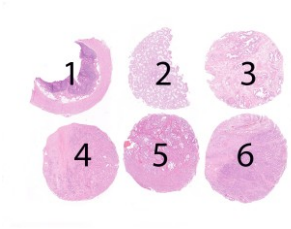
Assessment Run 77 2026

NKX3.1

Purpose
Evaluation of the technical performance, analytical sensitivity and specificity of NKX3.1 IHC assays among the NordiQC participants, typically used to identify prostate as origin for carcinomas of unknown primary (CUP). Relevant clinical normal and neoplastic tissues were selected to include a wide spectrum of NKX3.1 antigen expression levels (see below).

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



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

KEY POINTS FOR NKX3.1 IMMUNOASSAYS
- rmAb clone **EP356** was used by 82% of the participants with a total pass rate of 94%, 62% optimal. Optimal results could be obtained on all main IHC platforms.
- The Ventana/Roche RTU system **760-5086** provided an overall pass rate of 95%, 64% optimal when applied on a BenchMark platform.

Participation

Number of laboratories registered for NKX3.1, run 77	322
Number of laboratories returning slides	290 (90%)

Slides received after the assessment were not included in this report. However, all returned slides were assessed, and participating laboratories with insufficient results received advice.

Results
290 laboratories participated in this assessment. 264 (91%) achieved a sufficient mark (optimal or good), see Table 1a (see page 2). Tables 1b and 1c summarise the antibodies (Abs) used and assessment marks (see page 2 and 3).

The most frequent causes of insufficient staining reactions were:
- Inefficient HIER.
- Use of less sensitive detection systems.
- Less successful primary Ab.

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 of NKX3.1. Prostate is less reliable as a positive tissue control for NKX3.1, since the luminal epithelial cells express high levels of NKX3.1, making it difficult to evaluate the analytical sensitivity and reproducibility of the IHC protocol used.
Appendix or colon can be used as a 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 gradients in prostate resection materials have been seen (internal observations in NordiQC), which most likely is caused by delayed fixation and degradation of NKX3.1.

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 were 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-layer detection system gave the highest proportion of optimal results.

Table 1a. **Overall results for NKX3.1, run 77**

	n	Optimal	Good	Borderline	Poor	Sufficient results[1]	Optimal results
Concentrated antibodies	95	49	31	10	5	84%	51%
Ready-To-Use antibodies	195	116	68	9	2	94%	59%
Total	290	165	99	19	7		
Proportion		57%	34%	7%	2%	91%	

1) Optimal and good

Table 1b. **Concentrated antibodies and assessment marks for NKX3.1, run 77**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
rmAb clone **D6D2Z**	1	Cell Signaling	1	-	-	-	-	-
rmAb clone **EP356**	9	BioSB	1	4	4	-	89%	61%
	53	Cell Marque	37	13	1	2		
rmAb clone **ZR222**	1	Zeta Corporation	-	1	-	-	-	-
rmAb clone **QR055**	2	Quartett	1	-	1	-	-	-
rmAb clone **RM430**	1	BioSB	-	-	-	1	-	-
rmAb clone **GR20**	1	Genbio Solution	-	-	1	-	-	-
mAb clone **ZM95**	2	Zeta Corporation	1	1	-	-	-	-
mAb clone **361**	1	Diagnostic BioSystems	-	-	-	1	-	-
	1	Invitrogen	-	-	-	1		
pAb **CP422**	22	Biocare	8	12	2	-	91%	36%
pAb **AP10634C**	1	Gennova	-	-	1	-	-	-
Total	95		49	31	10	5		
Proportion			51%	33%	11%	5%	84%	

1) Proportion of sufficient results (optimal or good). For Laboratory Developed (LD) assays (≥5 assessed protocols)
2) Proportion of optimal results (≥5 asessed protocols).

Table 1c. **Ready-To-Use antibodies and assessment marks for NKX3.1, run 77**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **361 PDM569**	2	Diagnostic Biosystems	-	-	-	2	-	-
mAb clone **DY49306 4912422**	1	Unkown	1	-	-	-	-	-
Ab clone **SD471 AR0713**	1	Xiamen Talent Biomedical	-	-	1	-	-	-
rmAb clone **EP356 441R-17/18**	22	Cell Marque	17	5	-	-	100%	77%
rmAb clone **EP356 MAD-00071QD**	6	Master Diagnóstica	2	4	-	-	100%	33%
rmAb clone **EP356 8320-C010**	2	Sakura Finetek	2	-	-	-	-	-
rmAb clone **EP356 760-5086 (VRPS)[3]**	55	Ventana/Cell Marque	32	21	2	-	96%	58%
rmAb clone **EP356 760-5086 (LMPS)[4]**	81	Ventana/Cell Marque	55	23	3	-	96%	68%
rmAb clone **EP356 BSB-3113/4/5**	7	Bio SB	-	6	1	-	86%	0%
rmAb clone **EP356 CNR-0060**	1	Celnovte	1	-	-	-	-	-
rmAb clone **EP356 RMA-0753**	1	Fuzhou Maixin	1	-	-	-	-	-
rmAb clone **EP356 GT2260**	1	Gene Tech	1	-	-	-	-	-
rmAb clone **EP356 PR226/HAR226**	2	PathnSitu	-	2	-	-	-	-
rmAb clone **EP356 NOA-RBG080**	1	Zytomed Systems	-	-	1	-	-	-
rmAb clone **787G3C0 PA224**	1	Abcarta	1	-	-	-	-	-
rmAb clone **RM430 BSB-3785-3/7/15**	1	Bio SB	-	-	1	-	-	-
rmAb clone **BY079 BFM-0391**	1	Bioin Biotechnology	-	1	-	-	-	-
rmAb clone **IHC640 IHC640-7**	1	GenomeMe	-	1	-	-	-	-
rmAb clone **QR0055 QR0055**	1	Quartett	-	1	-	-	-	-
pAb **PP442 AA**	6	Biocare Medical	3	3	-	-	100%	50%
pAb **RBG062**	1	Zytomed Systems	-	1	-	-	-	-
Total	195		116	68	9	2		
Proportion			59%	35%	5%	1%	94%	

1) Proportion of sufficient results (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 77

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

rmAb clone **EP356**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) (11/18)*, Cell Conditioning 1 (CC1, Ventana/Roche) (20/32), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (6/9) or Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/2) as retrieval buffer. The rmAb was diluted in the range of 1:10-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 54 of 60 (90%) 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/Roche) (1/2), TRS pH 9 (Dako/Agilent) (5/16) or BERS2 (Leica Biosystems) (2/4) as retrieval buffer. The pAb was typically diluted in the range of 1:40-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 19 of 19 (100%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for NKX3.1 on the four main IHC systems by optimal titration settings as listed above**

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
rmAb clone **EP356** (1:10-200)	3/4*	-	8/14 (57%)	0/1	23/31 (74%)	-	6/9 (67%)	1/2
pAb **CP422** (1:40-100)	-	-	5/14 (36%)	-	1/1	-	2/4	-

1) Autostainer Classical, Link 48.
2) BenchMark XT, Ultra, Ultra plus
3) Bond III, Prime
* (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.), 12-60 min. incubation of the primary Ab and UltraView (760-500) +/- amplification kit or OptiView (760-700) as detection system. Using these protocol settings, 123 of 127 (97%) laboratories produced a sufficient staining result.
The product was used by 4 laboratories on a non-intended platform. This data is not included.

Table 3 summarises 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 according to the vendor recommendations and by laboratory modified systems with changes to basic protocol settings. Only protocols performed on the intended IHC stainer are included.

Table 3. **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 UltraView**	100% (35/35)	60% (21/35)	91% (10/11)	64% (7/11)
VMS Ultra/XT rmAb EP356 **760-5086 OptiView**	90% (18/20)	55% (11/20)	98% (65/66)	70% (46/66)

* 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 result was a too weak or false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 81% (21 of 26) of the insufficient results. The remaining insufficient results were characterized by a poor signal-to-noise ratio, excessive background staining and/or false positive staining reaction complicating interpretation (n=5). 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 adenocarcinomas and the Sertoli cells in seminiferous tubules of the testis. The majority of laboratories successfully demonstrated NKX3.1 in the majority of the epithelial cells of the prostate hyperplasia, with a high expression level of NKX3.1.

In this NordiQC assessment for NKX3.1, the rmAb clone EP356 was used by 82% (238 of 290) of the participants with a total pass rate of 94% (224 of 238), 62% optimal (n=148). When using a pAb, a slightly decreased pass rate of 90% (27 of 30) was obtained, 37% optimal (n=11) (see Tables 1b+c).

33% (95 of 290) of the laboratories used Abs as concentrated format within LD assays for NKX3.1 and obtained a pass rate of 84% (80 of 95), see Table 1b. 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 – see Table 2.

The **rmAb clone EP356** was most successful as 89% (55 of 62) of the laboratories using this clone produced a sufficient staining result and 61% were assessed as optimal. As seen in Table 2, optimal results could be obtained on all four main IHC platforms. 71% of the laboratories used a 3-layer detection system, with a pass rate of 95% (42 of 44), 70% optimal (n=31), being significantly higher compared to laboratories using a 2-layer detection system with a pass rate of 72% (13 of 18), 39% optimal results.

For the **pAb CP422**, an overall pass rate of 91% (20 of 22) was observed, 36% optimal. Efficient HIER in an alkaline buffer, careful calibration of the primary Ab and use of a 3-layer detection system were the most central protocol prerequisites for optimal results. Most laboratories used the pAb on the Dako Omnis platform (68%, 15 of 22). Sufficient results were obtained with use of both 2- and 3-layer detection systems, however, optimal results were only seen with EnVision Flex+ (mean titre of 1:70, range 1:50-100). However, when using EnVision Flex+ non-optimal results were due to excessive background staining, seen in protocols with a higher mean titre of 1:45, range 1:30-50.

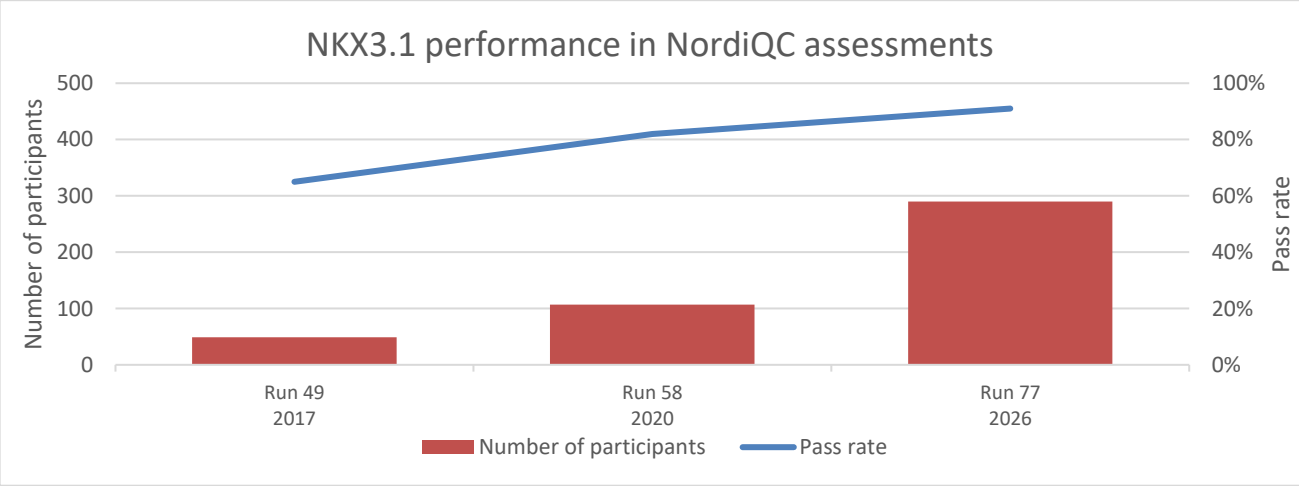
RTU antibodies were used by 67% (195 of 290) of the laboratories. In general, the RTU formats provided an increased pass rate of 94% (184 of 195) compared to LD assays – see Table 1a.

The RTU system from **Ventana/Roche (760-5086)** was the most widely used. When applying the vendor recommended protocol settings, pass rates of 100% and 90% were observed for UltraView and OptiView protocols, 60% and 55% optimal, respectively (see Table 3). The majority of laboratories modified the protocol settings, obtaining a pass rate of 91% and 98% for UltraView and OptiView protocols, 64% and 70% optimal, respectively (see Table 3). When using OptiView as detection system, modifications were typically prolonged HIER and incubation time of the primary Ab. VRPS are based on HIER for 32 min. and 16 min. incubation of primary Ab. By prolonging HIER to minimum 48 min. and incubation of primary Ab to minimum 32 min., a pass rate of 100% (30/30), 83% optimal (n=25), was seen.

The RTU format, **441R-17 (Cell Marque)** based on the rmAb EP356, was used by 22 laboratories and provided a pass rate of 100%, 77% optimal, see Table 1c. 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 concentrated format of rmAb EP356.

Performance history
This was the third NordiQC assessment of NKX3.1. The pass rate increased to 91% in this assessment compared with the previous assessment runs as shown in Graph 1.

Graph 1**. Proportion of sufficient results for NKX3.1 in the three NordiQC runs performed.**



Summary
This was the third NordiQC assessment of NKX3.1 (see Graph 1). The pass rate increased from 82% in run 58 to 91% in this assessment run 77 even though the number of participants has almost tripled with 183 new laboratories. From the previous runs, and in concordance to the general observations in the NordiQC

EQA scheme, a constant increase in laboratories using the RTU products was observed going from 24% (12 of 49) in run 49 (2017) and 43% (61 of 107) in run 58 (2020) to 67% (195 of 290) in this run.
From the main vendors, it is currently only Ventana/Roche that offers an RTU system, but the most widely used concentrate, rmAb clone EP356, produced optimal results on all platforms.

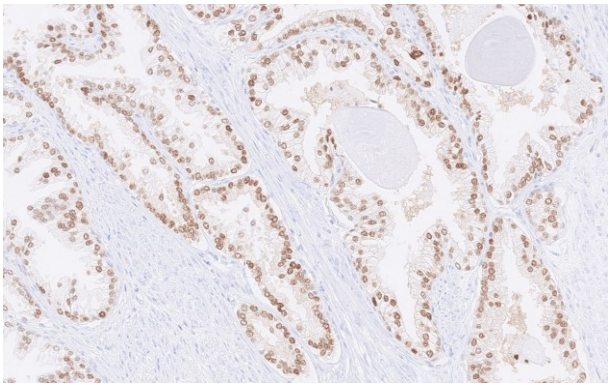


Fig. 1a
Optimal staining for NKX3.1 of the prostate with hyperplasia using the RTU system 760-5086, Ventana/Roche, based on rmAb EP356 with modified protocol settings with HIER in CC1 for 48 min., 32 min. incubation of primary Ab and OptiView as detection system. All the epithelial cells of the prostatic glands show a moderate nuclear staining reaction.
Also compare with Figs. 2a - 6a, same protocol.

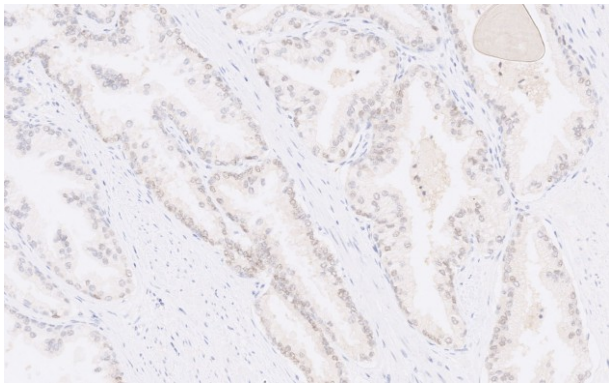


Fig. 1b
Insufficient staining for NKX3.1 of the prostate with hyperplasia using a concentrated format of rmAb clone EP356 (1:50) on a BenchMark Ultra, with HIER in CC1 for 52 min., 24 min incubation of primary Ab and UltraView as detection system. Scattered epithelial cells are demonstrated, but a significantly reduced intensity compared to the result seen in Fig. 1a.
Also compare with Figs. 2b – 4b, same protocol.

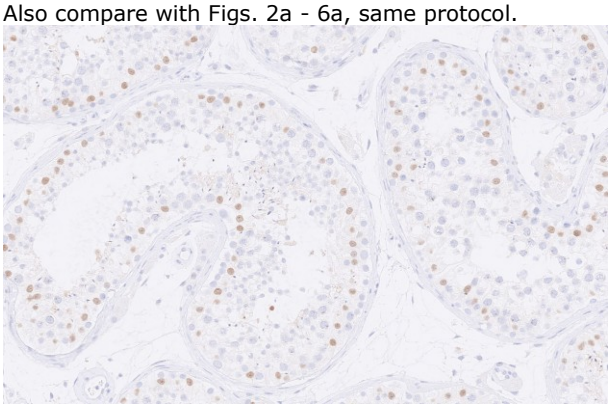


Fig. 2a
Optimal staining for NKX3.1 of normal testis using same protocol as in Fig. 1a.
Sertoli cells show a weak to moderate distinct nuclear staining reaction and no background staining is seen.
Also compare with Figs. 3a - 6a, same protocol.

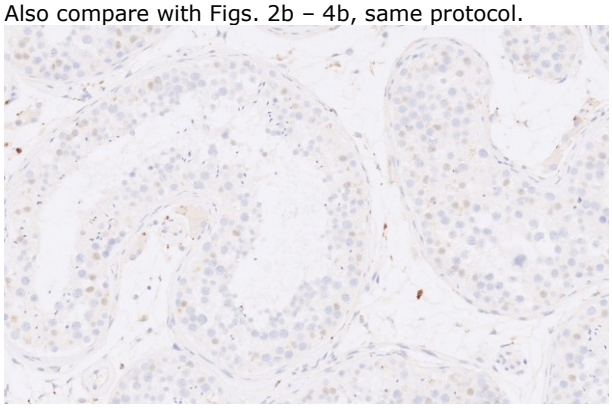


Fig. 2b.
Insufficient staining for NKX3.1 of normal testis using same protocol as in Fig. 1b.
The intensity and proportion of cells demonstrated are significantly reduced compared to the level expected - same field as in Fig. 2a.
Also compare with Figs. 3b and 4b, same protocol.

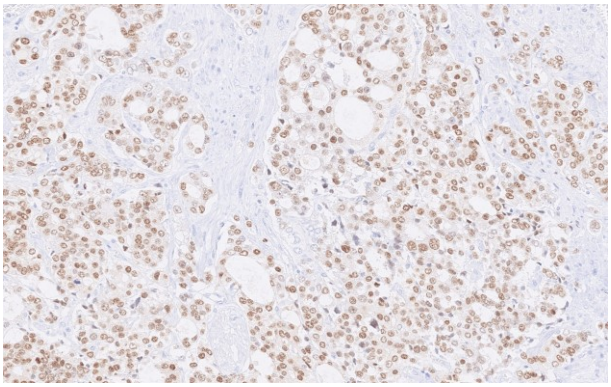


Fig. 3a
Optimal staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 4, using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a weak to moderate nuclear staining reaction.

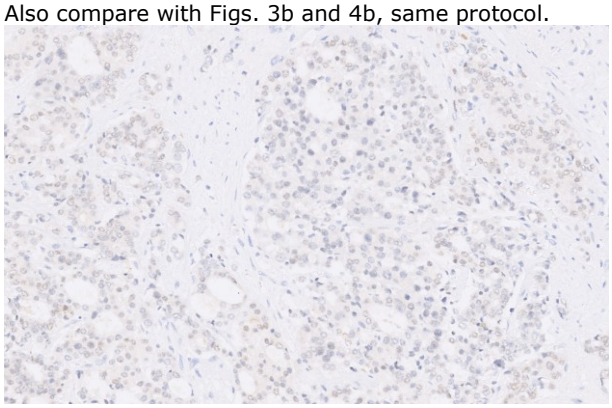


Fig. 3b
Insufficient staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 4, using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. Only scattered cells show a faint and dubious staining reaction. Also compare with Fig. 4b, same protocol.

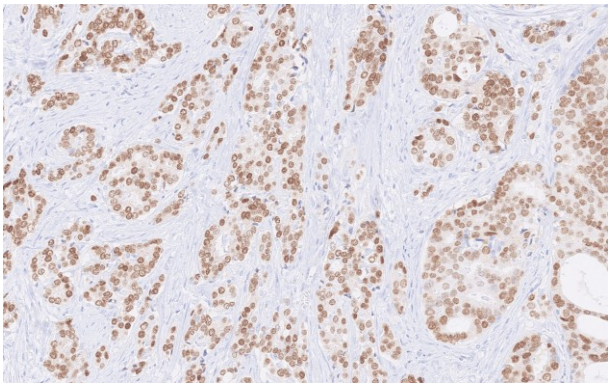


Fig. 4a
Optimal staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1a - 3a. The majority of the neoplastic cells show a moderate to strong nuclear staining reaction.

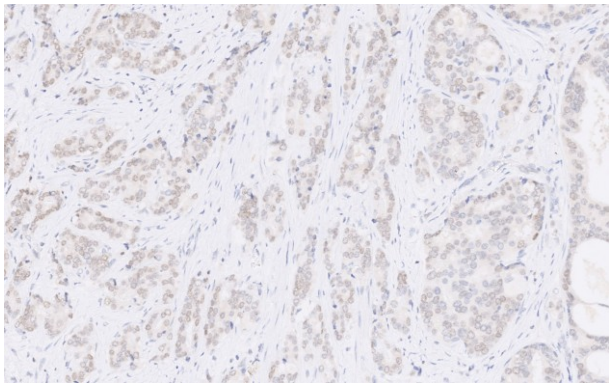


Fig. 4b
Insufficient staining for NKX3.1 of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1b – 3b. The neoplastic cells show a significantly reduced intensity compared to the result seen in Fig. 4a – same field.

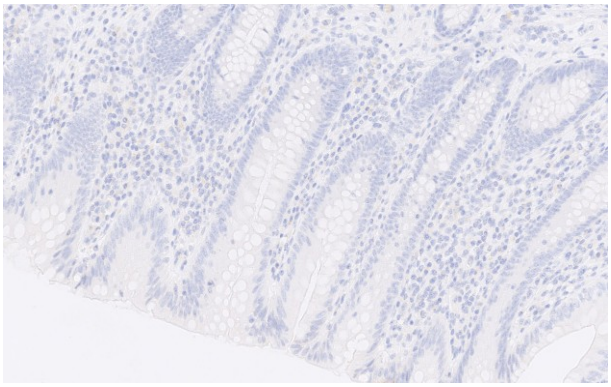


Fig. 5a
Optimal staining for NKX3.1 of the appendix using same protocol as in Figs. 1a – 4a. No staining reaction is seen. Appendix serves as negative tissue control to monitor a potential aberrant staining reaction e.g. caused by the primary Ab. Compare with Fig. 5b.

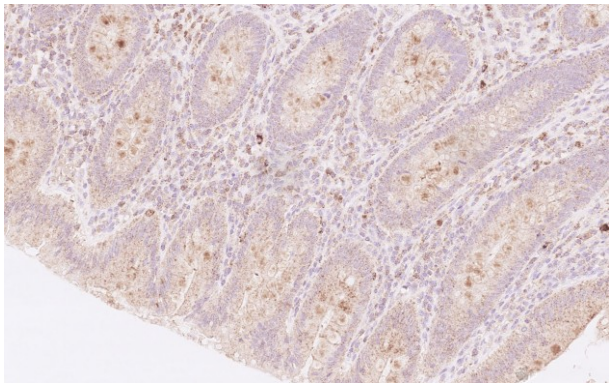


Fig. 5b
Insufficient staining for NKX3.1 of the appendix using the less successful mAb 361 giving a false positive staining reaction.
An aberrant cytoplasmic staining reaction is seen in epithelial cells, together with a generally poor signal-to-noise ratio. Also see Fig. 6b, same protocol.

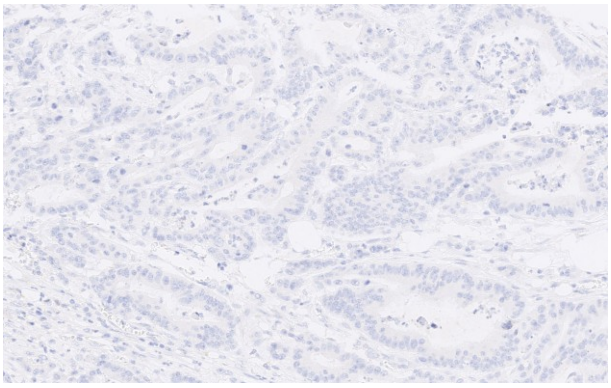


Fig. 6a
Optimal staining for NKX3.1 of the colon adenocarcinoma using same protocol as in Figs. 1a - 5a. No staining reaction is seen. Compare with Fig. 6b – same area.

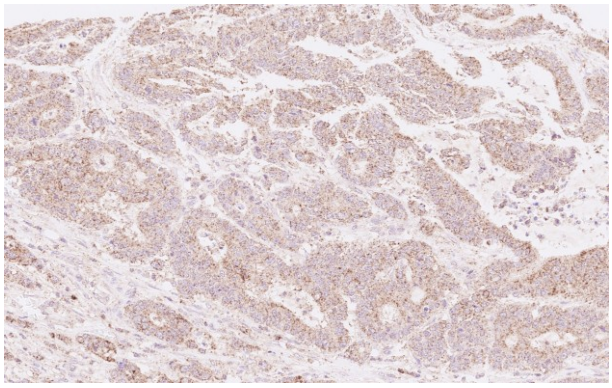


Fig. 6b
Insufficient staining for NKX3.1 of the colon adenocarcinoma using same protocol as in Fig. 5b. A weak diffuse staining reaction in the neoplastic cells is seen giving a poor signal-to-noise ratio and compromising the interpretation.

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