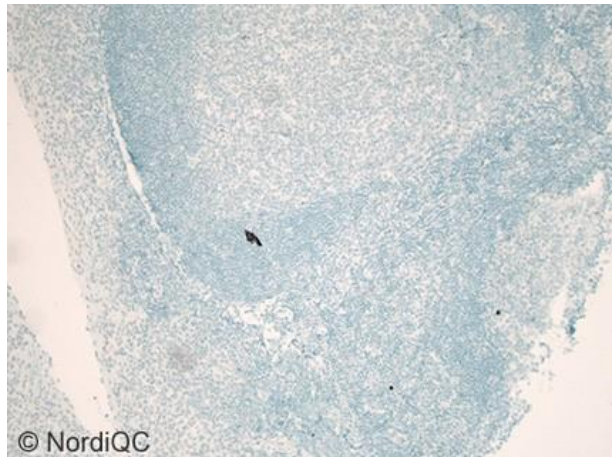


Fig. 4b (x100)
Uroplakin II/III staining of the tonsil using same insufficient protocol as in Figs. 1b - 3b. All cells are negative as expected. However, the analytical sensitivity is reduced as seen in Figs. 1b-3b.

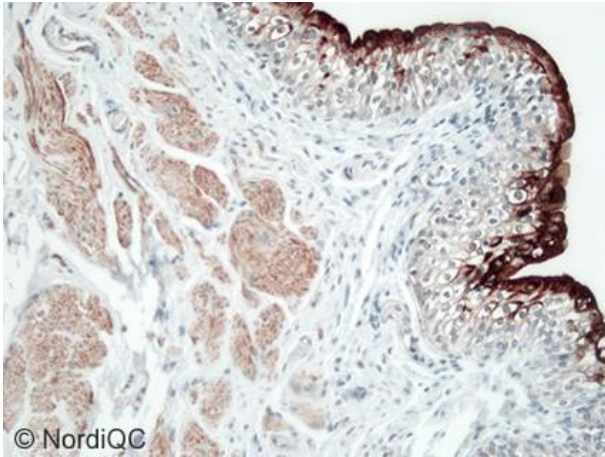


Fig. 5a (x200)
 Insufficient Uroplakin II/III staining of urethra using the rmAb clone SP73, diluted 1:100 (30 min. incubation), epitope retrieval using HIER in TRS high (20 min.) and a 2-step multimer based detection system (EnVision Flex) performed on Autostainer (Dako).
 The umbrella cells show a strong, plaque-like membranous predominantly apical staining reaction and a moderate cytoplasmic and membranous staining of the intermediate urothelial cells is seen. A weak to moderate cytoplasmic staining is seen in the muscle cells.

Same protocol used in Figs. 5b-6a-6b.

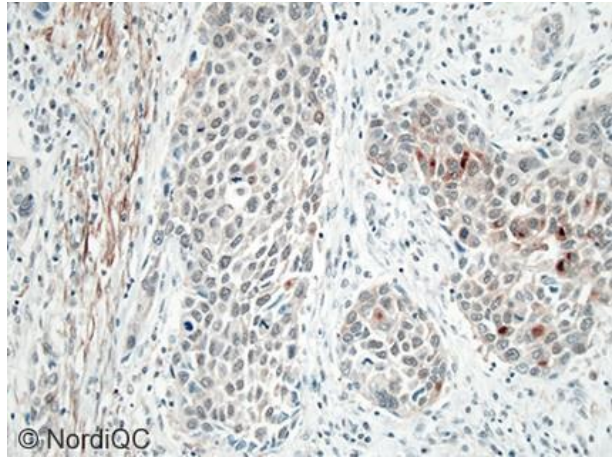


Fig. 5b (x200)
 Insufficient Uroplakin II/III staining of the urothelial carcinoma tissue core no 3 using same protocol as in Fig. 5a. The neoplastic cells show an aberrant faint nuclear staining reaction and only a weak cytoplasmic staining reaction is seen in the neoplastic cells. Compare with the expected level in Fig. 3a.

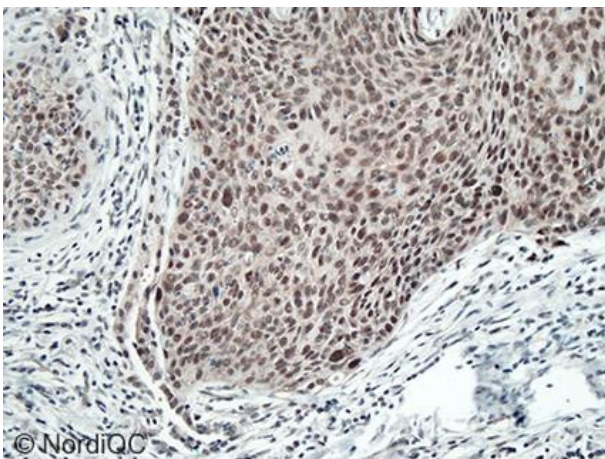


Fig. 6a (x200)
 Insufficient Uroplakin II/III staining of the lung squamous cell carcinoma using same protocol as in Figs. 5a and 5b.
 The neoplastic cells show a moderate false positive nuclear and cytoplasmic staining reaction.

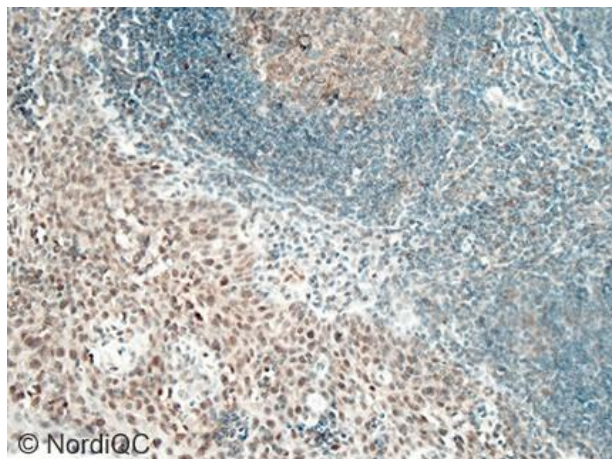
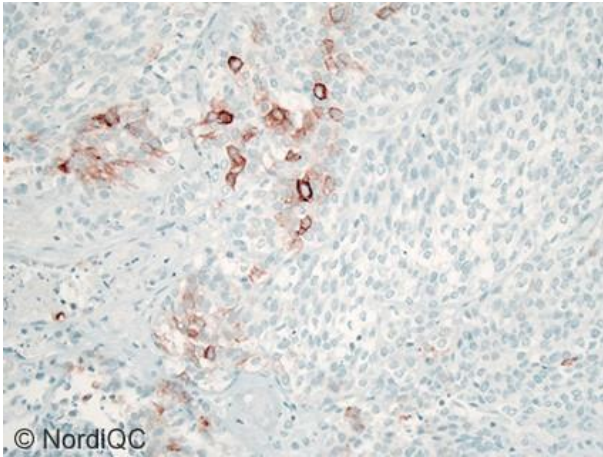


Fig. 6b (x200)
 Insufficient Uroplakin II/III staining of the tonsil using same protocol as in Figs. 5a, 5b and 6a.
 The squamous epithelial cells and germinal centers display a moderate false positive nuclear and cytoplasmic staining reaction.

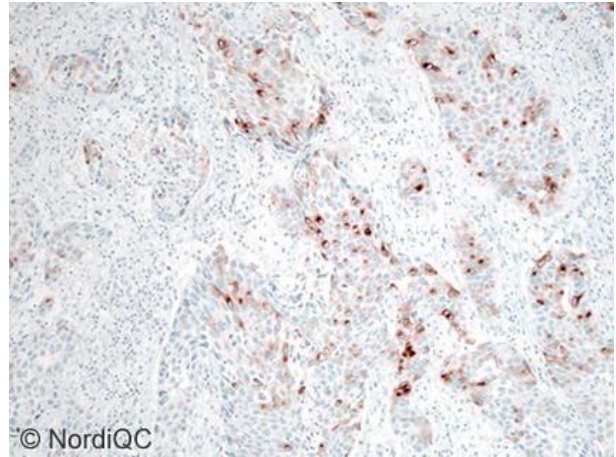


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Fig. 7a (x200)

Insufficient Uroplakin III staining of the urothelial carcinoma tissue core no 2 using the rmAb EP321 clone (Ventana), diluted, 1:50 (32 min. incubation), epitope retrieval using HIER in CC1 (36 min.) and a 2-step detection system (UltraView) on the Ventana platform. Same protocol as in Fig 7b.

A few scattered positive neoplastic cells display a strong membranous staining reaction, while the majority of the neoplastic cells remain negative (all other areas in the core showed no staining reaction).



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Fig. 7b (x100)

Insufficient Uroplakin III staining of the urothelial carcinoma tissue core no 3. Only this area includes neoplastic cells showing a moderate to strong plaque-like membranous staining reaction while the vast majority of neoplastic cells (>80-90%) remain negative (all other areas in the core showed no staining reaction).

TJ/RR/LE/SN 30.06.2020