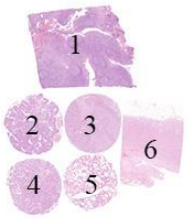
Assessment Run 65 2022

Cytokeratin 5 (CK5)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC assays among the NordiQC participants for CK5, typically used in the diagnostic work-up of prostate samples differentiating hyperplasia, prostate interepithelial neoplasia and carcinoma and also in lung samples to identify mesothelioma and to differentiate squamous cell carcinoma and adenocarcinoma. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for CK5 (see below).

Material

The slide to be stained for cytokeratin 5 (CK5) comprised:



1. Tonsil, 2. Pancreas, 3. Lung squamous cell carcinoma, 4. Lung adenocarcinoma, 5. Prostate hyperplasia, 6. Malignant epithelioid mesothelioma.

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 in virtually all squamous epithelial cells in the tonsil.
- A weak to moderate, predominantly membranous staining reaction of scattered cuboidal epithelial cells in the pancreatic intercalated ducts.
- A strong and distinct cytoplasmic staining reaction in the majority of basal cells in the hyperplastic prostate glands.
- An at least weak to moderate cytoplasmic staining reaction of virtually all neoplastic cells in the lung squamous cell carcinoma.
- An at least weak to moderate staining reaction in the majority of neoplastic cells in the malignant epithelioid mesothelioma.
- No staining of neoplastic cells in the lung adenocarcinoma.

Participation

Number of laboratories registered for CK5, run 65	329
Number of laboratories returning slides using appropriate antibodies	311 (95%)

Results
At the date of assessment, 95% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

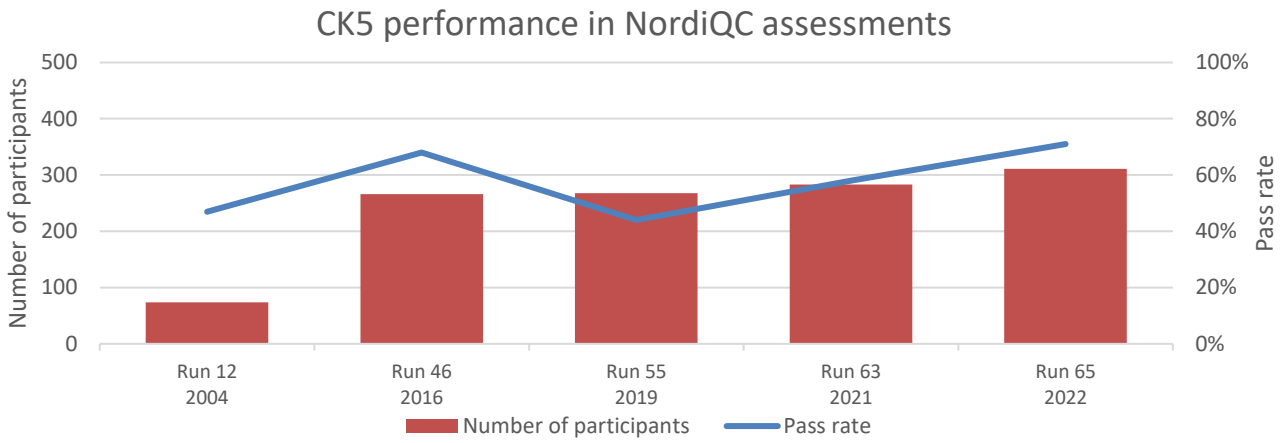
311 laboratories participated in this assessment. 220 (71%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Less successful performance of mouse monoclonal Ab (mAb) clone D5/16 B4 – both as concentrate and in Ready-To-Use (RTU) systems
- Use of less sensitive detection systems

Performance history
This was the fifth NordiQC assessment of CK5. An improvement in the pass rate was observed compared to the latest runs (Run 55 in 2019, Run 63 in 2021) as seen in Graph 1. No single explanation for the improved pass rate has been found. However, the combination of extended use of 3-step detection systems (67%, 62% and 55%, runs 65, 63 and 55, respectively) and reduced average dilution factor for concentrated primary Abs (1:86, 1:100 and 1:111, runs 65, 63 and 55, respectively) seems to have been successful protocol modifications. Further, the use of the less successful mAb clone D5/16 B4 has been reduced to 47% (146/311) in this run 65 compared to 61% (160/263) in run 55. Finally, the use of

pancreas as recommended control with low expression levels have given the laboratories a better opportunity to monitor the sensitivity of the protocol.

Graph 1. **Proportion of sufficient results for CK5 in the NordiQC runs performed**



Conclusion

The mAbs clones **XM26**, **D5/16 B4** and the rmAb clones **BSR55, EP42** and **SP27** could all be used as concentrate within a laboratory developed assay for the demonstration of CK5. The widely used mAb clone XM26 was significantly more successful compared to mAb clone D5/16 B4 with pass rates of 93% and 49%, respectively. The mAb clone D5/16 B4 typically provided a too low analytical sensitivity and as most frequently being produced as an ascites format with the risk of false positive staining reactions due to "Mouse Ascites Golgi" (MAG)[1] reaction, this complicates the optimization process for the antibody. Irrespective of the clone applied, efficient HIER (preferable in an alkaline buffer), careful calibration of the primary antibody and use of a sensitive 3-step polymer/multimer detection system were the most important prerequisites for an optimal staining result.

In this assessment, the Ventana/Roche RTU system based on rmAb clone **SP27**, 760-4935, was the most successful RTU system providing a pass rate of 100% of which 94% were optimal. However, SP27 has in NordiQC studies (ref; Thomsen et al[3]) shown positive reaction in lung adenocarcinomas being negative for other CK5 antibodies as well as p40, which has to be taken in account when used for subclassification of NSCLC.

Controls

Tonsil and pancreas can be recommended as positive tissue control[2]. In tonsil, virtually all squamous epithelial cells throughout all cell layers must show a moderate to strong cytoplasmic staining reaction. In pancreas, scattered cuboidal epithelial cells of intercalated ducts must show a weak to moderate predominantly membranous staining reaction. Liver can be recommended as negative tissue control; no staining reaction must be seen in hepatocytes and bile ducts.

Table 1. **Antibodies and assessment marks for CK5, run 65**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff[1]	OR[2]
mAb clone **D5/16 B4***	35 2 1 1 2	Dako/Agilent Cell Marque Millipore Epredia Zytomed	6	15	19	1	51%	15%
mAb clone **XM26**	2 3 64 3	Abcam Diagnostic BioSystems Leica Biosystems Monosan	56	11	4	1	93%	78%
mAb clone **IHC556***	1	GenomeMe	0	0	1	0	-	-
mAb clone **ZM186**	1	Zeta Corporation	0	0	1	0	-	-
rmAb clone **BSR55**	2	Nordic Biosite	1	0	1	0	-	-
rmAb clone **EP1601Y**	3	Cell Marque	0	1	2	0	-	-
rmAb clone **EP24/EP67***	2	Cell Marque	0	2	0	0	-	-
rmAb clone **EP24**	1	Epitomics	0	1	0	0	-	-
rmAb clone **EP42**	1	Epitomics	1	0	0	0	-	-
rmAb clone **SP27**	1	Immunologic	1	0	0	0	-	-
rmAb clone **QR027**	1	Quartett	0	1	0	0	-	-
mAb clone **XM26/SF13****	1	DCS Innovative Diagnostik-Systeme	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **D5/16 B4* 790-4554[3]**	6	Ventana/Roche	0	3	3	0	50%	0%
mAb clone **D5/16 B4* 790-4554[4]**	46	Ventana/Roche	9	23	11	3	70%	20%
mAb **D5/16 B4* GA780[3]**	13	Dako/Agilent	0	1	12	0	8%	0%
mAb **D5/16 B4* GA780[4]**	26	Dako/Agilent	0	9	16	1	35%	0%
mAb clone **D5/16 B4* IR/IS780[3]**	4	Dako/Agilent	0	1	2	1	-	-
mAb clone **D5/16 B4* IR/IS780[4]**	9	Dako/Agilent	1	1	4	3	22%	11%
mAb clone **D5/16 B4* 8295-C010**	1	Sakura Finetek	1	0	0	0	-	-
rmAb clone **RM226 8408-C010**	1	Sakura Finetek	0	1	0	0	-	-
mAb clone **XM26 PA0468[3]**	7	Leica Biosystems	2	4	1	0	86%	29%
mAb clone **XM26 PA0468[4]**	9	Leica Biosystems	8	1	0	0	100%	89%
mAb clone **XM26 PM234**	3	Biocare Medical	2	1	0	0	-	-
rmAb clone **EP1601Y 305R-17/18**	4	Cell Marque	0	2	2	0	-	-
rmAb clone **EP42 AN853-10M**	1	BioGenex	0	1	0	0	-	-
rmAb clone **EP24/EP67* MAD-000651QD**	1	Master Diagnostica	1	0	0	0	-	-
rmAb clone **EP24/EP67* MRH1159**	1	PathnSitu	0	1	0	0	-	-
rmAb clone **SP27 760-4935[3]**	21	Ventana/Roche	21	0	0	0	100%	100%
rmAb clone **SP27 760-4935[4]**	29	Ventana/Roche	26	3	0	0	100%	90%
rmAb clone **C9E33 CCR-0973**	1	Celnovte	0	0	1	0	-	-

mAb clone **150A8C1 PA018**	1	Abcarta	0	0	1	0	-	-
Total	311		136	84	81	10		
Proportion			44%	27%	26%	3%	71%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).
*) Cytokeratin 5 and 6.
**) Cytokeratin 5 and 14.

Detailed analysis of CK5, Run 65
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **D5/16 B4**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana/Roche) (6/22*). The mAb was typically diluted in the range of 1:25-1:100 in combination with a 3-step polymer/multimer detection system. Using these protocol settings, 10 of 13 (77%) 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 based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) (24/27), TRS pH 9 (3-in-1) (Dako/Agilent) (1/4), CC1 (Ventana/Roche) (18/25), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (12/14) or Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:200. Using these protocol settings, 64 of 68 (94%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for CK5 for the most commonly used antibodies as concentrates on the four main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**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	CC1 pH 8.5 + Protease 3	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **D5/16 B4**	0/2	-	0/2	-	5/12 (42%)	1/1	-	0/5 (0%)	0/2
mAb clone **XM26**	1/4	-	24/26 (92%)	-	17/24 (71%)	1/1	-	12/12 (100%)	1/1

* 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. **790-4554**, Ventana/Roche, BenchMark XT/Ultra:
Protocols with optimal results were based on HIER using CC1, efficient heating time 24-64 min. and 16-40 min. incubation of the primary Ab. OptiView (760-700) +/- Tyramide amplification kit (760-099 / 860-099) were used as detection systems. Using these protocol settings, 25 of 36 (69%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **XM26**, product no. **PA0468**, Leica Biosystems, Leica Bond-III/Bond-Max:
Protocols with optimal results were typically based on HIER using BERS2 pH 9 (efficient heating time 10-20 min. at 95-100°C), 15-20 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 14 of 15 (93%) laboratories produced an optimal staining result.

rmAb clone **SP27**, product no. **760-4935**, Ventana/Roche, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using CC1, efficient heating time 24-64 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, 48 of 48 (100%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. 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.

Table 3. **Proportion of sufficient and optimal results for CK5 for the most commonly used RTU IHC systems**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Ventana Benchmark mAb clone **D5/16 B4, 790-4554**	50% (3/6)	0% (0/6)	70% (32/46)	20% (9/46)
Dako Omnis mAb clone **D5/16 B4, GA780**	8% (1/13)	0% (0/13)	36% (9/25)	0% (0/25)
Dako Autostainer mAb clone **D5/16 B4, IR/IS780**	(1/4)	(0/4)	0% (0/6)	0% (0/6)
Leica Bond mAb clone **XM26, PA0468**	86% (6/7)	29% (2/7)	100% (9/9)	89% (8/9)
Ventana Benchmark rmAb clone **SP27, 760-4935**	100% (21/21)	100% (21/21)	100% (27/27)	89% (24/27)

* 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 integrated.

Comments

In this assessment and in concordance with the previous NordiQC assessments 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 92% of the insufficient results (84 of 91). The remaining 8% (7 of 91) insufficient results were characterized by either a false positive staining reaction (n=2) or poor signal-to-noise ratio/excessive background (n=5). Virtually all laboratories were able to demonstrate CK5 in high-level antigen expressing structures such as the squamous epithelial cells of tonsil, whereas demonstration of CK5 in low-level antigen expressing structures as the neoplastic cells in the malignant mesothelioma, lung squamous cell carcinoma and especially the cuboidal epithelial cells of intercalated ducts in pancreas was significantly more challenging and required a carefully calibrated protocol. The pass rate has increased in this run from 44% in run 55, 58% in run 63 to 71% in this run 65, which is the highest pass rate obtained for CK5 in the five NordiQC assessments conducted.

41% (127 of 311) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for CK5. The well-established mAb clones D5/16 B4 and XM26 for CK5/6 and CK5, respectively, were the two most widely used Abs. 32% (41 of 127) of the LD-assays were based on the mAb clone D5/16 B4 whereas 57% (72 of 127) were based on the mAb clone XM26.
Within a LD assay, mAb clone XM26 was by far the most successful of the two, and optimal results could be obtained on all four main IHC platforms from Dako/Agilent, Leica Biosystems and Ventana/Roche (see Table 2). In concordance with previous assessments the main prerequisites for a sufficient and optimal staining with mAb clone XM26 were efficient HIER typically in an alkaline buffer (alone or in combination with weak proteolysis (Protease 3, Ventana/Roche), careful calibration of the titre of the primary Ab and the use of a sensitive detection system, preferably a 3-step polymer/multimer based detection system. The proportion of sufficient staining results for the use of 3-step polymer/multimer based detection systems and 2-step polymer/multimer based systems was 96% and 81%, respectively.
The mAb clone D5/16 B4 in a LD assay had a low pass rate on all IHC platforms. The overall proportion of sufficient staining results was 51% (21 of 41), and optimal staining results were only obtained by 6 laboratories (15%). These were based on HIER in an alkaline buffer and 3-step polymer/multimer based detection systems. It was observed that mAb clone D5/16 B4 in addition to the low analytical sensitivity provided, occasionally also showed an inferior performance due to a distinct, aberrant (false positive) cytoplasmic staining reaction in pancreas, most likely caused by MAG reaction[1]. The two main vendors, Dako/Agilent and Cell Marque, of the mAb provides the product as an ascites format and it is well-known that this aberrant MAG reaction can be seen in tissues of blood type A patients. As CK5 is localized in the cytoplasmic compartment similar to the MAG reaction a genuine risk of false positivity and hereby misclassification of e.g. NSCLC is induced.

Ready-To-Use (RTU) antibodies were used by 59% (184 of 311) of the laboratories. The Ventana/Roche RTU system based on rmAb clone SP27, 760-4935, was most successful and provided a proportion of sufficient staining results of 100% (50 of 50), all assessed as optimal if using the vendor recommended

protocol settings. Optimal results could also be obtained by laboratory modified protocol settings (typically minor adjustments of HIER time and/or incubation time of the primary Ab).
The RTU system based on rmAb clone SP27 gave a very high qualitative result in this assessment and performed as expected in all tissue samples included.
However, the rmAb clone SP27 has in NordiQC studies[3] shown positive reaction in 23% of lung adenocarcinomas being negative for other CK5 antibodies as well as p40. The significance of this is uncertain but must be taken into account in the subclassification of NSCLC and emphasizes that a panel of markers must be applied to secure a correct diagnosis.

The Leica Biosystems RTU system based on mAb clone XM26, PA0468, also provided a high pass rate. Using the recommended protocol settings, the proportion of sufficient staining results was 86% (6 of 7) of which 29% (2 of 7) were assessed as optimal. As seen in Table 3, a pass rate of 100% (9 of 9), 89% optimal, was seen if modifying the protocol settings. Only minor changes for HIER time and/or incubation time of the primary Ab was made.

The Dako/Agilent RTU systems based on mAb clone D5/16 B4, IR/IS780 and GA780 for Autostainer and Omnis, respectively, both provided a low proportion of sufficient and optimal staining results similar to the observations and data generated in runs 55 and 63. For the Omnis platform the performance of the RTU system used as "plug-and-play" was inferior to the performance obtained by laboratory modified protocol settings as shown in Table 3. The insufficient results were characterized by too weak or false negative test results. The most successful modification was based on use of FLEX+ as detection system and not FLEX as recommended. Surprisingly, no MAG reaction was seen for the Dako/Agilent RTU systems, as observed for the conc. format of the mAb clone D5/16 B4 despite adding a linker (FLEX+) to the protocols and hereby increasing the technical and analytical sensitivity for the ascites-based antibody.
The Ventana/Roche RTU system based on mAb clone D5/16 B4, 790-4554, performed significantly better than the corresponding Dako/Agilent RTU systems with a pass rate of 67% if including both vendor recommended and laboratory modified protocol settings, but still inferior to the other Ventana/Roche RTU system based on rmAb clone SP27. 88% (46 of 52) of the laboratories used laboratory modified protocol settings and 70% (32 of 46) obtained sufficient staining results of which 20% (9 of 46) were assessed as optimal. The protocols producing optimal staining results were all based on a sensitive 3-step multimer system, OptiView, giving a pass rate of 87% (27 of 31), 29% optimal. If using iView or UltraView (as recommended in package insert), a pass rate of 38% (8 of 21), no optimal, was obtained.

[1]Kliman HJ, Feinberg RF, Schwartz LB, Feinman MA, Lavi E, Meaddough EL. A mucin-like glycoprotein identified by MAG (mouse ascites Golgi) antibodies. Menstrual cycle-dependent localization in human endometrium. Am J Pathol. 1995;146(1):166–81.

[2]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.

[3]Thomsen, C., Nielsen, O., Nielsen, S., Røge, R., & Vyberg, M. (2020). NordiQC Assessments of Keratin 5 Immunoassays. Applied Immunohistochemistry & Molecular Morphology, 28(7), 566-570. https://doi.org/10.1097/PAI.0000000000000855

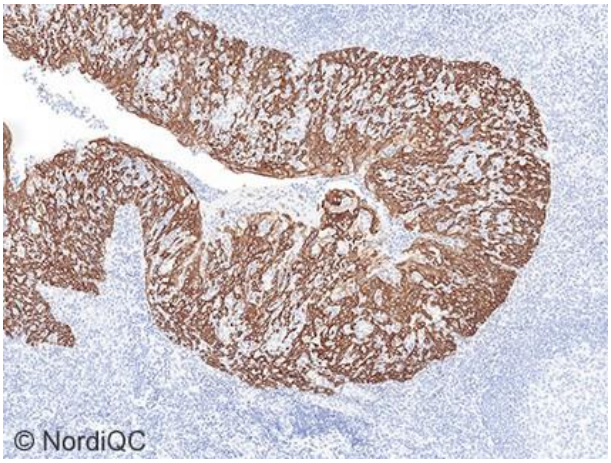


Fig. 1a
Optimal CK5 staining of the tonsil, tissue core no. 1, using the **rmAb clone SP27** in an RTU format (760-4935, Ventana/Roche) using the vendor recommended protocol settings on the BenchMark Ultra. A strong cytoplasmic staining reaction is seen in virtually all squamous epithelial cells in the tonsil. Also compare with Figs. 2a-6a, same protocol.

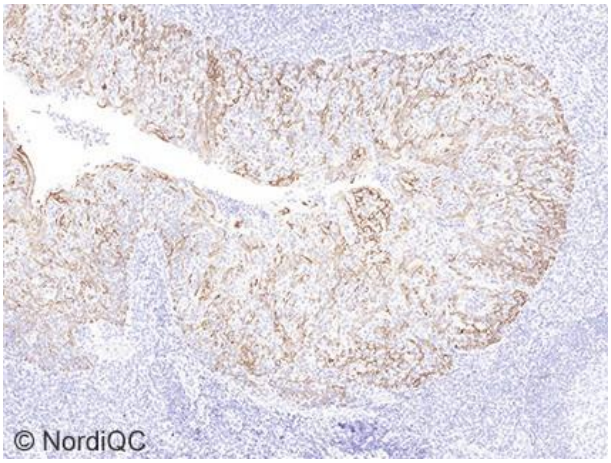


Fig. 1b
CK5 staining of the tonsil, tissue core no. 1, using the **mAb clone D5/16 B4** in an RTU format (IR780, Dako/Agilent) using the vendor recommended protocol settings on the Dako Autostainer. Using this protocol, the intensity and proportion of the epithelial cells demonstrated is reduced compared to the level expected and obtained in Fig. 1a, - same field as in Fig. 1a. However also compare with Figs. 2b-5b, same protocol.

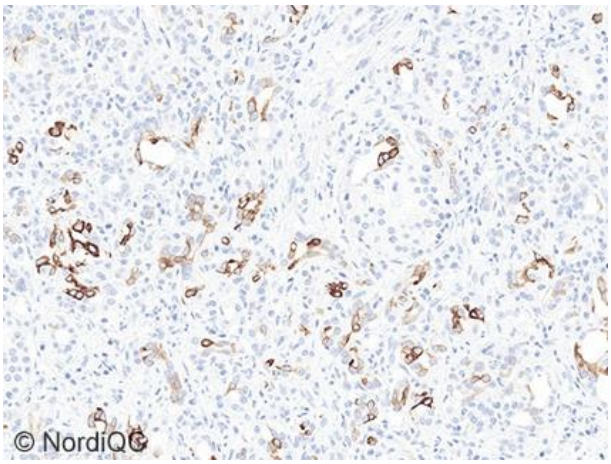


Fig. 2a
Optimal CK5 staining of pancreas, tissue core no. 2, with low-level CK5 expression using same protocol as in Fig. 1a. Scattered cuboidal epithelial cells of intercalated ducts display a weak to moderate predominantly membranous staining reaction. No background staining is seen.

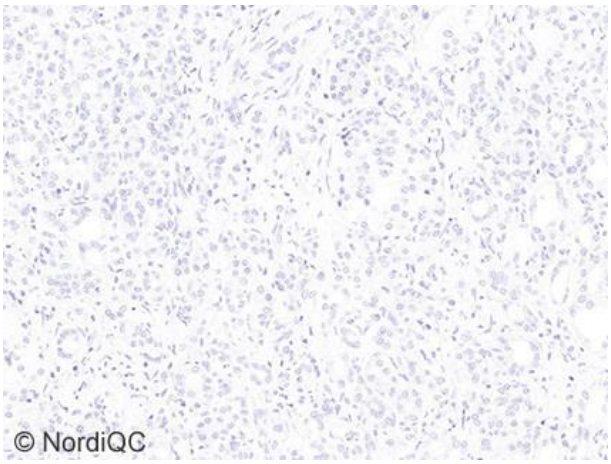


Fig. 2b
Insufficient CK5 staining of the pancreas, tissue core no. 2, with low-level CK5 expression using same protocol as in Fig. 1b – same field as in Fig. 2a. No staining reaction is seen in the epithelial cell of the intercalated ducts giving a false negative result – compare with Fig. 2a.

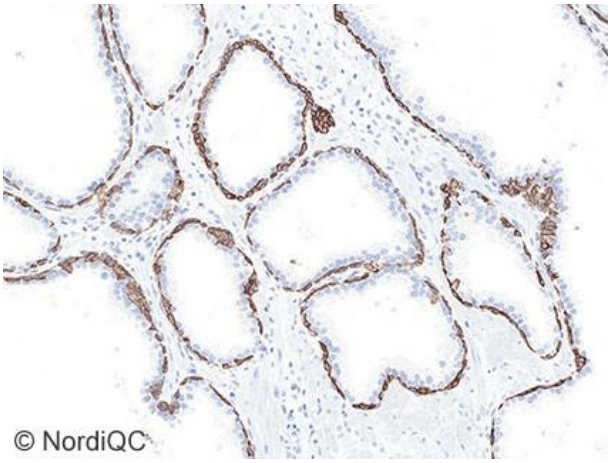


Fig. 3a
Optimal CK5 staining of the prostate hyperplasia, tissue core no. 5, using same protocol as in Figs. 1a-2a.
A strong and distinct cytoplasmic staining reaction is seen in the majority of basal cells in the hyperplastic prostate glands. No background staining is seen.

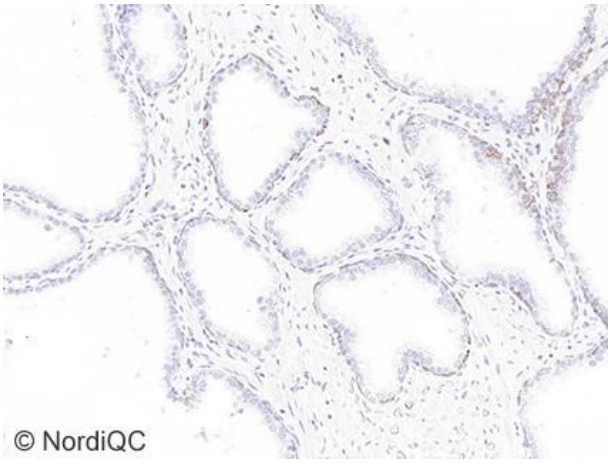


Fig. 3b
Insufficient CK5 staining of the prostate hyperplasia, tissue core no. 5, using same protocol as in Figs. 1b-2b – same field as in Fig. 3a. Only scattered basal cells in the hyperplastic prostate glands show a weak staining reaction, compromising the diagnostic utility of the test in prostate samples.

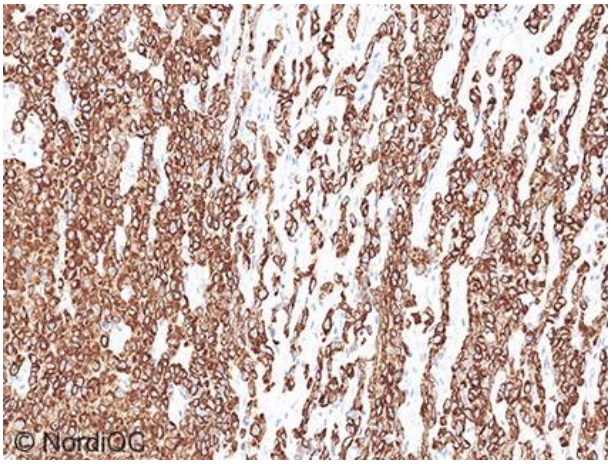


Fig. 4a
Optimal CK5 staining of the malignant mesothelioma, tissue core no. 6, with a medium to high level of CK5 expression using same protocol as in Figs. 1a-3a. All the neoplastic cells show a moderate to strong, distinct cytoplasmic staining reaction.

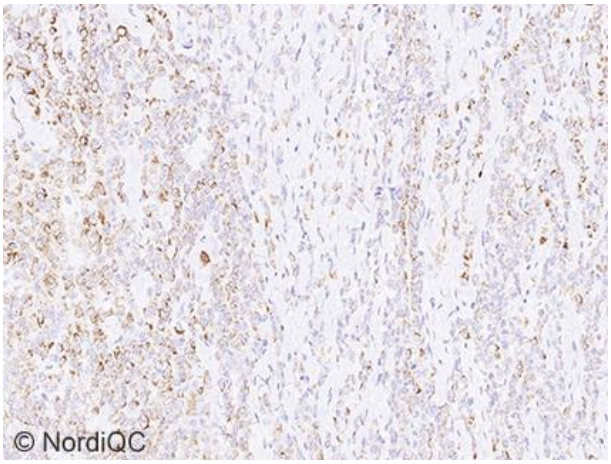


Fig. 4b
CK5 staining of the malignant mesothelioma, tissue core no. 6, using the same insufficient protocol as in Figs. 1b-3b – same field as in Fig. 4a. The intensity and proportion of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 4a.

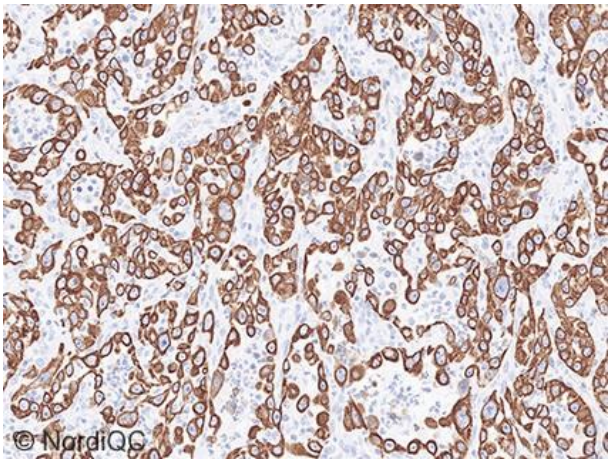


Fig. 5a
Optimal CK5 staining of the lung squamous cell carcinoma, tissue core no. 3, with moderate to high-level CK5 expression using same protocol as in Figs. 1a-4a. All the neoplastic cells show a strong and distinct cytoplasmic staining reaction.

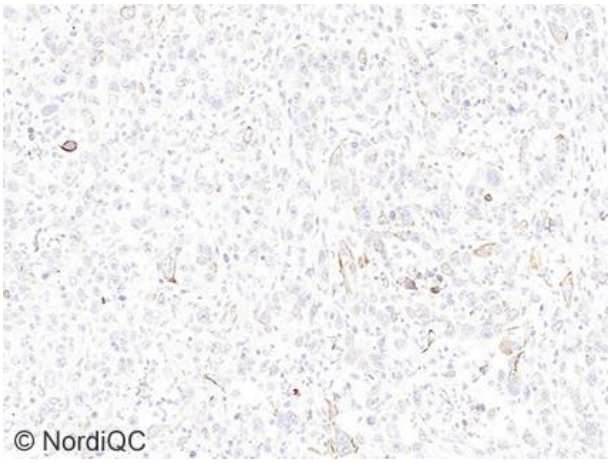


Fig. 5b
Insufficient CK5 staining of the lung squamous cell carcinoma, tissue core no. 3, with moderate to high-level CK5 expression using the same insufficient protocol as in Figs. 1b-4b – same field as in Fig. 5a. Virtually no staining reaction is seen in the neoplastic cells, giving a false negative staining reaction.

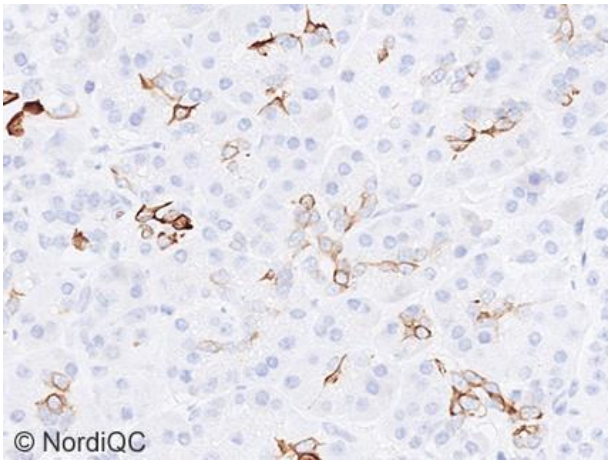


Fig. 6a
High magnification of the optimal CK5 staining of pancreas, tissue core no. 2, with low-level CK5 expression using same protocol as in Figs. 1a-5a. Scattered cuboidal epithelial cells of intercalated ducts display a weak to moderate predominantly membranous staining reaction. No background staining is seen.

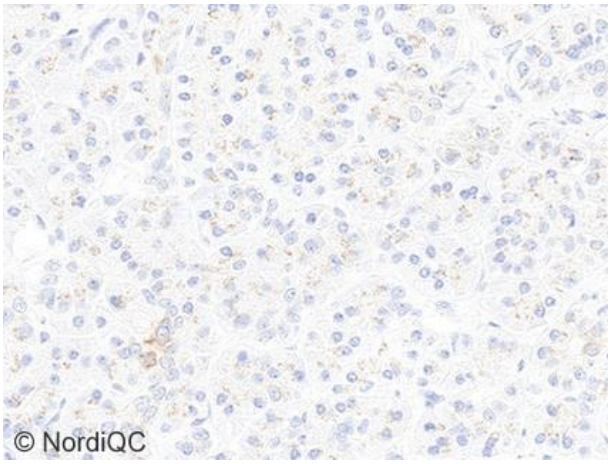


Fig. 6b
Insufficient CK5 staining of pancreas, tissue core no. 2, using the **mAb clone D5/16 B4** in a concentrated format, using a relatively high concentration (1:50), HIER in an alkaline buffer and a 3-layer detection system - same field as in Fig. 6a. No staining reaction is seen in the epithelial cells of the intercalated ducts. Instead, a weak but distinct cytoplasmic MAG reaction (Mouse Ascites Golgi) is seen in the majority of acinar cells. The staining result is thus both false negative and false positive.

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