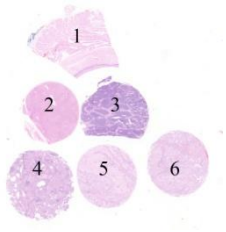
Assessment Run 41 2014

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. Lung adenocarcinoma, 5-6. Renal clear cell carcinoma (RCC).

All tissues were fixed in 10% neutral buffered formalin.

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 squamous epithelial cells throughout all cell layers in the esophagus
- A strong, distinct cytoplasmic staining reaction in the majority of the neoplastic cells in the lung adenocarcinoma.
- An at least moderate, distinct cytoplasmic, dot-like staining reaction in the majority of the neoplastic cells of the SCLC.
- An at least weak to moderate, distinct cytoplasmic staining reaction in the majority of the neoplastic cells of the RCC no. 5 and at least focally in the RCC no. 6.

Participation

Number of laboratories registered for CK-PAN, run 41	250
Number of laboratories returning slides	236 (94%)

Results
Of the 236 participating laboratories, 3 protocols were based on inappropriate antibodies (towards either high- or low-molecular weight CK). 67% (n=155) of the remaining participants 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 were:
- Inappropriate epitope retrieval
- Too low concentration of the primary antibody
- Less successful primary antibodies.

Performance history
This was the 7th NordiQC assessment of CK-PAN. The overall pass rate has remained almost constant in the last 5 runs performed, as shown in table 2.

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

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

Conclusion
The mAb clone cocktails **AE1/AE3** and **AE1/AE3/PCK26** can both 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 system from Dako based on the mAb clone cocktail AE1/AE3 were in this assessment most successful and provided a pass rate of 100%.
Liver and esophagus combined 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 41**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone cocktail **AE1/AE3**	76	Dako	30	26	13	7	74%	
	15	Thermo/NeoMarkers	3	8	3	1	73%	
	4	Leica/Novocastra	1	0	0	3		
	3	Cell Marque	1	1	1	0		
	2	Biocare	0	1	1	0		
	1	Biogenex	0	0	1	0		
	1	Diagnostic BioSystems	1	0	0	0		
	1	Genemed	0	0	0	1		
	1	ID labs	1	0	0	0		
	1	Millipore	1	0	0	0		
	1	Monosan	0	1	0	0		
mAb clone cocktail **AE1/AE3/5D3**	7	Biocare	0	5	2	0	72%	-
mAb clone cocktail **PAN CK Ab-2**	2	Thermo/NeoMarkers	0	1	0	1	-	-
mAb clone **BS5**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **C-11**	1	Leica/Novocastra	0	0	1	0	-	-
mAb clone **KL1**	3 1	Beckman Coulter AbD Serotec	0	0	3	1	-	-
mAb clone **Lu-5**	1	Immunologic	0	0	0	1	-	-
mAb clone **MNF116**	17	Dako	1	0	2	14	6%	100%
mAb clone **OSCAR**	1	Covance	0	1	0	0	-	-
"Laboratory made" antibody cocktails								
mAb clone cocktail **AE1/AE3/DC10**	1	Leica/Novocastra	0	1	0	0		
mAb clone cocktail **AE1/AE3/5D3**	1	Milipore/Leica	0	0	1	0	-	-
mAb clone cocktail **AE1AE3/CAM5.2**	1	Dako/BD	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone cocktail **AE1/AE3 IR053**	32	Dako	27	5	0	0	100%	100%
mAb clone cocktail **AE1/AE3 GA053**	6	Dako	6	0	0	0	100%	100%
mAb clone cocktail **AE1/AE3 313M-10**	2	Cell Marque	1	0	1	0	-	-
mAb clone cocktail **AE1/AE3 PDM 072**	1	Diagnostic BioSystems	0	1	0	0	-	-
mAb clone cocktail **AE1/AE3 PA0909**	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone cocktail **AE1/AE3 RTU-AE1/AE3**	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone cocktail **AE1/AE3/5D3 IP162**	1	Biocare	1	0	0	0	-	-
mAb clone cocktail **AE1/AE3/PCK26 760-2135/2595**	44	Ventana	24	4	1	15	64%	96%
mAb clone **KL1 PMD 073**	1	Diagnostic Biosystems	0	0	1	0	-	-
mAb clone **OSCAR MAD-000641QD**	1	Master Diagnostica	0	0	1	0	-	-
Total	233		100	55	32	46	-	
Proportion			43%	24%	14%	19%	67%	

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 41

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) (6/12)*, TRS pH 9 (Dako) (5/11), Cell Conditioning 1 (CC1; Ventana) (22/49), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (1/9) or Tris-EDTA/EGTA pH 9 (4/11) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:150 depending on the total sensitivity of the protocol employed. Using these protocol settings 62 of 71 (87%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **BS5**: One protocol with an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 as retrieval buffer. The dilution factor was 1:200 using a 2-step polymer based detection system (Nordic Biosite KDB-1007).

mAb clone **MNF116**: One protocol with an optimal result was based on enzymatic pre-treatment in Protease 1 (1/2) (Ventana) for 12 min. The dilution factor was 1:100 using a 3-step multimer based detection system (OptiView, Ventana).

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**	9/15** (60%)	-	21/37 (57%)	-	1/5 (20%)	0/2

* 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 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 30 of 30 (100%) 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 30 min. at 97°C) and 9-12.5 min. incubation of the primary Ab and EnVision FLEX (GV800/GV823) as detection system. Using these protocol settings 6 of 6 (100%) laboratories produced an optimal staining result.

mAb clone cocktail **AE1/AE3/5D3**, product no. **IP162**, Biocare, IntelliPath:
One protocol with an optimal result was based on HIER using Citrate pH 6 in a pressure cooker and MACH4 (M4U534) as detection system.

mAb clone cocktail **AE1/AE3/PCK26,** product no. **760-2135/2595**, Ventana, BenchMark XT/Ultra:
Protocols with optimal results were typically based on a combined pre-treatment using HIER (mild CC1) followed by 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 26 of 27 (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 two renal cell carcinomas (especially tissue core no. 6) was more difficult, and only seen when protocols with a high sensitivity and appropriate protocol settings were applied. The pass rate was highly influenced by the retrieval method, which underlines the necessity for individual optimization for each clone/clone cocktail used for the demonstration of CK. This correlation has been observed in the last six NordiQC CK-PAN assessments, summarized in table 4.

Table 4. **Pass rates for antibody cocktails and epitope retrieval methods in six NordiQC runs (combined)**

Pass rate for run 15, 20, 24, 30, 36 & 41								
	Total		HIER		Proteolysis		HIER + proteolysis	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
mAb AE1/AE3	569	408 (72%)	533	404 (76%)	35	4 (11%)	1	0
mAb AE1/AE3/5D3	30	27 (90%)	29	27 (93%)	1	0	0	0
mAb AE1/AE3/PCK26	114	60 (53%)	15	6 (40%)	30	0	69	54 (78%)
mAb MNF116	84	30 (36%)	38	9 (24%)	43	21 (49%)	3	2 (67%)

These data clearly supports that the choice of epitope retrieval has a significant impact on the performance of the antibody applied. For the most widely used clone cocktail AE1/AE3, the over-all pass rate in these 6 successive NordiQC runs was 72%. Using HIER, a pass rate of 76% 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 in Protease 3 (Ventana), provided a pass rate of 78%, compared to 40% and 0% using either HIER or proteolysis as single retrieval method, respectively.
The mAb clone MNF116 showed a higher pass rate when used with proteolytic pre-treatment compared to HIER. However, using proteolysis as pre-treatment the cumulated pass rate of 49% in the 6 runs performed is lower than the level obtained for the three other clones (cocktails) listed in table 4. In this context it also has to be emphasized that antibodies requiring HIER as pre-treatment as opposed to proteolytic pretreatment in general should be preferred, as efficient HIER provides consistent IHC results irrespective of the formalin fixation time, while proteolytic pre-treatment must be adjusted to the formalin fixation time for optimal performance.

In this run, the proportion of optimal results was unaffected by the choice of detection system, whereas the titre of the primary Ab had a high impact on the final results. 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) for optimal results was 1:90 (range 1:40 – 1:200, n=38 protocols), whereas an MDV of 1:220 (range 1:50 – 1:800, n=26 protocols) was seen for protocols with insufficient results (borderline and poor).

The Dako Ready-To-Use (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 100% was obtained and 87% were optimal. Optimal results were typically obtained by using the official protocol recommendations. Laboratory modified protocol settings (typically adjusting HIER, incubation time of the primary Ab and/or choice of detection system) could also provide 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 64% and 55% were optimal. Using optimal protocol settings a pass rate of 96% was obtained. Optimal results were obtained by modified and laboratory validated protocol settings typically using combined pre-treatment of HIER in mild CC1 followed by proteolysis in P3, 4-8 min. No sufficient results were obtained by using the official recommendations given in the package insert for the Ventana RTU format (proteolysis in P1 as single pre-treatment). The superior performance of the RTU format using the above mentioned modified protocol settings has been published by Ventana in a customer newsletter (Bioperspectives) in 2007, but yet no update of the official package insert has been effectuated.

The overall pass rate of 67% is still at a relatively low level and as seen in table 2, page 1 virtually no improvement has been achieved in the latest runs for CK-PAN.
A small difference in pass rates was observed for the laboratories participating in the CK-PAN assessment for the first time compared to the laboratories also participating in the latest assessments. For new laboratories the pass rate was 57%, whereas the pass rate was 69% for the laboratories participating in the latest runs.
The persistent use of less successful antibodies (e.g. mAb clone MNF116) and inappropriate epitope retrieval applied for the mAb clone cocktails AE1/AE3 and AE1/AE3/PCK26 have a high impact on the low pass rates seen.
At present the official data sheets for the most commonly used Abs towards CK-PAN from many vendors are misleading or imprecise regarding information on the epitope retrieval and protocol set-up. As an example, the general protocol for the mAb clone cocktail AE1/AE3 sold as a concentrate (Dako) is based on proteolytic pre-treatment, while HIER is recommended when the clone cocktail is sold as a RTU format from the same vendor. Thermo Scientific recommends protelytic pre-treatment for AE1/AE3 when applied with UltraVision LP as detection kit, but HIER using UltraVision Quanto as detection system. Leica Biosystems recommends HIER for the concentrated format of AE1/AE3 and proteolysis for the corresponding RTU format. These inconsistent guidelines from most vendors probably contribute to maintain the pass rate of CK-PAN at a low level.

Controls

As seen in the previous NordiQC assessments of CK-PAN, liver and esophagus in combination are recommendable as positive tissue controls. It is crucial that the majority of the 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 the esophagus virtually all squamous epithelial cells throughout all cell layers must show a strong distinct cytoplasmic staining reaction due to the expression of HMW cytokeratins. No staining should be seen in the stromal cells in the liver. Smooth muscle cells in vessels and in muscularisa mucosae in esophagus will typically show a weak to moderate patchy cytoplasmic staining reaction.

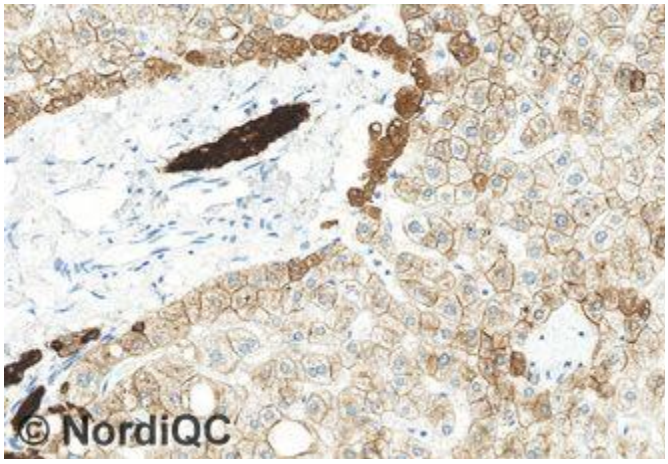


Fig. 1a
Optimal CK-PAN staining of the liver using the Dako Ready-To-Use format IR053 of the mAb clone cocktail AE1/AE3 with HIER in an alkaline buffer (TRS high pH) and a 2-step polymer based detection kit (EnVision FLEX). The majority of hepatocytes show a distinct, moderate staining reaction with membrane enhancement, while the columnar epithelial cells of bile ducts show a strong cytoplasmic staining reaction. Compare with Figs. 2a-4a, same protocol.

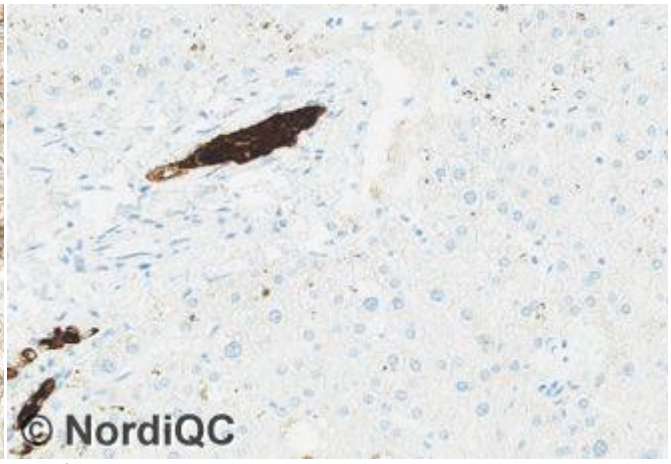


Fig. 1b
Insufficient CK-PAN staining of the liver, using the Ventana Ready-To-Use format of the mAb clone cocktail AE1/AE3/PCK26, Ventana, with proteolytic pre-treatment (Protease 1) and a 3-step multimer based detection kit (OptiView) – same field as in Fig. 1a. Only epithelial cells of bile ducts are demonstrated, while hepatocytes are unstained and thus false negative. Compare with Figs. 2b-4b, same protocol.

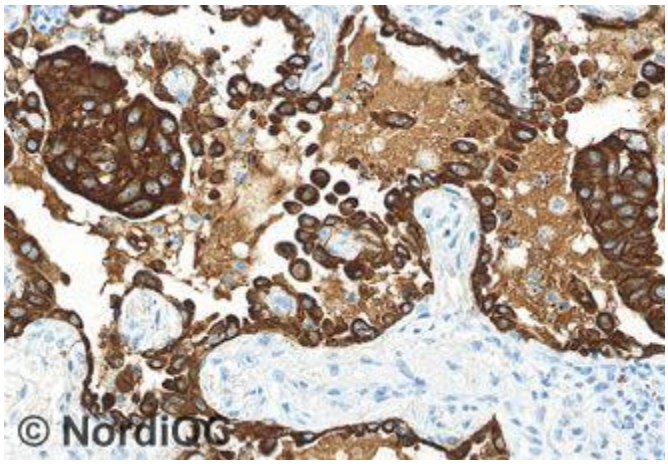


Fig. 2a
Optimal CK-PAN staining of the lung adenocarcinoma carcinoma using same protocol as in Fig. 1a. Virtually all neoplastic cells show a moderate to strong cytoplasmic staining reaction. No background staining is seen.

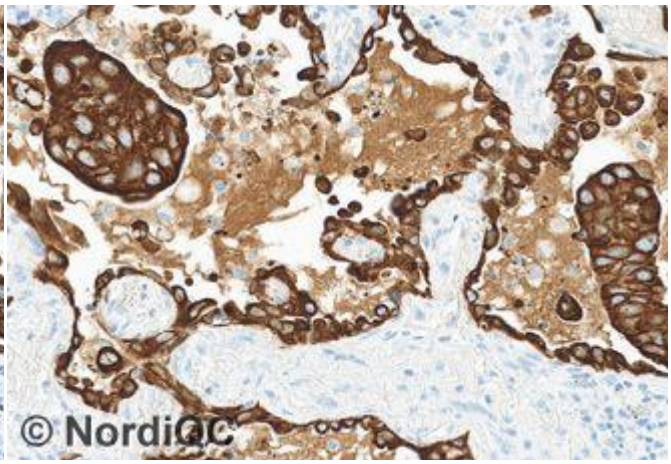


Fig. 2b
Staining for CK-PAN of the lung adenocarcinoma using same insufficient protocol as in Fig. 1b – same field as in Fig. 2a. The neoplastic cells of the lung adenocarcinoma are distinctively demonstrated. However also compare with Figs. 3b & 4b, same protocol.

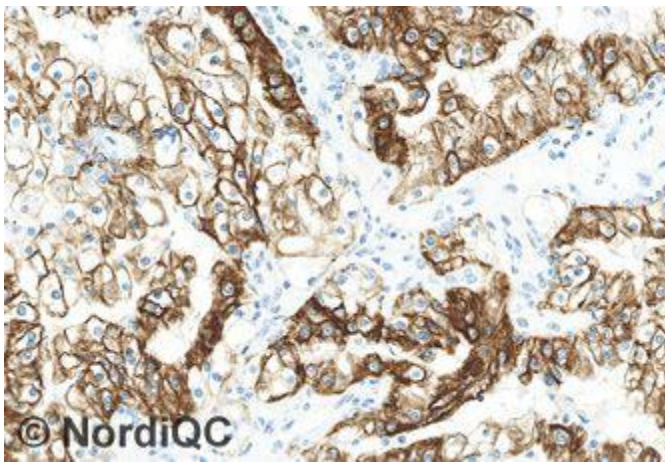


Fig. 3a
Optimal CK-PAN staining of the renal cell carcinoma tissue core no. 5 using same protocol as in Figs. 1a and 2a. The vast majority of the neoplastic cells show a weak to moderate and distinct predominantly membranous staining reaction.

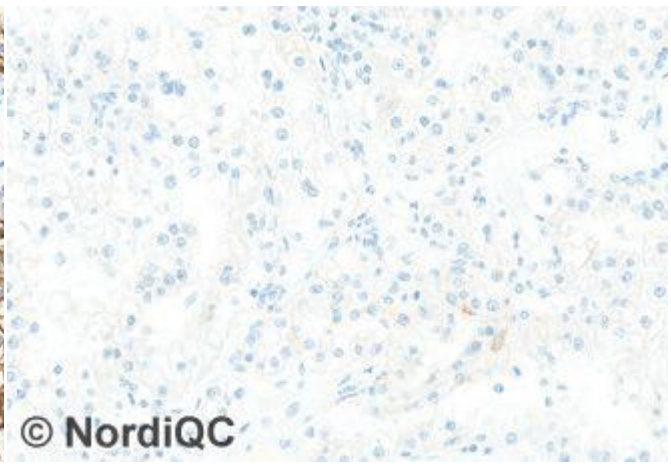


Fig. 3b
Insufficient CK-PAN staining of the renal cell carcinoma tissue core no. 5 using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.Virtually no staining reaction is seen.

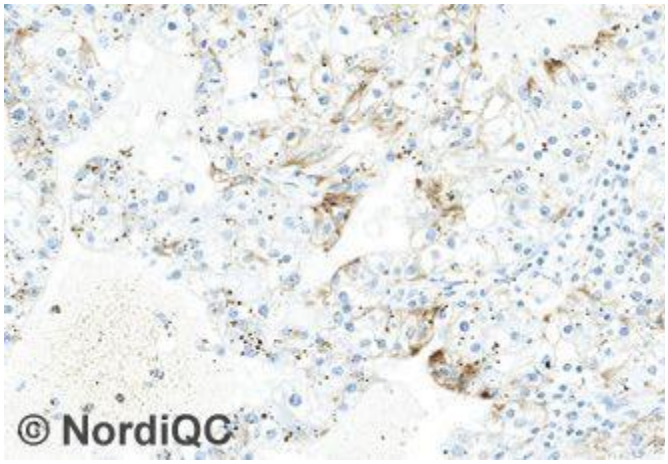


Fig. 4a
Optimal CK-PAN staining of the renal cell carcinoma tissue core no. 6 using same protocol as in Figs. 1a-3a. Focally the neoplastic cells show a weak but distinct predominantly membranous staining reaction.

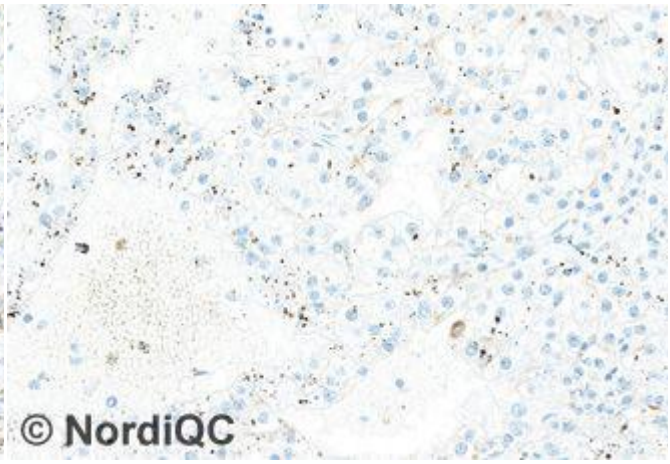


Fig. 4b
Insufficient CK-PAN staining of the renal cell carcinoma tissue core no. 5 using same protocol as in Figs. 1b-3b – same field as in Fig. 4a. Virtually no staining reaction is seen.

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