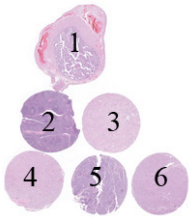
Assessment Run 56 2019

PAX8

Material
The slide to be stained for PAX8 comprised:



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

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a PAX8 staining as optimal included:

- A 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 in the ovarian serous adenocarcinoma.
- A moderate to strong, nuclear staining reaction of the majority of neoplastic cells in the renal clear cell carcinoma.
- No nuclear staining reaction of B-cells when antibodies were raised against the C-terminal part of PAX8 - e.g. mAb clone BC12 and rmAbs clones EP298 and ZR-1.
- No staining reaction of neoplastic cells in the lung adenocarcinoma.
- A nuclear staining reaction of B-cells in the tonsil and all other specimens included was expected and accepted for antibodies raised against the N-terminal part of PAX8 (1). This applied for all polyclonal Abs and mAbs clones MRQ-50 and DBM15.48 and the rmAb EP331.

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

Cross-reaction with other PAX epitopes will in coming runs be downgraded, due to interpretational challenges.

Participation

Number of laboratories registered for PAX8, run 56	278
Number of laboratories returning slides	264 (95%)

Results
264 laboratories participated in this assessment. 97 laboratories (37%) 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 reaction were:
- Less successful performance of the mAb MRQ-50 on the Ventana BenchMark and Dako Omnis platforms
- Use of detection systems with a low sensitivity
- Too short efficient Heat Induced Epitope Retrieval (HIER) time
- Too low concentration of the primary Ab.

Performance history
This was the fourth NordiQC assessment of PAX8. A decrease in pass rate was seen compared to run 51 in 2017 (see Table 2). The reason for this could be a combination of many new participants and an extensive use of the mAb clone MRQ-50 (67% of all participants) that was found to be less successful on the Ventana BenchMark and Dako Omnis platforms (used by 75% of all participants).

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

	Run 34 2012	Run 42 2014	Run 51 2017	Run 56 2019
Participants, n=	35	125	213	264
Sufficient results	63 %	70 %	56 %	37%

Conclusion
Optimal staining results could be obtained with the mAb clone **MRQ-50**, the rmAb clones **EP298**, **SP348** and **ZR-1** and the pAbs **10336-1-AP** and **363A-17/18**. Irrespective of the clone applied, efficient HIER, use of a sensitive 3-step polymer/multimer based detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result.
The rmAbs clones **EP298**, **SP348** and **ZR-1** gave encouraging results and a high proportion of sufficient results on the main fully automated platforms where no cross-reaction with e.g. PAX5 was observed. In contrast, the mAb clone **MRQ-50** provided a poor performance especially on the Ventana BenchMark and Dako Omnis platforms and at the same time also labelled PAX5 in B-cells. In coming assessments cross-reaction with other PAX epitopes will be downgraded.
Kidney and Fallopian tube are both recommendable as positive tissue controls for PAX8. In kidney, an at least weak to moderate, distinct nuclear staining reaction in the majority of the epithelial cells of the proximal, distal/collecting renal tubules and parietal epithelial cells of Bowman's capsule must be seen. 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. Tonsil can be used as negative tissue control for PAX8, as no staining should be seen in e.g. squamous epithelial cells and lymphocytes (positive nuclear staining in B-cells indicate cross reaction with PAX5).

Table 1. **Antibodies and assessment marks for PAX8, run 56**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BC12***	13 1	Biocare Zytomed Systems	-	4	4	6	29%	-
mAb clone **DBM15.48**	1	Diagnostic Biosystems	-	-	1	-	-	-
mAb clone **MRQ-50**	59 2	Cell Marque Master Diagnostica	8	19	22	12	44%	38%
mAb clone **MX062***	1	Maixin	-	1	-	-	-	-
mAb clone **PAX8R1**	1	abcam	-	-	-	1	-	-
rmAb clone **EP298***	7 5 1 2	Cell Marque Epitomics BIO SB Nordic Biosite	3	6	3	3	60%	100%
rmAb clone **EP331**	1	Path n situ	-	-	1	-	-	-
rmAb clone **SP348***	2 2	Spring Biosciences abcam	3	-	1	-	75%	100%
rmAb clone **ZR-1***	1 1 1	Zeta Corporation Abcam Gene tech	1	2	-	-	-	-
pAb, **10336-1-AP**	26	Protein Tech	9	6	11	-	58%	60%
pAb, **363A-15**	5	Cell Marque	-	2	1	2	-	-
pAb, **AP10903**	2	Gennova	-	1	-	1	-	-
pAb, **CP379**	5	Biocare	-	4	1	-	-	-
pAb, **Pax8**	1	Menapath	-	1	-	-	-	-
pAb, **RBG047**	1	Zytomed	-	-	-	1	-	-
pAb, **RBK047**	1	Diagomic	-	-	-	1	-	-
Ready-To-Use antibodies								
mAb clone **MRQ-50 760-4618**	91	Ventana/Cell Marque	3	16	41	31	21%	20%
mAb clone **MRQ-50 363M**	28	Cell Marque	3	4	12	9	25%	-
pAb **363A-17/363A-18**	3	Cell Marque	1	-	1	1	-	-
Total	264		31	66	99	68	-	
Proportion			12%	25%	37%	26%	37%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
*Clones that do not show cross reactivity with PAX5.

Detailed analysis of PAX8, Run 56
The following protocol parameters were central to optimal staining:

Concentrated Antibodies
mAb clone **MRQ-50**: Protocols with optimal results were all based on HIER using either Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/12)* or Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (5/16). The mAb was typically diluted in the range of 1:40-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 23 of 61 (38%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

rmAb clone **EP298**: Protocols with optimal results were based on HIER using TRS High pH (3/5) (Dako). The mAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP348**: Protocols with optimal results were based on HIER using TRS High pH (2/2) (Dako) or Cell Conditioning 1 (CC1, Ventana) combined with enzymatic pretreatment in protease 3 (Ventana) for 4 min. (1/1). The rmAb was diluted 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

rmAb clone **ZR-1**: One lab obtained an optimal result using the rmAb ZR-1 clone. The protocol was based on HIER using TRS High (Dako). The rmAb was diluted 1:50 and incubated for 30 min. and visualized with a 3-step polymer conjugated system (EnVision Flex+, Dako).

pAb **10336-1-AP**: Protocols with optimal results were all based on HIER using either CC1 (Ventana) (5/14) or TRS high (Dako) (3/6) as retrieval buffer. The pAb was diluted in the range of 1:200-1:1,600 depending on the total sensitivity of the protocol employed. Using these protocol settings, 12 of 20 (60%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for PAX8 for the most commonly used antibodies as concentrate on the four 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 **MRQ-50**	5/15** (33%)	0/2	0/7	-	0/17	0/1	3/12 (25%)	0/1
rmAb **EP298**	0/1	-	3/5 (60%)	-	0/8	-	0/1	-
pAb **10336-1-AP**	3/5 (60%)	0/3	3/6 (50%)	0/1	6/15 (40%)	-	0/1	-

* 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 **MRQ-50**, product no. **760-4618**, Ventana, BenchMark GT/XT/Ultra:
One lab produced an optimal result using this Ready-To-Use format for the Ventana platform. The protocol was based on HIER for 64 min. with CC1, 24 min. incubation of the primary Ab and OptiView (760-700) with amplification as detection system.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for PAX8 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT mAb MRQ-50 **760-4618**	0% (0/3)	0% (0/3)	20% (16/82)	1% (1/82)

* 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 >25%, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this assessment and in concordance with the previous NordiQC PAX8 assessments, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 98% of the insufficient results (164 of 167 laboratories). The remaining 2% of insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction compromising interpretation.
The majority of the participating laboratories were able to demonstrate PAX8 in high-level antigen expressing cell, such as secretory epithelia cells of the Fallopian tube and neoplastic cells of the ovarian serous adenocarcinoma, whereas demonstration of PAX8 in low-level antigen expressing cells as the neoplastic cells of the renal clear cell carcinoma, epithelial cells of renal distal convoluted 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 were more challenging and only seen with appropriate protocol settings (see Fig. 1a to Fig. 5b). Cases of insufficient staining reactions due to false positive cytoplasmic and/or aberrant nuclear reaction of cells not expressing PAX8 were also seen. This pattern was typically caused by use of a less successful primary antibody (see Fig. 6b).
In this assessment, the NordiQC assessor panel accepted cross-reactivity to PAX5 resulting in a distinct nuclear staining reaction of B-cells for antibodies raised against the N-terminal part of PAX8 (see Fig 5b and 6a). This applied for all polyclonal Abs and clones MRQ-50, DBM15.48 and EP331. Within the last couple of years well-performing rmAbs without cross reactivity has been introduced to the market (see Fig. 5a and Table 1). Based on this, cross reactivity with PAX5 will in future runs be downgraded due to the risk of misinterpretation.

54% (142 of 264) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for PAX8. The mAb clone MRQ-50 and the pAb 10336-1-AP were the most widely used antibodies (see Table 1). Both antibodies could be used to obtain an optimal staining result, but in general the pass rates for both antibodies were relatively low. For LD assays, the pass rate for mAb clone MRQ-50 and pAb 10336-1-AP was 44% (27 of 61) and 58% (15 of 26), respectively. Data focusing on the four main IHC systems (see Table 3) showed that the mAb clone MRQ-50 as a concentrate could not be used to provide an optimal result on the Ventana BenchMark and Dako Omnis systems. On the Ventana system, 0% (0 of 17) was evaluated as optimal. On the Dako Autostainer and Leica Bond systems, the proportion of optimal results was 33% (5 of 15) and 25% (3 of 12), respectively (see Table 3). This observation was also seen in the previous Run 51 and can potentially be related to the washing conditions and/or influence of elevated temperature settings (32°C on the Omnis and 36°C on the BenchMark) compared to systems using room temperature for incubation and washings. Whether this is the case with the mAb clone MRQ-50 is so far uncertain. The MRQ-50 protocols applied on Dako Autostainer and Leica Bond achieving optimal marks were all based on HIER at high pH and a 3-step polymer detection system. With the pAb 10336-1-AP, optimal staining results could be obtained on the Dako Autostainer (3 of 5), Dako Omnis (3 of 6) and Ventana Benchmark (6 of 15) systems, whereas no optimal staining results were seen on the Leica Bond (0 of 1) system.
Considering the overall low pass rate and low proportion of optimal scores obtained by the most widely used concentrated antibodies, mAbs clone BC12 and MRQ-50 and pAb 10336-1-AP, it is important to focus on some encouraging results obtained by the three rabbit monoclonal Abs. The clones EP298, SP348 and ZR-1 were all capable of producing optimal staining results within a LD assay for PAX8. The Dako Omnis showed only optimal results with these 3 clones, whereas the Ventana BenchMark and Dako Autostainer both showed sufficient results. 37% (3 of 8) of protocols based on EP298 on the Ventana platforms provided a suffcient result marked as good. None were optimal and at present no data on protocol modifications using e.g. combined pre-treatment might improve the result as seen for SP348.
Of the three rmAbs clones only the EP298 clone was tested on the Leica Bond but with an insufficient protocol. In addition to the good results the rmAb clones EP298, SP348 and ZR-1 do not cross-react with PAX5, leaving B-cells unstained and subsequently improving the signal to noise ratio (see Figs.1a-5a) and reducing the risk of clinical misdiagnosis.

46% (122 of 264) of the laboratories used Abs in Ready-To-Use (RTU) formats. This was a minor increase compared to the previous PAX8 assessment in 2017, where 40% of the laboratories used RTU formats. The most widely used RTU system for PAX8 were the mAb clone MRQ-50 based **760-4616** from Ventana/Cell Marque and the mAb clone MRQ-50 based **363M-18** from Cell Marque both intended for use on the Ventana BenchMark system. Both RTU products had an alarmingly low pass rate of 19% (16 of 85) and 11% (2 of 18), respectively. However, these data are in line with the observation for the MRQ-50 based LD assays and supports the claim that the mAb clone MRQ-50 is very difficult to optimize on the Ventana BenchMark system. In total, 103 laboratories used the Ventana BenchMark (see Table 4) with one of the two RTUs. Only 3 laboratories followed the vendors recommended protocol settings, and none of

these laboratories achieved a sufficient staining result. The remaining 100 laboratories modified the protocol settings and 19% (19 of 100) achieved a sufficient staining result with only one optimal.
In comparison, both clones were used on non-Ventana systems with a 50% (8 of 16) pass rate 31% (5 of 16) optimal.
Analyses of the data for the RTU products on the Ventana system did not reveal which modifications to the RTU protocol that would ensure optimal or even sufficient staining results. The only lab that obtained optimal staining results used the OptiView detection system with tyramide signal amplification, in combination with a 24 min. incubation of the primary Ab and a prolonged HIER pretreatment in CC1 (64 min.). This indicates that a highly sensitive detection system in combination with a careful recalibration of incubation time in primary Ab and HIER pretreatment might improve the general pass rate for the MRQ-50 RTUs on the Ventana system. In support of this was the fact that only 4% (2 of 57) showed a sufficient result using either UltraView or OptiView without tyramide signal amplification, whereas a 35% pass rate was seen among the labs using amplification. Only a slightly decreased pass rate was seen using OptiView 35% (11/31) instead of UltraView 33% (5/15).
In general, the recommendation would be to change to one of the rmAb concentrates listed in Table 1 that does not show a cross reactivity to PAX5.

Controls
Kidney and Fallopian tube are both recommended as positive tissue controls for PAX8. In kidney, optimally calibrated protocols must provide an at least weak to moderate, distinct nuclear staining reaction in the majority of 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 reaction in the same cells can be expected. 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. Internal observations show that inadequate fixation (too short time / delayed) in formalin can reduce epitope availability in low-level PAX8 expressing structures.
Tonsil can be used as negative tissue control for PAX8, as no staining should be seen in e.g. squamous epithelial cells and lymphocytes (positive nuclear staining in B-cells indicate cross reaction with PAX5)

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.

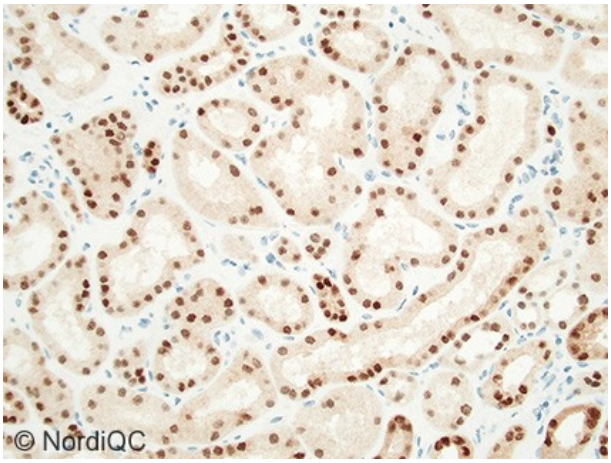


Fig. 1a
Optimal PAX8 staining of the kidney using the rmAb clone SP348 (Abcam) within a laboratory developed assay optimally calibrated, using HIER in an alkaline buffer and a 3-step polymer system (Dako/Agilent) and performed on the Dako Omnis stainer. A moderate to strong, distinct nuclear staining of the distal/collecting tubular cells in the kidney is seen. A weak to moderate nuclear staining is seen in the majority of proximal tubular cells. A weak cytoplasmic background staining is seen and accepted in the tubular cells (same protocol used in Figs. 1a - 6a) Compare with Fig. 1b.

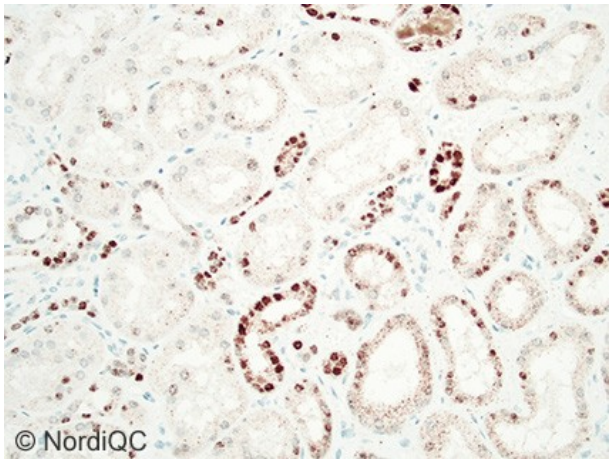


Fig. 1b
Insufficient PAX8 staining of the kidney using the mAb clone MRQ-50 (Ventana/Cell Marque) within a laboratory developed assay of the RTU using an alkaline HIER pretreatment and a 3-step polymer system with OptiView on the Ventana BenchMark system. A weak to moderate nuclear staining of the distal/collecting tubular cells is seen. The proximal tubular cells are virtually negative. Compare with Fig. 1a. This was the main issue for the MRQ-50 clone on the Ventana Benchmark and Dako Omnis platform. Also compare with Figs. 2b-6b – same protocol.

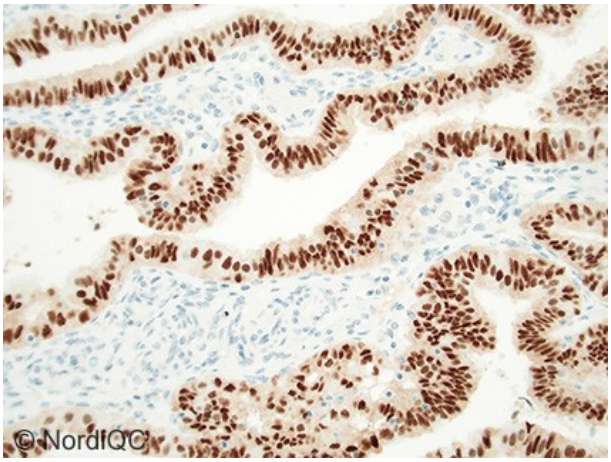


Fig. 2a
Optimal PAX8 staining of the Fallopian tube using the same protocol as in Fig. 1a. A weak to moderate, distinct nuclear staining of the majority of the ciliated epithelial cells and a strong nuclear staining of the intercalated secretory epithelial cells is seen. A weak cytoplasmic staining in these cells was seen and accepted. Compare with Fig. 2b.

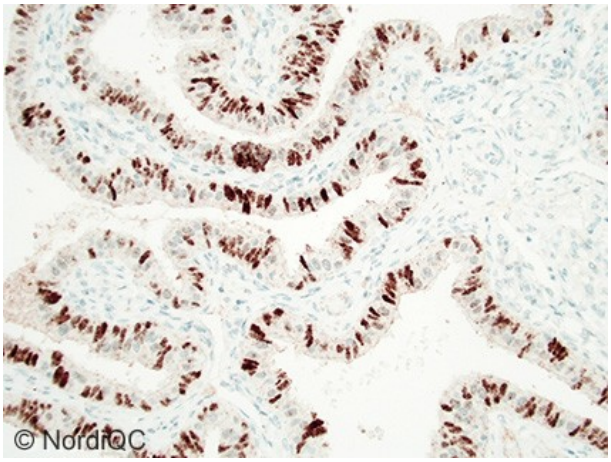


Fig. 2b
Insufficient PAX8 staining of the Fallopian tube using the same protocol as in Fig. 1b. A moderate nuclear staining of the intercalated secretory epithelial cells is seen whereas the ciliated epithelial cells are virtually negative. Compare with Fig. 2a.

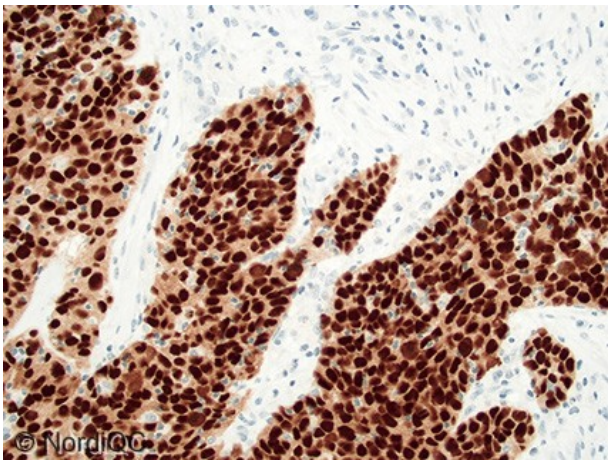


Fig. 3a
Optimal PAX8 staining of the ovarian serous adenocarcinoma using the same protocol as in Figs. 1a and 2a. A very strong, nuclear staining is seen in virtually all the neoplastic cells. Compare with Fig. 3b.

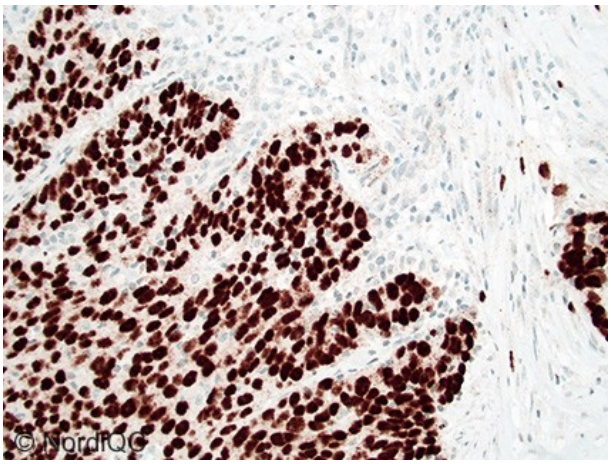


Fig. 3b
PAX8 staining of the ovarian serous adenocarcinoma using the same protocol as in Figs. 1b and 2b. The majority of the neoplastic cells display a moderate to strong nuclear staining reaction without cytoplasmic staining. Compare with Fig. 3a.

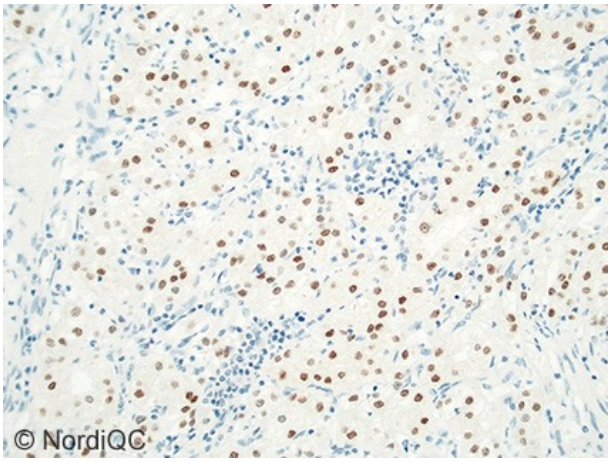


Fig. 4a
Optimal PAX8 staining in the renal clear cell carcinoma using the same protocol as in Figs. 1a-3a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen. Compare with Fig 4b.

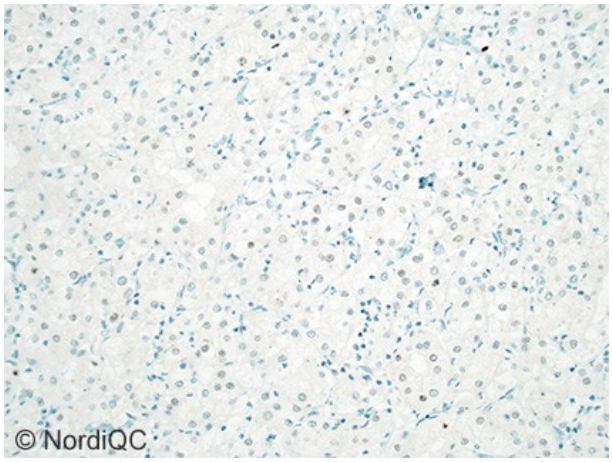


Fig. 4b
Insufficient PAX8 staining in the renal clear cell carcinoma using the same protocol as in Figs. 1b-3b. Only faint nuclear staining is seen and only in a minor fraction of the neoplastic cells. Compare with Fig. 4a.

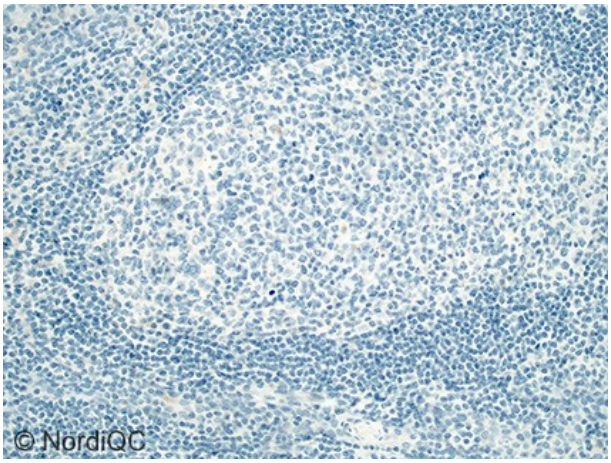


Fig.5a
PAX8 staining without PAX5 cross reactivity. PAX8 staining in tonsil using the same protocol as in Figs. 1a-4a. The rmAb clone SP348 do not cross-react with PAX5, leaving the B-cells unstained. Compare with Fig. 5b.

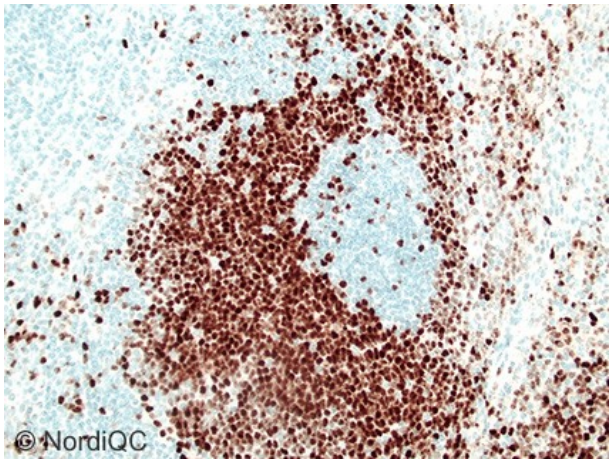


Fig. 5b
PAX8 staining with PAX5 cross reactivity. PAX8 staining in tonsil using the same protocol as in Figs. 1b-4b. The mAb clone MRQ-50 cross-reacts with PAX5 resulting in nuclear staining in virtually all B-cells. Compare with Fig. 5a.

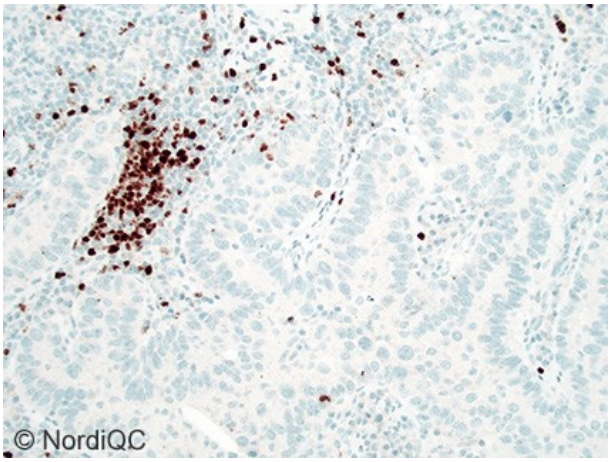


Fig. 6a

PAX8 staining with PAX5 cross reactivity. PAX8 staining in the lung adenocarcinoma using the mAb clone MRQ-50 (Cell Marque) same protocol as Figs 1b-5b. The mAb clone MRQ-50 cross-reacts with PAX5 resulting in strong nuclear staining in virtually all B-cell.

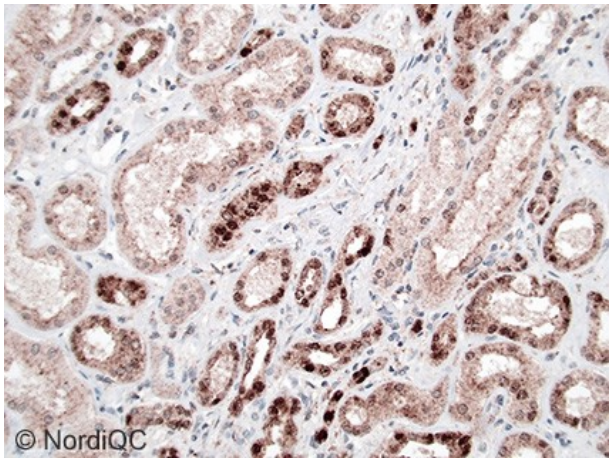


Fig. 6b

Insufficient PAX8 staining of the kidney using the mAb clone MRQ-50 (Cell Marque) on the Ventana Benchmark. Using a 3-step polymer detection system and increased HIER is causing a significant background staining without staining of the nuclei of the proximal tubules.

TJ/LE/RR/MV/SN 8.7.2019