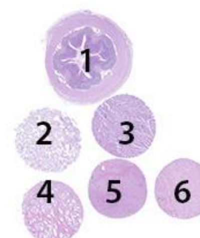
Assessment Run 58 2020
NKX3.1 (NKX3.1)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of the IHC assays for NKX3.1 performed by the NordiQC participants, identifying prostate as origin for carcinomas of unknown primary (CUP). Relevant clinical tissues, both normal and neoplastic, were selected to include a wide spectrum of NKX3.1 antigen densities (see below).

Material
The slide to be stained for NKX3.1 comprised:



1. Appendix 2. Testis 3. Colon adenocarcinoma 4. Prostate hyperplasia 5-6. Prostate adenocarcinoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing NKX3.1 staining as optimal included:

- A moderate to strong, distinct nuclear staining reaction of virtually all luminal epithelial cells in the prostate hyperplasia.
- An at least weak to moderate and distinct nuclear staining reaction of the majority of sertoli cells in seminiferous tubules of the testis.
- A strong, distinct nuclear staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma, tissue core no. 5.
- An at least weak to moderate nuclear staining reaction of the vast majority of neoplastic cells in the prostate adenocarcinoma, tissue core no. 6.
- No nuclear staining reaction of epithelial cells in appendix or the colon adenocarcinoma.

A weak to moderate cytoplasmic reaction in cells with strong nuclear staining was accepted.

Participation

Number of laboratories registered for NKX3.1, run 58	119
Number of laboratories returning slides	108 (91%)*

*One laboratory used an inappropriate antibody. Data is not included below.

Results
107 laboratories participated in this assessment. 78 (82%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody.
- Use of less sensitive detection systems.

Performance history
This was the second NordiQC assessment of NKX3.1 and an increased pass rate of 82% was observed.

Table 2. **Proportion of sufficient results for NKX3.1 in the two NordiQC runs performed**

	Run 49 2017	Run 58 2020
Participants, n=	49	107
Sufficient results	65%	82%

Conclusion
The widely used rmAb clone **EP356 and pAb CP422** could both be used to provide an optimal result for NKX3.1. The rmAb clone EP356 was most successful and used by the majority of laboratories. Optimal results was obtained both within a laboratory developed (LD) assay and as Ready-To-Use (RTU) formats. Within LD assays, efficient HIER in an alkaline buffer and use of a sensitive and specific 3-step polymer / multimer based detection system gave the highest proportion of optimal results.
Testis and normal prostate can be used as positive tissue controls for NKX3.1. In testis, a weak to moderate and distinct nuclear staining reaction must be seen in sertoli cells, while virtually all luminal epithelial cells of prostate glands must show a moderate to strong nuclear staining reaction. Appendix can be used as negative tissue control, in which no nuclear staining reaction in epithelial cells should be seen.

Table 1. **Antibodies and assessment marks for NKX3.1, run 58**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **OTI6E7**	1	ORIGENE	0	1	0	0	-	-
rmAb clone **EP356**	26 3 1	Cell Marque Bio SB Biocare Medical	16	10	3	1	87%	53%
mAb **ZM95**	1	Zeta Corporation	0	1	0	0	-	-
pAb **CP422**	24	Biocare	10	8	4	2	75%	42%
pAb **RBK062**	1	Zytomed	0	1	0	0	-	-
pAb **ZA-0172**	1	Zhongshanjinqiao	0	0	0	1	-	-
pAb **AP10634C**	2	Gennova	1	0	0	1	-	-
mAb clone **361**	1	Diagnostic BioSystems	0	0	0	1	-	-
Ready-To-Use antibodies								OR[2]
rmAb clone **EP356 API3189**	3	Biocare Medical	2	0	1	0	-	-
rmAb clone **EP356 BSB3114**	1	Bio SB	0	1	0	0	-	-
rmAb clone **EP356 441R-17**	10	Cell Marque	9	1	0	0	100%	90%
rmAb clone **EP356 MAD-00071QD**	2	Master Diagnóstica	0	1	0	1	-	-
rmAb clone **EP356 8320-C010**	1	Sakura Finetek	1	0	0	0	-	-
rmAb clone **EP356 760-5086 (VPRS)[3]**	10	Ventana/Cell Marque	8	1	1	0	90%	80%
rmAb clone **EP356 760-5086 (LMPS)[4]**	14	Ventana/Cell Marque	11	2	1	0	93%	79%
pAb **PP442 AA**	5	Biocare Medical	1	2	1	1	60%	20%
Total	107		59	29	11	8		
Proportion			55%	27%	10%	8%	82%	

1) Proportion of sufficient stains (optimal or good). For Laboratory Developed (LD) assays (≥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 NKX3.1, run 58

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

rmAb clone **EP356**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/6)*, Cell Conditioning 1 (CC1, Ventana) (13/20) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:40-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 23 of 26 (89%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

pAb **CP422**: Protocols with optimal results were based on HIER using CC1 (Ventana) (3/8), TRS pH 9 (3-in-1) (Dako) (3/4) or TRS High pH (Dako Omnis) (4/9) as retrieval buffer. The pAb was typically diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 15 of 21 (71%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for NKX3.1 for the most commonly used antibodies as concentrate on the main IHC systems***

Concentrated antibodies	**Dako Autostainer**		**Dako Omnis**		**Ventana BenchMark 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
rmAb clone **EP356**	2/6 (33%)	-	-	-	13/20 (65%)	-	0/3	-
pAb **CP422**	3/4	-	4/9 (44%)	-	3/8 (38%)	-	0/3	-

* 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
rmAb clone **EP356**, product no. **760-5086**, Ventana/Cell Marque, BenchMark XT/Ultra:
Protocols with optimal results were based on HIER using CC1 (efficient heating time 32-64 min.), 16-32 min. incubation of the primary Ab and UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 20 of 21 (95%) laboratories produced a sufficient staining result. Two laboratories applied amplification to the protocol settings mentioned above. Both obtained optimal results.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 assessed protocols). 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 intended IHC stainer device are included (in Table 1 laboratory modified protocol settings (LMPS) also includes off label use on deviant IHC stainers).

Table 4. **Comparison of pass rates for vendor recommended and laboratory modified RTU protocols**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT rmAb EP356 **760-5086**	9/10 (90%)	8/10 (80%)	13/14 (93%)	11/14 (79%)

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

Comments
The prevalent feature of an insufficient staining was a too weak or false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 74% (14 of 19) of the insufficient results. The remaining insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction complicating interpretation. Too weak staining was typically characterized by reduced staining reaction regarding both the intensity and proportion of cells expected to be demonstrated. This was in particular observed for the neoplastic cells of the prostate adenocarcinoma, tissue core no. 6, and the sertoli cells in seminiferous tubules of the testis. The majority of laboratories successfully demonstrated NKX3.1 in the majority of neoplastic cells of the prostate adenocarcinoma, tissue core no. 5, and the epithelial cells of the prostate hyperplasia, both with high expression levels of NKX3.1.

In this NordiQC assessment for NKX3.1, the rmAb clone EP356 was used by 66% (71 of 107) of the participants with a total pass rate of 89% (63 of 71), 66% optimal. When using a pAb, a decreased pass rate of 70% (23 of 33) was obtained, 36% optimal (see Table 1).

57% (61 of 107) of the laboratories used Abs as concentrated format within LD assays for NKX3.1 and obtained a pass rate of 79% (48 of 61). The rmAb clone EP356 and pAb CP422 were the two most widely used Abs, and both could be used to obtain an optimal staining result. The rmAb clone EP356 was most successful as 87% (26 of 30) of the laboratories using this clone produced a sufficient staining result and 53% were assessed as optimal. For the pAb CP422, an overall pass rate of 75% (18 of 24) was observed, 42% optimal. Efficient HIER in an alkaline buffer, careful calibration of the primary Ab and use of a 3-step polymer / multimer based detection system were the most central protocol prerequisites for optimal results.

RTU antibodies were used by 43% (46 of 107) of the laboratories. In general, the RTU formats provided an increased pass rate of 87% (40 of 46) compared to LD assays.

The RTU system from Ventana (760-5086) was the most widely used. The majority of laboratories using the Ventana RTU modified the protocol settings, obtaining a pass rate of 93% (13 of 14), 79% optimal. When applying the protocol settings recommended by Ventana, a pass rate of 90% (9 of 10) was obtained, 80% optimal (see Table 4).

The RTU format, 441R-17 (Cell Marque) based on the rmAb EP356, was used by 10 laboratories and provided a pass rate of 100%, 90% optimal. It must be emphasized that this RTU format is not developed for a particular automated IHC system/platform but was mostly used by laboratories for the Ventana Benchmark Ultra/XT platform. The successful protocol settings applied for the RTU format 441R-17 were similar to the settings described above for the Ventana RTU format 760-5086, also based on rmAb EP356.

Controls

Testis and normal prostate can be used as positive tissue controls. Virtually all luminal epithelial cells lining the prostate glands must show a moderate to strong and distinct nuclear staining reaction. In testis, a weak to moderate nuclear staining reaction must be seen in many sertoli cells of the seminiferous tubules. Testis seems to be the preferred critical positive tissue control to monitor the analytical sensitivity as the sertoli cells express low-levels NKX3.1.
Prostate is less reliable as positive tissue control for NKX3.1, since the luminal epithelial cells express high-level NKX3.1, making it difficult to evaluate the analytical sensitivity and reproducibility of the IHC protocol used.
Appendix or colon can be used as negative tissue control for NKX3.1, in which no nuclear staining reaction should be seen in epithelial cells. Dispersed lymphocytes can show a weak nuclear staining reaction.
Internal NordiQC data have indicated that the NKX3.1 antigen is influenced by pre-analytical conditions. Frequently, staining gradient in prostate resection materials has been seen (internal observations in NordiQC), which most likely is caused by delayed fixation and degrading of NKX3.1.

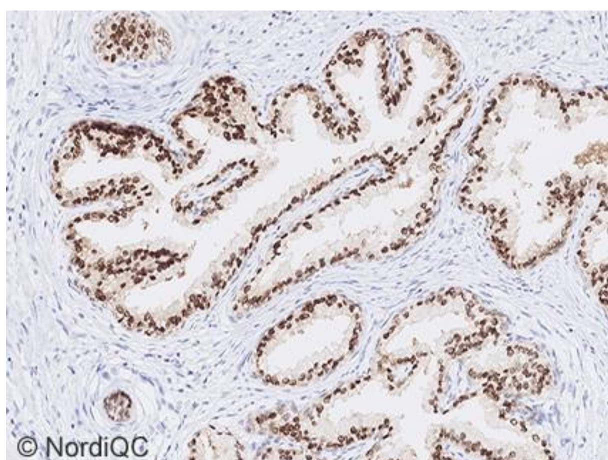


Fig. 1a
Optimal staining for NKX3.1 of the prostate with hyperplasia using the rmAb EP356 carefully calibrated with HIER in an alkaline buffer and a 3-step multimer based detection system.
All the epithelial cells of the prostatic glands show a moderate to strong nuclear staining reaction.
Also compare with Figs. 2a - 6a, same protocol.

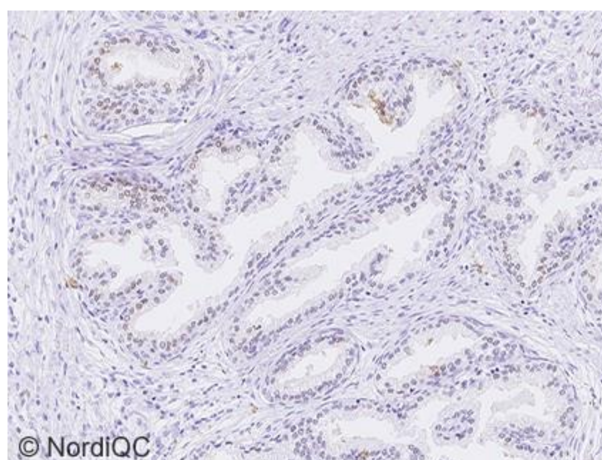


Fig. 1b
Insufficient staining for NKX3.1 of the prostate with hyperplasia using a polyclonal antibody with protocol settings providing too low sensitivity. Too low concentration of the primary Ab and a 2-step multimer based detection system - same field as in Fig. 1a.
Scattered epithelial cells are demonstrated, but a significant reduced intensity compared to the result seen in Fig. 1a.
Also compare with Figs. 2b – 4b, same protocol.

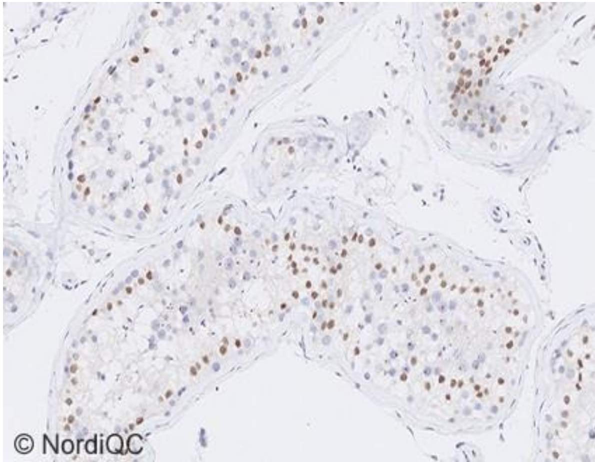


Fig. 2a
Optimal staining for NKX3.1 of normal testis using same protocol as in Fig. 1a.
Sertoli cells show a moderate distinct nuclear staining reaction and no background staining is seen.
Also compare with Figs. 3a and 6a, same protocol.

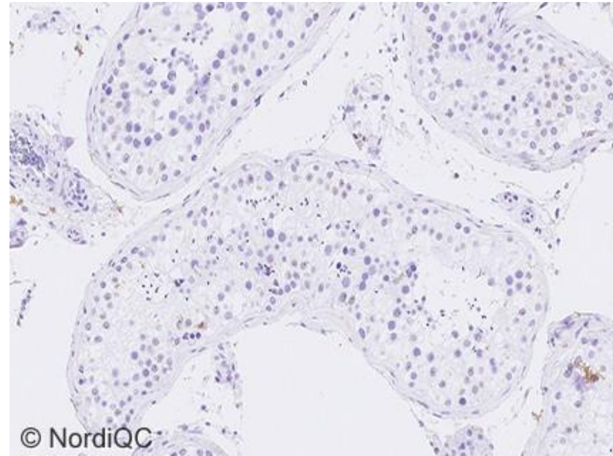


Fig. 2b.
Insufficient staining for NKX3.1 of normal testis using same protocol as in Fig. 1b.
The intensity and proportion of cells demonstrated is significant reduced compared to the level expected - same field as in Fig. 2a.
Also compare with Figs. 3b and 4b, same protocol.

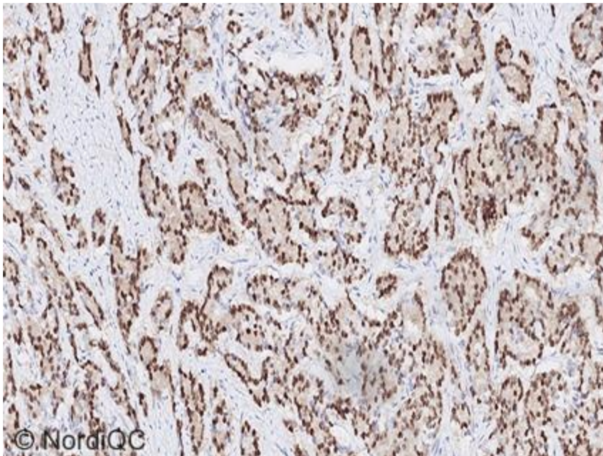


Fig. 3a
Optimal staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction.

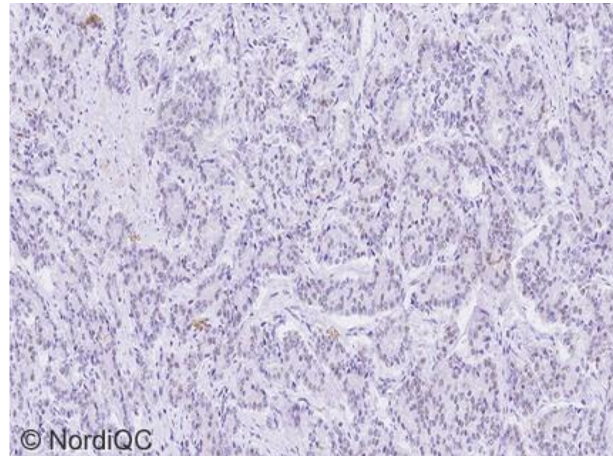


Fig. 3b
Staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. Only scattered cells show a faint and dubious staining reaction. Also compare with Fig. 4b, same protocol.

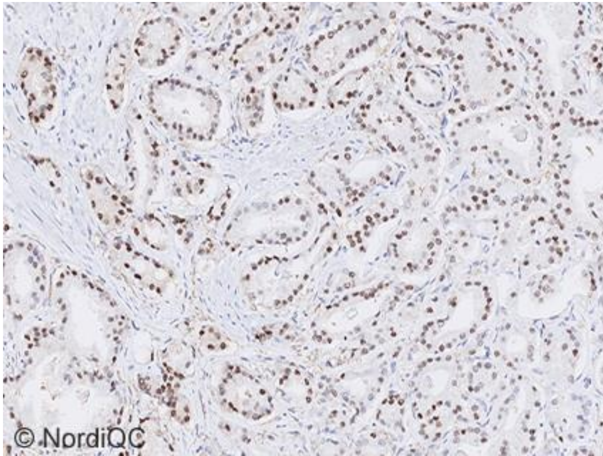


Fig. 4a
Optimal staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 6, using same protocol as in Figs. 1a - 3a. The majority of the neoplastic cells show a weak to moderate nuclear staining reaction.

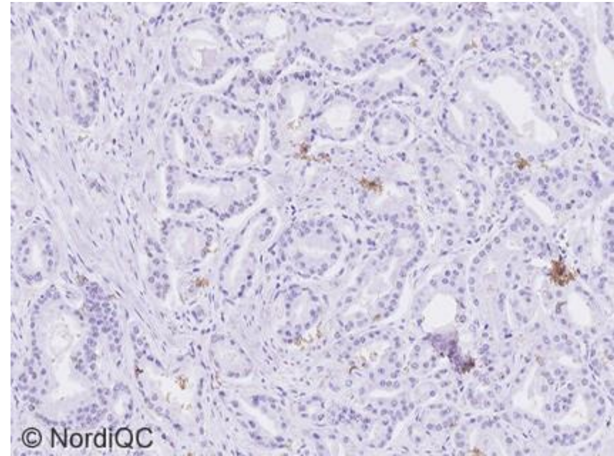


Fig. 4b
Insufficient staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 6, using same protocol as in Figs. 1b – 3b. – same field as in Fig. 4a.
Only scattered cells show a faint and dubious staining reaction. Clusters of unspecific dab precipitation is also seen.

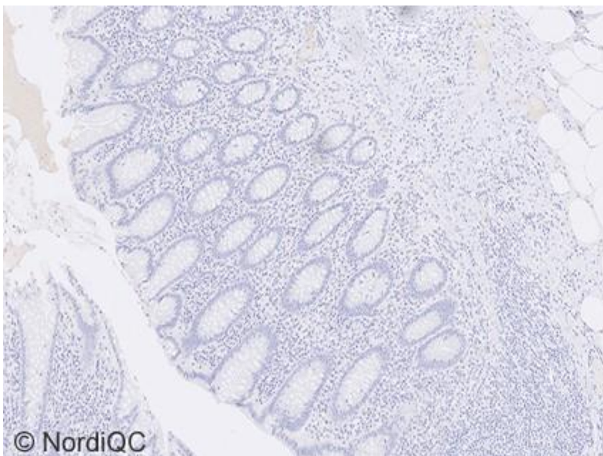


Fig. 5a
Optimal staining for NKX3.1 of the appendix using same protocol as in Figs. 1a – 4a. No staining reaction is seen. Appendix serves as negative tissue control to monitor a potential aberrant staining reaction e.g. caused by the primary Ab. Compare with Fig. 5b.

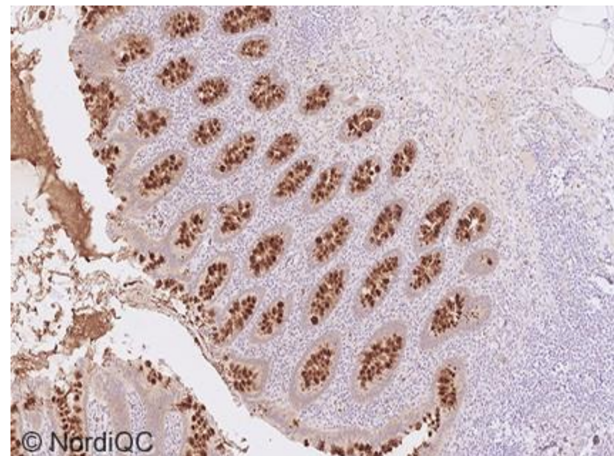


Fig. 5b
Insufficient staining for NKX3.1 of the appendix using the mAb 361 by protocols settings giving a false positive staining reaction, using same protocol as in Fig. 6b.
A strong aberrant cytoplasmic staining reaction in epithelial cells and a general poor signal-to-noise ratio is seen also see Fig. 6b, same protocol.

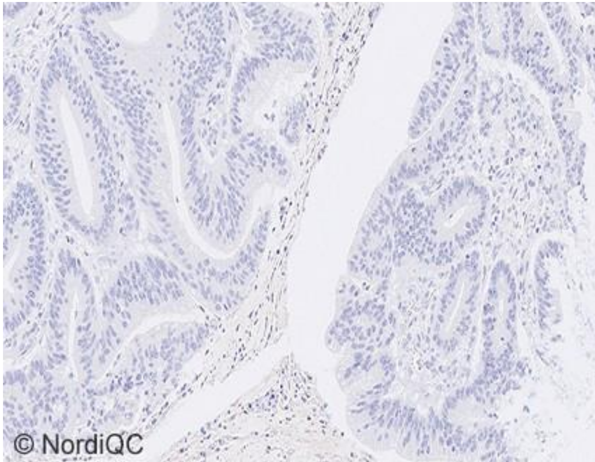


Fig. 6a
Optimal staining for NKX3.1 of the colon adenocarcinoma using same protocol as in Figs. 1a - 5a. No staining reaction is seen. Compare with Fig. 6b – same area.

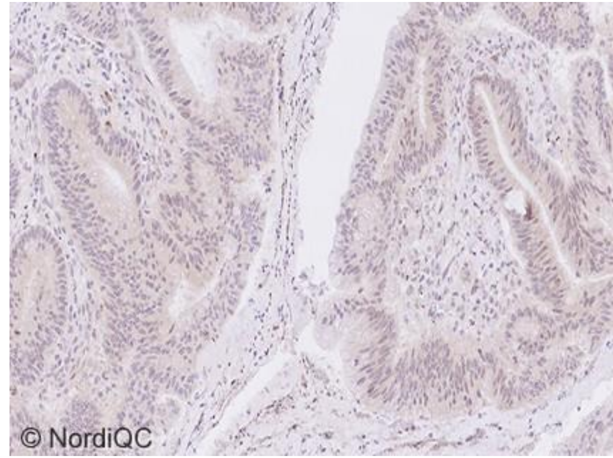


Fig. 6b
Insufficient staining for NKX3.1 of the colon adenocarcinoma using same protocol as in Fig. 5b. A weak diffuse staining reaction in the neoplstic cells is seen giving a poor signal-to-noise ratio and compromising the interpretation.

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