Assessment Run 67 2023
p40 (ΔNp63)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of the IHC assays for p40 performed by the NordiQC participants for the differentiation between lung squamous cell carcinoma and lung adenocarcinoma.
Relevant clinical tissues, both normal and neoplastic, were selected to include a wide spectrum of p40 antigen densities (see below).

Material
The slide to be stained for p40 comprised:

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

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.
- 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 virtually all neoplastic cells in the lung squamous cell carcinoma, tissue core no. 5.
- An at least weak to moderate staining reaction in 70-100%* of the neoplastic cells in the lung squamous cell carcinoma, tissue core no. 4.
- No staining reaction of the neoplastic cells in the lung adenocarcinoma.
- No staining reaction of other cells including lymphocytes in the tonsil.

**In some slides, a significant smaller proportion of neoplastic cells were positive. The participant slides were always compared to the nearest reference slide.*

Participation

Number of laboratories registered for p40, run 67	374
Number of laboratories returning slides	344 (92%)

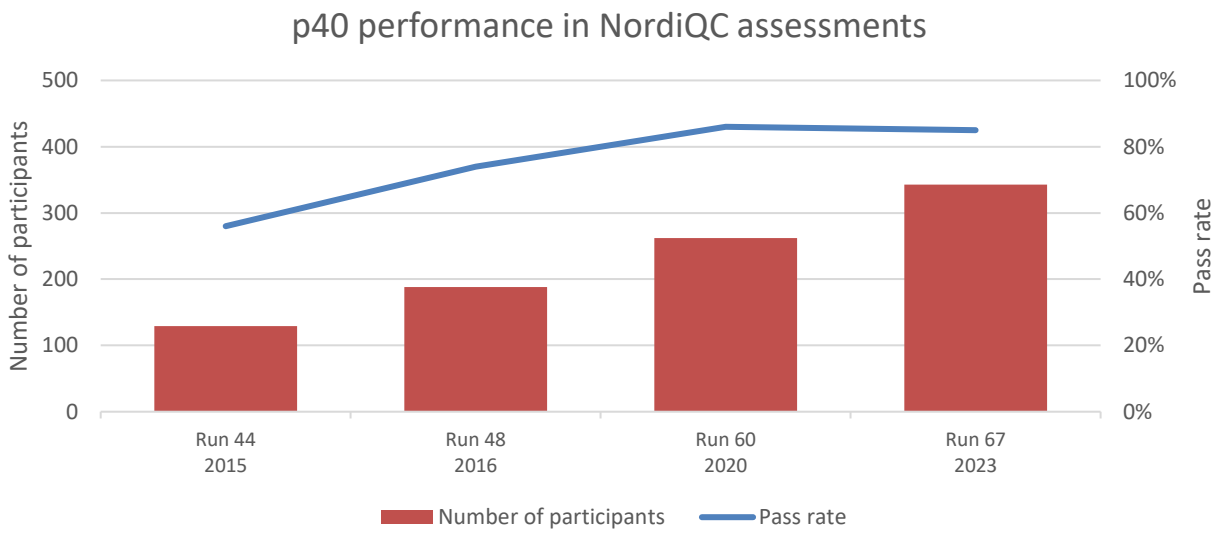
All slides returned after the assessment were assessed and received advice if the result being insufficient, but the data were not included in this report.

Results
344 laboratories participated in this assessment. 1 participant used an inappropriate antibody. The participant was not included in the analysis. Of the remaining 343 laboratories, 85% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining were:
- Inefficient HIER
- Too short incubation time of primary Ab
- Too low concentration of the primary antibody
- Less successful primary Ab
- Use of less sensitive detection systems

Performance history
This was the fourth NordiQC assessment of p40. A similar pass rate was observed in this run 67 compared to the last run 60 (see Graph 1).

Graph 1. **Proportion of sufficient results for p40 in four NordiQC runs**



Conclusion
Optimal staining results for p40 could be obtained with the mAb clone **BC28** and rmAb clones **DAK-p40** and **ZR8.** mAb clone **BC28** was the most commonly used p40 antibody, giving pass rates of 94% and 83% for the concentrated and RTU formats, respectively. The RTUs from Leica Biosystem based on mAb clone BC28 and from Dako/Agilent based on rmAb clone DAK-p40 were the most successful antibodies, giving pass rates of 100% both when using the vendor recommended protocol settings or modifying the protocols. The concentrated format of mAb clone **BC28** provided optimal staining results on the main platforms from Dako/Agilent, Leica Biosystems and Ventana/Roche. Irrespective of the clone applied, efficient Heat Induced Epitope retrieval (HIER) in an alkaline buffer and use of a sensitive and specific 3-step polymer / multimer based detection system gave the highest proportion of optimal results. The concentration of the primary antibody must be carefully calibrated. As seen in last assessment run 48 and 60, polyclonal Abs were less successful and should be avoided.

Controls
Placenta is recommended as primary critical 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 a low magnification (5x objective).
Supportive to placenta, tonsil can be used as positive and negative tissue control to guide analytical specificity. 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.

Table 1. **Antibodies and assessment marks for p40, run 67**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **BC28**	80 11 3 2	Biocare Medical Zytomed abcam Gennova	73	17	6	0	94%	76%
mAb clone **I.I**	1	DCS	0	0	1	0	-	-
mAb clone **IHC058**	1	GenomeMe	0	1	0	0	-	-
rmAb clone **ZR8**	4 4 2	Zeta Corporation Immunologic BioSB	4	4	2	0	80%	40%
rmAb clone **ZR303**	1	Zeta Corporation	1	0	0	0	-	-
pAb **AC13030**	2	Biocare Medical	1	0	1	0	-	-
pAb **RP163**	3	Diagnostic Biosystems	0	0	1	2	-	-
pAb **RBK054-05**	1	Zytomed	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **BC28** **API/AVI/VLTM 3066**	12	Biocare Medical	10	1	1	0	92%	83%
mAb clone **BC28** **790-4950 (VRPS)[3]**	33	Ventana/Roche	2	15	15	1	52%	6%
mAb clone **BC28** **790-4950 (LMPS)[4]**	112	Ventana/Roche	69	31	12	0	89%	62%
mAb clone **BC28** **MSG097/BMS050**	6	Zytomed	4	1	1	0	83%	67%
mAb clone **BC28** **PA0163 (VRPS)[3]**	8	Leica Biosystems	8	0	0	0	100%	100%
mAb clone **BC28** **PA0163 (LMPS)[4]**	6	Leica Biosystems	4	2	0	0	100%	67%
mAb clone **BC28** **8341-C010**	2	Sakura Finetek	2	0	0	0	-	-
mAb clone **C3B4** **CPM-0133**	1	Celnovte	1	0	0	0	-	-
mAb clone **ABT-p40** **01.09.70.16.08.02**	1	Zybio	0	0	1	0	-	-
Ab clone **513M2A7** **PA560**	1	abcarta	0	1	0	0	-	-
rmAb clone **BY004** **BFM-0062**	1	Bioin Biotechnology	1	0	0	0	-	-
rmAb clone **GR006** **GT233802**	1	Gene Tech	1	0	0	0	-	-
rmAb clone **DGR010** **DGR010**	1	Shanghai DG Diagnology Tec	0	1	0	0	-	-
rmAb clone **DAK-p40** **GA784 (VRPS)[3]**	16	Dako/Agilent	13	3	0	0	100%	81%
rmAb clone **DAK-p40** **GA784 (LMPS)[4]**	13	Dako/Agilent	9	4	0	0	100%	69%
rmAb clone **MXR010** **RMA-1006**	1	Fuzhou Maixin	1	0	0	0	-	-
rmAb clone **ZR8** **MAD-000686QD**	5	Master Diagnostica	0	0	5	0	0%	0%
rmAb clone **ZR8** **LS-C31213**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **ZR8** **Z2004RP**	1	Zeta Corporation	0	1	0	0	-	-
rmAb clone **ZR8** **BSB 2072**	1	BioSB	0	1	0	0	-	-
rmAb clone **ZR8** **483R-10/17/18**	1	Cell Marque	0	0	1	0	-	-
rmAb clone **TP40/3980R**	1	BioGenex	0	0	1	0	-	-

ANA43								
pAb **A00112-0007**	1	ScyTek Laboratories, Inc.	0	1	0	0	-	-
pAb **API 3030**	2	Biocare Medical	0	1	1	0	-	-
Total	343		206	85	50	3		
Proportion			60%	25%	15%	1%	85%	

1) Proportion of sufficient stains (optimal or good) (≥5 asessed protocols).
2) Proportion of Optimal Results (≥5 asessed protocols).
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 (≥5 assessed protocols).

Detailed analysis of p40, Run 67
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/Roche) (33/42)*, Target Retrieval Solution (TRS) High pH (Dako/Agilent) (27/38), TRS Low pH (Dako/Agilent) (2/2) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (11/12). 72 of 73 optimal protocols applied a 3-layer detection system. The mAb was typically diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 88 of 94 (94%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **ZR8**: Protocols with optimal results were typically based on HIER using BERS2 (Leica Biosystems) (2/2) or CC1 (Ventana/Roche) (2/4). The rmAb was typically diluted in the range of 1:50-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 6 of 8 (75%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for the two most commonly applied p40 antibodies as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT/Ultra**		**Leica Biosystems 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**	6/10** (60%)	-	23/30 (77%)	-	33/42 (79%)	-	11/12 (92%)	-
rmAb clone **ZR8**	0/2	-	-	-	2/4	-	2/3	-

* 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/Roche, BenchMark GX / XT / ULTRA:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min.), 16-48 min. incubation of the primary Ab and UltraView (760-500), UltraView (760-500) with amplification (760-080), OptiView (760-700) or OptiView (760-700) with OptiView Amplification Kit (760-099 / 860-099) as detection system. Using these protocol settings 110 of 136 (81%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **BC28**, product no. **PA0163**, Leica Biosystems, Bond III:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 20-30 min.), 15-30 min. incubation of the primary Ab and Bond Refine (DS9800) as detection system. Using these protocol settings 12 of 12 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **DAK-p40**, product no. **GA784**, Dako/Agilent, Omnis:
Protocols with optimal results were based on HIER using TRS High pH 9 (efficient heating time 20-30 min. at 97°C), 10-30 min. incubation of the primary Ab and EnVision FLEX or EnVision FLEX+ (GV800 + GV809) as the detection system. Using these protocol settings, 27 of 27 (100%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 assessed protocols). The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems

changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included (in Table 1 LMPS also includes off label use on deviant IHC stainers).

Table 3. **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**	17/33 (52%)	2/33 (6%)	100/112 (89%)	69/112 (62%)
Leica BOND III mAb **BC28 PA0163**	8/8 (100%)	8/8 (100%)	6/6 (100%)	4/6 (67%)
Dako Omnis rmAb **DAK-p40 GA784**	16/16 (100%)	13/16 (81%)	10/10 (100%)	8/10 (80%)

* Protocol settings recommended by vendor – retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer were included.

Comments

In concordance with the previous NordiQC assessments for p40, 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 81% of the insufficient results (43 of 53 laboratories). The remaining insufficient results were typically characterized by a general poor signal-to-noise ratio or false positive nuclear staining reactions.
Too weak staining result 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 and a significantly reduced intensity and/or proportion of positive neoplastic cells of the lung squamous cell carcinoma, tissue core no. 4. 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.

34% (115 of 343) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for p40 with a total pass rate of 88% (101 of 115), 69% optimal.
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 2. Efficient HIER in an alkaline buffer in combination with a 3-step polymer/multimer based detection system provided the highest proportion of optimal results. In particular, the choice of detection system influenced the performance for the mAb clone BC28 within LD assays for p40. 88% of protocols (72 of 82) based on a 3-layer detection system provided an optimal result being in clear contrast and superior to the use of 2-layer detection systems only giving an optimal result in 7% (1 of 14).

Sufficient and optimal results could also be achieved with the rmAb clone ZR8, but the data from both the prevous and current assessment suggests that this antibody can be difficult to optimize. In this assessment 8 of 10 (80%) laboratories achieved sufficient results, but only 40% were assessed as optimal.

3 different polyclonal Abs (pAb) were used as concentrates within LD assays (6 protocols in total). Despite protocol settings, as retrieval conditions, detection systems and IHC stainer platforms were identical to the mAb clone BC28 and rmAb clone ZR8, only one sufficient result was provided. The insufficient results were typically characterized by a false negative staining reaction, a poor signal-to-noise ratio and/or false positive staining reaction. This observation was concordant to data generated in runs 48 and 60.

Ready-To-Use (RTU) antibodies were used by 66% (228 of 343) of the laboratories. The most frequently used RTU systems for p40 were the Ventana/Roche 790-4950 system for BenchMark and the newly launched GA784 Dako/Agilent for Omnis.

The mAb clone BC28 (790-4950) was successfully applied as RTU system from Ventana/Roche. Following the vendor protocol recommendations, a relatively low pass rate of 52% was obtained. A significant higher pass-rate of 89% was seen for the participants applying laboratory modified RTU protocols (see Table 3). The most common and successful modification was a prolonged incubation time of the primary Ab to 24-40 min. (recommended 16 min.). The majority of laboratories using OptiView as detection system also

successfully prolonged the HIER time to 48-64 min. (recommended 32 min.). These "positive" modifications of the official RTU protocol, resulted in a noticeable increase in optimal results, as 62% of the laboratories achieved optimal results compared to 6% of the laboratories using the official RTU protocol (see Table 3).

The newly launched GA784 based on rmAb clone DAK-p40 from Dako/Agilent obtained a 100% pass rate both when following the vendor protocol recommendations, and when modifying the protocol, with similar proportion of optimal results (see Table 3). The modifications were related to minor adjustments in incubation time in HIER and/or primary Ab.

The Leica Biosystems RTU system PA0163 based on mAb clone BC28 was used by 14 participants, and all produced a sufficient result, 86% optimal. Laboratories applying the vendor recommended protocol settings, using HIER in BERS2 for 20 min., 15 min. incubation of the primary Ab and Refine as detection system all obtained optimal results (see Table 3).

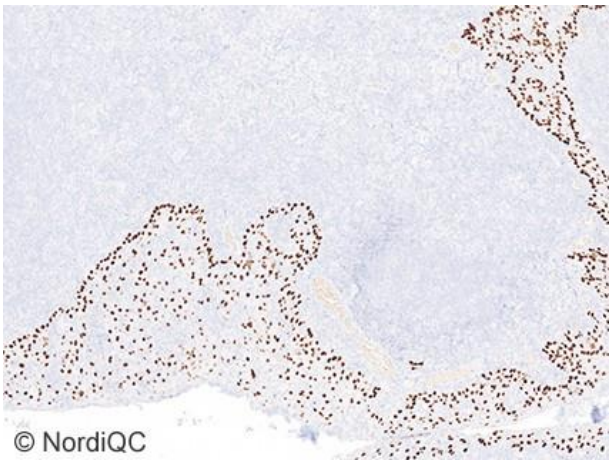


Fig. 1a
Optimal p40 staining of the tonsil using the Ventana/Roche RTU format 790-4950 based on mAb clone BC28 with 32 min. incubation time, HIER in an alkaline buffer (CC1 pH 8.5, 64 min.), and a 3-step polymer-based detection system (OptiView, Ventana). A moderate to strong nuclear staining reaction is seen in virtually all the squamous epithelial cells. No background staining is seen.
Same protocol used in Figs. 2a - 5a.

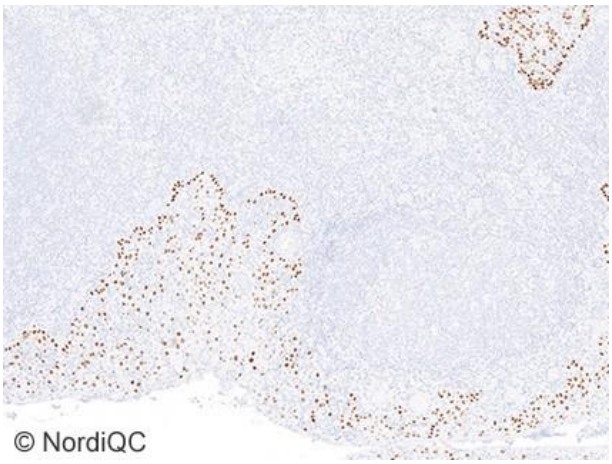


Fig. 1b
Insufficient p40 staining of the tonsil using the Ventana/Roche RTU format 790-4950 based on mAb clone BC28 with the vendor recommended protocol settings with 16 min. incubation time, HIER in CC1 for 32 min., and OptiView as detection system.
Compare with Fig. 1a (same field). The intensity and proportion of cells demonstrated is reduced.
Also compare with Figs. 2b - 4b, same protocol.

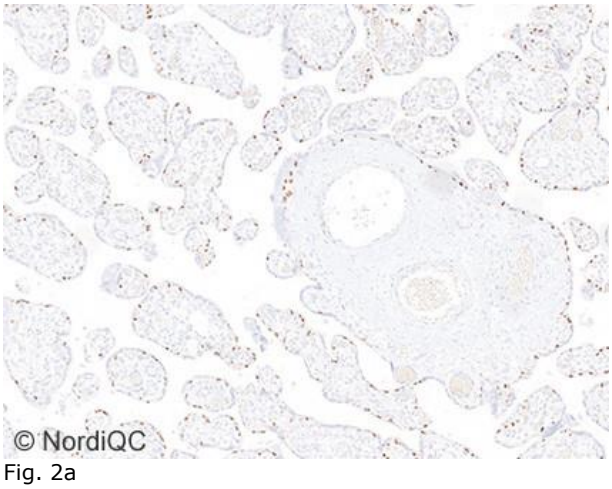


Fig. 2a
Optimal p40 staining of the placenta using same protocol as in Fig. 1a. Scattered cytothrophoblastic cells show a weak to moderate, distinct nuclear staining reaction. Can easily be identified at low magnification (5x)

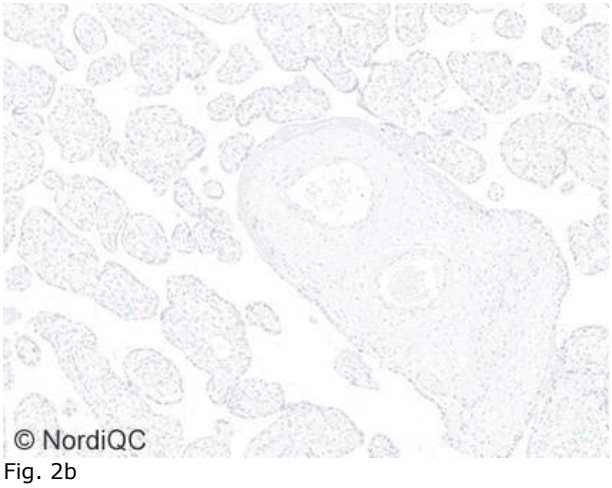


Fig. 2b
Insufficient p40 staining of the placenta using same protocol as in Fig. 1b. Virtually no nuclear staining reaction of cytothrophoblastsic cells is seen. Compare with Fig. 2a (same field).
Also compare with Figs. 3b and 4b, same protocol.

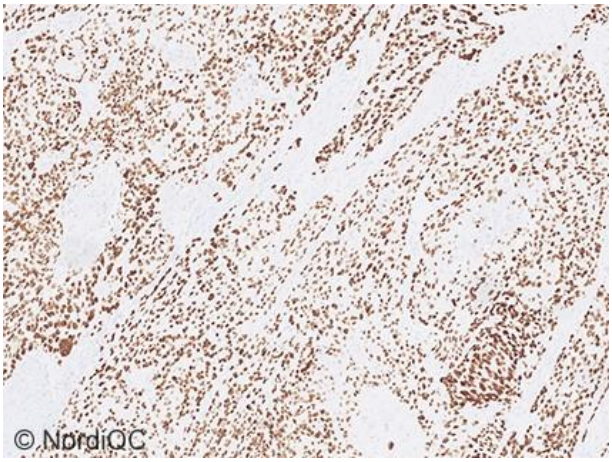


Fig. 3a
Optimal p40 staining of the lung squamous cell carcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction.
No background staining is seen.

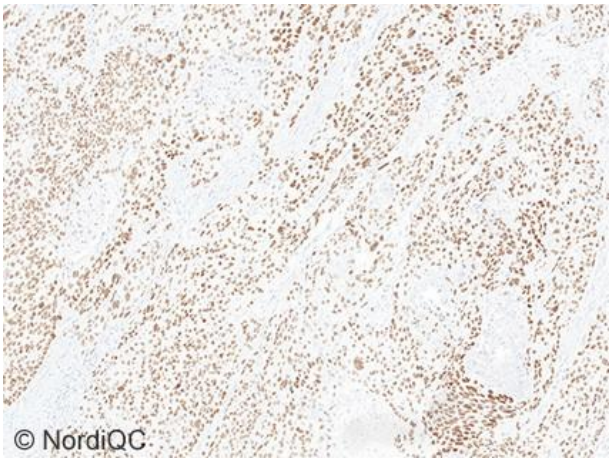


Fig. 3b
p40 staining of the lung squamous cell carcinoma, tissue core no. 5, using same protocol as in Figs. 1b and 2b. The neoplastic cells are demonstrated, though the intensity is reduced compared to the level expected and shown in Fig. 3a (same field). However also compare with Fig. 4b, same protocol.

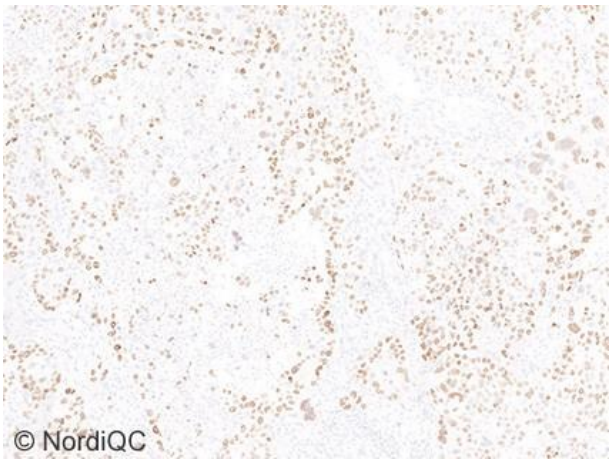


Fig. 4a
Optimal p40 staining of the lung squamous cell carcinoma, tissue core no. 4, using same protocol as in Figs. 1a - 3a.
The majority of neoplastic cells show a weak to moderate nuclear staining reaction.

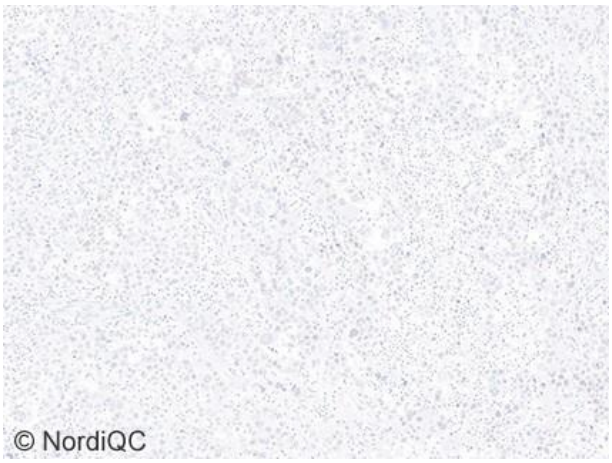


Fig. 4b
Insufficient p40 staining of the lung squamous cell carcinoma, tissue core no. 4, using same protocol as in Figs. 1b – 3b.
Only scattered neoplastic cells shows a faint nuclear staining reaction. Compare with Fig. 4a (same field).

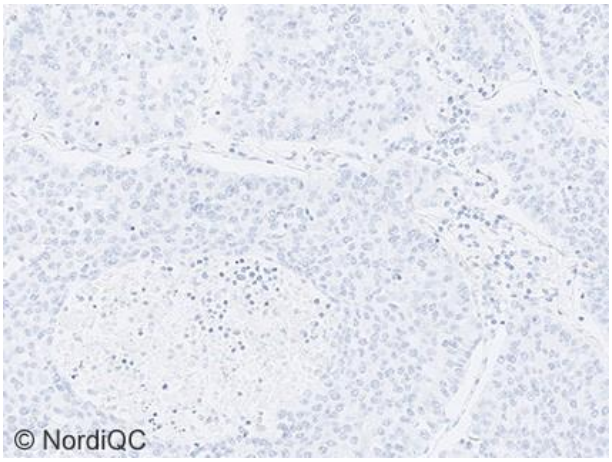


Fig. 5a
Optimal staining of the lung adenocarcinoma, using same protocol as in Figs. 1a-4a. No staining is observed.

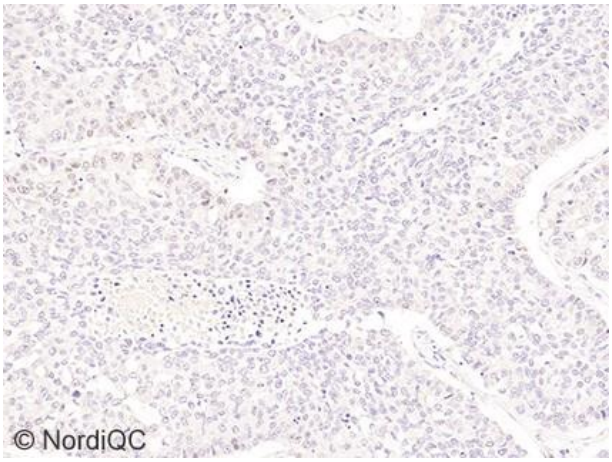


Fig. 5b
Insufficient staining of the lung adenocarcinoma, using a polyclonal Ab, with HIER in an alkaline buffer and a 3-step polymer-based system. Dispersed neoplastic cells show a weak nuclear staining reaction. Compare with Fig. 5a (same field).

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