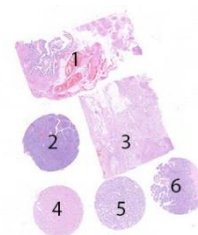


Material

The slide to be stained for PAX8 comprised:

1. Fallopian tube, 2. Tonsil, 3. Renal clear cell carcinoma, 4. Kidney, 5. Lung adenocarcinoma 6. Serous ovarian carcinoma.



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a PAX8 staining as optimal included:

- An at least weak to moderate, distinct nuclear staining reaction of the majority of ciliated epithelial cells and a strong nuclear staining of intercalated secretory epithelial cells in the fallopian tube.
- An at least weak to moderate, distinct nuclear staining reaction in the majority of epithelial cells of the proximal, distal/collecting renal tubules, loops of Henle and the parietal epithelial cells of Bowman’s capsule in the kidney.
- A strong, nuclear staining reaction of virtually all neoplastic cells of the serous ovarian carcinoma.
- A moderate to strong, nuclear staining reaction of the majority of the neoplastic cells in the renal clear cell carcinoma.
- Strong, nuclear staining of virtually all mantle zone B-cells, germinal centre B-cells and the interfollicular peripheral B-cells in the tonsils were expected for all antibodies used (except for antibodies raised against the C-terminal part of PAX8 e.g. mAb clone BC12 and rAb clone ZR-1).
- No staining reaction in the tumor cells in the lung adenocarcinoma

In cells with strong specific nuclear staining reaction, faint cytoplasmic staining was accepted.

Participation

Number of laboratories registered for PAX8, run 42	134
Number of laboratories returning slides	125 (93%)

Results

125 laboratories participated in this assessment. 88 laboratories (71%) achieved a sufficient mark (optimal or good). Abs used and assessment marks are summarized in Table 1 (see page 2)

The most frequent causes of insufficient staining were:

- Less successful performance of the mAb MRQ-50 on the Ventana BenchMark platform.
- Use of low sensitive detection systems
- Use of too short efficient HIER time
- Too low concentration of the primary Ab
- Too high concentration of the primary Ab

Performance history

This was the second NordiQC assessment of PAX8. A minor increase in pass rate was seen compared to run 34 in 2012 (see Table 2).

Table 2. **Proportion of sufficient results for PAX8 in the two NordiQC runs performed**

	Run 34 2012	Run 42 2014
Participants, n=	36	125
Sufficient results	64%	71%

Conclusion

The mAbs clones **MRQ-50**, **BC12**, **ZR-1** and **ILQ-150** and the pAb **10336-1-AP**, **CP379**, **NBP1-32440** and **363A** are all recommendable Abs for demonstrating PAX8. Irrespective of the antibodies applied, HIER in an alkaline buffer for at least 20 min. (or at least 32 min. for Ventana BenchMark users) and use of a sensitive and specific 3-step polymer/multimer based detection system gave the highest proportion of optimal results. The concentration of the primary antibody must be carefully calibrated. The most widely used PAX8 antibody, mAb clone **MRQ-50**, had a relatively low pass rate on the Ventana BenchMark platform, whereas the pAb **10336-1-AP** provided sufficient result on all main IHC systems. Fallopian tube

and kidney are recommendable positive tissue controls for PAX8. In fallopian tube an at least weak to moderate, distinct nuclear staining reaction of the majority of ciliated epithelial cells and a strong nuclear staining of intercalated secretory epithelial cells must be seen. In kidney an at least weak to moderate, distinct nuclear staining reaction in the epithelial cells of the proximal and distal/collecting renal tubules and parietal epithelial cells of Bowman's capsule must be seen.

Table 1. Antibodies and assessment marks for PAX8, run 42

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone MRQ-50	33	Cell Marque	19	8	6	0	82%	81%
mAb clone BC12	7	BioCare	1	3	1	2	57%	-
mAb clone ILQ-150	1	Immunologic	1	0	0	0	-	-
mAb clone PAX8R1	1	Abcam	0	1	0	0	-	-
rmAb clone ZR-1	1	Abcam	2	0	0	1	-	-
	1	Zeta						
	1	Zhongshan						
pAb, 363A	11	Cell Marque	0	4	7	0	36%	-
pAb, 10336-1-AP	11	Protein Tech	5	5	0	1	91%	100%
pAb, CP379	4	Biocare	1	2	1	0	-	-
pAb, RBK047	2	Zytomed Systems	0	1	1	0	-	-
pAb, HPA030062	1	Atlas Antibodies	0	0	0	1	-	-
pAb, ILP3633-C05	1	Immunologic	0	1	0	0	-	-
pAb, ABE671	1	Millipore	0	0	1	0	-	-
pAb, NBP1-32440	1	Novus	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone MRQ-50 760-4618	36	Ventana/Cell Marque	2	20	12	2	61%	73%
mAb clone MRQ-50 MAD-000550QD	3	Master Diagnostica	3	0	0	0	-	-
mAb clone MRQ-50 363M	2	Cell Marque	1	1	0	0	-	-
mAb clone BC12 API438	3	BioCare	3	0	0	0	-	-
mAb clone BC12 PDM 180	1	Diagnostic Biosystems	0	0	0	1	-	-
mAb clone ILQ-150 ILM4403	1	Immunologic	1	0	0	0	-	-
Total	125		41	47	29	8	-	
Proportion			33%	38%	23%	6%	71%	

1) Proportion of sufficient stains (optimal or good),

2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of PAX8, Run 42

The following protocol parameters were central to optimal staining:

Concentrated Antibodies

mAb clone **MRQ-50**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Bond Epitope Retrieval Solution 2 (Leica) (6/6) *, Target Retrieval Solution pH 9 (3-in-1) (Dako) (5/5), Tris-EDTA/EGTA pH 9 (3/5), Target Retrieval Solution pH 9 (Dako) (2/3), Citrate buffer pH 6.7 (1/1), Bond Epitope Retrieval Solution 1 (Leica) (1/1) or Cell Conditioning 1 (Ventana) (1/10) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 25 of 31 (81%) laboratories produced a sufficient staining result (optimal or good).

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

rmAb clone **ZR-1**: Two protocols with optimal results were both based on HIER using either Bond Epitope Retrieval Solution 2 (Leica) (1/1) or a combination of Protease 3 pretreatment (Ventana) and HIER in Cell

Conditioning 1 (Ventana) (1/1). The mAb was diluted in the range of 1:25-1:800 depending on the total sensitivity of the protocol employed.

mAb clone **BC12**: One protocol with optimal result was based on HIER in Borg Decloaker pH 9.5 (Biocare) using a pressure cooker. The mAb was diluted 1:25 and incubated for 30 min. at room temperature and visualized with a 3-step polymer conjugate system (Mach4, Biocare).

mAb clone **ILQ-150**: One protocol with optimal result was based on HIER in Tris-EDTA/EGTA pH 9. The mAb was diluted 1:50 and incubated for 60 min. at room temperature and visualized with a 2-step polymer conjugate system (BrightVision, Immunologic).

pAb **10336-1-AP**: Protocols with optimal results were all based on HIER using either, Cell Conditioning 1 (Ventana) (2/3), Tris-EDTA/EGTA pH 9 (1/1), EDTA/EGTA pH 8 (1/1) or Cell Conditioning 2 (Ventana) (1/2) as retrieval buffer. The pAb was typically diluted in the range of 1:100-1:800 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 of 6 (100%) laboratories produced a sufficient staining result.

pAb **CP379**: One protocol with optimal result was based on HIER in Cell Conditioning 1 (Ventana). The pAb was diluted 1:25 and incubated for 32 min. at 36°C and visualized with a 2-step multimer conjugate system (UltraView, Ventana).

pAb **NBP1-32440**: One protocol with an optimal result was based on HIER in Bond Epitope Retrieval Solution 2 (Leica). The pAb was diluted 1:100 and incubated for 15 min. at room temperature and visualized with a 2-step polymer conjugate system (Bond Refine, Leica).

Table 3 summarizes the overall proportion of optimal staining results for the most frequently used concentrated antibodies on the three most commonly used IHC stainer platforms.

Table 3. Proportion of optimal results for PAX8 using concentrated antibodies on the 3 main IHC systems*

Concentrated antibodies	Dako Autostainer Link / Classic / 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 MRQ-50	6/7 (85%)**	0/2	1/10 (10%)	-	6/6 (100%)	1/1
mAb clone BC12	0/2	-	0/2	-	0/1	-
pAb 10336-1-AP	1/2	0/1	2/3	1/2	0/2	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.

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

Ready-To-Use (RTU) Antibodies

mAb clone **MRQ-50** (product no. 760-4618, Ventana/Cell Marque): Protocols with optimal results were typically based on 32-80 min. HIER using Cell Conditioning 1 (Ventana), 16-32 min. incubation of the primary Ab and OptiView (Ventana 760-700 with or without amplification) as detection system. Using these protocol settings 8 of 11 (73%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **MRQ-50** (product no. MAD-000550QD, Master Diagnostica): Protocols with optimal results were typically based on HIER using Bond Epitope Retrieval Solution 2 (Leica), Tris-EDTA/EGTA pH 9, or EDTA/EGTA pH 8 (efficient heating time 20-30 min. at 95°C – 100°C) and 10-30 min incubation of the primary Ab and Bond Refine (Leica) or Quanto (Master Diagnostica) as detection system. Using these protocol settings 3 of 3 (100%) laboratories produced an optimal staining.

mAb clone **MRQ-50** (product no. 363M, Cell Marque): One protocol with an optimal result was based on 56 min. HIER using Cell Conditioning 1 (Ventana), 24 min. incubation of the primary Ab and OptiView with amplification (Ventana 760-700) as detection system.

mAb clone **BC12** (product no. API438, Biocare): Protocols with optimal results were typically based on HIER using Bond Epitope Retrieval Solution 2 (Leica), Tris-EDTA/EGTA pH 9, or EDTA/EGTA pH 8 (efficient heating time 20-30 min. at 95°C – 100°C) and 10-30 min. incubation of the primary Ab and Bond Refine

(Leica) or Quanto (Master Diagnostica): as detection system. Using these protocol settings 3 of 3 (100%) laboratories produced an optimal staining.

mAb clone **ILQ-150** (product no. ILM4403, Immunologic): One protocol with an optimal result was based on 64 min. HIER using Cell Conditioning 1 (Ventana), 48 min. incubation of the primary Ab at room temperature and UltraView with amplification (Ventana 760-500) as detection system.

pAb **363A** (product no. 363A, Cell Marque): The protocol giving an optimal result was based on HIER in TRS pH 9 (3-in-1) (Dako) with an efficient heating time of 15 min. at 97°C. The pAb was incubated for 20 min. at room temperature and visualized in a 2-step polymer conjugate system (EnVision, Dako).

Comments

In concordance with the previous assessment of PAX8 in NordiQC (run 34, 2012), the prevalent feature of an insufficient results was a too weak or false negative staining of the structures expected to be demonstrated. The majority of the participating laboratories were able to demonstrate PAX8 in the secretory epithelia cells of the fallopian tube and in neoplastic cells of the serous ovarian carcinoma, whereas the demonstration of PAX8 in neoplastic cells of the renal clear cell carcinoma, epithelial cells of collecting ducts, parietal cells lining the Bowman capsules of the kidney and in particular ciliated epithelial cells of the fallopian tube and epithelial cells of the proximal tubules in the kidney was more challenging and only seen with appropriate protocol settings (Fig. 1a - Fig. 4b) A few cases of insufficient staining due to false positive cytoplasmic and/or aberrant nuclear reaction of cells not expressing PAX8 were also seen (Fig. 5b and Fig. 6b). This pattern was typically caused by a too high concentration of the primary Ab or the use of a less successful primary antibody.

Optimal staining results could be obtained with the mAbs clones MRQ-50, BC12, ZR-1 and ILQ-150 and the pAb 10336-1-AP, CP379, NBP1-32440 and 363A (see Table 1). All laboratories used HIER or HIER in combination with short proteolytic pretreatment. The mAb clone MRQ-50 was the most widely used antibody for demonstration of PAX8. Used as a concentrate, mAb clone MRQ-50 gave an overall pass rate of 82% (27 of 33). Data focusing on the 3 main IHC systems showed that the proportion of sufficient and optimal results using the mAb clone MRQ-50 as a concentrate was significantly lower on the Ventana system compared to the Dako and Leica systems. On the Ventana system a pass rate of 50% (5 of 10) was seen and only 10% (1 of 10) were evaluated as optimal. On the Dako and Leica systems the pass rate was 92% (12 of 13) and 100% (7 of 7) respectively of of which 62% and 100% were optimal (see Table 3). The reasons for this difference are unclear, but similar findings were observed with the RTU format of MRQ-50 (see later). Using mAb clone MRQ-50 as a concentrate on non-Ventana platforms, optimal mark could be achieved with both 2- and 3-step polymer systems and with high and low pH HIER – although the combination of high pH HIER and 3-step polymer system was clearly the most successful with 11 out of 11 (100%) achieving optimal mark. Of the remaining 11 labs that did not use this combination, only 6 (55%) achieved optimal mark. On the BenchMark platform only one very sensitive protocol achieved optimal mark. This protocol combined a relative long HIER at high pH (CC1 for 64 min.) and the use of OptiView with amplification.

The RTU format of mAb clone MRQ-50 from Ventana/Cell Marque (760-4618) was the most widely used RTU in this assessment. 36 laboratories used this product on the Ventana BenchMark platform, where it showed a relatively disappointing pass rate of 61% (22 of 36) and with just 2 laboratories (6%) achieving optimal marks. One of the reasons for this might be related to less precise recommendation for the protocol given in the official package insert for the product. The datasheet recommends a relative short HIER treatment in CC1 (Ventana) (24 min. for OptiView and 36 min. for UltraView) in combination with short incubation times with the primary (8 min. for OptiView and 16 min. for UltraView). This is in contrast to the fact, that the typical protocols, that passed in this assessment, used incubation of the primary Ab for minimum 24 min. and HIER in CC1 for 48 min. or longer. Furthermore, the 2 labs that achieved optimal mark, combined these settings with the use of a 3-step multimer system with amplification.

The mAb clone BC12 and the rmAb ZR-1 are both raised against the C-terminal part of the PAX8 protein and do not show PAX5 cross reactivity (1). Consequently B-cells are unstained when mAb clone BC12 and rmAb ZR-1 are used for PAX8 staining. The general pass rate for mAb clone BC12 (RTU and concentrate) was 64% (7 of 11), but for non-Ventana users applying mAb clone BC12 in a 3-step polymer system after HIER in an alkaline buffer or in Diva pH 6.2 (BioCare), the pass rate was 100% (7 of 7). Two laboratories, using mAb clone BC12 in similar settings at the Ventana BenchMark platform, both achieved poor marks. A recent introduced rmAb clone ZR-1 was used by 3 laboratories. Two of these combined a 3-step multimer/polymer system with an efficient HIER protocol and both achieved optimal marks.

In this assessment the pAb 10336-1-AP (Protein Tech) was the most successful polyclonal antibody with a pass rate of 91% (10 of 11). Sufficient staining was achieved on all major staining platforms. Sufficient staining could be achieved using HIER in both alkaline and citrate based buffers and with 2-step and 3-step polymer/multimer detection systems, but the typical protocols achieving optimal marks, were protocols utilizing HIER in an alkaline buffer and detection in 3-step polymer/multimer systems. Although the numbers are small, data indicate, that pAb 10336-1-AP from Protein Tech is easier to optimize on the Ventana BenchMark platform, than other PAX8 antibodies (e.g. mAb clone MRQ-50, mAb clone BC12 and pAb 363A). With pAb 10336-1-AP 3 of 5 (60%) Ventana BenchMark users achieved optimal marks, whereas only 4 of 56 (7%) Ventana BenchMark users applying mAb MRQ-50, mAb clone BC12 or pAb 363A achieved optimal marks. In contrast to the high pass rate of the pAb 10336-1-AP (91%), the pAb 363A - in concentrated format - managed only a pass rate of 36% (4 out of 11) none of which achieved optimal marks.

Controls

Fallopian tube and kidney are both recommended as positive tissue controls for PAX8. In fallopian tube the protocol must be calibrated to provide an at least weak to moderate, distinct nuclear staining of the majority of the ciliated epithelial cells and a strong nuclear staining of the intercalated secretory epithelial cells. A weak cytoplasmic staining in the intercalated secretory epithelial cells can be expected and must be accepted. In kidney, optimally calibrated protocols must show an at least weak to moderate, distinct nuclear staining in the epithelial cells of the proximal and distal renal tubules, loops of Henle, collecting ducts, and the parietal epithelial cells of Bowman's capsule. A weak cytoplasmic staining in the same cells can be expected.

1: Moretti L, Medeiros LJ, Kunkalla K, Williams MD, Singh RR, Vega F. N-terminal PAX8 polyclonal antibody shows cross-reactivity with N-terminal region of PAX5 and is responsible for reports of PAX8 positivity in malignant lymphomas. *Mod. Pathol.* 2012 Feb;25(2):231-6. doi: 10.1038/modpathol.2011.162. Epub 2011 Oct 28. PubMed PMID: 22037256.

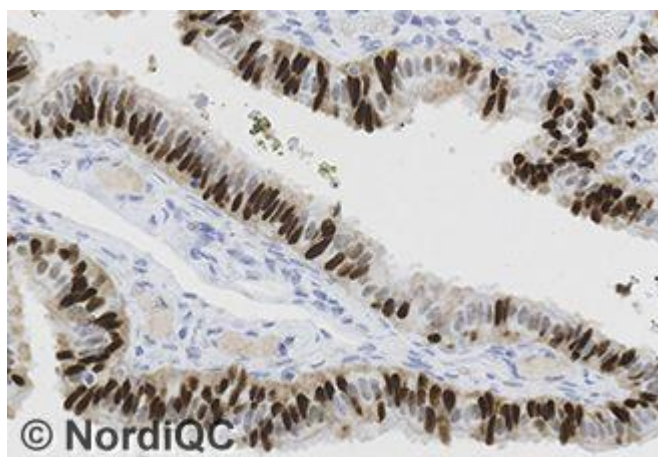


Fig. 1a
Optimal PAX8 staining of the fallopian tube using the mAb clone MRQ-50 (Cell Marque 1:200) with HIER in an alkaline buffer (Tris-EDTA/EGTA pH 9) using a 2-step polymer system (Immunologic) and performed on the LabVision Autostainer. A weak to moderate, distinct nuclear staining reaction of the majority of the ciliated epithelial cells and a strong nuclear staining of the intercalated secretory epithelial cells is seen. A weak cytoplasmic staining in the epithelial cells is seen - (same protocol used in Figs. 1a - 4a). Compare with Fig. 1b.

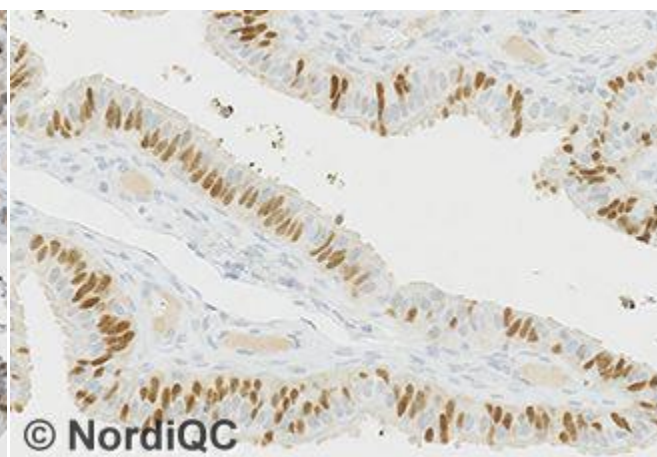


Fig. 1b
Insufficient PAX8 staining of the fallopian tube using the mAb clone MRQ-50 (Ventana/Cell Marque, RTU) with HIER in an alkaline buffer (CC1, Ventana) using a 2-step multimer system (Ventana) and performed on the BenchMark Ultra. A moderate nuclear staining of the intercalated secretory epithelial cells is seen whereas the ciliated epithelial cells are virtually negative. Compare with Fig. 1a – same field. Also compare with Figs. 2b, 3b and 4b – same protocol

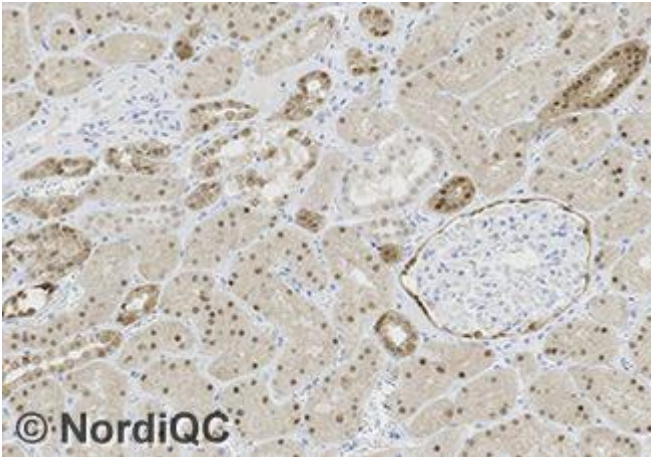


Fig. 2a
Optimal PAX8 staining of the kidney using the same protocol as in Fig. 1a. A moderate, distinct nuclear staining of the epithelial cells lining the Bowman capsule and of the distal/collecting tubular cells in the kidney is seen. A weak but distinct nuclear staining is seen in the majority of proximal tubular cells. A weak cytoplasmic staining is seen and accepted in both the epithelial cells lining the Bowman capsule and the tubular cells. Compare with Fig. 2b.

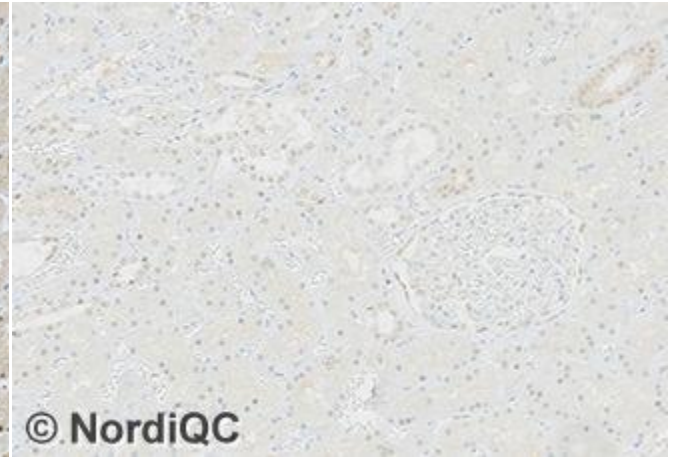


Fig. 2b
Insufficient PAX8 staining of the kidney using the same protocol as in Fig. 1b. Only a weak nuclear staining of the epithelial cells lining the Bowman capsule and of the distal/collecting tubular cells is seen. The proximal tubular cells are virtually negative. Compare with Fig. 2a - same field.

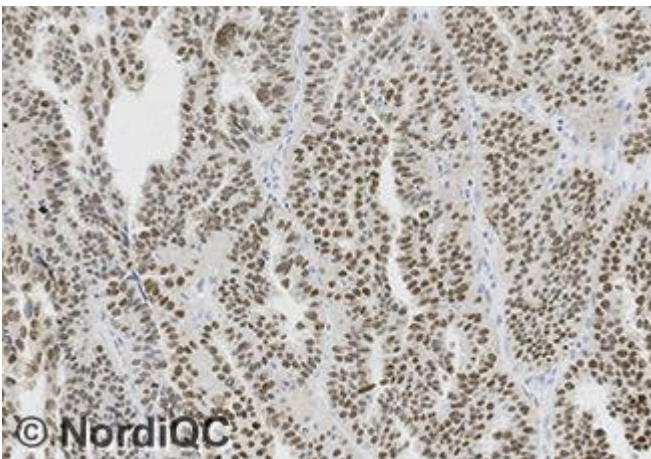


Fig. 3a
Optimal PAX8 staining of the serous ovarian carcinoma using the same protocol as in Figs. 1a & 2a. A strong, nuclear staining seen in virtually all the neoplastic cells is seen. Compare with Fig. 3b.

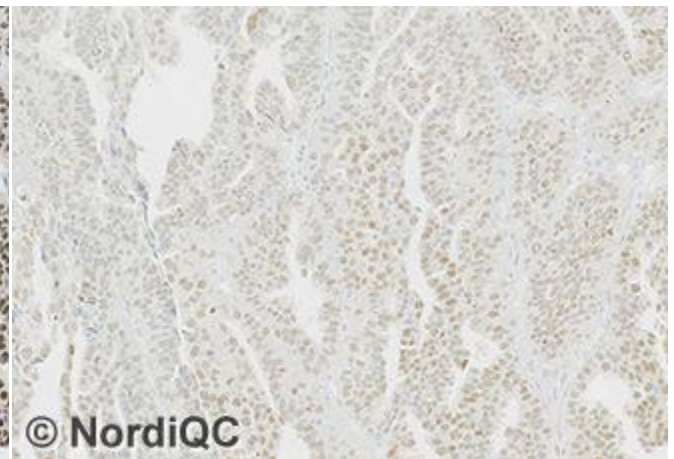


Fig. 3b
Insufficient PAX8 staining of the serous ovarian carcinoma using the same protocol as in Figs. 1b & 2b. The neoplastic cells display only a weak nuclear staining reaction. Compare with Fig. 3a - same field.

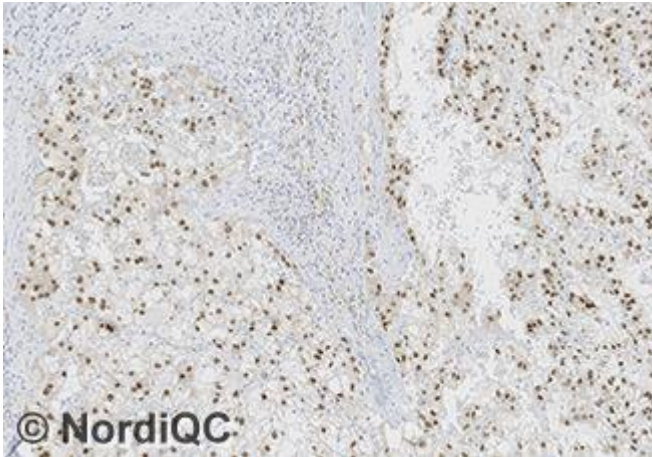


Fig. 4a
Optimal PAX8 staining in the renal clear cell carcinoma using the same protocol as in Figs. 1a, 2a & 3a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen. Compare with Fig 4b.



Fig. 4b
Insufficient PAX8 staining in the renal clear cell carcinoma using the same protocol as in Figs. 1b, 2b & 3b. Only faint nuclear staining is seen and only in a minor fraction of the neoplastic cells. Compare with Fig. 4a – same field.

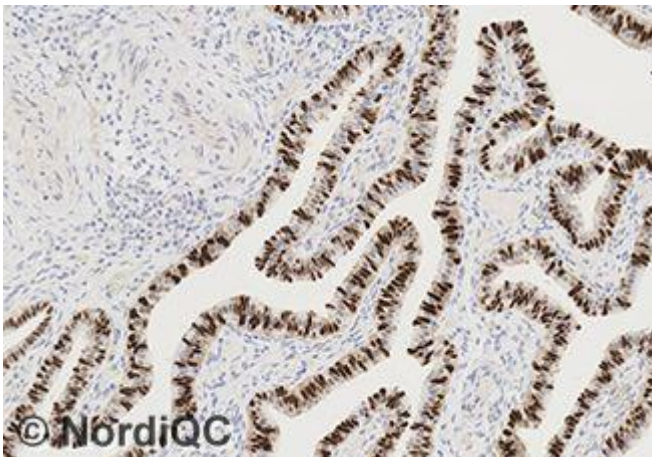


Fig. 5a
Optimal PAX8 staining of the fallopian tube using the pAb 10336-1-AP (ProteinTech, 1:500) with HIER in an alkaline buffer (CC1, Ventana) using a 3- step multimer system (OptiView, Ventana) and performed on the BenchMark Ultra. A moderate, distinct nuclear staining of the majority of the ciliated epithelial cells and a strong nuclear staining of the intercalated secretory epithelial cells is seen (same protocol used in Figs. 5a and 6a). Compare with Fig. 5b.

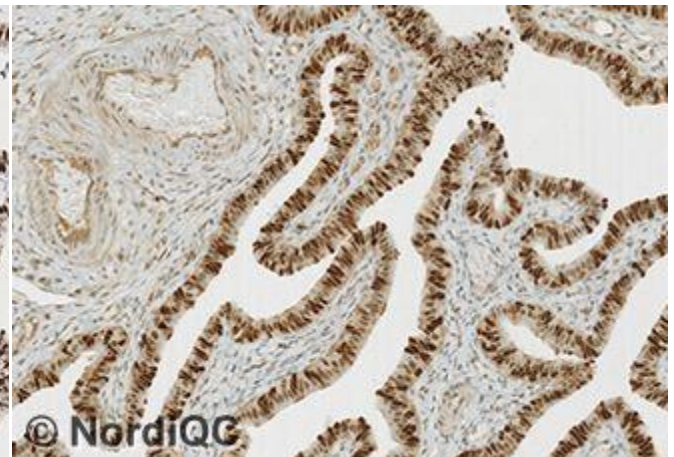


Fig. 5b
Insufficient PAX8 staining of the fallopian tube using the pAb 10336-1-AP (ProteinTech, 1:20) with HIER in an citrate based buffer (CC2, Ventana) using a 3-step multimer system (OptiView, Ventana) and performed on the BenchMark Ultra. A too high concentration of the primary Ab is applied causing a poor signal-to-noise ratio (same primary Ab as in Fig. 5a). In addition to the expected specific reaction a moderate, unspecific nuclear and cytoplasmic staining is seen in the majority of stromal cells. Compare with Fig. 5a - same field. Also compare with Fig. 6b – same protocol.

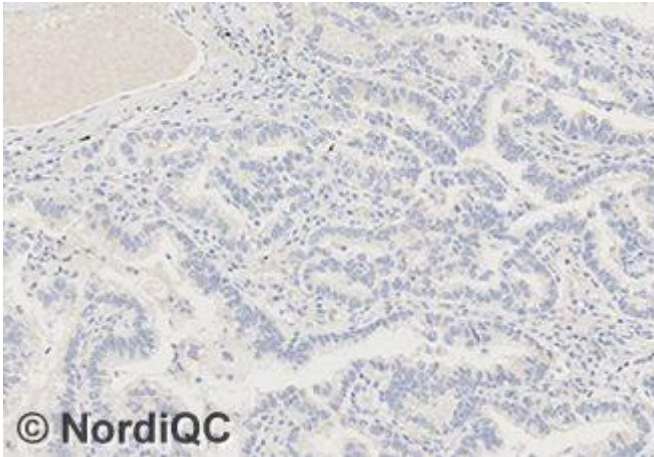


Fig. 6a
Optimal PAX8 staining of the lung adenocarcinoma using the same protocol as in Fig 5a. The neoplastic cells are negative. Only scattered B-cells in the stroma display a nuclear staining reaction. Compare with Fig. 6b.

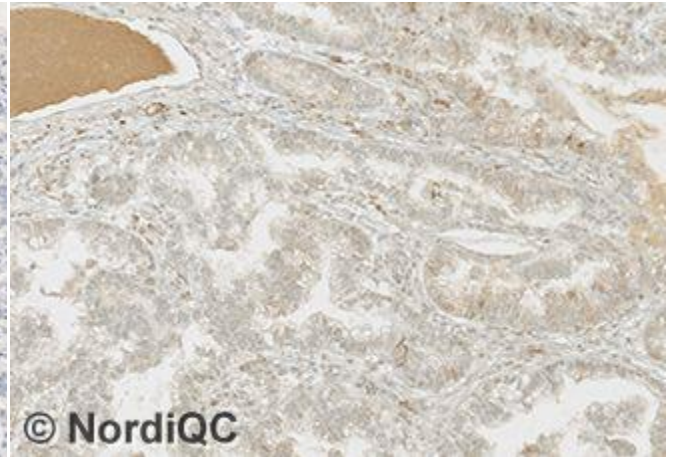


Fig. 6b
Insufficient PAX8 staining of the lung adenocarcinoma. Due to a too high concentration of the primary Ab the majority of the neoplastic cells are false positive. Compare with Fig. 6a - same field.

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