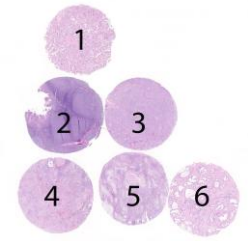
Assessment Run 48 2016
p40 (ΔNp63)

Material
The slide to be stained for p40 comprised:



1. Placenta, 2. Tonsil, 3. Lung adenocarcinoma, 4-5. Lung squamous cell carcinoma, 6. Prostate hyperplasia

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing p40 staining as optimal included:

- A moderate to strong, distinct nuclear staining reaction of virtually all squamous epithelial cells in the tonsil and basal cells lining the hyperplastic glands in the prostate
- An at least weak to moderate, distinct nuclear staining reaction of dispersed cytotrophoblastic cells in the placenta
- A moderate to strong, distinct nuclear staining reaction of the vast majority of neoplastic cells in the lung squamous cell carcinoma
- No staining reaction of neoplastic cells in the lung adenocarcinoma
- No staining reaction of other cells including lymphocytes in the tonsil

Participation

Number of laboratories registered for p40, run 48	209
Number of laboratories returning slides	188 (90%)

Results
188 laboratories participated in this assessment, 74% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

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

Performance history
This was the second NordiQC assessment of p40. A significantly increased pass rate was observed (table 2).

Table 2. **Proportion of sufficient results for p40 in two NordiQC runs**

	Run 44 2015	Run 48 2016
Participants, n=	129	188
Sufficient results	56%	74%

The increased pass rate in this run may be explained by an extended use of the highly sensitive and robust mAb clone BC28 both as concentrate and as Ready-To- Use (RTU) format. Additionally, the proportion of laboratories using less successful polyclonal antibodies has been reduced from 41% in run 44 to 16% in the current run.

Conclusion
Optimal staining results for p40 could be obtained with the mAb clones **BC28** and **ZR8.** mAb clone **BC28** was by far the most commonly used and successful p40 antibody, giving pass rates of 86% and 91% for the concentrated and RTU formats, respectively. The concentrated formats of mAb clone **BC28** provided optimal staining result on the three main platforms from Dako, Leica and Ventana. Irrespective of the clone applied, efficient HIER 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.
All polyclonal antibodies applied in this assessment gave less successful results and should be avoided.

Placenta is recommended as positive tissue control for p40 where an at least a weak to moderate, distinct nuclear staining reaction of dispersed cytotrophoblasts must be seen. The cytotrophoblasts should be visible even at low magnification (5x objective). Tonsil can serve as both positive and negative tissue control. No nuclear staining reaction in lymphocytes should be seen, whereas virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction.

Table 1. **Antibodies and assessment marks for p40, run 48**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BC28**	77 6 2 2 1	Biocare Zytomed Menarini abcam Nordic Biosite	52	24	10	2	86%	89%
rmAb clone **ZR8**	12 1 1	Immunologic Zeta Corporation BioSB	1	6	2	5	50%	67%
pAb **AC13030**	8	Biocare	0	2	6	0	-	-
pAb **RP163**	5	Diagnostic Biosystems	0	1	1	3	-	-
pAb **PC373**	4	Calbiochem, Merck	0	1	0	3	-	-
pAb **RBK054**	3	Zytomed	0	0	1	2	-	-
pAb **PI049**	1	DCS	0	1	0	0	-	-
pAb **PP123**	1	Pathnsitu	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **BC28 API/IPI/AVI 3066**	13	Biocare	5	8	0	0	100%	100%
mAb clone **BC28 790-4950**	39	Ventana	19	15	5	0	87%	94%
mAb clone **BC28 MSG097**	1	Zytomed	1	0	0	0	-	-
mAb clone **ZR8 MAD-000686QD**	3	Master Diagnostica	0	2	1	0	-	-
pAb **API 3030**	6	Biocare	0	0	4	2	-	-
pAb **RAB-066**	1	Maixin	0	1	0	0	-	-
pAb **A00112**	1	Loxo GmbH	0	0	1	0	-	-
Total	188		78	61	32	17	-	
Proportion			42%	32%	17%	9%	74%	

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

Detailed analysis of p40, Run 48
The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies
mAb clone **BC28**: Protocols with optimal results were all based on HIER using Cell Conditioning 1 (CC1, Ventana) (30/43)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (7/20), TRS pH 9 (Dako) (6/8), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/6), Tris-EDTA/EGTA pH 9 (3/6), TRS pH 6.1 (Dako) (1/1), TRS pH 6.1 (3-in-1) (Dako) (1/1) or Diva Decloaker pH 6.2 (BioCare) (1/2) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 71 of 80 (89%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **ZR8**: One protocol with an optimal result was based on 48 min. HIER using CC1 (Ventana) (1/4), 32 min. incubation of the primary Ab, a titre of 1:200 and OptiView (Ventana) with amplification (760-099 / 860-099) as detection system.

Table 3. **Proportion of optimal results for p40 for the BC28 antibody as concentrate on the 4 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 clone **BC28**	7/20** (35%)	1/1	6/8 (75%)	1/1	30/42 (71%)	-	3/6 (50%)	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 **BC28**, product no. **790-4950**, Ventana, BenchMark GX / XL / ULTRA:
Protocols with optimal results were typically based on 32-64 min. HIER using Cell Conditioning 1 (Ventana), 16-32 min. incubation of the primary Ab and UltraView (Ventana 760-500) with amplification (760-080), OptiView (Ventana 760-700) or OptiView (Ventana 760-700) with amplification (760-099 / 860-099) as detection system. Using these protocol settings 30 of 32 (94%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **BC28**, product no. **AVI 3066 KG**, BioCare, BenchMark ULTRA:
One protocol with an optimal result was based on 32 min. HIER using CC1 (Ventana), 32 min. incubation of the primary Ab and UltraView-AP (Ventana 760-501) with amplification (760-080) as detection system.

mAb clone **BC28** product no. **API 3066**, Biocare, intelliPATH:
One protocol with an optimal result was based on HIER using Diva Decloaker pH 6.2 in a Pressure Cooker (efficient heating time 15 min. at 110°C), 30 min. incubation of the primary Ab and MACH4 Universal HRP-Polymer (M4U534) as detection system.

Table 4. **Proportion of sufficient and optimal results for p40 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 BC28 **790-4950**	87% (13/15)	40% (6/15)	91% (21/23)	57% (13/23)

* 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 concordance with the previous NordiQC assessment for p40 (run 44, 2015), the prevalent feature of an insufficient result was a too weak or completely false negative staining reaction of the cells expected to be demonstrated. This pattern was seen in 71% of the insufficient results (35 of 49 laboratories). The remaining insufficient results were characterized by a generally poor signal-to-noise ratio, excessive background reaction and/or aberrant cytoplasmic staining reaction in e.g. cytotrophoblasts, lymphocytes and smooth muscle cells complicating the interpretation. Too weak staining was typically characterized by a reduced staining reaction regarding both the intensity and proportion of cells expected to be demonstrated. This was in particular observed in the cytotrophoblasts of placenta, basal cells of prostate glands and to some extent the neoplastic cells of the lung squamous cell carcinoma tissue core no. 4 with a moderate expression level of p40. Virtually all laboratories successfully demonstrated p40 in the majority of neoplastic cells of the lung squamous cell carcinoma tissue core no. 5 with high expression level of p40. Too weak staining reaction was most frequently caused by too low titre of an otherwise well performing primary antibody as mAb clone BC28 often in the combination with the use of 2-step polymer/multimer systems (Fig. 1 – Fig. 4). Poor signal-to-noise ratio was typically caused by a less successful primary antibody (Fig. 5 – Fig. 6)

66% (124 of 188) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for p40. The mAb clone BC28 was the most widely used antibody and had the highest proportion of sufficient and optimal results, as seen in table 1. Optimal results could be obtained on the 4 most widely used IHC platforms, as shown in table 3. Efficient HIER in an alkaline buffer in combination with a 3-step polymer/multimer based detection system provided the highest proportion of optimal results. As an example, on the Ventana BenchMark platform, 26 of 30 protocols (87%) provided an optimal result if mAb clone BC28 was used as a concentrate in the range of 1:20-100, HIER was performed in CC1 for 32-64 min. and OptiView (760-700) or UltraView (760-500) with amplification (760-080) used as detection

system. Using the same settings but applying UltraView (760-500) (2-step multimer) as detection system only 1 of 7 protocols (14%) provided optimal results. Similar observation was seen for the Dako platforms. On the Dako Autostainer/OMNIS platforms 13 of 19 protocols (68%) provided an optimal result when mAb clone BC28 was used as a concentrate in the range of 1:25-1:200, HIER was performed in TRS pH 9 for 10-30 min. and 3-step EnVision (polymer: K8000/K8002/K8012/GV800 and linker: K8021/K8022/GV821) used as detection system. Using similar settings but applying 2-step EnVision (K8000/K8002/K8012/GV800) as detection system none of 9 protocols (0%) provided optimal results. Sufficient and optimal results could also be achieved with the rmAb clone ZR8, but the data from the current assessment suggests that this antibody can be difficult to optimize. 7 of 14 (50%) laboratories achieved sufficient results, but only one was assessed as optimal. The optimal result was obtained by efficient HIER (CC1 in 48 min.) and a very sensitive detection system (OptiView with tyramide based amplification).

Six different polyclonal Abs (pAb) were used as concentrates within LD assays (22 protocols in total). Despite protocol settings, as retrieval conditions, detection systems and IHC stainer platforms, were identical to the mAb clones BC28 and ZR8, no optimal results were provided and the overall pass rate for laboratories using a pAb within a LD assay was only 23% (5 of 22). The insufficient results were typically characterized by a poor signal-to-noise ratio and aberrant staining reaction compromising the interpretation. The most commonly used pAb AC13030 (BioCare) gave a cytoplasmic staining reaction in cytotrophoblasts and smooth muscle cells (Fig. 6b), whereas the pAb RP163 gave an aberrant cross reaction with B-cells (Fig. 5b). From the protocols submitted it seemed impossible to eliminate the aberrant staining reactions e.g. by reducing the titre of the primary Ab as this compromised the sensitivity to identify p40 in low-level expressing structures such as cytotrophoblasts of placenta and basal cells of prostate glands.

Ready-To-Use (RTU) antibodies was used in 34% (64 of 188) of the laboratories. The RTU formats basically provided the same results as seen for the concentrated formats. Optimal results were obtained by the RTU system from Biocare based on the mAb clone BC28 (API3066) using the vendor protocol recommendation, which in brief was based on HIER in Diva buffer, MACH4 as detection system and intelliPATH™ as IHC stainer. mAb clone BC28 (790-4950) was also successfully applied as RTU system from Ventana. The vendor protocol recommendations for the Ventana BenchMark platform are based on either OptiView as detection system with HIER in CC1 for 32 min. and primary antibody incubation for 16 min. or UltraView with amplification as detection system with HIER in CC1 for 64 min. and primary antibody incubation for 16 min. Following the vendor protocol recommendations, the pass rate was 87% and 40% optimal (table 4). A similar pass rate was seen for the laboratory modified RTU protocols (91%) whereas the proportion of optimal was increased to 57%. Among the 23 laboratories using modified RTU protocols, 6 laboratories failed to meet the minimum requirements by using protocols based on UltraView without amplification as detection system or too short HIER. None of these 6 laboratories achieved optimal results. The remaining 17 laboratory modified RTU protocols were all based on the recommended detection systems, but with prolonged incubation times of the primary antibody and/or prolonged HIER in CC1. These "positive" modifications of the official RTU protocol, resulted in a noticeable increase in optimal results, 71% of the laboratories (12 out of 17) achieved optimal results compared to 40% of the laboratories using the official RTU protocol.

Controls

Placenta is recommended as positive tissue control for p40, where an at least weak to moderate, distinct nuclear staining reaction of cytotrophoblasts must be seen. The cytotrophoblasts should be visible even at low magnification (5x objective).

Supportive to placenta, tonsil can be used as positive and negative tissue control. Virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction. No nuclear or cytoplasmic staining reaction should be seen in other cell types.

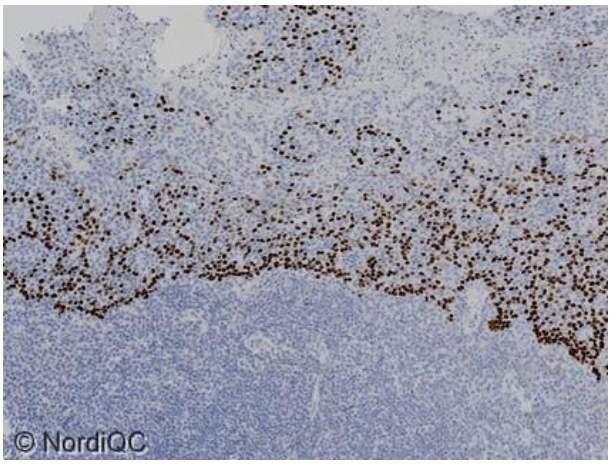


Fig. 1a (x100)
Optimal p40 staining of the tonsil using the mAb clone BC28 diluted 1:100 with 30 min. incubation time, HIER in an alkaline buffer (TRS pH 9.0, Dako), and a 3-step polymer based detection system (EnVision FLEX+, Dako). A moderate to strong nuclear staining reaction is seen in the majority of the squamous epithelial cells. No background staining is seen.
Same protocol used in Figs. 1a - 4a.

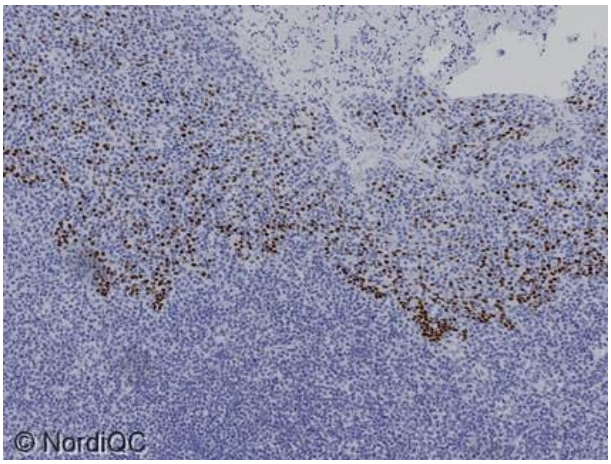


Fig. 1b (x100)
Insufficient p40 staining of the tonsil using the mAb clone BC28. Protocol settings was similar to the settings used in Fig. 1a. The primary differences were the use of a 2-step polymer detection system (EnVision FLEX, Dako) and a relative strong haematoxylin nuclear counter stain compromising the identification of a weak p40 reaction.
The protocol provided an overall too low sensitivity. Compare with Fig. 1a (same field). The intensity and proportion of cells demonstrated is reduced.
Also compare with Figs. 2b - 4b, same protocol.

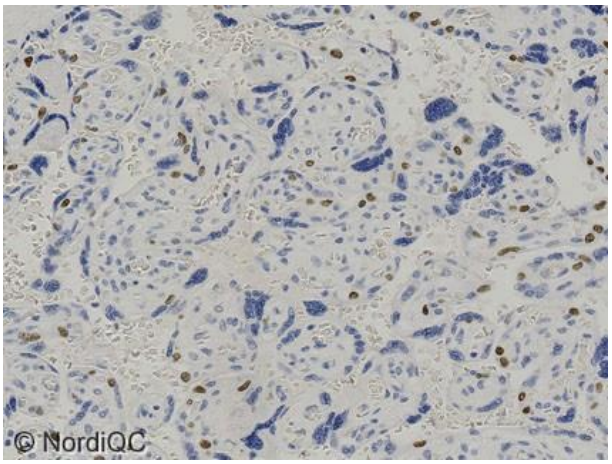


Fig. 2a (x200)
Optimal p40 staining of the placenta using same protocol as in Fig. 1a. Scattered cytothrophoblastic cells show a weak to moderate, distinct nuclear staining reaction.

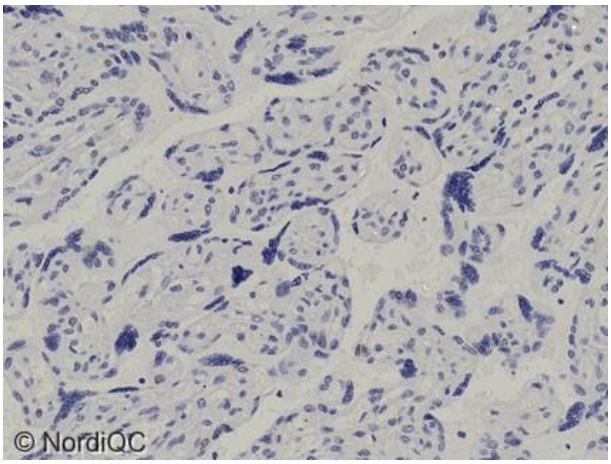


Fig. 2b (x200)
Insufficient p40 staining of the placenta using same protocol as in Fig. 1b. Virtually no nuclear staining reaction of cytothrophoblastsic cells is seen.
Also compare with Figs. 3b and 4b, same protocol.

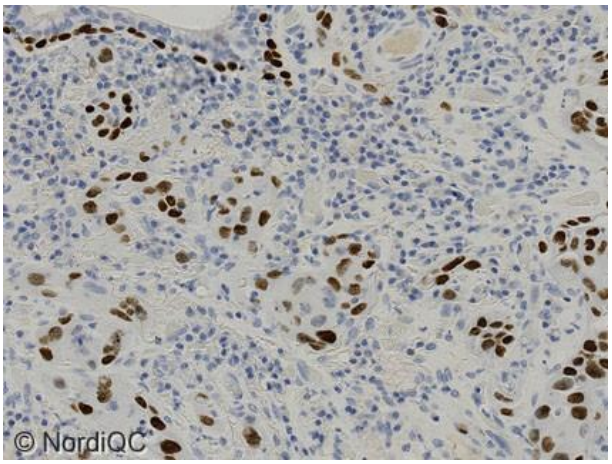


Fig. 3a (x200)
Optimal p40 staining of the lung squamous cell carcinoma (tissue core no. 4) using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction.
No background staining is seen.

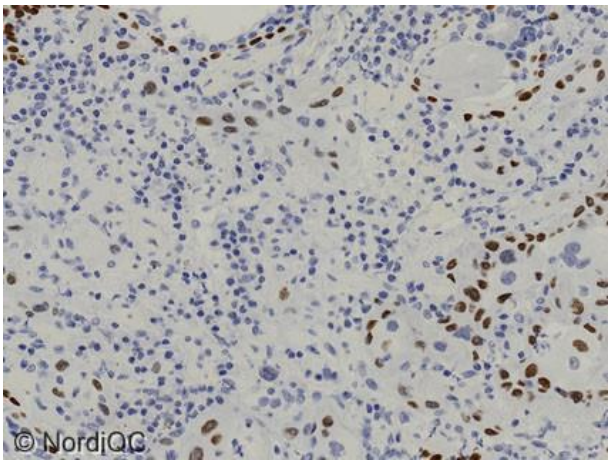


Fig. 3b (x200)
Insufficient p40 staining of the lung squamous cell carcinoma (tissue core no. 4) using same protocol as in Figs. 1b and 2b. The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected and shown in Fig. 3.

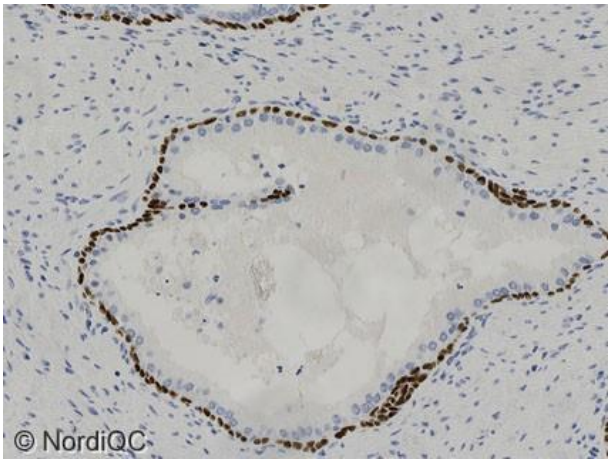


Fig. 4a (x200)
Optimal p40 staining of the prostate hyperplasia using same protocol as in Figs. 1a - 3a.
The basal cells are distinctively demonstrated as a moderate to strong nuclear staining reaction is observed.

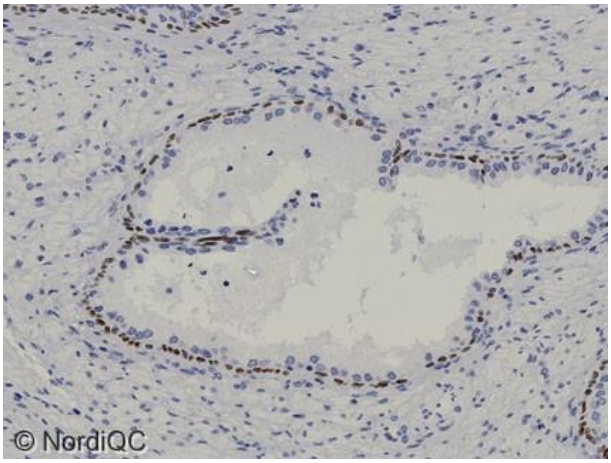


Fig. 4b (x200)
Insufficient p40 staining of the prostate hyperplasia using same protocol as in Figs. 1b – 3b.
Only a weak and equivocal nuclear staining reaction in the basal cells is observed.

Fig. 5a (x100)
Optimal p40 staining of the tonsil using the mAb clone BC28 in an optimally calibrated protocol on the Ventana BenchMark platform. A moderate to strong nuclear staining reaction is seen in the majority of the squamous epithelial cells. No background staining is seen.

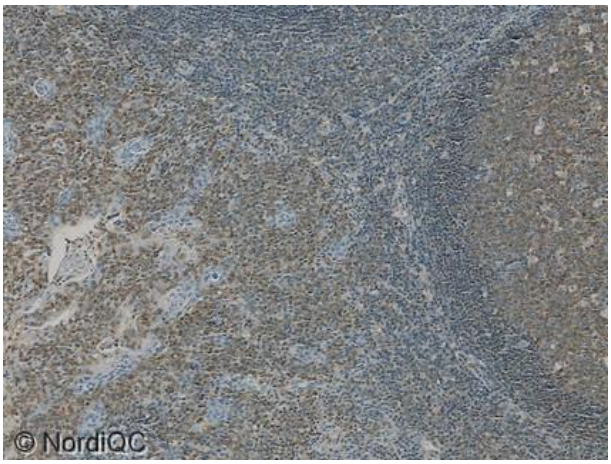


Fig. 5b (x100)
Insufficient p40 staining of the tonsil using a pAb (RP163, Diagnostic BioSystems) on the Ventana BechMark platform. The interpretation is compromised primarily due to a poor signal-to-noise ratio and a general background reaction but also to an aberrant staining reaction of B-cells. Compare with Fig. 5a (same field).

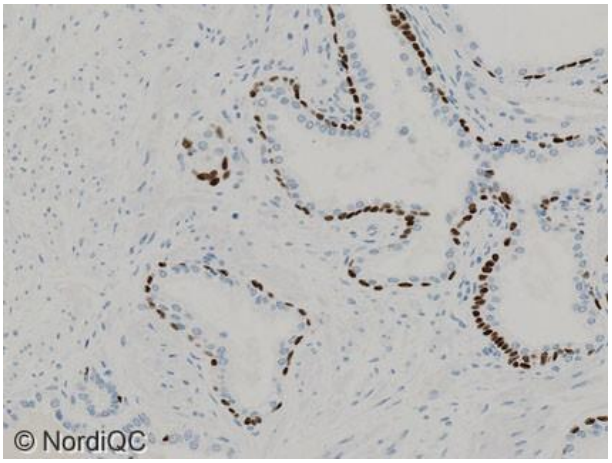


Fig. 6a (x200)
Optimal p40 staining of the prostate hyperplasia using same protocol as in Fig. 5a. The basal cells are distinctively demonstrated as a moderate to strong nuclear staining reaction is observed. No background staining is seen.

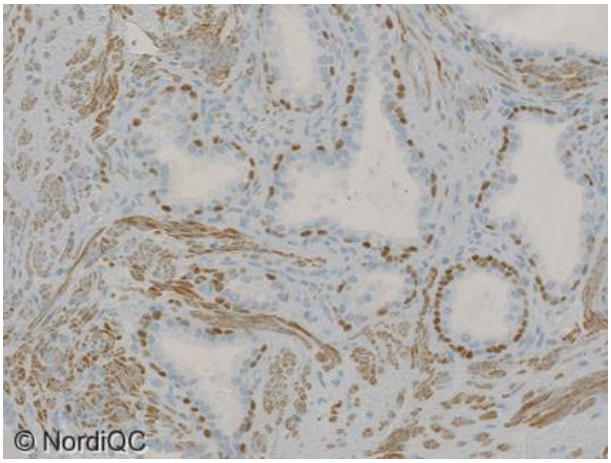


Fig. 6b (x200)
Insufficient p40 staining of the prostate hyperplasia using a pAb (AC13030, Biocare). An excessive cytoplasmic staining reaction in smooth muscle cells compromises the interpretation in the basal cells. This pattern was frequently seen for pAbs AC13030, RBK054 and PC373. Compare with Fig. 6a (same field).

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