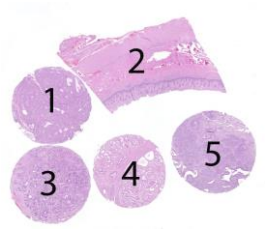
Assessment Run 46 2016
CK5

Material
The slide to be stained for CK5 comprised:



1: Lung squamous cell carcinoma 2: Esophagus 3: Lung adenocarcinoma
4: Prostate hyperplasia 5: Lung squamous cell carcinoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CK5 staining as optimal included:

- A moderate to strong and distinct cytoplasmic staining reaction of all squamous epithelial cells in esophagus throughout all the cell layers.
- A strong and distinct cytoplasmic staining reaction of the majority of basal cells in the hyperplastic prostate glands.
- A moderate to strong cytoplasmic staining reaction of virtually all neoplastic cells in the lung squamous cell carcinoma, tissue core no. 1.
- An at least weak to moderate cytoplasmic staining reaction of the majority of neoplastic cells in the lung squamous cell carcinoma, tissue core no. 5.
- No staining of neoplastic cells in the lung adenocarcinoma.

Participation

Number of laboratories registered for CK5, run 46	281
Number of laboratories returning slides	266 (95%)

Results
266 laboratories participated in this assessment. 181 (68%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful CK5 antibodies
- Too low concentration of the primary Ab
- Insufficient HIER - too short efficient HIER time

Performance history
This was the second NordiQC assessment of CK5. The pass rate in this run was improved compared to the previous run from 2004 as shown in table 2.

Table 2. **Proportion of sufficient results for CK5 in the two NordiQC runs performed**

	Run 12 2004	**Run 46 2016**
Participants, n=	74	266
Sufficient results	47%	68%

Conclusion
The mAb clone **XM26**, rmAb **EP1601Y** and rmAb clone **SP27** are all recommendable antibodies for the demonstration of CK5. The mAb clone D5/16 B4 was less successful.
Irrespective of the clone applied, efficient HIER, preferable in an alkaline buffer, and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result.
The Ventana Ready-To-Use system based on rmAb clone SP27, 760-4935 was most successful providing both a pass rate and proportion of optimal results of 92%.
Esophagus in combination with pancreas can be recommended as positive tissue controls for CK5.

Table 1. **Antibodies and assessment marks for CK5, run 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **10C11E6**	1	Immunologic	0	1	0	0	-	-
mAb clone **D5/16 B4**	74 1 6 3 1 2 1	Dako/Agilent Invitrogen Cell Marque Zytomed Thermo Scientific Biocare Immunologic	21	34	28	5	63%	66%
mAb **BS42**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **XM26**	49 2 1	Leica/Novocastra Zytomed Sanbio	25	15	11	1	77%	79%
mAb clone **XM26/LL002**	1	Zytomed	0	1	0	0	-	-
rmAb clone **EP1601Y**	8 1	Cell Marque Biocare	6	3	0	0	100%	100%
rmAb clone **SP27**	3	Immunologic	3	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **D5/16 B4 1072**	1	Monosan	0	1	0	0	-	-
mAb clone **D5/16 B4 BMS017**	1	Zytomed	0	1	0	0	-	-
mAb clone **D5/16 B4 IR/IS780**	36	Dako/Agilent	1	8	22	5	25%	67%
mAb **D5/16 B4 GA780**	11	Dako/Agilent	1	8	2	0	82%	82%
mAb clone **D5/16 B4 790-4554**	38	Ventana/Roche/Cell Marque	15	15	5	3	79%	85%
mAb clone **XM26 PA0468**	2	Leica/Novocastra	0	2	0	0	-	-
mAb clone **XM26 PM234**	1	Biocare	0	1	0	0	-	-
rmAb clone **EP1601Y/LL002 760-4939**	1	Ventana/Cell Marque	1	0	0	0	-	-
rmAb clone **EP1601Y/LL002 905H-8**	3	Cell Marque	1	1	1	0	-	-
rmAb clone **EP1601Y 305R-18**	2	Cell Marque	0	1	1	0	-	-
rmAb clone **EP24/EP67 MAD-000651QD**	2	Master Diagnostica	2	0	0	0	-	-
rmAb clone **SP27 760-4935**	12	Ventana /Cell Marque	11	0	1	0	92%	92%
rmAb clone **SP27 RMA-0612**	1	Maixin	0	0	1	0	-	-
Total	266		88	92	72	14	-	
Proportion			33%	35%	27%	5%	68%	

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

Detailed analysis of CK5, Run 46
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **D5/16 B4**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (2/3)*, Target Retrieval Solution (TRS) pH 9 (3-in-1)(Dako) (3/6), Cell Conditioning 1 (CC1, Ventana) (13/50), Tris-EDTA pH 9 (2/3) or Citrate pH 6 (1/2) as retrieval buffer. The

mAb was typically diluted in the range of 1:20-1:200. Using these protocol settings, 44 of 67 (66%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **XM26**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (2/5), Target Retrieval Solution (TRS) pH 9 (3-in-1)(Dako) (5/6), Cell Conditioning 1 (CC1, Ventana) (7/21), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (7/10), Tris-EDTA pH 9 (4/1) or Citrate pH 6 (1/2) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:200. Using these protocol settings, 34 of 43 (79%) laboratories produced a sufficient staining result.

mAb clone **BS42**: One protocol with an optimal result was based on HIER using Tris-EGTA/EDTA pH 9 (efficient heating time 20 min. at 98°C), 30 min. incubation of the primary Ab, a titre of 1:100 and BioSiteHisto Plus HRP Polymer anti-Mouse kit (KDB-10007, Nordic Biosite) as detection system.

rmAb clone **EP1601Y:** Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (4/4), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:200. Using these protocol settings, 8 of 8 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP27:** Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (1/1), Cell Conditioning 1 (CC1, Ventana) (2/2) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:250. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for CK5 for the two most commonly used antibodies as concentrate 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 **D5/16 B4**	5/17** (29%)	-	13/50 (26%)	-	0/5 (0%)	-
mAb clone **XM26**	9/16 (56%)	-	7/21 (33%)	-	7/10 (70%)	-

* 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 **D5/16 B4**, product no. **IR/IS780**, Dako, Autostainer Link:
One protocol with an optimal result was based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1)(Dako) (1/32), efficient heating time 20 min. at 97°C, 20 min. incubation of the primary Ab and FLEX+ as detection system. Using these protocol settings, 4 of 6 (67%) laboratories produced a sufficient staining result.

mAb clone **D5/16 B4**, product no. **GA621**, Dako, Omnis:
One protocol with an optimal result was based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (1/10), efficient heating time 30 min. at 97°C, 20 min. incubation of the primary Ab and FLEX as detection system. Using these protocol settings, 9 of 11 (82%) laboratories produced a sufficient staining result.

mAb clone **D5/16 B4**, product no. **790-4554**, Ventana/Cell Marque, BenchMark:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1, efficient heating time 30-64 min. and 16-32 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings, 28 of 33 (85%) laboratories produced a sufficient staining result.

rmAb clone **EP1601Y/LL002**, product no. **760-4939**, Ventana, BenchMark:
One protocol with an optimal result was based on HIER using Cell Conditioning 1, efficient heating time 32 min., 32 min. incubation of the primary Ab and OptiView (760-700) as detection system.

rmAb clone **EP24/EP67**, product no. **MAD-000651QD-7/N**, Master Diagnostica, LabVision:
Protocols with optimal results were based on HIER using EDTA/EGTA pH 8, efficient heating time 20 min. at 95-97°C, 10 min. incubation of the primary Ab and (MAD-000237QK/N) as detection system. Using these protocol settings, 2 of 2 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP27**, product no. **760-4935**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1, efficient heating time 24-64 min. and 16-32 min. incubation of the primary Ab. UltraView (760-500) or OptiView (760-700) were used as detection systems. Using these protocol settings, 11 of 12 (92%) laboratories produced a sufficient staining result.

Comments
In this second NordiQC assessment of CK5, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells and structures expected to be demonstrated. This pattern was observed in 93% of the insufficient results (80 of 86). The remaining insufficient results were characterized by a poor signal-to-noise ratio or a false positive staining reaction.
Virtually all laboratories were able to demonstrate CK5 in high level antigen expressing structures such as neoplastic cells of the lung squamous cell carcinoma, tissue core no. 1 and squamous epithelial cells of esophagus. Demonstration of CK5 in low level antigen expressing structures as neoplastic cells of the lung squamous cell carcinoma, tissue core no. 5 was significantly more challenging and required a carefully calibrated protocol.

The well-established mAb clones D5/16 B4 and XM26 for CK5/6 and CK5, respectively, were two most widely used Abs.
Within a laboratory developed (LD) assay, mAb clone XM26 was most successful and optimal results could be obtained on all three main IHC platforms from Dako, Leica and Ventana, see table 3. The two main prerequisites for a sufficient staining were efficient HIER typically in an alkaline buffer and careful calibration of the titre of the primary Ab. Use of 3-step polymer/multimer based detection systems seemed to provide higher proportions of optimal results compared to 2-step polymer/multimer based systems.
mAb clone D5/16 B4 in a LD assay showed inferior performance compared to XM26, as a reduced proportion of both sufficient and optimal results was seen. As for XM26, HIER in an alkaline buffer and 3-step polymer/multimer based detection systems were main requirements for an optimal staining result. However, despite identical and optimal protocol settings were applied for the two clones, D5/16 B4 provided a reduced proportion of cells demonstrated in the lung squamous cell carcinoma, tissue core no. 5 compared to level expected and seen for the other successful Abs for CK5. Simultaneously, it was noticed that mAb clone D5/16 B4, Dako, occasionally showed an inferior performance especially on the Bond IHC stainer, Leica. A distinct aberrant cytoplasmic staining reaction was thus seen in the colon adenocarcinoma and most likely caused by MAG reaction (Mouse Ascites Golgi). Dako provides the mAb as an ascites format and it is well-known that this aberrant MAG reaction can be seen in tissues of blood-type A.

Interestingly, the three recently launched Abs for CK5, mAb clone BS42 and rmAbs clones EP1601Y and SP27 grouped together provided a pass pate of 100% (13 of 13) of which 71% was optimal. All three clones, and in particular rmAb clone SP27, seemed to increase the analytical sensitivity for CK5 compared to both mAb clone XM26 and in particular for mAb clone D5/16 B4. In this assessment and the material included, the same analytical specificity as seen for the established clones was observed. Additional studies must be performed to evaluate the level of sensitivity and specificity for the newly introduced clones. At this point all three clones seem very promising for IHC demonstration of CK5.

In this assessment, the Ventana Ready-To-Use system based on rmAb clone SP27 (760-4935) was most successful and provided a pass rate and proportion of optimal results of 92% (11 of 12)
Optimal results could both be obtained using the protocol recommendations given by Ventana and by laboratory modified protocol settings (typically adjusting HIER time and/or incubation time of the primary Ab.

The Dako RTU systems based on mAb clone D5/16 B4, IR/IS780 and GA780 both provided a reduced proportion of optimal results compared to corresponding LD assays based on same clone. The Omnis RTU system gave a higher proportion of sufficient results compared to the Autostainer RTU system. Only a laboratory modified protocol based on FLEX+ as detection system provided an optimal result on the Autostainer system.

Controls
Esophagus can be recommended as positive tissue control for CK5. Virtually all squamous epithelial cells must show a moderate to strong cytoplasmic staining reaction in all cell layers. The most superficial cells may show a reduced staining reaction, whereas basal cells will display the strongest intensity. As esophagus does not contain any reliable structures with low level CK5 expression, supplemental positive tissues may be required. This is needed to identify and monitor an adequately calibrated protocol for the purpose to demonstrate CK5 in neoplastic tissues with a reduced CK5 expression as seen in this

assessment for the lung squamous cell carcinoma, tissue core no 5. As a supplement to esophagus, pancreas can be used as positive tissue control. This is recommended in the new guidelines published by the International Ad Hoc Expert Committee[1]. In pancreas, scattered columnar epithelial cells of intercalated ducts must show a weak to moderate predominantly membranous staining reaction.
Liver or appendix can be used as negative tissue control, in which no staining reaction of hepatocytes or epithelial cells should be seen.

[1]Torlakovic EE, Nielsen S, Francis G, Garratt J, Gilks B, Goldsmith JD, Hornick JL, Hyjek E, Ibrahim M, Miller K, Petcu E, Swanson PE, Zhou X, Taylor CR, Vyberg M. Standardization of positive controls in diagnostic immunohistochemistry: recommendations from the International Ad Hoc Expert Committee. Appl Immunohistochem Mol Morphol. 2015 Jan;23(1):1-18. doi: 10.1097/PAI.0000000000000163. Review. PubMed PMID: 25474126.

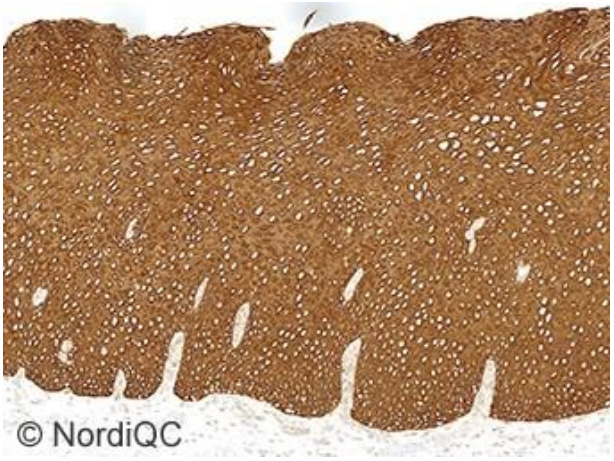


Fig. 1a
Optimal staining for CK5 of the esophagus using the mAb clone XM26 optimally calibrated and with HIER in an alkaline buffer.
Virtually all the squamous epithelial cells show a distinct, moderate to strong cytoplasmic staining reaction. A weak hue of the stromal compartment in the close vicinity of the basal epithelial cells is seen, most likely caused by chromogen diffusion.

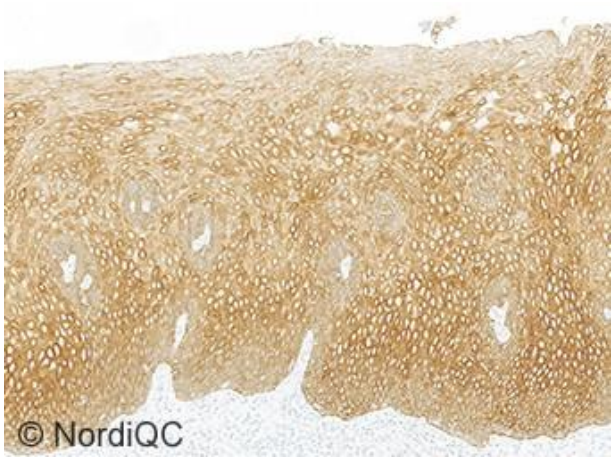


Fig. 1b
Staining for CK5 of the esophagus based on the mAb clone XM26 with a protocol giving a too low analytical sensitivity, most likely caused by a too low titre of the primary Ab and insufficient HIER - same field as in Fig. 1a.
The intensity of cells demonstrated is reduced compared o the level expected and obtained in Fig. 1a.
Also compare with Figs. 2b - 4b, same protocol.

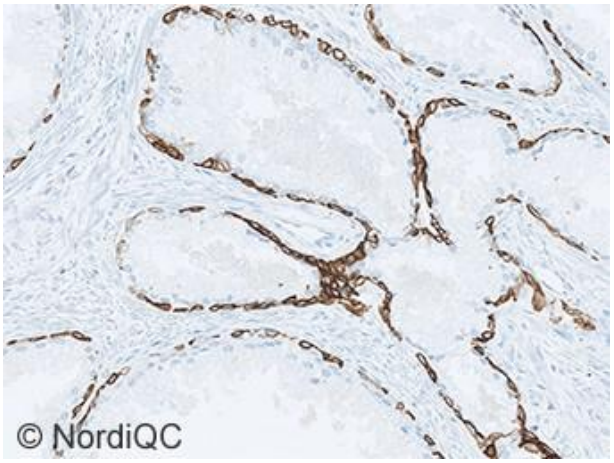


Fig. 2a
Optimal staining for CK5 of the prostate hyperplasia using same protocol as in Fig. 1a.
Virtually all the basal cells decorating the glands show a strong and distinct cytoplasmic staining reaction.
No background staining is seen.

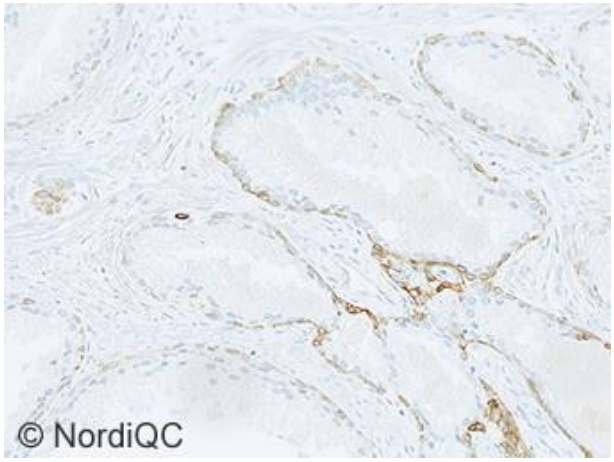


Fig. 2b
Insufficient staining for CK5 of the prostate hyperplasia using same protocol as in Fig. 1b – same field as in Fig. 2a.
The basal cells show a weak and equivocal staining reaction and the identification of these cells is compromised complicating the intereptation.

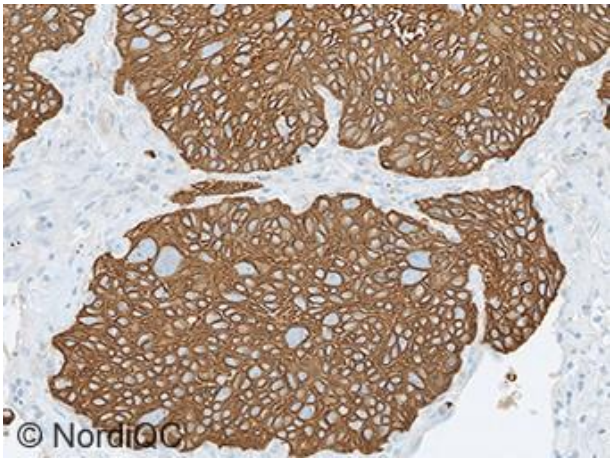


Fig. 3a
Optimal staining for CK5 of the lung squamous cell carcinoma, tissue core no. 1 using same protocol as in Figs. 1a and 2b.
Virtually all the neoplastic cells show a strong cytoplasmic staining reaction.
No background staining is seen.

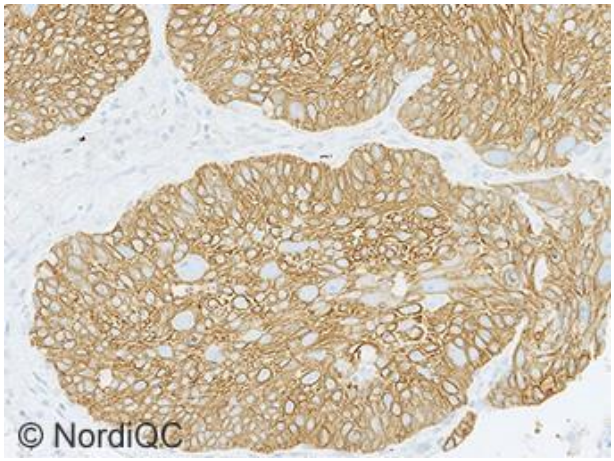


Fig. 3b
Staining for CK5 using same insufficient protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The vast majority of the neoplastic cells are demonstrated, but the intensity is reduced compared to the optimal result shown in Fig. 2b.
Also compare with Fig. 4b – same protocol.

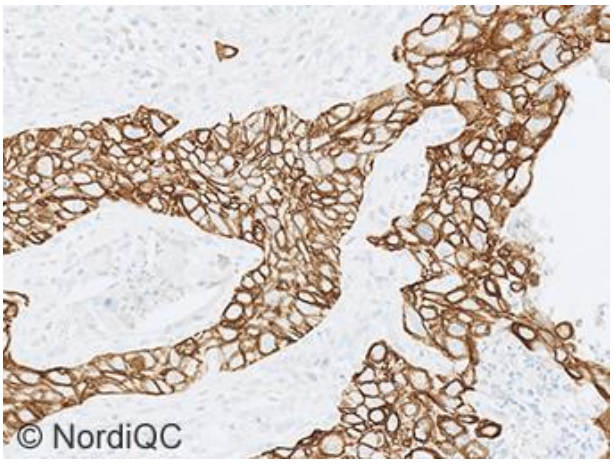


Fig. 4a
Optimal staining for CK5 of the lung squamous cell carcinoma, tissue core no. 5 using same protocol as in Figs. 1a - 3a.
A moderate and distinct cytoplasmic staining reaction is seen in the vast majority of neoplastic cells.

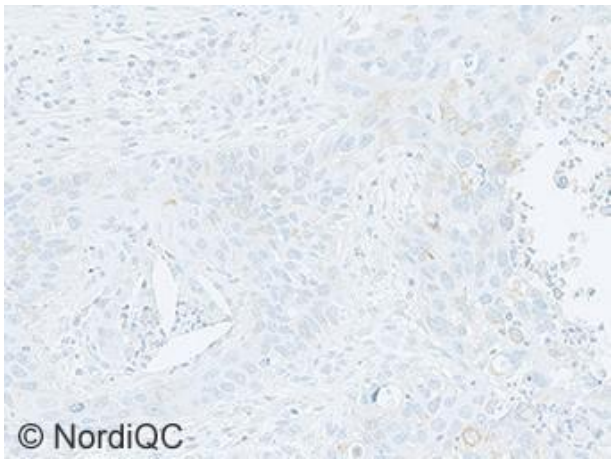
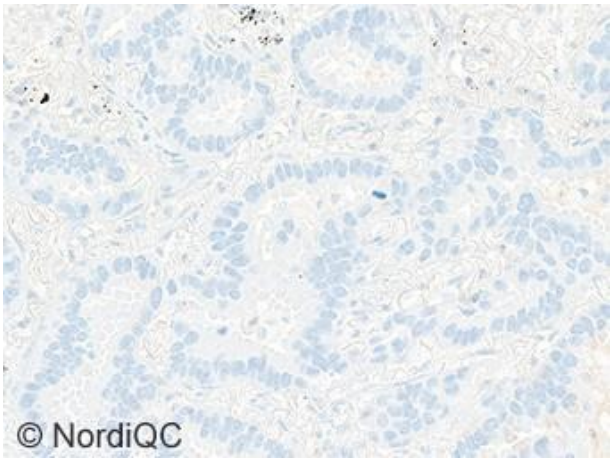
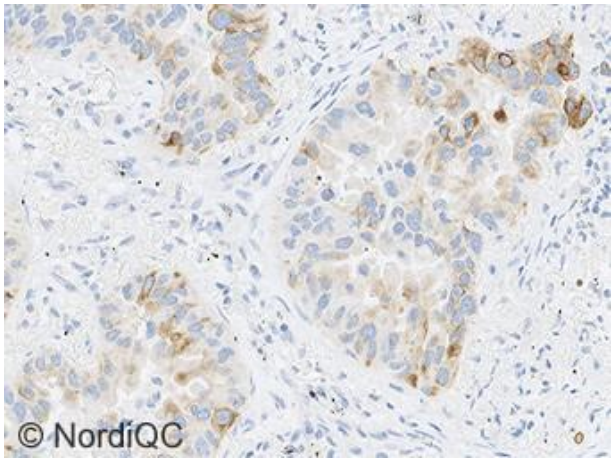


Fig. 4b
Insufficient staining for CK5 of the lung squamous cell carcinoma, tissue core no. 5 using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
Only dispersed neoplastic cells show a faint staining reaction.



Optimal staining for CK5 of the lung adenocarcinoma using same protocol as in Figs. 1a - 4a.
The neoplastic cells are unstained.
No background staining is seen.
The tumour was classified as adenocarcinoma both by conventional histological features and IHC profile.
CK5, CK14 and p63 were thus all uniformly negative by IHC performed by the NordiQC reference laboratory.



Insufficient false positive staining for CK5 of the lung adenocarcinoma using the mAb clone D5/16 B4 as a concentrate (Dako).
A moderate aberrant cytoplasmic staining reaction is seen in many neoplastic cells.
The protocol was based on efficient HIER in an alkaline buffer (BERS2, pH9) and a 3-step polymer based detection system (Refine, Leica).
From many vendors including Dako (most common vendor) the Ab is provided as an ascites format and the aberrant staining reaction might be caused by a cross reaction to Golgi associated proteins (MAG-reaction) or to another unspecified structure.

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