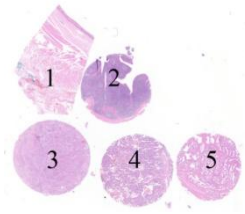
Assessment Run 41 2014
p63

Material
The slide to be stained for p63 comprised:



1. Esophagus, 2. Tonsil, 3. Lung squamous cell carcinoma, 4. Lung adenocarcinoma
5. Prostate hyperplasia.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a p63 staining as optimal included:

- A moderate to strong, distinct nuclear staining in the majority of the squamous epithelial cells in the esophagus.
- A moderate to strong, distinct nuclear staining in almost all the 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 in the basal cells in the prostate hyperplasia.
- A moderate to strong, distinct nuclear staining in virtually all tumor cells in the lung squamous cell carcinoma
- No staining reaction in the vast majority of tumor cells in the lung adenocarcinoma
- No staining reaction in secretory cells of the prostate hyperplasia
- No or only a week cytoplasmic reaction.

Participation

Number of laboratories registered for p63, run 41	255
Number of laboratories returning slides	239 (94%)

Results
239 laboratories participated in this assessment. 3 laboratories used inappropriate antibodies (Abs). Of the remaining 236 laboratories, 166 (70%) achieved a sufficient mark (optimal or good). 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 Ab
- Insufficient heat induced epitope retrieval
- Detection systems with low sensitivity
- Too low concentration of the primary Ab

Performance history
This was the 3rd NordiQC assessment of p63. A decrease in pass rate was seen (table 2).

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

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

The reduced pass rate (70%) in this run may be explained by a large proportion of new participants, and new and more challenging tissue material circulated.

Conclusion
The mAbs clones **4A4** and **DAK-p63** are both recommendable Abs for demonstrating 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. Both clones performed slightly better in the Ready-To-Use format than in concentrate. Prostate and tonsil are recommendable positive tissue controls for p63. In prostate, moderate to strong nuclear reaction should be present in virtually all basal

cells. In tonsil optimal protocols displays strong nuclear reaction in virtually all the squamous epithelial cells and a weak but distinct nuclear reaction in scattered lymphocytes.

Table 1. **Antibodies and assessment marks for p63, run 41**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4A4**	38 14 6 5 3 2 1 1 1 1 1 1	BioCare Medical Dako ImmunoLogic Zeta Corporation Santa Cruz Zytomed Systems BioLogo BioGenex BioSite Bio SB Minarini NeoMarkers Thermo Scientific	21	25	27	2	61 %	67 %
mAb clone **DAK-p63**	26	Dako	13	9	4	0	85 %	95 %
mAb clone **7JUL**	14	Leica/Novocastra	0	0	10	4	0 %	-
rmAb clone **EP174**	1	Bio SB	0	0	1	0	-	-
mAb clone **SFI-6**	1	DCS Immunoline	0	0	0	1	-	-
Ab	1	Unknown	0	0	1	0	-	-
Ready-To-Use Abs								
mAb clone **4A4 790-4509**	74	Ventana	37	25	11	1	84 %	87 %
mAb clone **DAK-p63 IR662**	36	Dako	24	9	3	0	92 %	92 %
mAb clone **4A4 PM163**	3	BioCare	0	2	1	0	-	-
mAb clone **7JUL PA0103**	2	Leica/Novocastra	0	0	2	0	-	-
mAb clone **4A4 AM418**	1	BioGenex	0	0	1	0	-	-
mAb clone **4A4 PDM136**	1	DBS	0	0	0	1	-	-
mAb clone **4A4 MAD-000479QD**	1	Master Diagnostica SL	0	1	0	0	-	-
Total	236		95	71	61	9	-	
Proportion			40 %	30 %	26 %	4 %	70 %	

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 41

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) (14/29)*, Tris-EDTA/EGTA pH 9 (2/9), Bond Epitope Retrieval Solution 2 (Leica) (2/11), Target Retrieval Solution pH 9 (Dako) (2/12), or Target Retrieval Solution pH 9 (3-in-1) (Dako) (1/6) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:600 depending on the total sensitivity of the protocol employed. Using these protocol settings, 42 of 62 (68%) 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) (9/11), Tris-EDTA/EGTA pH 9 (2/4), Target Retrieval Solution pH 9 (3-in-1) (Dako) (1/5), or EDTA/EGTA pH 8 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings 19 of 20 (95%) laboratories produced a sufficient staining result (optimal or good).

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 Link / Classic		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 **4A4**	2/17 (12%)**	-	14/26 (54%)	-	2/11 (18%)	0/1
mAb clone **DAK-p63**	1/9 (11%)	0/1	9/10 (90%)	-	-	-

* 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
mAb clone **4A4** (product no. 790-4509, Ventana): Protocols with optimal results were typically based on 32-64 min. HIER using Cell Conditioning 1 (Ventana), 16-52 min. incubation of the primary Ab and iView (790-091), UltraView (Ventana 760-500), UltraView (Ventana 760-500) with amplification (760-080) or OptiView (Ventana 760-700) as detection system. Using these protocol settings 62 of 71 (87%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-p63** (product.no. IR662, Dako): Protocols with optimal results were typically based on 10-20 min. HIER using Tris-EDTA/EGTA pH 9, Target Retrieval Solution pH 9 (3-in-1) (Dako), Target Retrieval Solution pH 9 pH 9 (Dako) or Target Retrieval Solution pH 6.1 (Dako), 15-30 min. incubation of the primary Ab and EnVision Flex/Flex+ (GV800/K8000/K8002) as detection system. Using these protocol settings 33 of 36 (92%) laboratories produced a sufficient staining result.

In concordance with previous assessments for p63 in NordiQC (run 16, 2006 and run B8, 2009), the prevalent feature of the insufficient results was a too weak or false negative staining of the cells/structures expected to be demonstrated. The majority of participants were capable to detect p63 in squamous epithelial cells in the tonsil and esophagus, whereas demonstration of p63 in basal cells in the prostate hyperplasia, scattered lymphocytes in tonsil and tumor cells in the squamous cell lung carcinoma was more difficult and only seen using protocols with a high sensitivity.
A few cases of aberrant cytoplasmic or false positive nuclear reaction of cells not expressing p63 were also seen. This pattern was typically caused by too high concentration of the primary Ab or use of less successful primary antibody.

Optimal staining results could be obtained with both the mAb clone 4A4 and the recently introduced mAb clone DAK-p63 (see table 1). The mAb clone 4A4 was the most widely used. Used as a concentrate, mAb clone 4A4 gave an overall pass rate of 61% (46 of 75). The proportion of sufficient and optimal results using the mAb clone 4A4 as a concentrate was higher on the Ventana system compared to the Dako and Leica system. On the Ventana system a pass rate of 88% (23 of 26) was seen and 54% were evaluated as optimal. On the Dako and Leica system the pass rate was 29% (5 of 17) and 58% (7 of 12) respectively and 13% and 18% were optimal.
The reason for these differences is unclear, but might be related to the fact, that the majority of protocols performed on the Ventana system were based on a relatively long HIER pretreatment 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.
The importance of a relatively long HIER pretratment on the Ventana system is illustrated in Fig. 5a and Fig. 5b.

The recently introduced mAb clone DAK-p63 provided a pass rate of 85% when applied as a concentrate. Similar to mAb clone 4A4, the proportion of optimal results using the concentrated mAb DAK-p63 was much higher on the Ventana system (90%) compared to the Dako system (11%) (DAK-p63 was not used on Leica systems) - see table 3. As for mAb clone 4A4, the reason for this difference could be related to the typically longer HIER pretreatment in alkaline buffer (CC1) on the Ventana system compared to the Dako system (TRS pH 9.0). Furthermore a 3-step multimer based detection system was typically applied on the Ventana system compared to 2-step polymer based system most frequently used on the Dako system. On the Ventana system 8 of 11 laboratories used a 3-step multimer based system of which all 8 laboratories produced a sufficient staining result and 6 (75%) were assessed as optimal.
The typical protocol settings for the 6 laboratories achieving optimal staining on the Ventana system were quite similar: mAb clone DAK-p63 applied in a titer range of 1:50 – 1:150, 28-32 min. incubation of the

primary Ab at 36°C, HIER pretreatment in CC1 for 48 - 64 min. at 95°C - 100°C and a 3-step multimer based detection system (OptiView). In contrast to these findings, only 4 of 10 laboratories used a 3-step polymer based detection system on the Dako system. All of these produced a sufficient staining result and 1 was assessed as optimal. The remaining 6 laboratories used 2-step polymer based detection system and only 2 of these produced a sufficient staining (33%) and none were assessed as optimal. Not surprisingly, the DAK-p63 data indicates that combination of efficient HIER pretreatment and a highly sensitive detection system (e.g. 3-step multimer/polymer) facilitates the process of optimizing IHC-protocols using concentrated antibodies.

For the other p63 antibodies mAb clone 7JUL (Leica/Novocastra), mAb SFI-6 (DCS Immunoline) and rmAb clone EP174 (Bio SB) no sufficient staining result was obtained. Only 1 protocol of each of the last 2 antibodies was submitted and obviously no conclusion could be draw from these data. The mAb clone 7JUL was used by 14 laboratories as a concentrate and despite similar protocol settings as e.g. HIER, detection systems etc. were applied as for the mAbs clones 4A4 and DAK-p63, all produced insufficient staining results (borderline or poor) as shown in table 1. Identical findings were also observed for the corresponding RTU format. 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 and tumor cells in the lung squamous cell carcinoma, as shown in Fig 1b – Fig 4b. The mAb clone 7JUL also showed low pass rates in previous p63 assessments run 16 (2006) and B8 (2009) 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.

The most frequently applied Ready-To-Use assays for p63 in this assessment were based on the mAb clone 4A4 (790-4509, Ventana) and the mAb clone DAK-p63 (IR662, Dako). Both RTU systems provided a higher pass rate compared to the corresponding pass rates using the concentrates within a laboratory developed assay. The RTU system from Dako (IR662) provided the highest pass rate of 92% (33 of 36) and 67% were assessed as optimal. The RTU system from Ventana (790-4509) gave a slightly lower pass rate of 84% (62 of 74) and 50% were assessed as optimal.
For both RTU systems, optimal results could be obtained by using the official protocol recommendations given by the respective companies. Laboratory modified protocol settings (typically adjusting HIER time, incubation time of the primary Ab and/or choice of detection system) could also provide optimal result.
For the Ventana system a pass rate of 100% (25 of 25) was seen of which 80% were optimal, when the RTU format was applied in combination with a 3-step multimer based detection system. A pass rate of 73% (30 of 41) and 34% optimal was seen using a 2-step detection system as recommended in the official data sheet for the RTU format.

Controls
Both tonsil and prostate were found recommendable as positive tissue controls for p63.
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.
No staining should be seen in secretory cells of the prostate or in the vast majority of lympohocytes.

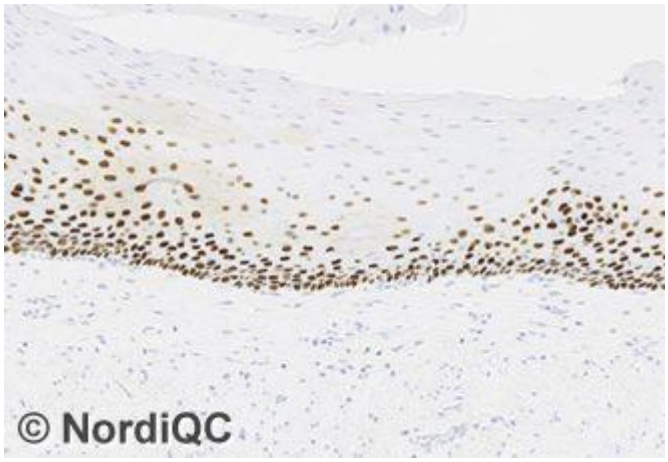


Fig. 1a
Optimal p63 staining of the esophagus using the mAb clone DAK-p63 (Dako RTU) with HIER in an alkaline buffer (TRS pH 9.0, Dako) and performed on the Dako Autostainer. A strong nuclear staining reaction is seen in the majority of the squamous epithelial cells in the esophagus. No background staining is seen. Same protocol used in Figs. 1a - 4a.

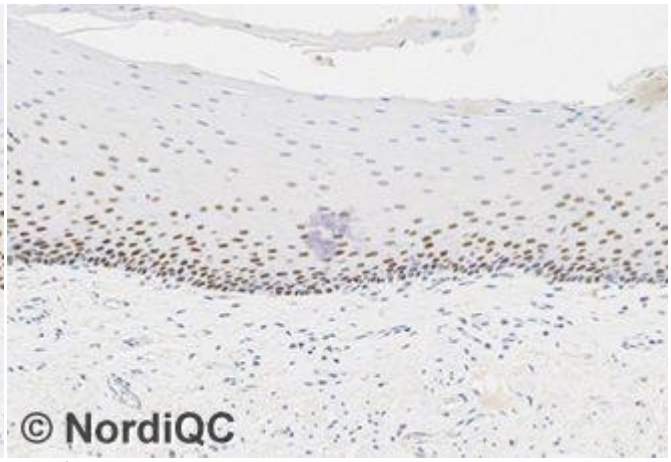


Fig. 1b
Insufficient p63 staining of the esophagus using the mAb clone 7JUL (Leica/Novocastra, 1:100) with HIER in an alkaline buffer (BERS2, Bond) and performed on the Bond III, Leica. A moderate nuclear staining reaction is seen in the majority of the squamous epithelial cells in the esophagus. Compare with Fig. 1a – same field. Also compare with Figs. 2b, 3b and 4b – same protocol.

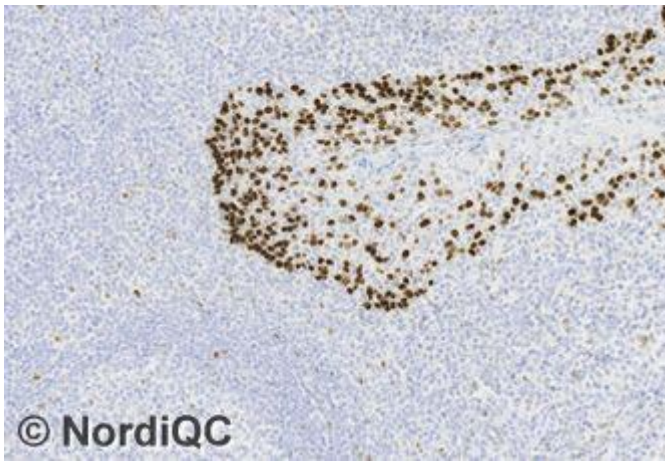


Fig. 2a
Optimal p63 staining of the tonsil using the same protocol as in Fig. 1a. A moderate to strong, distinct nuclear staining is seen in virtually all the squamous epithelial cells in the tonsil. In addition to the epithelial staining a weak but distinct nuclear reaction is present in scattered lymphocytes in the tonsil.

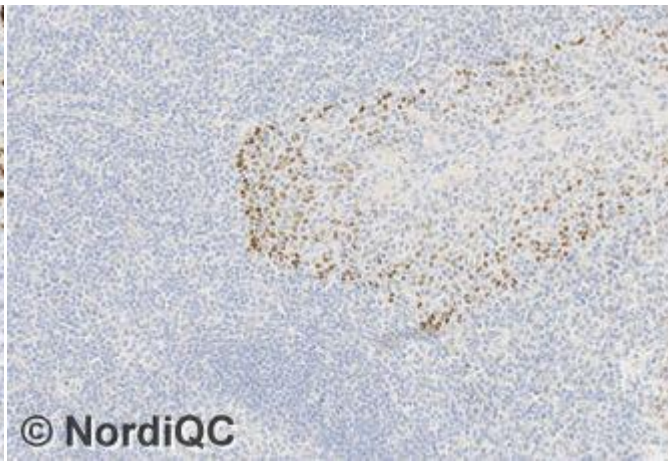


Fig. 2b
Insufficient p63 staining of the tonsil using the same protocol as in Fig. 1b. A weak to moderate, distinct nuclear staining is seen in the majority of the squamous epithelial cells in the tonsil. But in the insufficient protocol no staining is seen in lymphocytes. Compare with Fig. 2a. - same field.

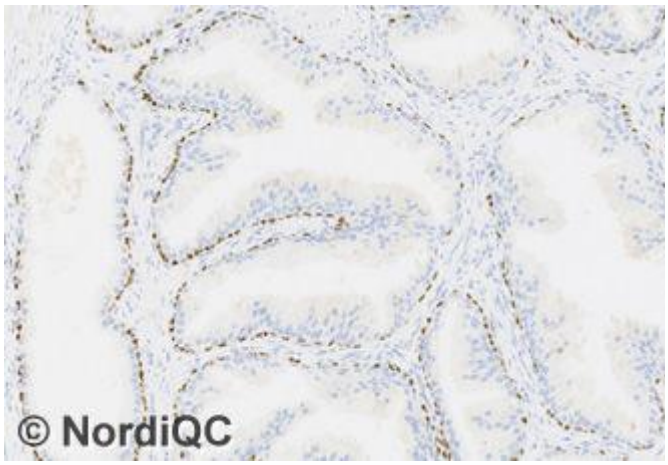


Fig. 3a
Optimal p63 staining in the prostate hyperplasia using the same protocol as in Figs. 1a & 2a. Virtually all the basal cells show a moderate to strong distinct nuclear staining reaction. No background staining is seen.

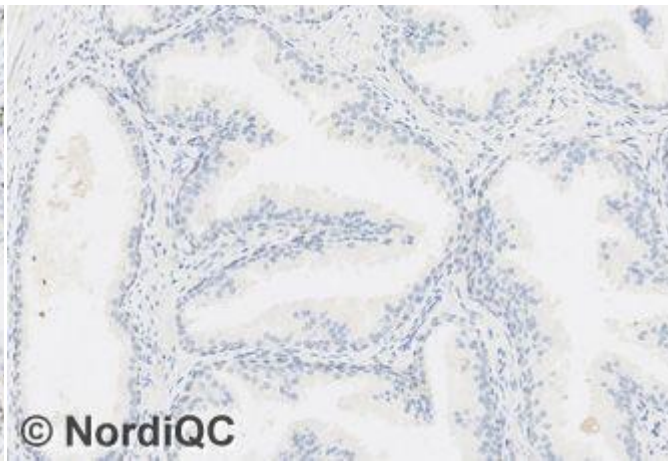


Fig. 3b
Insufficient p63 staining in the prostate hyperplasia using the same protocol as in Figs. 1b & 2b. Virtually all basal cells in the prostate hyperplasia are negative. Compare with Fig. 3a – same field.

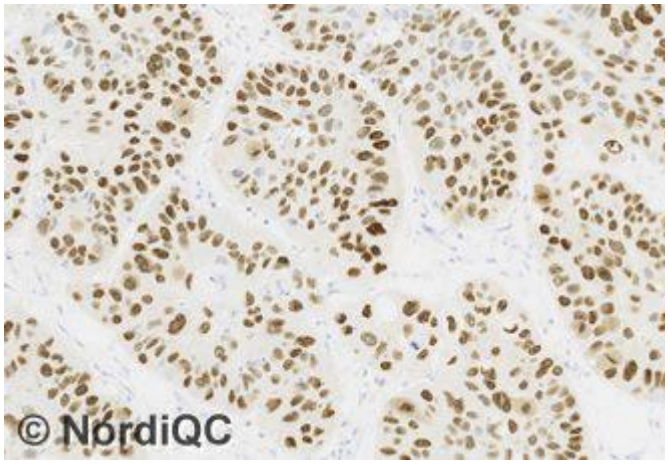


Fig. 4a
Optimal p63 staining of the lung squamous cell carcinoma using the same protocol as in Figs. 1a, 2a & 3a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen.

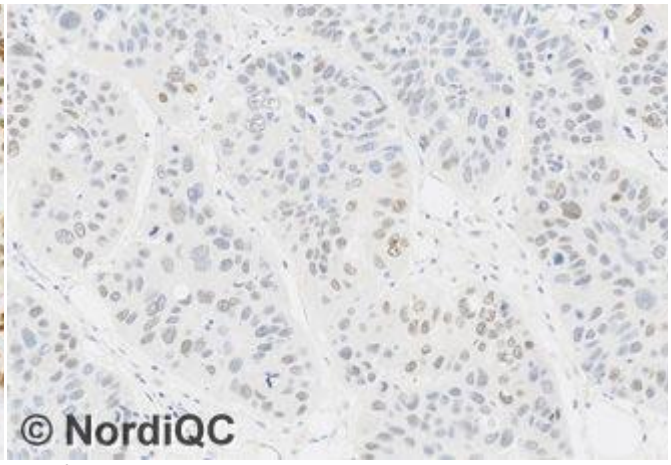


Fig. 4b
Insufficient P63 staining of the lung squamous cell carcinoma using the same protocol as in Figs. 1b, 2b & 3b. Only faint nuclear staining is seen and only in a minor fraction of the neoplastic cells. Compare with Fig. 4a – same field.

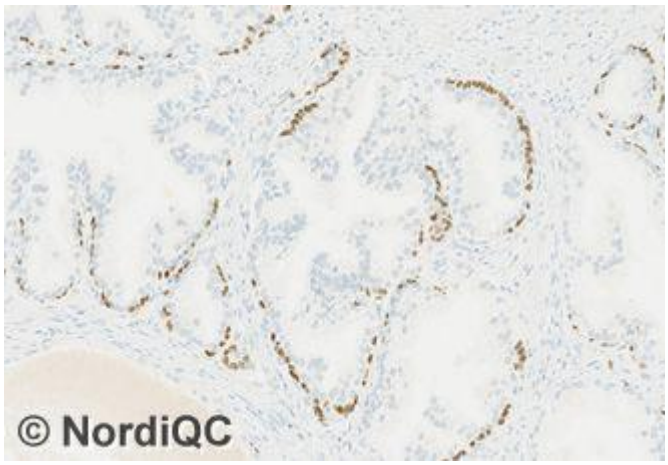


Fig. 5a
Optimal p63 staining of the prostate hyperplasia using the mAb clone 4A4 (Ventana, RTU) with HIER in CC1 (Ventana) for 64 min. Moderate to strong nuclear reaction is seen in virtually all basal cells. Efficient HIER pretreatment is essential to optimal P63 staining. Compare with Fig. 5b.

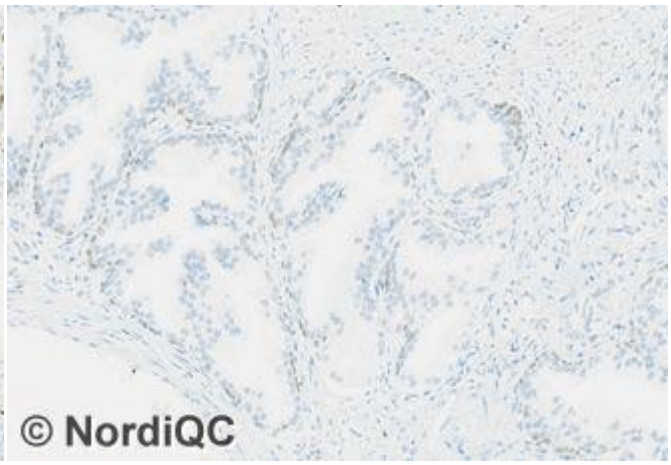


Fig. 5b
Insufficient p63 staining in the prostate hyperplasia using the mAb clone 4A4 (Ventana, RTU) in the same protocol as in Fig. 5a, except for the reduction in HIER pretreatment to 24 min compared to the 64 min i Fig 5a. Consequenctly a dramatic reduction in staining intensity is seen making the identification of the basal cell difficult. Compare with Fig. 5a – same field.

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