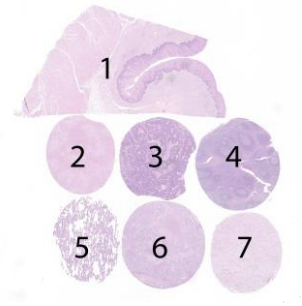
Assessment Run 47 2016

Pan Cytokeratin (CK-PAN)

Material

The slide to be stained for CK-PAN comprised:
1. Esophagus, 2. Liver, 3. Small cell lung carcinoma (SCLC), 4. Tonsil,
5. Lung adenocarcinoma, 6. Lung squamous cell carcinoma, 7. Renal clear cell carcinoma (RCC).



Criteria for assessing a CK-PAN staining as optimal were:

- A strong, distinct cytoplasmic staining reaction of all bile ductal epithelial cells and at least a moderate cytoplasmic staining reaction with membrane accentuation of the vast majority of hepatocytes.
- A strong, distinct cytoplasmic staining reaction of all squamous epithelial cells throughout all cell layers in the esophagus.
- A strong, distinct cytoplasmic staining reaction of the majority of neoplastic cells in the lung adenocarcinoma and squamous cell carcinoma.
- An at least moderate, distinct cytoplasmic, dot-like staining reaction of the majority of neoplastic cells in the SCLC.
- An at least weak to moderate, distinct cytoplasmic and membranous staining reaction of the majority of neoplastic cells in the RCC.

All tissues were fixed in 10% neutral buffered formalin.

Participation

Number of laboratories registered for CK-PAN, run 47	298
Number of laboratories returning slides	276 (93%)

Results

276 laboratories participated in this assessment. One laboratory used an inappropriate antibody (CK-HMW). Of the remaining 275 laboratories, 72% achieved a sufficient mark. Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining were:

- Too low concentration of the primary antibody
- Insufficient HIER – too short efficient heating time and/or use of non-alkaline HIER buffers
- Inappropriate epitope retrieval
- Less successful primary antibodies.

Performance history

This was the eighth NordiQC assessment of CK-PAN. The overall pass rate was slightly improved compared to previous runs performed, as shown in table 2.

Table 2. **Proportion of sufficient results for CK-PAN in the eight NordiQC runs performed**

	Run 8 2003	Run 15 2005	Run 20 2008	Run 24 2008	Run 30 2010	Run 36 2012	Run 41 2014	Run 47 2016
Participants, n=	72	85	103	123	168	202	233	275
Sufficient results	53%	58%	62%	60%	65%	65%	67%	72%

Conclusion

The mAb clone cocktails **AE1/AE3, AE1/AE3/5D3, AE1/AE3/PCK26** and mAb clone **BS5** can all be recommended for demonstration of CK-PAN. The epitope retrieval method must be specifically tailored to each of the clones/cocktails applied. The Ready-To-Use systems from Dako based on mAb clone cocktail AE1/AE3 were in this assessment most successful and provided the highest proportion of sufficient and optimal results.
Liver and esophagus in combination are recommendable as positive tissue controls irrespective of the antibody applied. The vast majority of hepatocytes must show a distinct cytoplasmic staining reaction with membrane enhancement, while virtually all squamous epithelial cells of the esophagus throughout all cell layers must show a strong cytoplasmic staining reaction. No staining reaction should be seen in the stromal cells in the liver.

Table 1. **Antibodies and assessment marks for CK-PAN, run 47**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone cocktail **AE1/AE3**	85	Dako/Agilent	33	28	17	7	72%	83%
	11	Thermo/NeoMarkers	2	3	4	2	45%	100%
	5	Cell Marque	0	3	0	2	60%	-
	5	Leica/Novocastra	1	0	3	1	20%	50%
	3	Biocare	1	2	0	0	-	-
	2	Zytomed	0	2	0	0	-	-
	1	Biosystems	1	0	0	0	-	-
	1	Genemed	0	1	0	0	-	-
	1	Gennova	0	1	0	0	-	-
	1	Immunologic	0	0	1	0	-	-
	1	Millipore	1	0	0	0	-	-
	1	Monosan	0	0	0	0	-	-
mAb clone cocktail **AE1/AE3/ks 13.2**	1	Linaris	0	0	0	1	-	-
mAb clone cocktail **AE1/AE3/5D3**	2 1	Biocare Zytomed	1	2	0	0	-	-
mAb clone cocktail **PAN CK Ab-2**	1	Thermo/NeoMarkers	0	1	0	0	-	-
mAb clone **BS5**	1 1	Monosan Nordic Biosite	2	0	0	0	-	-
mAb clone **C-11**	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone **Lu-5**	2	Immunologic	0	0	1	1	-	-
mAb clone **MNF116**	7	Dako/Agilent	0	0	4	3	0%	-
mAb clone **OSCAR**	1 1	Signet "In-house"	0	2	0	0	-	-
Unknown	3		1	1	0	1	-	-
"Laboratory made" antibody cocktails								
mAb clone cocktail **AE1/AE3/5D3**	2	Leica/Novocastra & Milipore	1	1	0	0	-	-
mAb clone cocktail **AE1/AE3/5D3**	1	Leica/Novocastra	1	0	0	0	-	-
mAb clone cocktail **AE1AE3/CAM5.2**	1	Dako/Agilent & BD	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone cocktail **AE1/AE3 IR053**	36	Dako/Agilent	28	5	2	1	92%	95%
mAb clone cocktail **AE1/AE3 GA053**	19	Dako/Agilent	18	0	1	0	95%	100%
mAb clone cocktail **AE1/AE3 313M-18**	3	Cell Marque	0	1	0	2	-	-
mAb clone cocktail **AE1/AE3 MAD 001000QD**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone cocktail **AE1/AE3 Kit-0009**	1	Maixin	1	0	0	0	-	-
mAb clone cocktail **AE1/AE3 PA0909**	5	Leica/Novocastra	0	1	3	1	20%	-
mAb clone cocktail **AE1/AE3 RTU-AE1/AE3**	2	Leica/Novocastra	0	0	2	0	-	-
mAb clone cocktail **AE1/AE3/5D3 IP162**	2	Biocare	1	1	0	0	-	-
mAb clone cocktail **AE1/AE3/PCK26 760-2135/2595**	62	Ventana/Roche	37	8	5	12	73%	96%
rmAb clone cocktail **EP24/EP67/B22.1/B23.1 MAD-000680QD**	2	Master Diagnostica	0	2	0	0	-	-
Total	275		132	65	43	35	-	
Proportion			48%	24%	16%	12%	72%	

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

Detailed analysis of CK-PAN, Run 47
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone cocktail **AE1/AE3**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (4/8)*, TRS pH 9 (Dako) (4/6), Cell Conditioning 1 (CC1; Ventana) (27/61) or Tris-EDTA/EGTA pH 9 (3/10) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 61 of 74 (82%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone cocktail **AE1/AE3/5D3** (Biocare): One protocol with an optimal result was based on HIER in pressure cooker using Tris-EDTA/EGTA pH 9 as retrieval buffer. The dilution factor was 1:200 using a 2-step polymer based detection system (Dako K8000).

mAb clone **BS5**: Two protocols with optimal results were both based on HIER using either Tris-EDTA/EGTA pH 9 or Bond Epitope Retrieval Solution 2 pH 9 (Leica) as retrieval buffer. The dilution factor was 1:200. Both a 2-step polymer based detection system (Nordic Biosite KDB-1007) and a 3-step system (Refine DS9800, Leica) was used.

Table 3. **Proportion of optimal results for CK-PAN using the mAb clone cocktail AE1/AE3 as concentrate 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 **AE1/AE3**	8/13** (62%)	-	27/52 (52%)	-	0/9	0/4

* 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 cocktail **AE1/AE3**, product no. **IR053**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 95-99°C) and 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 21 of 22 (95%) laboratories produced a sufficient staining result.

mAb clone cocktail **AE1/AE3**, product no. **GV053**, Dako, OMNIS:
Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 24-30 min. at 97°C) and 12.5 - 30 min. incubation of the primary Ab and EnVision FLEX (GV800/GV823) as detection system. Using these protocol settings 18 of 18, (100%) laboratories produced an optimal staining result.

mAb clone cocktail **AE1/AE3/PCK26,** product no. **760-2135/2595**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on a combined pre-treatment using HIER in CC1 for 24-36 min. followed by enzymatic pre-treatment in Protease 3 (4-8 min.), 8-32 min. incubation of the primary Ab and UltraView (760-500, Ventana) or OptiView (760-700, Ventana) as detection system. Using these protocol settings, 22 of 23 (96%) laboratories produced a sufficient staining result.

Comments
In concordance with the previous NordiQC assessments for CK-PAN, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells and structures expected to be demonstrated. Virtually all the participating laboratories were able to stain CK in epithelial cells of bile ducts in the liver and neoplastic cells of the lung adenocarcinoma, whereas demonstration of CK in neoplastic cells of the SCLC and renal cell carcinoma was more difficult, and only obtained by protocols with a high sensitivity and appropriate protocol settings. The pass rate was highly influenced by the choice of Ab and retrieval method applied, which underlines the necessity for individual optimization for each clone/clone cocktail used for the demonstration of CK. This correlation, observed in the last seven NordiQC CK-PAN assessments, is summarized in table 4.

Table 4. **Pass rates for antibody cocktails combined with epitope retrieval methods in seven NordiQC runs**

Pass rate for run 15, 20, 24, 30, 36, 41 & 47								
	Total		HIER		Proteolysis		HIER + proteolysis	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
mAb AE1/AE3	752	542 (72%)	693	535 (77%)	44	5 (11%)	5	2 (40%)
mAb AE1/AE3/5D3	37	34 (92%)	36	34 (94%)	1	0	0	0
mAb AE1/AE3/PCK26	176	105 (60%)	25	13 (48%)	34	0	117	92 (79%)
mAb MNF116	91	30 (33%)	40	9 (23%)	47	21 (45%)	4	2 (50%)

These data clearly supports that choice of epitope retrieval has significant impact on performance of the applied antibodies. For the most widely used clone cocktail AE1/AE3, the over-all pass rate in these 7 successive NordiQC runs was 72%. Using HIER, a pass rate of 77% was obtained, significantly higher than the pass rate of 11% when proteolytic pre-treatment was applied for AE1/AE3. For the second most commonly used antibody cocktail, AE1/AE3/PCK26, combined epitope retrieval using HIER in CC1 (Ventana) followed by proteolysis e.g. in P3, provided a pass rate of 79%, compared to 48% and 0% using either HIER or proteolysis as single retrieval method.
The mAb clone MNF116 has in these seven consecutive runs provided an inferior overall performance compared to the 3 other antibody cocktails listed in table 4. No significant improvement of the performance could be identified by any of the different retrieval methods. Consequently mAb clone MNF116 should be substituted by one of the mentioned Ab cocktails.

Too weak or false negative staining result was the main feature of an insufficient result and was typically caused by protocols with too low technical and analytical sensitivity. The titre of the primary Ab is one of the most central parameters influencing technical and analytical sensitivity. Consequently, titre must be carefully calibrated to provide an IHC protocol, which will "fit-for-the-purpose". For CK-PAN this means that the protocol must be able to demonstrate CK in structures with both low-level and high-level CK expression, which is the range seen in carcinomas. Using protocol settings based on the concentrated format of the mAb clone cocktail AE1/AE3, HIER performed in an alkaline buffer and applying a standard 2- or 3-step polymer/multimer based detection system, the Median Dilution Value (MDV) of the primary Ab for optimal results was 1:90 (range 1:40 – 1:200, n=39). In comparison a MDV of 1:290 (range 1:50 – 1:1.500, n=30) was seen for protocols with insufficient results (borderline or poor).

The use of Ready-To-Use (RTU) formats is consistently increasing and in this run 48% of all protocols (n=133 of 275) were based on RTU format, compared to 25% and 9% in run 30, 2010 and run 15, 2005 respectively. Ideally, a RTU format of a primary Ab is used within a system with precise information on vendor recommended protocol settings, equipment, reagents and results expected.

In this assessment the Dako RTU systems **IR053** and **GA053** based on the mAb clone cocktail AE1/AE3 provided the highest number of sufficient and optimal results. For laboratories using one of these systems, a pass rate of 93% was obtained, 84% of which were optimal. Optimal results were primarily obtained by using the RTU system accordingly to the vendor protocol recommendations. Laboratory modified protocol settings (typically adjusting HIER and incubation time of the primary Ab) also provided sufficient and optimal results.
The Ventana RTU system **760-2135/2595** based on the mAb clone cocktail AE1/AE3/PCK26 gave an overall pass rate of 73% and 60% were optimal. However, using optimal protocol settings a pass rate of 96% was obtained. Optimal results were typically obtained by the newly updated recommended protocol settings from Ventana. In 2015, the recommended epitope retrieval method for the mAb clone cocktail AE1/AE3/PCK26 was changed from proteolysis to a combined pre-treatment based on HIER in mild CC1 followed by proteolysis in P3, 4 min. Laboratory modified protocol settings prolonging incubation time of the primary Ab and change of detection kit did not affect the overall result, whereas omission of HIER and too short HIER (< 20-24 min.) was less successful and provided a high proportion of insufficient and false negative results. As seen in previous CK-PAN assessments, it was not possible to obtain optimal results by the previously recommended protocol settings using proteolysis as single retrieval method.

The overall pass rate of 72% obtained in this run is the highest level observed to date. The improvement was in part related to increased use of AE1/AE3, AE1/AE3/5D3 and AE1/AE3/PCK26 at the expense of the less successful clones MNF116 and KL1. In the current run 92% (253 of 275) used AE1/AE3, AE1/AE3/5D3 or AE1/AE3/PCK26 and only 3% (7 of 275) used mAb clone MNF116. In run 15, 2005 22% of the laboratories used MNF116 or KL1, while 69% used AE1/AE3, AE1/AE3/5D3 or AE1/AE3/PCK26.
At the same time, harmonization of protocol settings has been observed. In run 15, 20% of the laboratories used proteolytic pre-treatment as epitope retrieval for AE1/AE3, AE1/AE3/5D3 or AE1/AE3/PCK26 compared to 4% in this run.

Controls

As seen in the previous NordiQC assessments, liver and esophagus in combination are recommendable as positive tissue controls for CK-PAN. It is crucial that the vast majority of hepatocytes (expressing only a limited amount of the primary LMW CK types 8 and 18) show an at least moderate, distinct cytoplasmic and membranous staining reaction. In esophagus, virtually all squamous epithelial cells throughout all cell layers must show a strong distinct cytoplasmic staining reaction due to expression of HMW CK. No staining should be seen in stromal cells in the liver. Smooth muscle cells in vessels and in muscularis mucosae in esophagus will typically show a weak to moderate patchy cytoplasmic staining reaction. Occasionally a nuclear staining reaction in scattered lymphocytes can be observed. No explanation for this staining pattern has been identified. The reaction is seen with all the mentioned ab cocktails providing an otherwise optimal staining result.

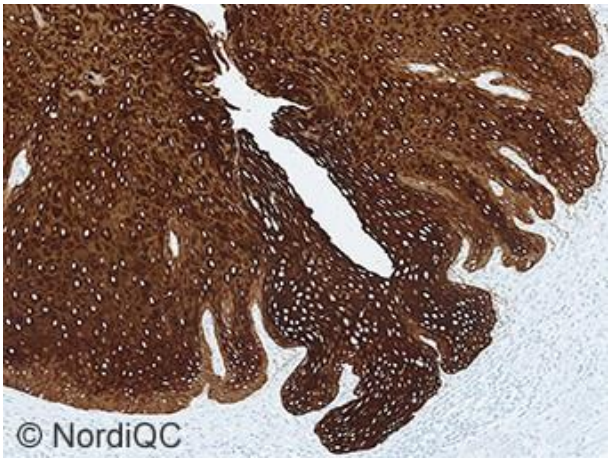


Fig. 1a
Optimal CK-PAN staining of esophagus using the Ventana Ready-To-Use format 760-2595, mAb clone cocktail AE1/AE3/PCK26, with HIER in CC1 pH 8,5 for 32 min followed by proteolysis in Protease 3 for 4 min (Ventana) and a 2-step multimer based detection kit (UltraView).
All squamous epithelial cells show a strong, distinct cytoplasmic staining recation.
No background reaction is seen
Compare with Figs. 2a-5a, same protocol.

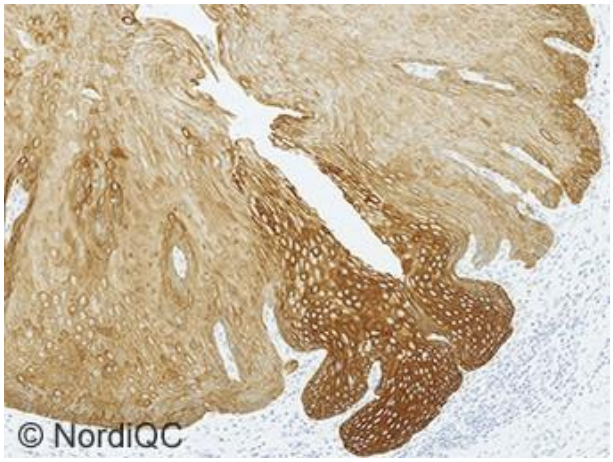


Fig. 1b
CK-PAN staining of esophagus using an insufficient protocol based on the mAb clone MNF116 with HIER in an alkaline buffer pH 9 and a 3-step polymer based detection kit (Refine) – same field as in Fig. 1a.
The majority of squamous epithelial cells are demonstrated, but the intensity is reduced compared to the level expected.
The protocol was capable to demonstrate CK-HMW in normal and neoplastic cells, but inadequate for CK-LMW.
Compare with Figs. 2b-5b, same protocol.

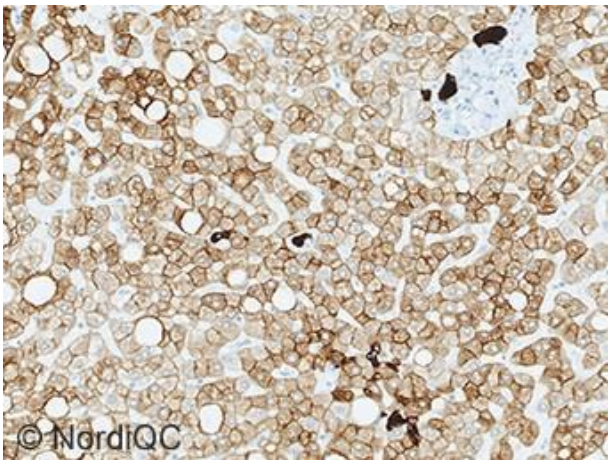


Fig. 2a
Optimal CK-PAN staining of liver using same protocol as in Fig. 1a.
The vast majority of hepatocytes show a distinct, moderate staining reaction with membrane enhancement, while the columnar epithelial cells of bile ducts show a strong cytoplasmic staining reaction.

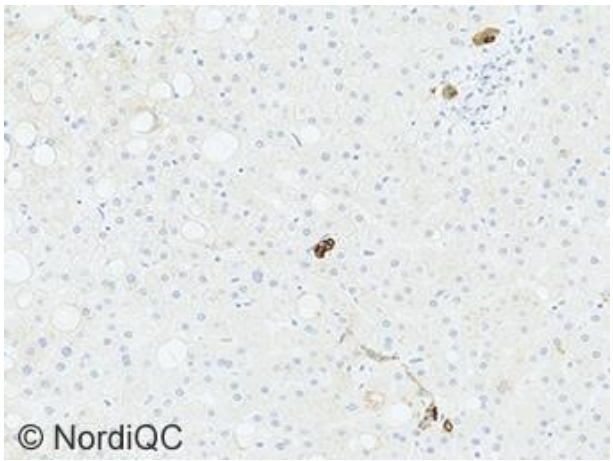


Fig. 2b
Insufficient staining for CK-PAN of liver
same protocol as in Fig. 1b – same field as in Fig. 2a.
Only epithelial cells of bile ducts with high level CK-LMW expression are demonstrated, while hepatocytes with low level CK-LMW expression (type 8 and 18) are unstained and thus false negative.

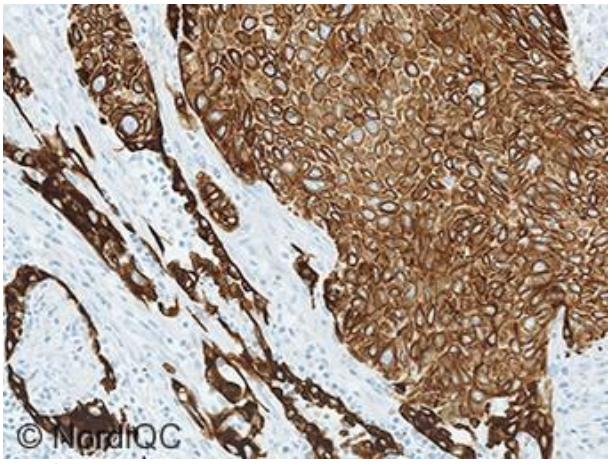


Fig. 3a
Optimal CK-PAN staining of the lung squamous cell carcinoma using same protocol as in Figs. 1a and 2a.
Virtually all neoplastic cells show a moderate and distinct cytoplasmic staining reaction.
Remnants of lung columnar epithelial cells – bottom left – show a strong staining reaction.

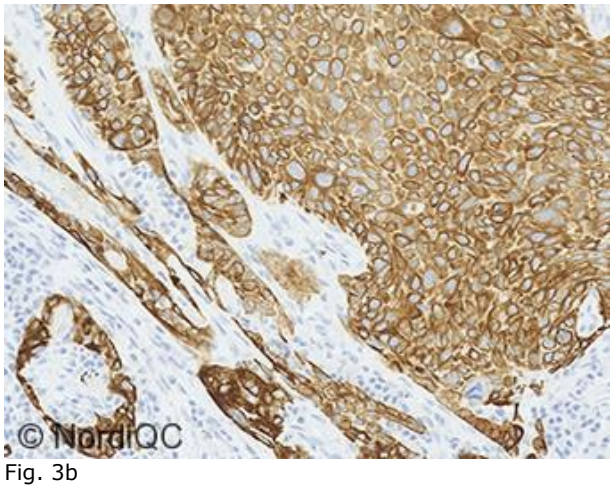


Fig. 3b
CK-PAN staining of the lung squamous cells carcinoma using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
Virtually all squamous epithelial cells are demonstrated, and the intensity is slightly reduced compared to the level expected. The protocol is useful for CK-HMW, however also compare with Figs. 4b and 5b, where same protocol gives a false negative result in neoplasias expressing CK-LMW.

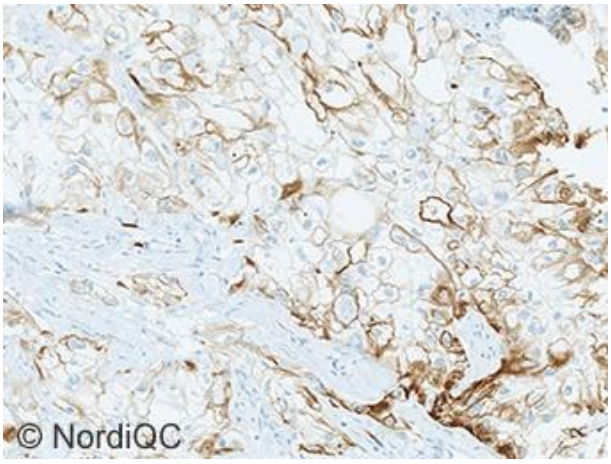


Fig. 4a
Optimal CK-PAN staining of the renal clear cell carcinoma using same protocol as in Figs. 1a-3a.
The majority of neoplastic cells show a weak but distinct predominantly membranous staining reaction.

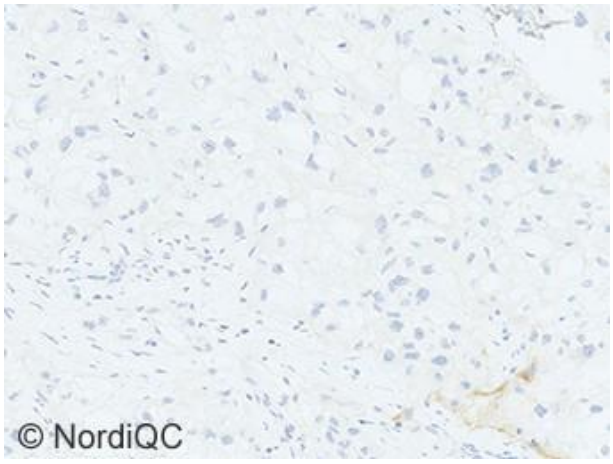


Fig. 4b
Insufficient CK-PAN staining of the renal clear cell carcinoma using same protocol as in Figs. 1b-3b – same field as in Fig. 4a.
Virtually no staining reaction is seen.

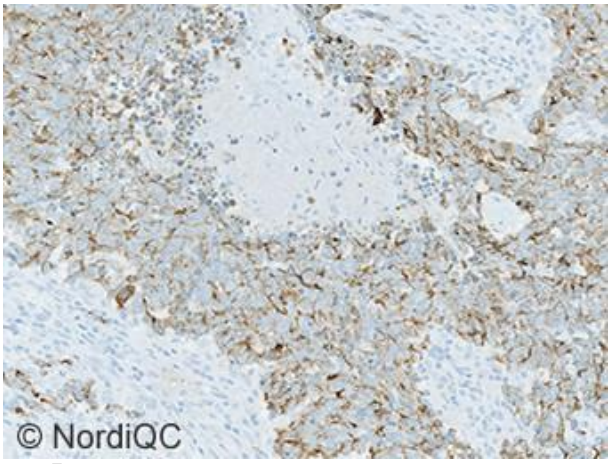


Fig. 5a
Optimal CK-PAN staining of the SCLC using same protocol as in Figs. 1a-4a.
The majority of neoplastic cells show a weak but distinct cytoplasmic staining reaction with a dot-like accentuation.

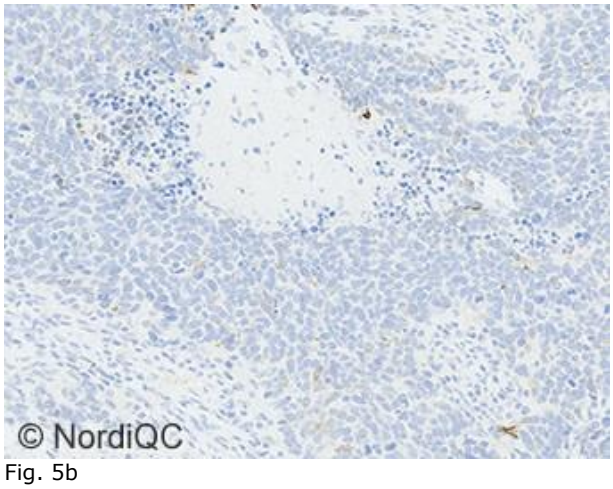


Fig. 5b
Insufficient CK-PAN staining of the SCLC using same protocol as in Figs. 1b-4b – same field as in Fig. 4a.
Only few cells show a weak equivocal staining reaction.

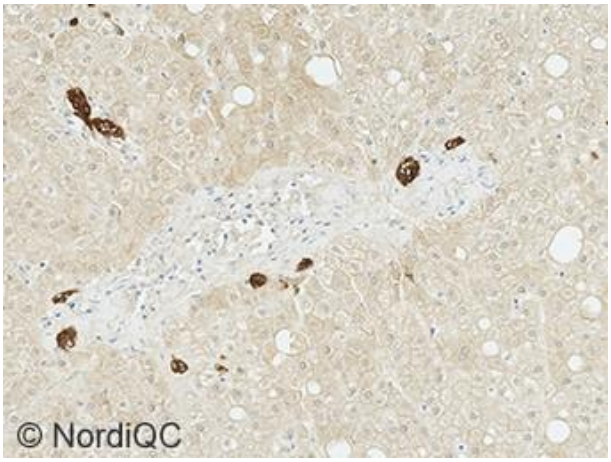


Fig. 6a
Insufficient CK-PAN staining of liver the mAb clone cocktail AE1/AE3 as concentrate within a laboratory developed assay. The protocol was based on short efficient HIER in Citrate pH 6 (10 min at 96°C and a 2-step polymer based detection system.
Insufficient HIER in combination with a less sensitive detection system provided an overall reduced technical sensitivity and a poor signal-to-noise ratio compromising the interpretation. Hepatocytes only show a weak and diffuse staining reaction.
Also compare with Fig. 6b, same protocol.

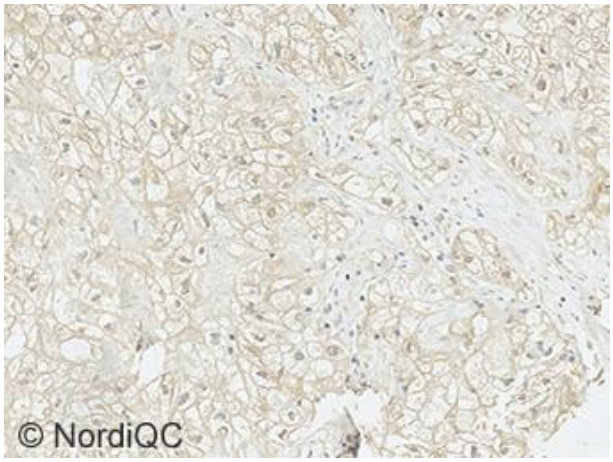


Fig. 6b
Insufficient CK-PAN staining of the renal clear cell carcinoma using same protocol as in Fig. 6a.
A poor signal-to-noise ratio is seen and both nuclei and the membranes of neoplastic cells show a weak staining reaction.

SN/LE/MV/RR 10.06.2016