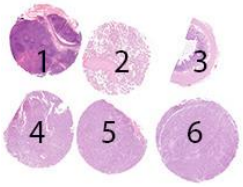
Assessment Run 60 2020
p40 (ΔNp63)

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, 3. Placenta, 3. Appendix, 4. Lung adenocarcinoma, 5-6. 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. 6.
- No staining reaction of the neoplastic cells in the lung adenocarcinoma.
- No staining reaction of other cells including lymphocytes in the tonsil and appendix.

**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 60	297
Number of laboratories returning slides	267 (90%)

Results
The number of laboratories returning slides has decreased in this run 60 compared to previous assessments, due to the COVID-19 pandemic. All slides returned after the assessment were assessed and received advice if the result being insufficient but were not included in this report.
During the assessment a limited number of participants have experienced issues with the circulated NordiQC slides, providing a partial or entire aberrant/false negative staining result in some cases. During the assessment, this observation was taken into account and for p40, 24 slides were potentially affected. 19 were assessed and five excluded. If performance was characterized by uneven staining or a completely false negative result that could be related to the quality of the slide and not the protocol submitted, this was commented in the individual assessment feed-back.
262 laboratories participated in this assessment. 86% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

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

Performance history
This was the third NordiQC assessment of p40. A significantly increased pass rate was observed (see Table 2).

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

	Run 44 2015	Run 48 2016	Run 60 2020
Participants, n=	129	188	262
Sufficient results	56%	74%	86%

The increased pass rate in this run may be explained by an extended use of the highly sensitive and robust mAb clone BC28 both as concentrate and as Ready-To- Use (RTU) format. Additionally, the proportion of laboratories using less successful polyclonal antibodies has been reduced from 41% in run 44 and 16% in run 48 to 7% in this current run 60.

Conclusion

Optimal staining results for p40 could be obtained with the mAb clones **BC28** and **ZR8.** mAb clone **BC28** was the most commonly used and successful p40 antibody, giving pass rates of 93% and 85% for the concentrated and RTU formats, repectively. The concentrated formats of mAb clone **BC28** provided optimal staining results on the main platforms from Dako, Leica and Ventana. 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. All polyclonal antibodies applied in this assessment gave less successful results and should be avoided.

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

Table 1. **Antibodies and assessment marks for p40, run 60**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **BC28**	91 9 1 6 4 1 3	Biocare Medical Zytomed Menarini abcam Nordic Biosite Diagomics Gennova	72	35	7	1	93%	63%
rmAb clone **ZR8**	3 1 1	Immunologic Zeta Corporation BioSB	1	3	1	-	80%	20%
Ab **GR006**	1	Gene Tech	-	1	-	-	-	-
pAb **AC13030**	2	Biocare Medical	-	-	1	1	-	-
pAb **RP163**	4	Diagnostic Biosystems	-	-	-	4	-	-
pAb **PA5-28477**	1	ThermoFischer Scientific	-	-	1	-	-	-
Ready-To-Use antibodies								
mAb clone **BC28 API/AVI/VLTM 3066 (VRPS)[3]**	1	Biocare Medical	1	-	-	-	-	-
mAb clone **BC28 API/AVI/VLTM 3066 (LMPS)[4]**	17	Biocare Medical	14	1	1	1	88%	82%
mAb clone **BC28 790-4950 (VRPS)[3]**	27	Ventana	4	17	6	-	78%	15%
mAb clone **BC28 790-4950 (LMPS)[4]**	77	Ventana	40	26	11	-	86%	52%
mAb clone **BC28 AIB-VLJKAX**	2	Nordic Biosite	2	-	-	-	-	-
mAb clone **BC28 MSG097/BMS050**	3	Zytomed	3	-	-	-	-	-
mAb clone **BC28 PA0163**	1	Leica Biosystems	1	-	-	-	-	-
Ab clone **BP6033 I10172**	1	Tuling Biotechnology	1	-	-	-	-	-
rmAb clone **ZR8 MAD-000686QD**	1	Master Diagnostica	-	1	-	-	-	-
rmAb clone **ZR8 SBS 2072**	1	BioSB	-	-	1	-	-	-
pAb **A00112-0007**	1	ScyTek Laboratories, Inc.	-	1	-	-	-	-
pAb **API 3030**	2	Biocare Medical	-	2	-	-	-	-

Total	262		139	87	29	7		
Proportion			53%	33%	11%	3%	86%	

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 60
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) (33/43)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (7/14), TRS pH 9 (Dako/Agilent) (19/33) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (13/17). 67 of 72 optimal protocols applied a 3-layer detection system. The mAb was diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 96 of 100 (96%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **ZR8**: One protocol with an optimal result was based on 20 min. HIER using BERS2 (Leica) (1/3), 30 min. incubation of the primary Ab, a titre of 1:80 and Bond Refine (Leica) (DS9800) as detection system.

Table 3. **Proportion of optimal results for p40 for the BC28 antibody as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer / Autostainer Link 48+**		**Dako Omnis**		**Ventana BenchMark GX / XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **BC28**	7/14** (50%)	-	19/31 (61%)	0/1	33/39 (85%)	-	13/16 (81%)	0/3

* 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, BenchMark GX / XT / ULTRA:
Protocols with optimal results were typically based on 32-64 min. HIER using CC1, 16-32 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 amplification (760-099 / 860-099) as detection system. Using these protocol settings 70 of 80 (83%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **BC28**, product no. **AVI 3066 KG**, Biocare Medical, BenchMark ULTRA:
Protocols with optimal results were based on 32-36 min. HIER using CC1, 32 min. incubation of the primary Ab and UltraView with amplification (760-500 / 760-080) or OptiView (760-700) as detection system. Using these protocol settings 2 of 2 laboratories produced a sufficient staining result.

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

mAb clone **BC28**, product no. **PA0163**, Leica Biosystems, Bond III:
One protocol with an optimal result was based on 30 min. HIER using BERS2, 15 min. incubation of the primary Ab and Bond Refine (DS9800) as detection system.

Table 4 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 4. **Proportion of sufficient and optimal results for p40 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT mAb BC28 **790-4950**	21/27 (78%)	4/27 (15%)	62/73 (85%)	38/73 (52%)

* 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 89% of the insufficient results (32 of 36 laboratories). The remaining insufficient results were characterized by a general poor signal-to-noise ratio or false positive nuclear staining reaction in lymphocytes in tonsil and appendix.
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. 6. Virtually all laboratories successfully demonstrated p40 in the majority of neoplastic cells of the lung squamous cell carcinoma, tissue core no. 5, with high expression level of p40. Too weak staining reaction was most frequently caused by too low titre of an otherwise well performing primary antibody as mAb clone BC28 often in the combination with the use of 2-step polymer/multimer systems.

49% (128 of 262) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for p40 with a total pass rate of 88% (112 of 128), 57% 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 3. Efficient HIER in an alkaline buffer in combination with a 3-step polymer/multimer based detection system provided the highest proportion of optimal results. It was thus observed that only protocols using HIER in an alkaline buffer obtained an optimal result. The choice of detection system also influenced the performance. 78% of protocols (67 of 86) based on a 3-layer detection system provided an optimal result being in clear contrast and superior the use of 2-layer detection systems only giving an optimal result in 17% (5 of 29).

Sufficient and optimal results could also be achieved with the rmAb clone ZR8, but the data from both the latest and current assessment suggests that this antibody can be difficult to optimize. In this assessment 4 of 5 (80%) laboratories achieved sufficient results, but only one was assessed as optimal (20%).

3 different polyclonal Abs (pAb) were used as concentrates within LD assays (7 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, no sufficient results were provided. The insufficient results were typically characterized by a false negative staining reaction and a poor signal-to-noise ratio. This observation was concordant to data generated in run 48, in which the overall pass rate for laboratories using a pAb within a LD assay was only 23% (5 of 22), none optimal.

Ready-To-Use (RTU) antibodies was used by 51% (134 of 262) of the laboratories. The RTU formats basically provided the same results as seen for the concentrated formats.
Optimal results were obtained by the RTU system from Biocare Medical based on the mAb clone BC28 (API/AVI/VLTM 3066). Both protocols used as recommended on the intended IHC stainer, modified protocols and protocols used on a different IHC stainer gave optimal and sufficient results, with a total pass-rate of 89% (16 of 18).

The mAb clone BC28 (790-4950) was also successfully applied as RTU system from Ventana. Following the vendor protocol recommendations, the pass rate was 78%. A similar pass-rate was seen for the laboratory modified RTU protocols (85%) (see Table 4). The most common and successful modification was a prolonged incubation time of the primary Ab to 24-32 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 52% of the laboratories (38 out of 73) achieved optimal results compared to 15% of the laboratories using the official RTU protocol (see Table 4).

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. Virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction. No nuclear or cytoplasmic staining reaction should be seen in other cell types.

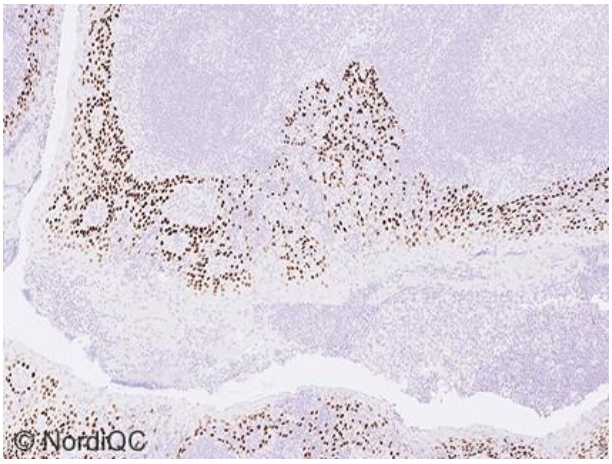


Fig. 1a
Optimal p40 staining of the tonsil using the Ventana RTU format 790-4950 based on mAb clone BC28 with 32 min. incubation time, HIER in an alkaline buffer (CC1 pH 8.5, 64 min. Ventana), and a 3-step polymer-based detection system (OptiView, Ventana). A moderate to strong nuclear staining reaction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

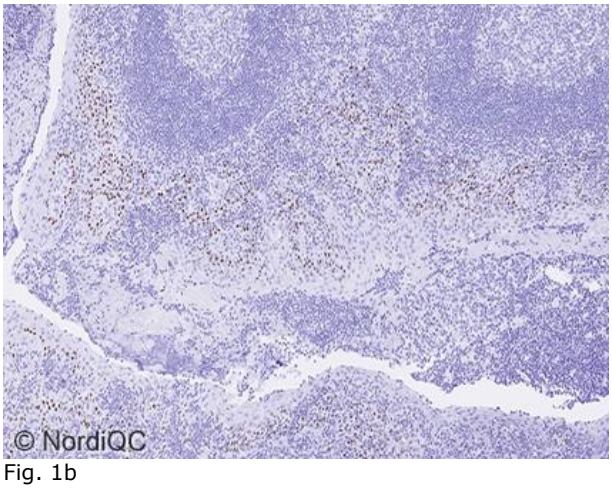


Fig. 1b
Insufficient p40 staining of the tonsil using a concentrated polyclonal Ab diluted 1:50 with 15 min. incubation time, HIER in an alkaline buffer (TRS 3-in-1, pH 9.0, Dako), a 2-step polymer-based detection system (EnVision FLEX, Dako) and a relative strong - counter staining compromising the identification of the p40 reaction.
The protocol provided an overall too low analytical sensitivity. Compare with Fig. 1a (same field). The intensity and proportion of cells demonstrated is reduced.
Also compare with Figs. 2b - 4b, same protocol.

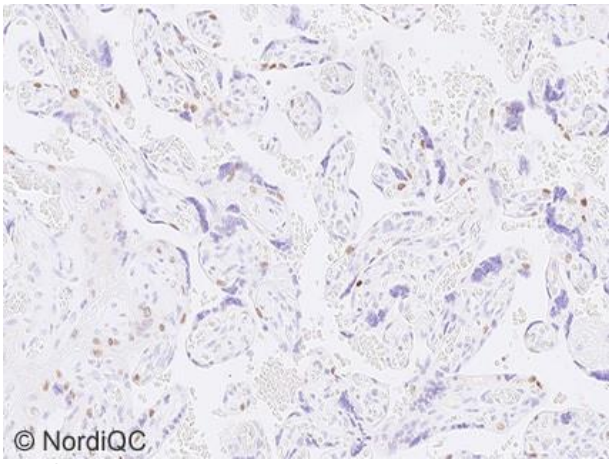


Fig. 2a
Optimal p40 staining of the placenta using same protocol as in Fig. 1a. Scattered cytothrophoblastic cells show a weak to moderate, distinct nuclear staining reaction.

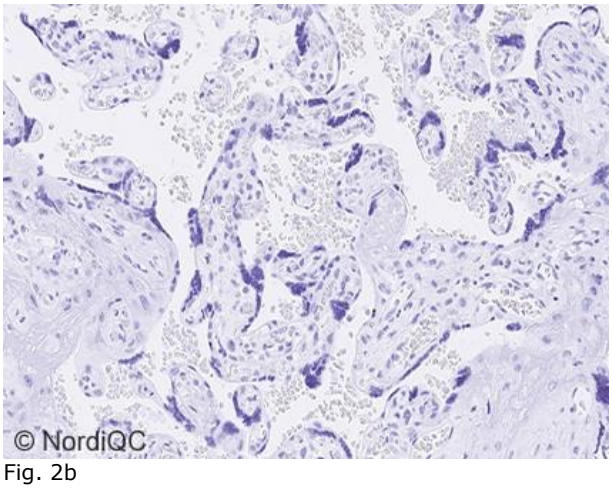


Fig. 2b
Insufficient p40 staining of the placenta using same protocol as in Fig. 1b. Virtually no nuclear staining reaction of cytothrophoblastsic cells is seen. Compare with Fig. 2a (same field).
Also compare with Figs. 3b and 4b, same protocol.

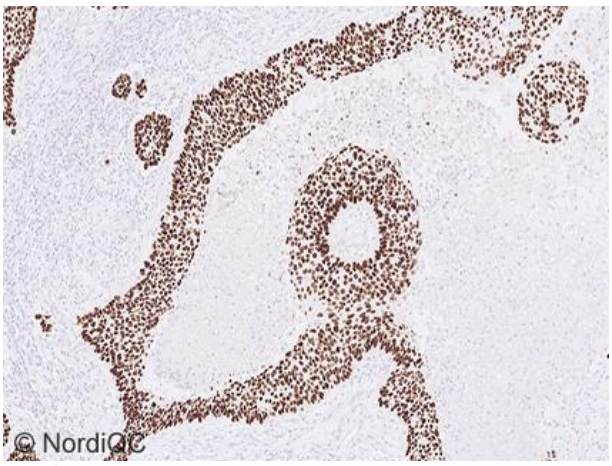


Fig. 3a
Optimal p40 staining of the lung squamous cell carcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction.
No background staining is seen.

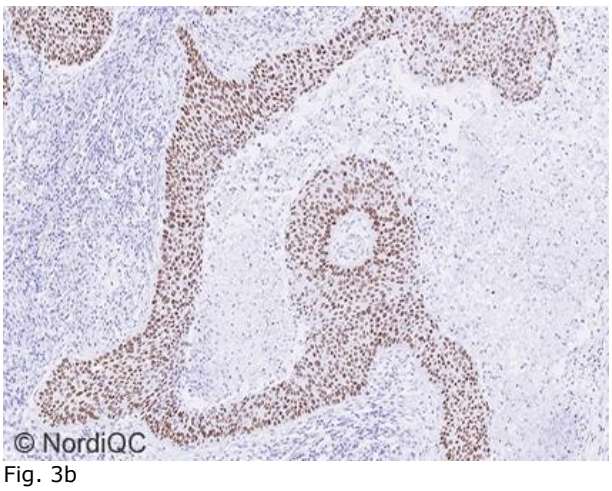


Fig. 3b
p40 staining of the lung squamous cell carcinoma, tissue core no. 5, using same protocol as in Figs. 1b and 2b. The neoplastic cells are demonstrated, though the intensity is reduced compared to the level expected and shown in Fig. 3a (same field). However also compare with Fig. 4b, same protocol.

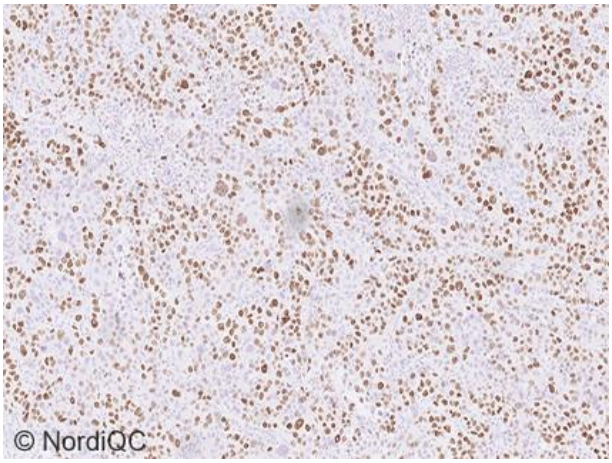


Fig. 4a
Optimal p40 staining of the lung squamous cell carcinoma, tissue core no. 6, using same protocol as in Figs. 1a - 3a.
The majority of neoplastic cells show a weak to moderate nuclear staining reaction.

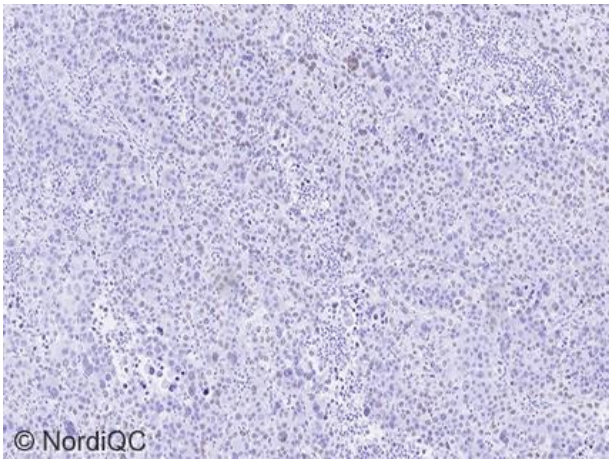


Fig. 4b (x200)
Insufficient p40 staining of the lung squamous cell carcinoma, tissue core no. 6, using same protocol as in Figs. 1b – 3b.
Only scattered neoplastic cells shows a faint nuclear staining reaction. Compare with Fig. 4a (same field).

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