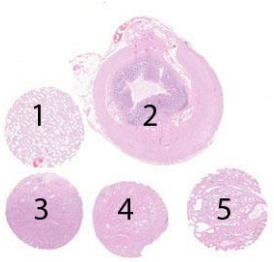
Assessment Run 49 2017
NKX3.1 (NKX3.1)

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



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

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 of the hyperplastic prostate glands.
- An at least weak to moderate and distinct nuclear staining reaction of dispersed spermatogonia in seminiferous tubules of the testis.
- A strong, distinct nuclear staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma, tissue core no. 3.
- An at least weak to moderate nuclear staining reaction of the majority of neoplastic cells in the prostate adenocarcinoma, tissue core no. 4.
- No nuclear staining reaction of other cellular structures including epithelial cells in appendix.

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

Participation

Number of laboratories registered for NKX3.1, run 49	56
Number of laboratories returning slides	49 (88%)

Results
49 laboratories participated in this assessment. 32 (65%) 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.
- Insufficient heat induced epitope retrieval (HIER).

Performance history
This was the first NordiQC assessment of NKX3.1 and a pass rate of 65% was observed.

Table 2. **Proportion of sufficient results for NKX3.1 in the first NordiQC run performed**

	Run 49 2017
Participants, n=	49
Sufficient results	65%

Conclusion
The rmAb clone **EP356** and pAb **CP422** were the most successful antibodies for immunohistochemical demonstration of NKX3.1.
rmAb clone EP356 was used by the majority of laboratories and optimal results was obtained both within a laboratory developed (LD) assay on the main IHC systems and as Ready-To-Use (RTU) format.
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 dispersed spermatogonia, 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 any cells should be seen.

Table 1. **Antibodies and assessment marks for NKX3.1, run 49**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **UMAB196**	1	ORIGENE	1	0	0	0	-	-
rmAb clone **EP356**	14 1	Cell Marque Zeta Corporation	10	3	2	0	87%	92%
pAb **0315**	2	Athena Enzyme System	0	2	0	0	-	-
pAb **CP422**	17	Biocare	5	2	4	6	41%	100%
pAb **RBK062**	1	Zytomed	0	0	1	0	-	-
Unknown	1	ZSGB-BIO	0	0	1	0	-	-
Ready-To-Use antibodies								
rmAb clone **EP356 API3189**	1	Biocare	1	0	0	0	-	-
rmAb clone **EP356 441R-17**	6	Cell Marque	3	2	1	0	83%	-
mAb clone **EP356 H0322226tTA**	1	PathnSitu	0	0	1	0	-	-
rmAb clone **EP356 760-5086**	2	Ventana/Cell Marque	2	0	0	0	-	-
pAb **PP422 AA**	2	Biocare	0	1	1	0	-	-
Total	49		22	10	11	6	-	
Proportion			45%	20%	23%	12%	65%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of NKX3.1, run 49
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **UMAB196**: One protocol with an optimal result was based on HIER for 64 min. at 91°C using Cell Conditioning 2 (CC2, Ventana) as retrieval buffer. The mAb was diluted 1:1,300 and a 2-step multimer based detection system (UltraView, Ventana) applied as detection system.

rmAb clone **EP356**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/1)*, Cell Conditioning 1 (CC1, Ventana) (8/12) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (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, 12 of 13 (92%) 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) (2/9) or Target Retrieval Solution, High pH (Dako Omnis) (3/3) as retrieval buffer. The pAb was typically diluted in the range of 1:25-1:30.
Using these protocol settings, 6 of 6 (100%) 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 3 main fully automated IHC systems***

Concentrated antibodies	**Dako 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
rmAb clone **EP356**	-	-	8/10 (80%)	-	1/1	-
pAb **CP422**	3/3	-	2/3	-	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
rmAb clone **EP356**, product no. **760-5086**, Ventana/Cell Marque, BenchMark Ultra:
Protocols with optimal results were based on HIER using Cell Conditioning 1 (efficient heating time 32-48 min.), 16 min. incubation of the primary Ab and OptiView (760-700) as detection system.
Using these protocol settings, 2 of 2 (100%) laboratories produced an optimal staining result.

rmAb clone **EP356**, product no. **API3189**, Biocare, IntelliPath:
One protocol with an optimal result was based on HIER in a Pressure Cooker using Diva Decloaker pH 6.2, 30 min. incubation time of the primary Ab and MACH 4 HRP-polymer kit as detection system.

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

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Optimal	Sufficient	Optimal	Sufficienct
VMS Ultra/XT rmAb EP356 **760-5086**	-	-	2/2	2/2

* 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 are included.

Comments
In this first NordiQC assessment for NKX3.1, the prevalent feature of an insufficient staining was a too weak or completely false negative staining reaction of cells expected to be demonstrated.
This pattern was seen in 88% of the insufficient results (15 of 17 laboratories). 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. 4 and the spermagonia in seminiferous tubules of the testis. Virtually all laboratories successfully demonstrated NKX3.1 in the majority of neoplastic cells of the prostate adenocarcinoma, tissue core no. 3, with high expression level of NKX3.1.

76% (37 of 49) of the laboratories used Abs as concentrated format within LD assays for NKX3.1. 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. rmAb clone EP356 was most successful as 87% of the laboratories using this clone produced a sufficient staining result and 67% were assessed as 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 parameters for optimal results.
For the pAb CP422, an overall inferior pass rate of 41% was observed. However, inadequate protocol settings providing reduced analytical sensitivity were frequently applied. Especially too low titre of CP422, insufficient HIER and/or 2-step polymer / multimer based detection systems were the main parameters causing an insufficient result. Focusing on the titre of CP422 the Median Dilution Value (MDV) for optimal results was 1:25 (range 1:25-1:30), whereas MDV of 1:50 (range 1:20-1:100) was seen in protocols with insufficient results.

RTU formats were used by 24% (12 of 49) of the laboratories. In general the RTU formats provided the same results as the concentrated formats.
The RTU format, 441R-17 (Cell Marque) based on the rmAb EP356 was most widely used and provided a pass rate of 83% (5 of 6), and 50% of the protocols were 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. Protocols performed on the Ventana Benchmark Ultra/XT providing optimal results, were based on HIER in CC1 pH 8.5 (efficient heating time 32 min. at 95-100°C), 16-24 min. incubation time of primary Ab and OptiView (760-700) as detection system. One protocol performed on Bond III (Leica) gave an optimal result. The protocol was based on HIER in ER2 pH 9 (efficient HIER time 15 min. at 100°C), 15 min. incubation time of primary Ab and Refine (DS9800) as detection system.
rmAb clone EP356 was also successfully applied as RTU system from Ventana (760-5083). The vendor protocol recommendation for the Ventana BenchMark platform is based on OptiView as detection system with HIER in CC1 for 64 min. and primary Ab incubation for 32 min. However, none of the two laboratories using the RTU product used the vendor protocol settings but laboratory modified settings reducing both the HIER time (to 32-48 min.) and the primary Ab incubation time (to 16 min.). Both laboratories produced optimal results.
One laboratory used the RTU system from Biocare based on the rmAb clone EP356 (API3189) obtained an optimal result. The protocol was based on HIER in Diva buffer, MACH4 as detection system and

intelliPATH™ as IHC stainer. It was not possible to identify the RTU product on the Biocare homepage and thus no data was available on vendor protocol recommendations.

Controls

From the observations generated in this first assessment for NKX3.1, testis and normal prostate seem to be recommendable 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 dispersed spermagonia of the seminiferous tubules. Testis seems to be the preferred positive tissue control as the spermagonia 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 sensitivity and consistency of the protocol used.

Appendix or colon can be used as negative tissue control for NKX3.1, in which no nuclear staining reaction should be seen.

Internal NordiQC data have indicated that the NKX3.1 antigen is influenced by pre-analytical conditions. Frequently, a staining gradient in prostate resection materials has been seen, which most likely is caused by delayed fixation and degrading of NKX3.1.

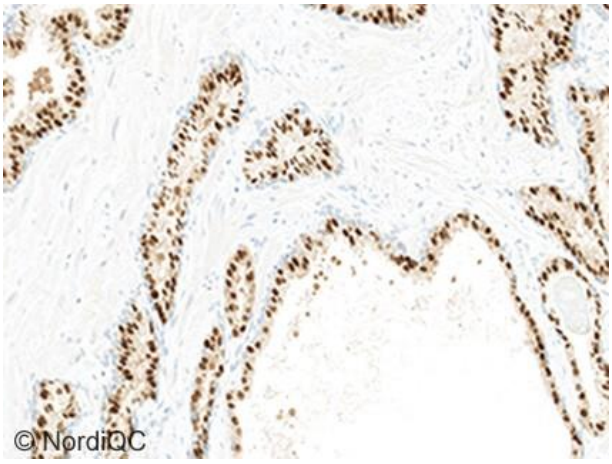


Fig. 1a
Optimal NKX3.1 staining of the prostate hyperplasia using the rmAb EP356 carefully calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana)(x100).
All the epithelial cells of the prostatic glands show a moderate to strong nuclear staining reaction. A weak cytoplasmic staining reaction in the epithelial cells is seen, but no general background staining. Also compare with Figs. 2a - 5a, same protocol.

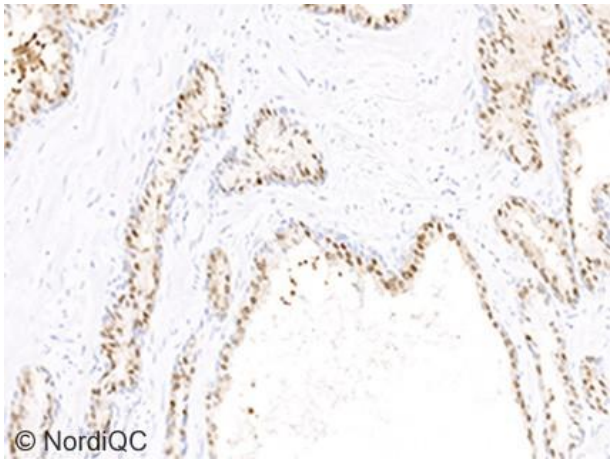


Fig. 1b
Staining for NKX3.1 of the prostate hyperplasia using an insufficient protocol based on the rmAb EP356 with protocol settings giving a too low sensitivity. Too low concentration of the primary Ab and 2-step multimer (UltraView, Ventana) - same field as in Fig. 1a (x100). The epithelial cells are demonstrated, but a reduced intensity compared to the result seen in Fig. 1a is seen. Also compare with Figs. 2b – 4b, same protocol.

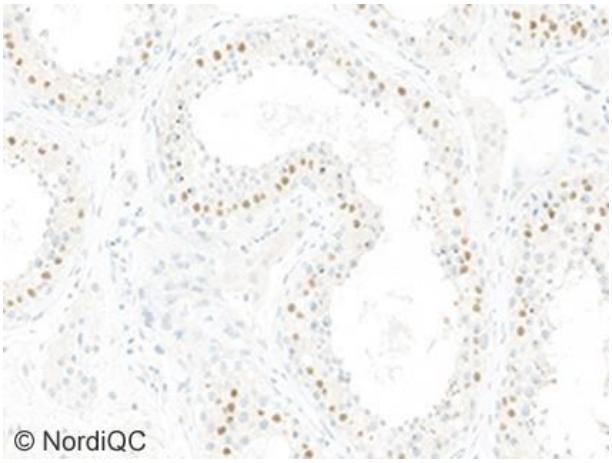


Fig. 2a
Optimal NKX3.1 staining of normal testis using same protocol as in Fig. 1a.
Spermatogonia at the basement membrane of the tubules show a moderate distinct nuclear staining reaction and no background staining is seen.
Also compare with Figs. 3a and 4a, same protocol.

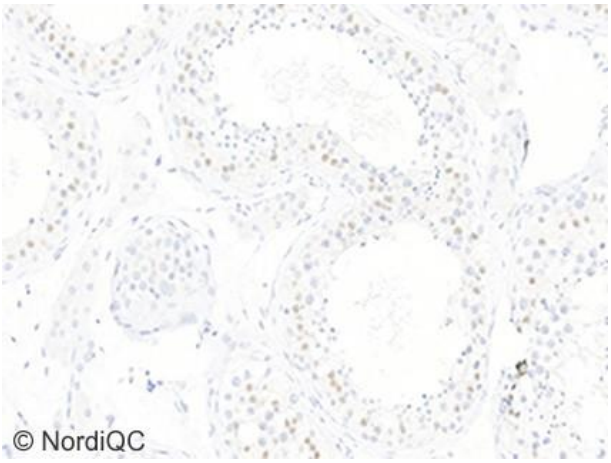


Fig. 2b.
Insufficient staining of normal testis using same protocol as in Fig. 1b.
The intensity and proportion of cells demonstrated is reduced compared to the level expected - same field as in Fig. 2a.
Also compare with Figs. 3b and 4b, same protocol.

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

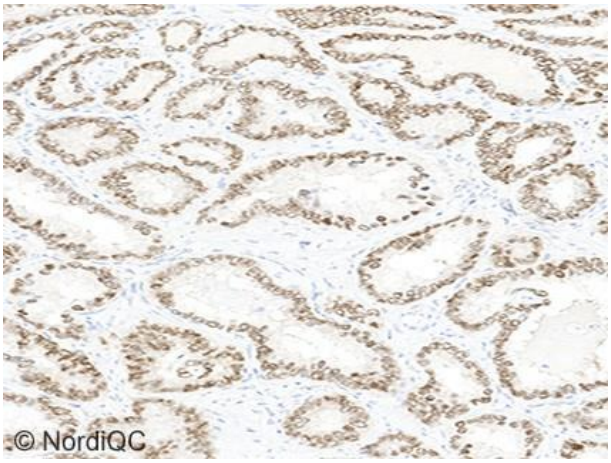


Fig. 3b
Staining for NKX3.1 of the prostate adenocarcinoma no. 3, using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
Virtually all neoplastic cells are demonstrated, but with significantly reduced intensity compared to the level expected.
Also compare with Fig. 4b, same protocol.

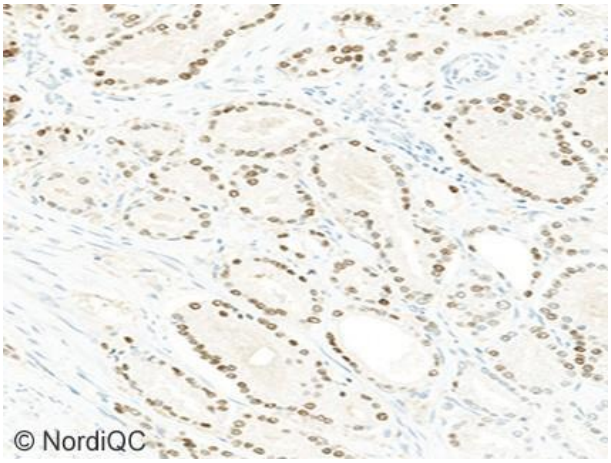


Fig. 4a
Optimal NKX3.1 staining of the prostate adenocarcinoma no. 4, using same protocol as in Figs. 1a - 3a.
The majority of the neoplastic cells show a weak to moderate nuclear staining reaction.

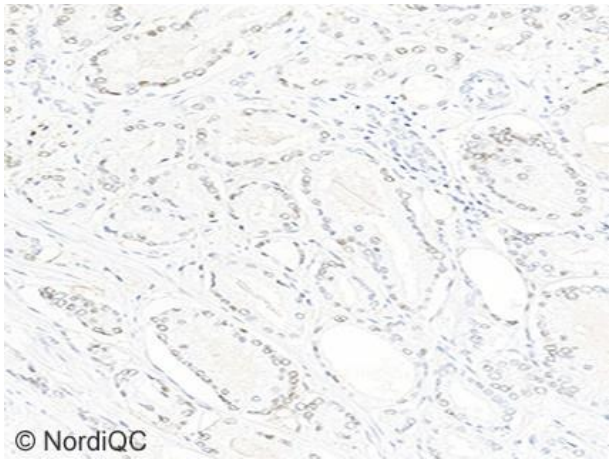


Fig. 4b
Insufficient staining for NKX3.1 of the prostate adenocarcinoma no. 4, using same protocol as in Figs. 1b – 3b. – same field as in Fig. 4a.
Only scattered cells show a faint and dubious staining reaction.

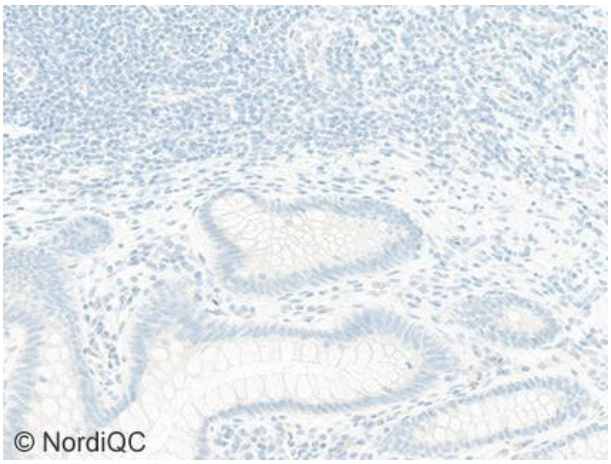


Fig. 5a
Optimal staining for NKX3.1 of the appendix using same protocol as in Figs. 1a – 4a. No staining reaction is seen.
Appendix serves as negative tissue control to monitor a potential aberrant staining reaction e.g. caused by the primary Ab.
Compare with Fig. 5b.

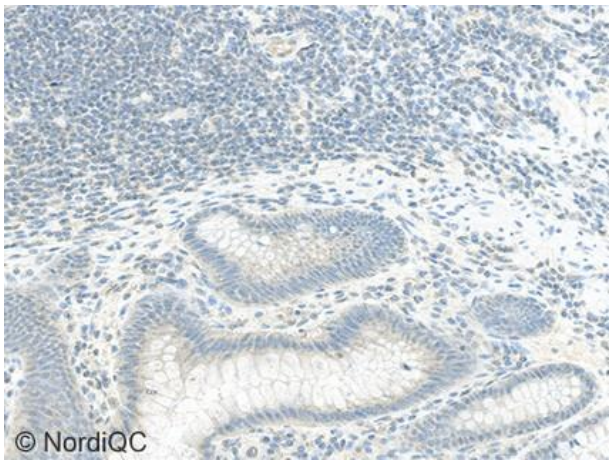


Fig. 5b
Insufficient staining for NKX3.1 of the appendix using the pAb CP422 by protocols settings giving a poor-signal-to-noise ratio.
A weak to moderate diffuse staining reaction in both epithelial cells and lymphocytes is seen.
The staining pattern most likely was caused by using the primary Ab too concentrated.

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