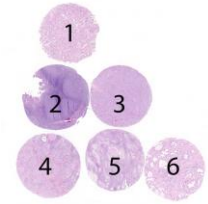
Assessment Run 48 2016
p63

Material
The slide to be stained for p63 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 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 in the tonsil.
- A moderate to strong, distinct nuclear staining reaction in basal cells in the hyperplastic prostate glands.
- A moderate to strong, distinct nuclear staining reaction in virtually all tumour cells of the lung squamous cell carcinomas.
- No staining reaction in the vast majority of tumour cells of the lung adenocarcinoma.
- No staining reaction in 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 48	299
Number of laboratories returning slides	274 (92%)

Results
274 laboratories participated in this assessment. 224 (82%) 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 fourth NordiQC assessment of p63. Compared to the previous run in 2014 (Run 41), an increase in pass rate was seen (table 2).

Table 2. **Proportion of sufficient results for p63 in four NordiQC runs**

	Run 16 2006	Run B8 2009	Run 41 2014	Run 48 2016
Participants, n=	68	113	236	274
Sufficient results	83%	95%	70%	82%

Conclusion
mAbs clones **4A4** and **DAK-p63** and rmAb **DBR16.1** are all 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 Ready-To-Use format of mAbs clones **4A4** and **DAK-p63** performed slightly better compared to laboratory developed assays using the concentrated format. Prostate, placenta and tonsil are all recommendable positive tissue controls for p63. In prostate, moderate to strong nuclear staining reaction should be present in virtually all basal cells. In placenta, an at least weak to moderate, distinct nuclear staining reaction of dispersed cytotrophoblastic cells should be seen. In tonsil, virtually all squamous epithelial cells must show a moderate to strong nuclear staining reaction, whereas a weak but distinct nuclear reaction in scattered lymphocytes must be seen. No staining should be seen in the vast majority of lymphocytes.

Table 1. **Antibodies and assessment marks for p63, run 48**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4A4**	26 4 3 3 2 2 1 1 1 1 1	BioCare Medical ImmunoLogic Dako Zeta Corporation Thermo Scientific Zytomed Systems BioGenex Diagnostic BioSystems Klinipath Minarini Nordic Biosite Santa Cruz	13	20	11	2	72%	76%
mAb clone **DAK-p63**	47	Dako	20	21	6	0	87%	91%
mAb clone **7JUL**	12	Leica/Novocastra	0	1	3	8	8%	-
mAb clone **SFI-6**	2	DCS Immunoline	0	0	2	0	-	-
rmAb clone **BSR6**	1	Nordic Biosite	0	0	1	0	-	-
rmAb clone **DBR16.1**	1	Diagnostic Biosystems	1	0	0	0		
rmAb clone **EPR5701**	1	Epitomics	0	0	1	0	-	-
Unknown Ab	1	Unknown	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **4A4 790-4509**	102	Ventana	59	36	5	2	93%	95%
mAb clone **DAK-p63 IR662**	46	Dako	21	23	2	0	96%	94%
mAb clone **4A4 PM163**	3	BioCare	1	1	1	0	-	-
mAb clone **7JUL PA0103**	5	Leica/Novocastra	0	0	3	2	-	-
mAb clone **4A4 AM418**	2	BioGenex	0	1	0	1	-	-
mAb clone **4A4 ARB-56695**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **MX013 MAB-0694**	1	Maixin	0	1	0	0	-	-
mAb clone **4A4 MAD-000479QD**	3	Master Diagnostica SL	3	0	0	0	-	-
Total	274		120	104	35	15	-	
Proportion			44 %	38 %	13 %	5 %	82 %	

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

Detailed analysis of p63, Run 48

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

Concentrated Antibodies

mAb clone **4A4**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Cell Conditioning 1 (CC1, Ventana) (9/23)*, Tris-EDTA/EGTA pH 9 (2/3) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (2/8) as retrieval buffer. The mAb was typically diluted in the range of 1:30-1:1,000 depending on the total sensitivity of the protocol employed. Using these protocol settings, 26 of 34 (76%) 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) (14/20), Target Retrieval Solution, High pH (Dako) (3/7), Tris-EDTA/EGTA pH 9 (2/3), or BERS2 (Leica) (1/4) 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 30 of 33 (91%) laboratories produced a sufficient staining result.

rmAb clone **DBR16.1**: One protocol with an optimal result was based on 20 min. HIER using Montage EDTA Antigen Retrieval Solution (Diagnostic Biosystems), 30 min. incubation of the primary Ab diluted 1:100 and PolyVue (Diagnostic Biosystems) as detection system.

Table 3 summarizes the overall proportion of optimal staining results for the most frequently used concentrated antibodies on the three most commonly used IHC stainer systems.

Table 3. **Proportion of optimal results for p63 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer / 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
mAb clone **DAK-p63**	3/15 (20%)**	0/1	14/20 (70%)	-	1/4 (25%)	-
mAb clone **4A4**	0/6 (0%)	-	9/22 (41%)	-	2/8 (25%)	0/1

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.
** (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, BenchMark GX / XL / Ultra:
Protocols with optimal results were typically based on 32-92 min. HIER using Cell Conditioning 1 (Ventana), 16-52 min. incubation of the primary Ab and UltraView (Ventana 760-500), UltraView (Ventana 760-500) with amplification (760-080) or OptiView (Ventana 760-700) as detection system. Using these protocol settings 76 of 80 (95%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-p63,** product.no. IR662, Dako, Dako Autostainer / Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-99°C), 20-30 min. incubation of the primary Ab and EnVision Flex/Flex+ (K8000/K8002) as detection system. Using these protocol settings 29 of 31 (94%) laboratories produced a sufficient staining result (optimal or good).

Table 4 summarises 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, whereas e.g. Dako RTU Ab formats applied on a Ventana stainer were excluded.

Table 4. **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
Dako AS48 mAb DAK-p63 **IR662**	93% (14/15)	60% (9/15)	94% (16/17)	24% (4/17)
VMS Ultra/XT mAb 4A4 **790-4509**	60% (3/5)	20% (1/5)	95% (89/94)	60% (56/94)

* 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 included.

In this assessment and in concordance with the previous assessment for p63 in NordiQC (run 16, 2006, run B8, 2009 and run 41, 2014) 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 96% of the insufficient results (48 of 50 laboratories). In 15% of these cases, the interpretation of the nuclear p63 staining reaction was further complicated by excessive counterstaining (Fig. 1 – Fig. 3). The remaining insufficient results were characterized by excessive background reaction and/or false positive staining reaction compromising the interpretation. Most participants were capable of detecting p63 in squamous epithelial cells in tonsil and in the majority of neoplastic cells of the lung squamous cell carcinoma, tissue core no. 5 (high level p63 expression), whereas demonstration of p63 in basal cells of the hyperplastic prostate glands, scattered lymphocytes in tonsil, cytotrophoblastic cells in placenta and tumour cells in the

squamous cell lung carcinoma, tissue core no. 4 (low level p63 expression) was more difficult. In a few cases an aberrant cytoplasmic or false positive nuclear reaction of cells not expressing p63 was seen. This pattern was typically caused by too high concentration of the primary Ab or use of a less successful primary antibody.

41% (111 of 274) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for p63. Optimal staining results could be obtained with both mAb clone DAK-p63, mAb clone 4A4 and the rmAb clone DBR16.1 (see table 1). The mAb clone DAK-p63 was the most widely used. 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 3 main IHC systems, but the proportion of sufficient and optimal results using the mAb clone DAK-p63 as a concentrate was higher on the Ventana system compared to the Dako and Leica systems. On the Ventana system an impressive pass rate of 100% (20 of 20) was seen of which 70% were evaluated as optimal. On the Dako and Leica system, the pass rates were 81% (13 of 16) and 75% (3 of 4), respectively, but only 19% and 25% were optimal. The reason for the higher proportion of optimal results on the Ventana system is unclear. 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 48-64 min. in CC1) compared to a reduced HIER pretreatment time of typically 10-20 min. in TRS High pH and ER2 on Dako and Leica systems. Furthermore, a sensitive 3-step multimer based detection system was used in 85% (17 of 20) of the laboratories using the Ventana system, whereas only 56% (9 of 16) 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 46 laboratories and the pass rate for the LD assays was 72% (33 of 46) with 28% achieving optimal results. Optimal results were seen on the Ventana (41%) and Leica (25%) systems, but not on the Dako system (table 3). On the Ventana system, 73% (16 of 22) of laboratories achieved a sufficient results. In these laboratories, the average dilution factor of clone 4A4 was 1:196 and the average heating time in CC1 was 52 min., whereas the average dilution factor was decreased to 1:333 and the average heating time reduced to 42 min. in the 6 laboratories that got insufficient results. This indicates that both the concentration of the mAb clone 4A4 and the retrieval protocol should be carefully calibrated. The importance of a relative long HIER pretreatment on the Ventana system is illustrated in Fig. 6a and 6b.

The mAb clone 7JUL was used by 12 laboratories as a concentrate. Despite applying similar protocol settings (e.g. HIER and detection systems) as for the mAbs clones 4A4 and DAK-p63, 11 (92%) produced insufficient staining results (borderline or poor) as shown in table 1. Identical findings were also observed for the corresponding RTU format, where all 5 laboratories produced insufficient staining results. The prevalent feature of the insufficient results was a too weak or false negative staining of basal cells in the prostate hyperplasia, scattered lymphocytes in tonsil, cytotrophoblastic cells in placenta and tumor cells in the lung squamous cell carcinomas (Fig. 4 – Fig. 5). The mAb clone 7JUL also showed low pass rates in previous p63 assessments run 16 (2006), B8 (2009) and run 41 (2014) 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 59% (163 of 274) of the laboratories.
The Ventana RTU system based on mAb clone 4A4 (790-4509) was the most widely used RTU system applied by 99 laboratories (3 laboratories used 790-4509 on non-Ventana systems). An overall pass rate of 93% was seen and 58% were optimal. Optimal results could be achieved with both the Ventana recommended protocol settings and by laboratory modified protocol settings. Ventana 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 5 of 99 laboratories followed the Ventana recommendations, giving a pass rate of 60% with only 20% optimal (table 4). In contrast, the 94 laboratories that used laboratory modified protocol settings had a pass rate of 95% (89 out of 94) with 60% optimal (56 of 94). 65% (61 of 94) of the laboratories with modified protocol settings had changed from 2-step multimer (UltraView) to a 3-step multimer (OptiView or UltraView with amplification) and 81% (76 out of 94) had prolonged the incubation time of the primary antibody (790-4509) to 24 min. or more. A total of 39 laboratories using both a 3-step multimer detection system and a prolonged incubation time of the primary antibody had a pass rate of 100%, with 85% (33 of 39) being optimal. These data suggest that Ventana may well change the recommended protocol settings in order to improve the performance of the RTU format.

The Dako RTU system based on mAb clone DAK-p63 (IR662) was applied by 32 laboratories on the Dako Autostainer Link platform. An overall pass rate of 94% was seen and 41% were optimal. Optimal results could be achieved with both the official Dako protocol settings and laboratory modified protocol settings.

The official Dako protocol recommends HIER in TRS High for 20 min., 20 min. incubation of the primary Ab with FLEX (2-step polymer) as detection system. 15 laboratories followed the recommended protocol settings and a pass rate of 93% (14 of 15) was seen and 60% (9 of 15) were optimal. The pass rate of the laboratory modified protocols was equally high, reaching 94% (16 of 17), but the proportion of optimal marks was only 24% (4 of 17). This could be the result of too short HIER pretreatment, as 7 of the modified protocols included reductions in HIER pretreatment to 10 min. compared to the recommended 20 min.
14 laboratories used the RTU format on Dako Omnis by protocol settings like the Dako recommendations for Autostainer but modified to "Omnis RTU" settings (using HIER for 30 min. and 20 min. incubation of the primary Ab and EnVision FLEX+ polymer GV800+GV823 as detection system). A pass rate of 100% was seen, 57% being optimal.

Controls
Tonsil, prostate and placenta are all recommendable as positive tissue controls for p63. In placenta, an at least weak to moderate, distinct nuclear staining reaction of dispersed cytotrophoblastic cells should be seen. Virtually all basal cells of prostate glands and squamous epithelial cells of tonsil must show a moderate to strong distinct nuclear staining reaction. In the tonsil scattered lymphocytes must show a weak to moderate and distinct nuclear staining reaction. The combination of both high- and low-level p63 expressing cells in the same tissue makes tonsil especially suitable as positive tissue control.
No staining should be seen in secretory cells of the prostate or in the vast majority of lymphocytes of the tonsil.

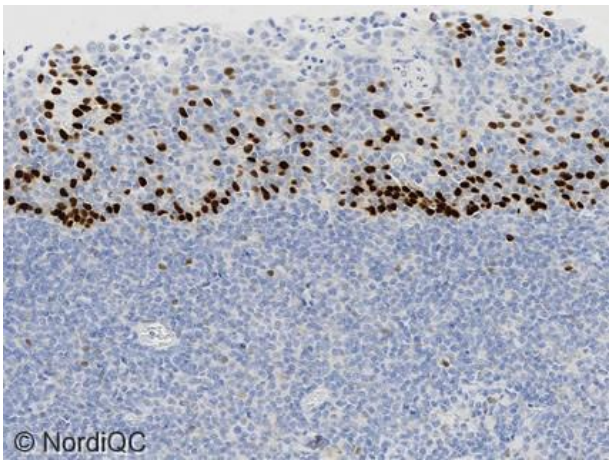


Fig. 1a (x200)
Optimal p63 staining of the tonsil using the mAb clone DAK-p63 (Dako RTU) with HIER in an alkaline buffer (TRS pH 9.0, Dako) and performed on the Dako Autostainer. Incubation time for both primary Ab and Flex polymer was 30 min. A strong nuclear staining reaction is seen in the majority 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)

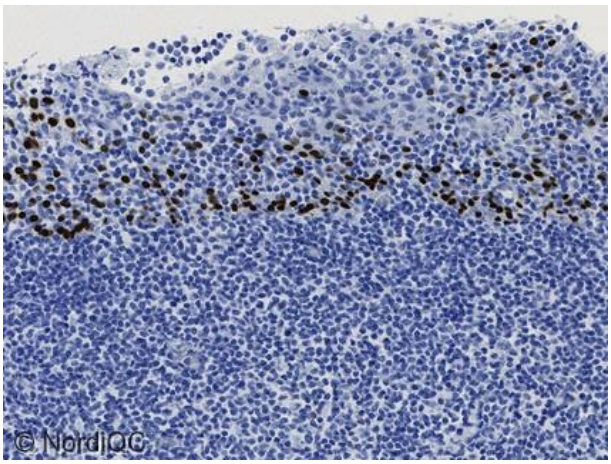


Fig. 1b (x200)
Insufficient p63 staining of the tonsil using the mAb clone DAK-p63 (Dako RTU) with similar protocol settings as used in Fig. 1a, but with a reduced incubation time of 20 min. for both the primary Ab and the Flex polymer. The reduced sensitivity of the protocol in combination with a too strong counterstain complicates the reading of the nuclear p63 signals. 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 – same field. Also, compare with Figs. 2b and 3b – same protocol.

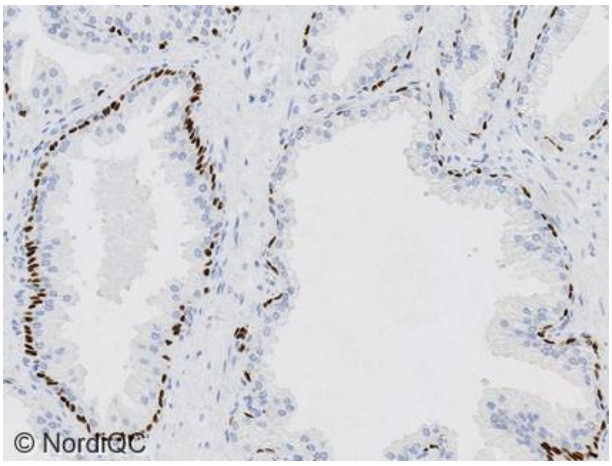


Fig. 2a (x200)
Optimal p63 staining in the prostate hyperplasia using the same protocol as in Fig. 1a. Virtually all the basal cells show a moderate to strong distinct nuclear staining reaction. No background staining is seen.

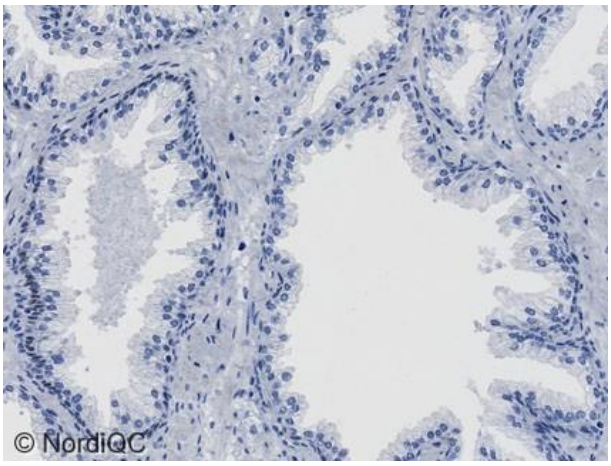


Fig. 2b (x200)
Insufficient p63 staining in the prostate hyperplasia using the same protocol as in Fig. 1b. The combination of reduced sensitivity of the protocol and strong counterstain leaves virtually all basal cells in the prostate hyperplasia negative. Compare with Fig. 2a – same field.

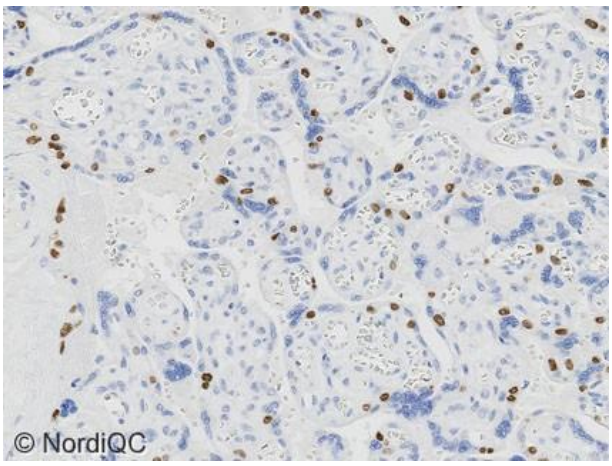


Fig. 3a (x200)
Optimal p63 staining of the placenta using same protocol as in Figs. 1a and 2a. Scattered cytotrophoblastic cells show a weak to moderate, distinct nuclear staining reaction. No background staining is seen.

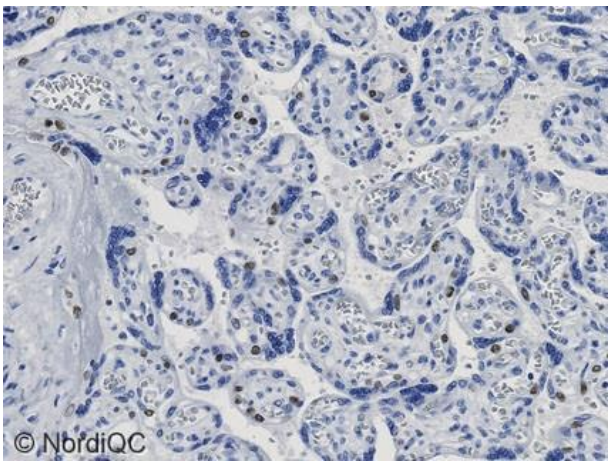


Fig. 3b (x200)
Insufficient p63 staining of the placenta using same protocol as in Figs. 1b and 2b. A reduced number of cytotrophoblastic cells is seen. Compare with Fig. 3a – same field.

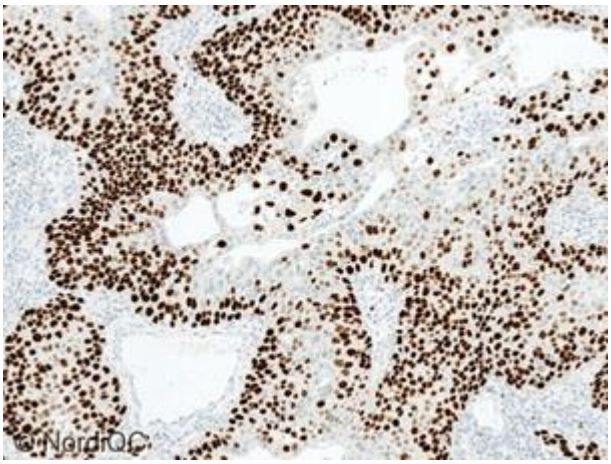


Fig. 4a (x100)
Optimal p63 staining of the lung squamous cell carcinoma (tissue core no. 4) using the mAb clone DAK-p63 (1:100 for 32 min.), HIER in an alkaline buffer (CC1 56 min.) and performed on the BenchMark Ultra, using 3-step multimer detection system. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen. Same protocol used in Fig. 5a

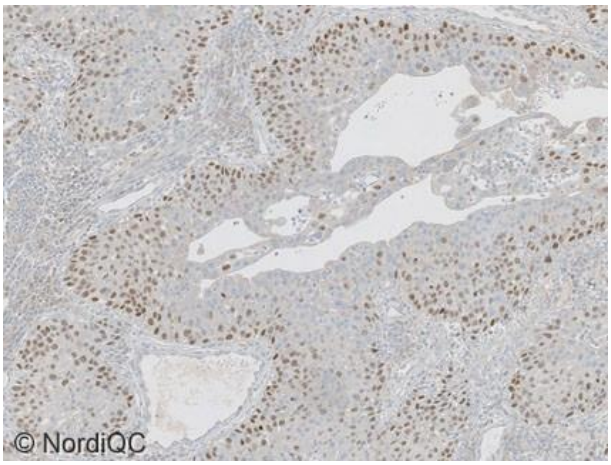


Fig. 4b (x100)
Insufficient p63 staining of the lung squamous cell carcinoma (tissue core no. 4) using mAb clone 7JUL with similar protocol settings on the BenchMark Ultra as in Fig. 4a. Only weak nuclear staining is seen and in a reduced number of neoplastic cells. Compare with Fig. 4a – same field.
Also, compare with Fig. 5b – same protocol.

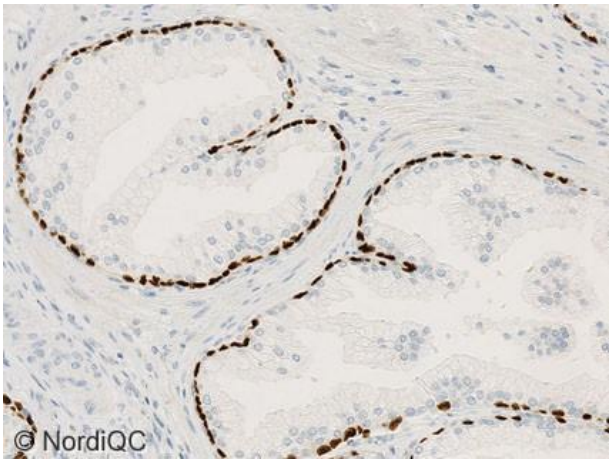


Fig. 5a (x200)
Optimal p63 staining in the prostate hyperplasia using the same protocol as in Fig. 4a. Virtually all the basal cells show a moderate to strong distinct nuclear staining reaction. No background staining is seen.

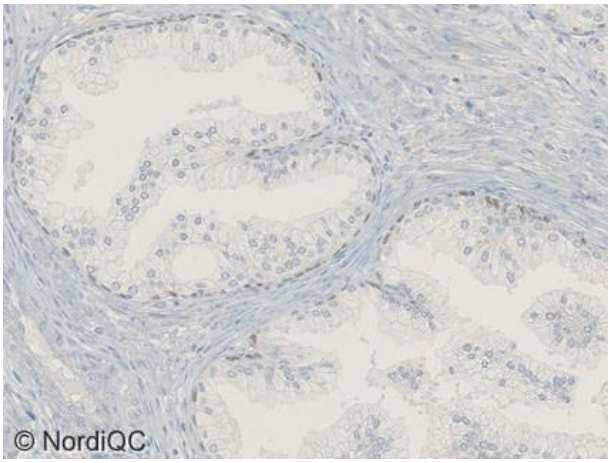


Fig. 5b (x200)
Insufficient p63 staining in the prostate hyperplasia using the same protocol as in Fig. 4b. The majority of basal cells in the prostate hyperplasia are negative. Compare with Fig. 5a. – same field.

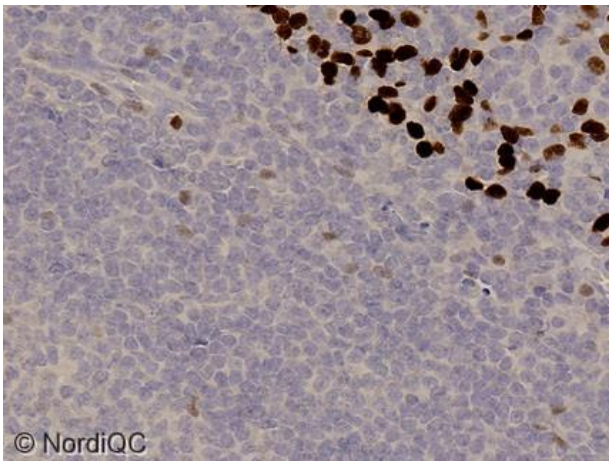


Fig. 6a (x400)
Optimal p63 staining of the tonsil using the mAb clone 4A4 (1:100 for 32 min.) after HIER in an alkaline buffer (CC1 64 min.) and performed on the BenchMark Ultra, using 3-step multimer detection system (OptiView). A strong nuclear staining reaction is seen in the majority of the squamous epithelial cells and a weak but distinct nuclear reaction is seen in scattered lymphocytes. Compare with Fig. 6b.

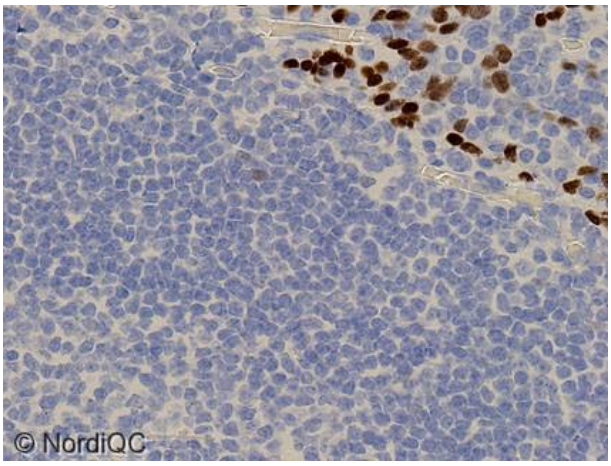


Fig. 6b (x400)
Insufficient p63 staining in the tonsil using the mAb clone 4A4 (Ventana, RTU) by similar protocol settings as in Fig. 6a, except for a reduction in HIER pretreatment to 32 min. and a reduction in the incubation time of the primary ab to 20 min. Too short HIER pretreatment (in combination with a reduction in incubation time of primary ab) results in a slightly weaker staining reaction in the squamous epithelial cells in the tonsil, but a loss of nuclear staining reaction in lymphocytes. Compare with Fig. 6a – same field.

ON/SN/LE/MV/RR 05.12.2016