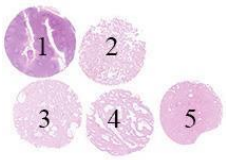
Assessment Run 61 2021

p63

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for p63, used to identify and differentiate prostate hyperplasia, PIN and prostate adenocarcinoma. Relevant clinical tissues, both normal and neoplastic, were selected displaying a broad spectrum of antigen densities for p63 (see below). This was the fifth NordiQC assessment of p63, focusing only on intended use in relation to the diagnosis of prostate lesions.

Material
The slide to be stained for p63 comprised:

1. Tonsil, 2. Placenta, 3. Prostate hyperplasia, 4. Prostate intraepithelial neoplasia (PIN), 5. Prostate adenocarcinoma



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a p63 staining as optimal included:

- 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 in almost all squamous epithelial cells in the tonsil and an at least weak nuclear reaction in scattered lymphocytes.
- A moderate to strong, distinct nuclear staining reaction in basal cells in the hyperplastic prostate glands and PIN
- No staining reaction in the neoplastic glands of the prostate adenocarcinoma.
- No staining reaction in the secretory cells of the hyperplastic prostate glands.
- No or only a week cytoplasmic reaction in cells with strong p63 expression.

Participation

Number of laboratories registered for p63, run 61	375
Number of laboratories returning slides	324 (86%)

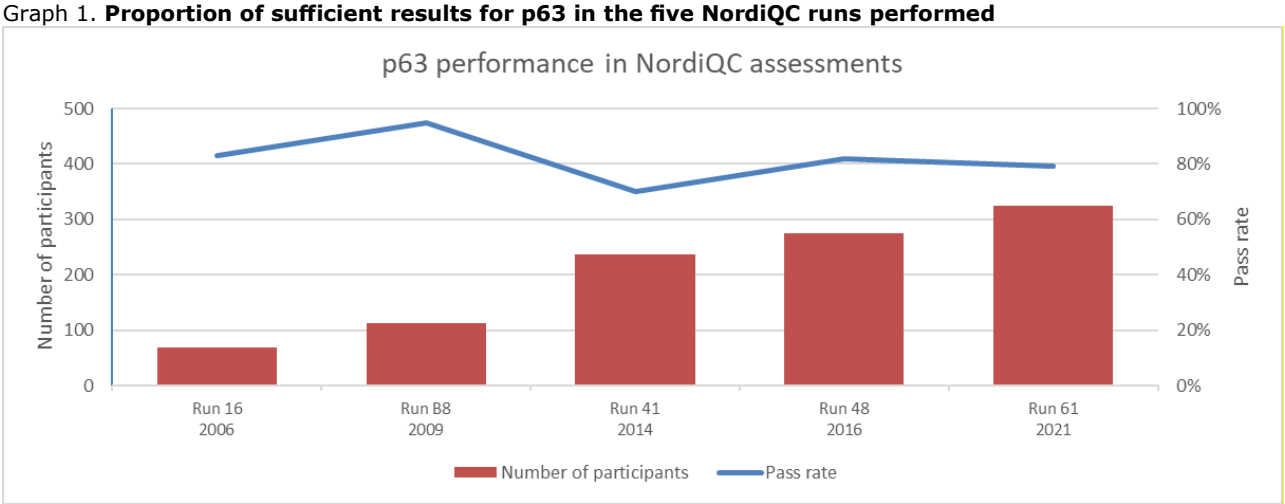
The number of laboratories returning slides has decreased in this run 61 compared to previous assessments, due to the COVID-19 pandemic and associated postal delays. All slides returned after the assessment were assessed and received advice if the result being insufficient but were not be included in this report.

Results
324 laboratories participated in this assessment. 256 (79%) achieved a sufficient mark (optimal or good). Antibodies (Abs) used and assessment marks are summarized in Table 1 (see page 2)

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibodies
- Too low concentration of the primary antibody
- Insufficient Heat Induced Epitope Retrieval (HIER)
- Detection systems with low sensitivity

Performance history
This was the fifth NordiQC assessment of p63. Compared to the previous run 48 in 2016, a slightly decreased pass rate was seen (graph 1). Fluctuation in pass rates can be influenced by different materials circulated in the respective runs and also by many new participants enrolling. In this run 25% (n=76) more participants were registered compared to run 48.

Graph 1. **Proportion of sufficient results for p63 in the five NordiQC runs performed**



Conclusion

Monoclonal antibodies (mAbs) clones **4A4** and **DAK-p63** are both recommendable Abs for the demonstration of p63. Irrespective of the clone applied, HIER in an alkaline buffer for at least 20 min. 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
The mAb clone 7JUL was found to be less successful giving an overall pass rate of only 10%.
The Ready-To-Use formats of mAb clones 4A4 and DAK-p63 being increasingly used by the participants provided slightly higher pass rate compared to corresponding concentrated formats. However, as observed for the RTU systems for Dako Omnis and Ventana BenchMark, laboratory modified protocol settings were more successful compared to the vendor recommended settings. Placenta is recommended as primary and critical positive tissue control for p63 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 a low magnification (5x objective). Tonsil can serve as both supplementary positive and also as negative tissue control. In tonsil virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction, while dispersed lymphocytes and endothelial cells must show a weak but distinct staining reaction. The vast majority of lymphocytes should be negative.

Table 1. **Antibodies and assessment marks for p63, Run 61**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **4A4**	3 25 1 1 3 3 1 2	Bio SB Biocare Medical BioGenex Genova Immunologic Zeta Zytomed Systems Dako	12	16	8	3	72%	31%
mAb clone **DAK-p63**	47	Dako/Agilent	21	19	5	2	85%	45%
mAb clone **7JUL**	10	Leica Biosystems	-	1	2	7	10%	-
mAb clone **SFI-6**	2	DCS	-	1	1	-	-	-
mAb clone **GR004**	1	Gene Tech	1	-	-	-	-	-
mAb clone **IHC063**	1	GenomeMe	-	1	-	-	-	-
mAb clone **p63**	1	Menapath	-	-	1	-	-	-
rmAb clone **BSR6**	1	Nordic Biosite	-	1	-	-	-	-
rmAb clone **DBR16.1**	2	Diagnostic Biosystems	1	-	1	-	-	-
Ready-To-Use antibodies								
mAb clone **4A4 790-4509**[3]	7	Ventana/Roche	-	4	3	-	57%	-
mAb clone **4A4 790-4509**[4]	115	Ventana/Roche	60	41	13	1	88%	52%
mAb clone **DAK-p63 IR662**[3]	12	Dako/Agilent	2	9	1	-	91%	17%

mAb clone **DAK-p63 IR662**[4]	29	Dako/Agilent	7	17	5	-	83%	24%
mAb clone **DAK-p63 GA662**[3]	20	Dako/Agilent	5	12	3	-	85%	25%
mAb clone **DAK-p63 GA662**[4]	15	Dako/Agilent	9	6	-	-	100%	60%
mAb clone **7JUL PA0103**[3]	4	Leica Biosystems	-	1	2	1	-	-
mAb clone **7JUL PA0103**[4]	6	Leica Biosystems	-	-	5	1	0%	0%
mAb clone **4A4 PM/IP/VP163**	4	BioCare Medical	-	2	1	1	-	-
mAb clone **MX013 MAB-0694**	2	Maixin	1	1	-	-	-	-
mAb clone **4A4 MAD-000479QD**	2	Master Diagnostica SL	1	1	-	-		
mAb clone **MX013 8312-C010**	1	Sakura Finetek	-	1	-	-	-	-
mAb clone **4A4 PM105**	1	PathnSitu	-	1	-	-		
mAb clone **13H4+4A4**	1	Biologo	-	-	-	-	-	-
mAb clone **C2C10**	1	Celnovte	1	-	-	-	-	-
Total	324		121	135	51	16		
Proportion			37%	42%	16%	5%	79%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
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 applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Detailed analysis of p63, Run 61

The following protocol parameters were central to obtain an optimal staining:

Concentrated Antibodies

mAb clone **4A4**: Protocols with optimal results were all based on HIER using either Cell Conditioning 1 (CC1, Ventana/Roche) (9/20)*, Target Retrieval Solution, High pH (Dako/Agilent) (1/5) Tris-EDTA/EGTA pH 9 (1/1), or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/7) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:250 depending on the total sensitivity of the protocol employed. Using these protocol settings, 23 of 28 (82%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mAb clone **DAK-p63**: Protocols with optimal results were all based on HIER using either CC1 (Ventana) (17/24) or Target Retrieval Solution, High pH (Dako) (4/12) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 26 of 28 (93%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for p63 for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche 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 **4A4**	0/3**	0/1	1/2	-	9/20 (45%)	-	1/7 (14%)	0/1
mAb clone **DAK-p63**	0/3	-	4/9 (44%)	0/1	17/24 (71%)	-	0/9	-
mAb clone **7JUL**	-	-	-	-	0/4	-	0/6	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 **4A4,** product no. 790-4509, Ventana/Roche, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min.), 16-36 min. incubation of the primary Ab and UltraView (760-500), UltraView (760-500) with amplification (760-080) or OptiView (760-700) as detection system. Using these protocol settings 87 of 98 (89%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-p63,** product.no. IR662, Dako/Agilent, Autostainer / Autostainer Link:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 20 min. at 97°C), 20 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) as detection system. Using these protocol settings 11 of 12 (91%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-p63,** product.no. GA662, Dako/Agilent, Omnis:
Protocols with optimal results were typically based on HIER using TRS High or Low pH (efficient heating time 20-30 min. at 95-97°C), 15-25 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 29 of 32 (91%) laboratories produced a sufficient staining result (optimal or good).

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance is evaluated both as a true plug-and-play system performed according to the recommendations provided by the vendor and by a laboratory modified system changing basal protocol settings. Only protocols performed on the specific IHC stainer device were included.

Table 3. **Proportion of sufficient and optimal results for p63 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 4A4 **790-4509**	57% (4/7)	0/7	88% (100/114)	52% (59/114)
Dako AS48 mAb DAK-p63 **IR662**	91% (11/12)	17% (2/12)	57% (4/7)	0/7
Dako Omnis mAb DAK-p63 **GA662**	85% (17/20)	25% (5/20)	100% (13/13)	62% (8/13)
Leica Bond mAb 7JUL **PA0103**	1/4	0/4	0/6	0/6

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

Comments
In this assessment and in concordance with the four previous assessment for p63 in NordiQC 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 97% of the insufficient results (65 of 67 laboratories). In 19% of these cases, the interpretation of the nuclear p63 staining reaction was further complicated by excessive counterstaining (Figs 6a - 6b).
A too weak staining reaction was in particular observed in lymphocytes in tonsil, cytotrophoblasts in placenta and a significantly reduced intensity and/or proportion of positive basal cells in prostate glands within the hyperplastic and PIN lesions. Virtually all laboratories successfully demonstrated p63 in the majority of squamous epithelial cells in the tonsil.
Especially an equivocal staining in basal cells of prostate glands was found critical, as a false negative reaction could have a diagnostic consequence to differentiate a benign/precursor lesion from an invasive lesion.

32% (104 of 324) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for p63, which is a decrease from the latest run 48, 2016 where 41% were using concentrated formats.

Optimal staining results could be obtained with mAb clone DAK-p63, mAb clone 4A4, mAb clone GR004 and the rmAb clone DBR16.1 (see Table 1). The mAb clone DAK-p63 was the most widely used clone. Used as a concentrate, mAb clone DAK-p63 gave an overall pass rate of 87% (41 of 47). Optimal results could be achieved on the two IHC systems Ventana BenchMark and Dako Omnis, but the proportion of

sufficient and optimal results using the mAb clone DAK-p63 as a concentrate was higher on the Ventana BenchMark system compared to the Dako Omnis system. On the Ventana system an impressive pass rate of 100% (24 of 24) was seen of which 71% were evaluated as optimal. On the Dako Omnis the pass rate was 80% (8 of 10), but only 40% were optimal. The reason for the higher proportion of optimal results on the Ventana system is unclear. As mentioned in previous reports it might be related to the observation, that the majority of protocols performed on the Ventana system were based on a relatively long HIER time in an alkaline buffer (most cases 32-64 min. in CC1) compared to a reduced HIER pretreatment time of typically 10-20 min. in TRS High pH and BERS2 on Dako and Leica systems. Furthermore, a sensitive 3-step multimer based detection system was used by 88% (21 of 24) of the laboratories using the Ventana system, whereas only 54% (7 of 13) of the laboratories using the Dako system applied an equally sensitive 3-step polymer-based detection system.

mAb clone 4A4 was used as a concentrate by 39 laboratories and the pass rate for the LD assays was 72% (28 of 39), 31% being optimal results. Optimal results were seen on all 3 fully automated platforms from Dako/agilent, Leica and Ventana/Roche (see Table 3). The mAb clone was most successful on the Ventana BenchMark platforms as 95% (19 of 20) of laboratories achieved a sufficient result. In the BenchMark protocols the average heating time in CC1 was 53 min., which support the importance of a relative long HIER pretreatment which seems to improve the score of the concentrated formats of Abs for p63.

The mAb clone 7JUL was used by 10 laboratories as a concentrate. Despite applying similar protocol settings (i.e. HIER and detection systems) as for the mAbs clones 4A4 and DAK-p63, 9 (90%) produced insufficient staining results (borderline or poor) as shown in Table 1. Identical findings were also observed for the corresponding RTU format, where 9/10 laboratories produced insufficient staining results. The prevalent feature of the insufficient results was a too weak or false negative staining of basal cells in glands of prostate hyperplasia, PIN and scattered lymphocytes in the tonsil (Figs. 4a-4b). The mAb clone 7JUL also showed low pass rates in previous p63 assessments with no laboratories obtaining optimal marks. Consequently, laboratories using mAb clone 7JUL should consider changing to one of the more sensitive/robust clones, 4A4 or DAK-p63.

Ready-To-Use (RTU) antibodies were used in 68% (220 of 324) of the laboratories.
The Ventana/Roche RTU system based on mAb clone 4A4 (790-4509) was the most widely used RTU system applied by 122 laboratories (1 laboratory used 790-4509 on a non-Ventana system). An overall pass rate of 86% was seen and 49% being optimal. Optimal results could be achieved with both the Ventana/Roche recommended protocol settings and by laboratory modified protocol settings. Ventana/Roche recommends HIER in CC1 for 64 min. at 95-100°C and 16-20 min. incubation time of the primary antibody (790-4509) using UltraView (2-step multimer) as detection system. Only 7 of 122 laboratories followed the Ventana/Roche recommendations, giving a pass rate of 57%, but no optimal results (see Table 3). In contrast, for the 115 laboratories that used laboratory modified protocol settings a cumulated pass rate of 88% (101 out of 115) was observed of which 52% being optimal (60 of 115). 63% (72 of 115) of the laboratories using modified protocol settings exchanged the recommended 2-step multimer system (UltraView) with a 3-step multimer system (OptiView or UltraView with amplification) and 82% (94 out of 115) prolonged the incubation time of the primary antibody (790-4509) to 24 min. or more.

The Dako/Agilent RTU system based on mAb clone DAK-p63 (IR662) was applied by 19 laboratories on the Dako Autostainer Link platform and an overall pass rate of 79% was seen, but only 11% were optimal. The two optimal results on the Autostainer platform were based on the vendor recommended protocol settings using HIER in TRS High pH for 20 min., 20 min. incubation of the primary Ab with EnVision FLEX (2-step polymer) as detection system. 10 other laboratories followed the exact same protocol, and for unknown reasons 9 of these "only" received a sufficient mark and one borderline.

In some (n=6) protocols based on IR662 (mAb clone DAK-p63) an aberrant moderate cytoplasmic staining reaction in the secretory cells of the prostate glands in tissue cores no. 4 and 5 was seen and at present no conclusion generated if being a lot or protocol issue – see Fig 5a. These was all stained on the Autostainer platform except for a single protocol being performed on the Ventana Benchmark Ultra. Even though the cytoplasmic staining in some cases displayed a moderate intensity, it did not interfere with the overall interpretation of p63 and therefore the pattern not downgraded to insufficient in any of the 6 cases.

17 participants used the IR662 format on the Dako Omnis platform with a pass-rate of 88% and 24% optimal. The higher pass-rate on Omnis compared to Autostainer may indicate that the clone like the DAK-p63 conc. perform better with a prolonged HIER time. This was also supported by the 3 participants applying the IR662 clone to the Ventana/Roche platform with an average HIER time at 55 min. and all receiving optimal results.

35 laboratories used the RTU format GA662 of mAb clone DAK-p63 on the Omnis platform. The vendor recommended protocol is based on HIER in TRS Low for 30 min., 25 min. incubation of the primary Ab and EnVision FLEX GV800 as detection system. Using these settings, a pass rate of 85% was seen, 25% being optimal. In this assessment the most successful protocol modification was based on HIER in TRS High giving a pass rate of 100% and 62% optimal.

Controls

Placenta is recommended as primary and critical positive tissue control for p63 where an at least weak to moderate, distinct nuclear staining reaction of dispersed cytotrophoblasts must be seen. The cytotrophoblasts should be visible even at a low magnification (5x objective).

Tonsil can serve as both supplementary positive and also as negative tissue control. In tonsil virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction, while dispersed lymphocytes and endothelial cells must show a weak but distinct staining reaction. The vast majority of lymphocytes should be negative.

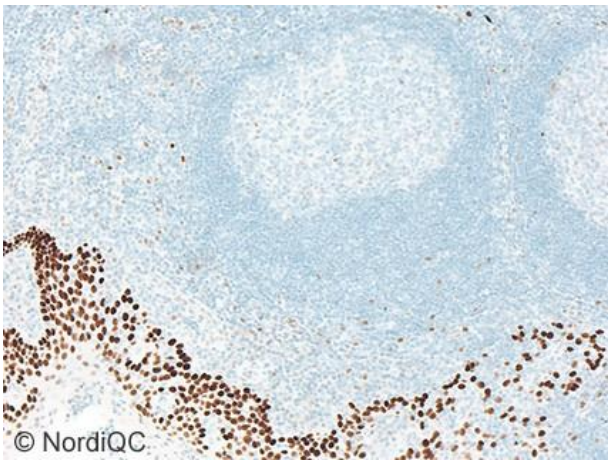


Fig. 1a (x100)
Optimal p63 staining of the tonsil using the mAb clone 4A4 (790-4509, Ventana/Roche RTU) with HIER in an alkaline buffer for 64 min. (CC1, Ventana/Roche) and performed on the Ventana Benchmark Ultra. Incubation time for primary Ab was 32 min. and UltraView with amplification was used as detection system. A strong nuclear staining reaction is seen in the majority of the squamous epithelial cells in the tonsil. A weak but distinct nuclear reaction is seen in scattered lymphocytes. No background staining is seen (same protocol used in Figs. 1a - 3a).

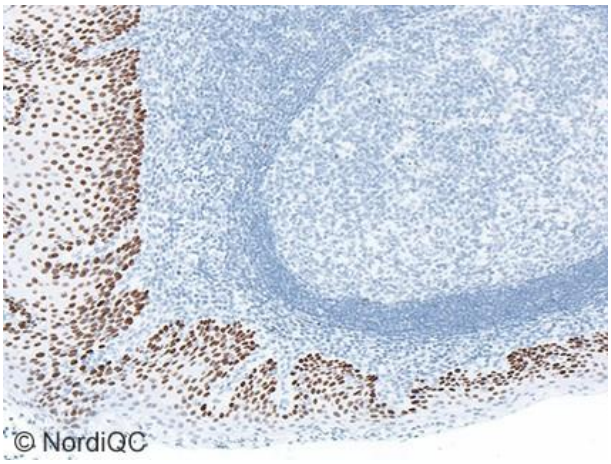


Fig. 1b (x100)
Insufficient p63 staining of the tonsil using mAb clone 4A4 (790-4509, Ventana/Roche RTU) with HIER in an alkaline buffer 64 min. (CC1, Ventana/Roche) and performed on the Ventana Benchmark Ultra. Incubation time for primary Ab was 48 min. and UltraView was used as detection system. The reduced sensitivity of the protocol using a 2-step detection system challenged the demonstration of p63 in cells with low expression levels. A moderate nuclear staining reaction is recognized in most of the squamous epithelial cells in the tonsil, whereas no staining is seen in the lymphocytes. Compare with Fig. 1a. Also, compare with Figs. 2b and 3b – same protocol.

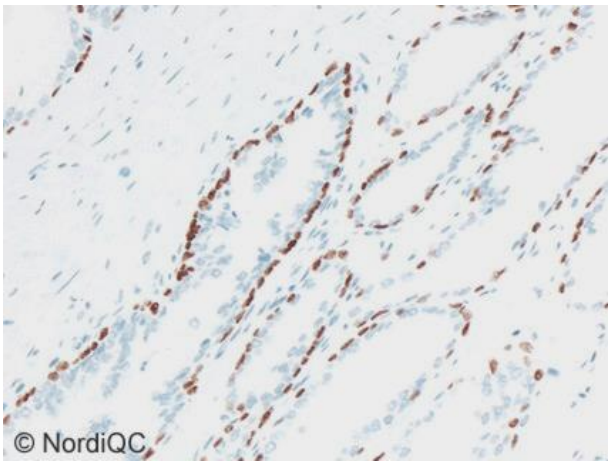


Fig. 2a (x200)
Optimal p63 staining in the prostate hyperplasia using the same protocol as in Fig. 1a. Virtually all the basal cells show a moderate to strong distinct nuclear staining reaction. No background staining is seen.

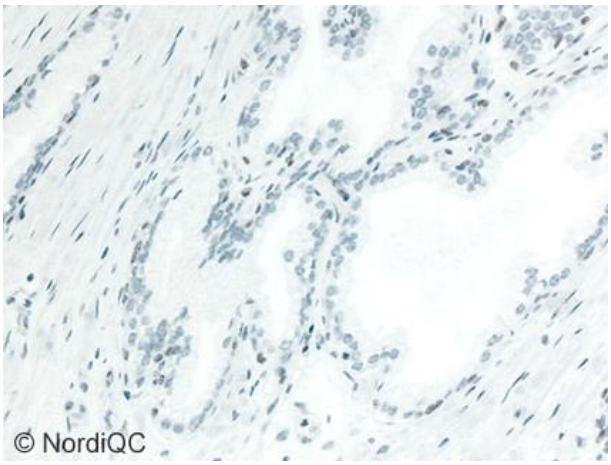


Fig. 2b (x200)
Insufficient p63 staining in the prostate hyperplasia using the same protocol as in Fig. 1b. Due to the reduced sensitivity of the protocol, virtually all basal cells in the prostate hyperplasia are false negative. Compare with Fig. 2a – same lesion.

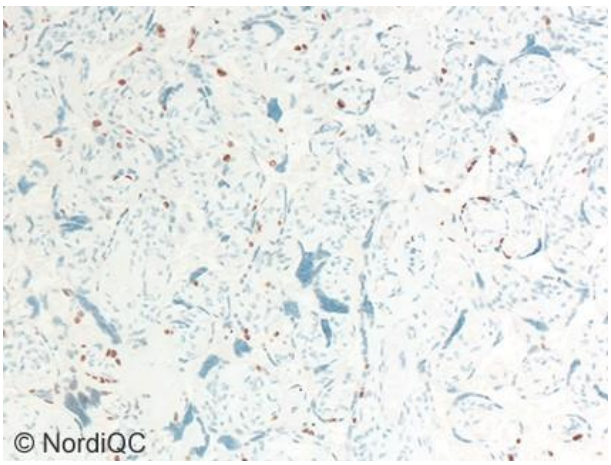


Fig. 3a (x100)
Optimal p63 staining of the placenta using same protocol as in Figs. 1a and 2a. Scattered cytotrophoblastic cells show a weak to moderate, distinct nuclear staining reaction. No background staining is seen.

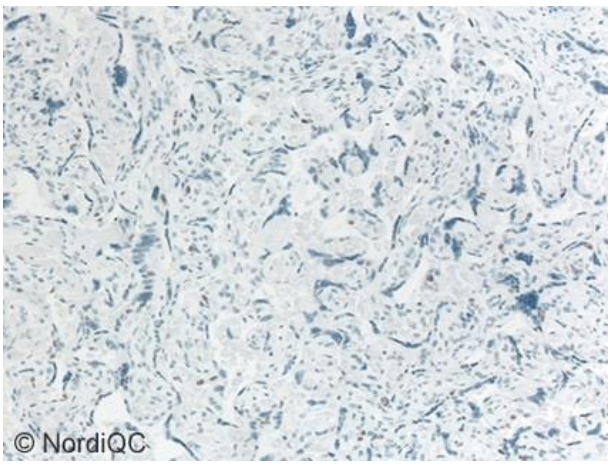


Fig. 3b (x100)
Insufficient p63 staining of the placenta using same protocol as in Figs. 1b and 2b. A significantly reduced number of cytotrophoblastic cells is seen and hardly identifiable. Compare with Fig. 3a – same lesion.

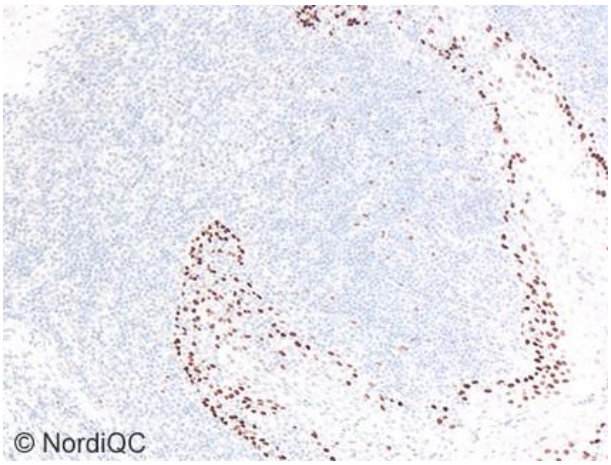


Fig. 4a (x100)
p63 staining of the tonsil using mAb clone 7JUL (PA0103, Leica RTU) with HIER in an alkaline buffer 30 min. (BERS2, Leica) and performed on the Leica Bond. Incubation time for primary Ab was 15 min. and Bond Refine was used as detection system.
The general level of analytical sensitivity is reduced compared to level expected and obtained e.g. by mAb clone 4A4.
A moderate nuclear staining reaction is seen in most of the squamous epithelial cells in the tonsil, and few lymphocytes show a weak nuclear staining reaction.
Compare with Fig. 1a – same field. Also compare with fig 4b.

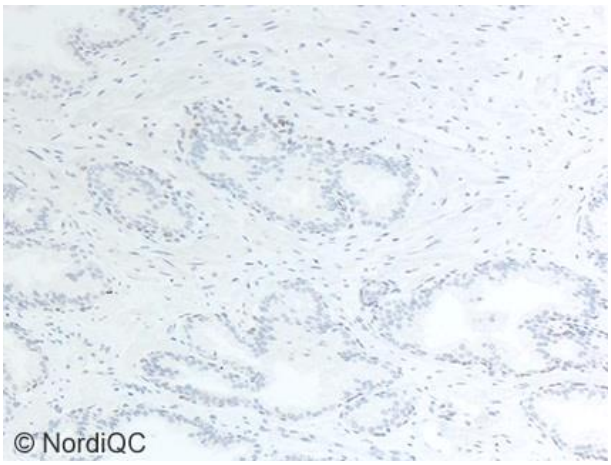


Fig. 4b (x100)
Insufficient p63 staining of the prostate hyperplasia using same protocol as in Fig. 4a. Virtually all basal cells in the prostate hyperplasia are negative.
The reduced sensitivity for clone 7JUL was in particular observed in the prostate lesions and placenta. The use of tonsil as primary positive tissue control can, as described in the report, NOT be recommended.
Tonsil should be used together with placenta to guide the low limit of detection for p63.

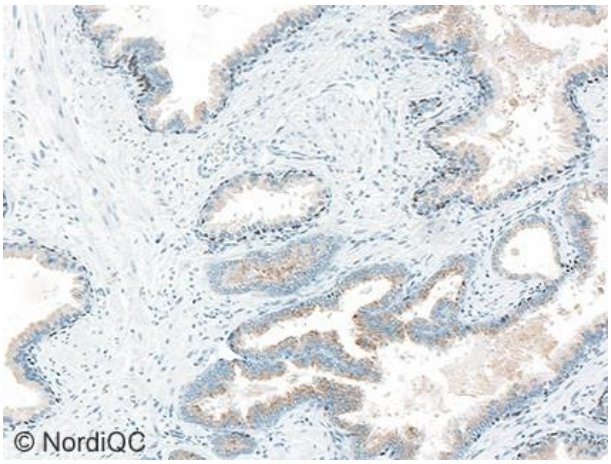


Fig. 5a (x100)
Sufficient p63 staining of the PIN using the mAb clone DAK-p63 (IR662, Dako/Agilent) with HIER in an alkaline buffer 20 min (TRS High, Dako/Agilent) on the Dako Autostainer, Incubation of the primary antibody was 30 min. and Envision FLEX used as detection system. The secretory cells of the prostate glands show a moderate cytoplasmic staining reaction. The basal cells still display a moderate nuclear staining reaction.

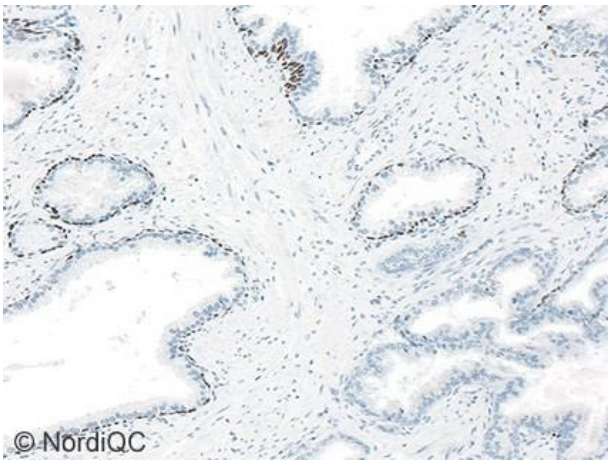


Fig. 5b (x100)
Sufficient p63 staining of the PIN using a similar protocol as in Fig. 5a but a different lot number.
The cytoplasmic compartment of the secretory cells of the prostate glands is negative as expected. The basal cells display a moderate nuclear staining reaction.

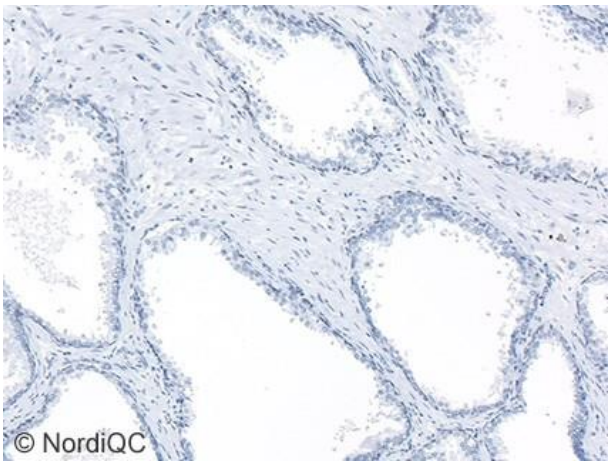


Fig. 6a (x100)
Insufficient p63 staining of the prostate hyperplasia using the mAb clone 4A4 (790-4509, Ventana/Roche RTU) with HIER in an alkaline buffer for 52 min. (CC1, Ventana/Roche) and performed on the Ventana Benchmark Ultra. Incubation time for primary Ab was 32 min. and UltraView was used as detection system. Despite using almost the same protocol as Figs. 1a-3a, the combination of excessive counterstain and low sensitivity gave a weak p63 nuclear reaction in the basal cells complicating the interpretation.

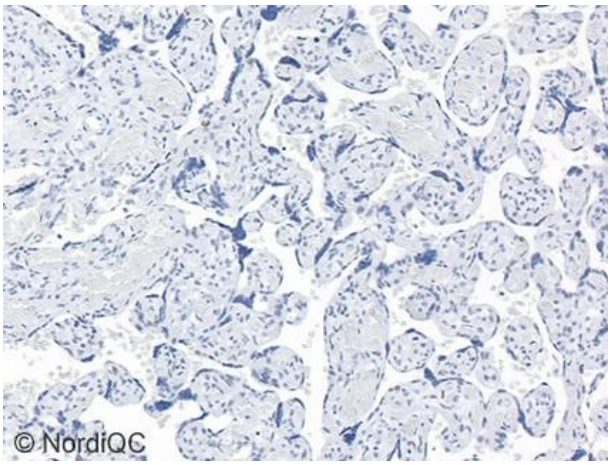


Fig 6b (x100)
Insufficient p63 staining of the placenta using same protocol as in Fig. 6a. A significantly reduced number of cytotrophoblastic cells is seen and hardly identifiable due to the strong counterstain.

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