Assessment Run 30 2010

Cytokeratin, pan (CK-Pan)

The slide to be stained for CK-Pan comprised:

1. Liver, 2. Esophagus, 3. Renal cell carcinoma, 4. Lung adenocarcinoma, 5. Small cell lung carcinoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CK-Pan staining as optimal included:

- A strong, distinct cytoplasmic reaction of virtually all the bile ductal epithelial cells, and at least a moderate cytoplasmic reaction with membrane accentuation of the large majority of hepatocytes.
- A strong, distinct cytoplasmic reaction of the squamous epithelial cells throughout all cell layers in the esophagus (a weak reaction in the basal cells was accepted with the mAb clone KL1).
- A strong, distinct cytoplasmic reaction in the majority of the neoplastic cells of the lung adenocarcinomas.
- An at least moderate, distinct cytoplasmic reaction in the majority of the neoplastic cells of the renal cell carcinoma and the lung small cell carcinoma.

168 laboratories participated in this assessment. 65 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for CK-PAN, run 30**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone cocktail **AE1/AE3**	62 10 6 3 2 1 1 1 1	Dako NeoMarkers Leica/Novocastra BioGenex Millipore BioCare ID labs Monosan Zytomed	34	29	18	6	72 %	85 %
mAb clone **MNF116**	14	Dako	0	4	4	6	29 %	-
mAb clone **KL1**	7 1	Beckman Coulter AbD-Serotec	1	5	2	0	75 %	100 %
mAb clone cocktail **AE1/AE3/5D3**	4	BioCare	2	2	0	0	-	-
mAb clone cocktail **5D3/LP34**	1 1	Master Diagostica Monosan	0	1	0	1	-	-
mAb clone cocktail **MNF116/DC10/ AE1AE3/CAM5.2**	1	Dako/BD (home-made cocktail)	1	0	0	0	-	-
mAb clone **Lu-5**	2 1	NeoMarkers BMA Biomedicals	0	0	2	1	-	-
mAb clone **C-11**	1 1	Neomarkers Leica/Novocastra	0	1	1	0	-	-
mAb clone cocktail **PAN CK Ab-2**	2	NeoMarkers	0	0	0	2	-	-
mAb clone cocktail **MNF116/LP34**	1	Dako/Monosan (home-made cocktail)	0	0	0	1	-	-
pAb **Z0622**	2	Dako	0	0	1	1	-	-
Ready-To-Use Abs								
mAb clone cocktail **AE1/AE3/PCK26, 760-2595 & 760-2135**	19	Ventana	8	4	6	1	63 %	100 %

mAb, clone cocktail **AE1/AE3 IR053**	14	Dako	11	2	0	1	93 %	100 %
mAb, clone cocktail **AE1/AE3, 313M-18**	2	Cell Marque	1	1	0	0	-	-
mAb, clone cocktail **AE1/AE3, PA0909**	2	Leica/Novocastra	0	0	2	0	-	-
mAb clone cocktail **AE1/AE3 PM011**	1	BioCare	0	1	0	0	-	-
mAb clone cocktail **AE1/AE3 E006**	1	Linaris	0	0	1	0	-	-
mAb clone cocktail **AE1/AE3+Ks13.2 E020**	1	Linaris	0	0	1	0	-	-
mAb clone cocktail **AE1/AE3+5D3 PM162**	1	BioCare	0	0	1	0	-	-
mAb clone **MNF116, N1523**	1	Dako	0	0	0	1	-	-
Total	168		58	50	39	21	-	-
Proportion			35 %	30 %	23 %	12 %	65 %	-

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

Following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs
mAb clone cocktail **AE1/AE3**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Tris-EDTA/EGTA pH 9 (9/18)*, Target Retrieval Solution (TRS) pH 9 (Dako) (5/9), TRS pH 9 (3-in-1,Dako) (4/9), Cell Conditioning 1 (BenchMark, Ventana) (8/26), Bond Epitope Retrieval Solution 1 (Bond, Leica) (1/2), Diva Decloaker (Biocare) (2/3), EDTA/EGTA pH8 (1/1) or Citrate pH 6 (4/10) as the 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 56 out of 66 (85 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone cocktail **AE1/AE3/5D3**: The protocols giving an optimal result were all based on HIER using either Tris-EDTA/EGTA pH 9 (1/1) or TRS pH 9 (3-in-1, Dako) (1/1) as the retrieval buffer. The mAb was typically diluted in the range of 1:200– 1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings both of 2 laboratories produced a sufficient staining (optimal or good).

mAb clone "home-made" cocktail **MNF116/DC10/AE1AE3/CAM5.2**: The protocol giving an optimal result was based on HIER using Cell Conditioning 1 (BenchMark, Ventana) (1/1) as the retrieval buffer. The dilutions of the mAbs is unknown.

mAb clone **KL1**: The protocol giving an optimal result was based on HIER using TRS pH 9 (3-in-1, Dako) (1/3) as the retrieval buffer. The mAb was diluted 1:20.

Ready-To-Use Abs
mAb clone cocktail **AE1/AE3/PCK26** (prod. no. 760-2595 & 760-2135, Ventana): The protocols giving an optimal result were based on a combined proteolysis and HIER using Protease 3 for 4 min and mild Cell Conditioning 1, an incubation time of 8-32 min in the primary Ab and UltraView (760-500) as the detection system. 1 lab used amplification. Using these protocol settings 9 out of 9 (100 %) laboratories produced a sufficient staining (optimal or good).

mAb, clone cocktail **AE1/AE3** (prod. no. IR053, Dako): The protocols giving an optimal result were all based on HIER in PT-Link using TRS pH 9 or TRS pH 9 (3-in-1) and an incubation time of 10-30 min in the primary Ab and EnVision Flex (K8000) or Flex+ (K8002) as the detection system. Using these protocol settings 12 out of 12 (100 %) laboratories produced a sufficient staining (optimal or good).
1 laboratory used HIER in standard Cell Conditioning 1 (Benchmark, Ventana), an incubation time of 32 min. in the primary Ab and UltraView (760-500) as the detection system.

mAb, clone cocktail **AE1/AE3** (prod. no. 313M-18, Cell Marque): The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) and an incubation time of 15 min in the primary Ab and BOND Polymer Refine Detection (DS9800) as the detection system. Using this protocol setting 1 out of 1

laboratory produced an optimal staining.

The most frequent causes of insufficient staining reactions were:

- Inappropriate epitope retrieval (e.g., proteolysis for the mAb clone AE1/AE3)
- Too low concentration of the primary antibody
- Less successful primary Ab.

In this assessment and in concordance with the previous CK-Pan assessments in NordiQC the prevalent feature of an insufficient staining was a too weak or false negative reaction of the cells and structures expected to be demonstrated. The majority of the laboratories were able to demonstrate CK in the columnar epithelial cells of the bile ducts, the neoplastic cells of the lung adenocarcinoma and the lung small cell carcinoma. However, the demonstration of CK in the neoplastic cells of the renal cell carcinoma and the hepatocytes was more difficult and only seen for protocols with a high sensitivity and appropriate protocol settings, e.g. as obtained with a correct titre of the mAb clone cocktails AE1/AE3 and AE1/AE3/5D3 combined with efficient HIER. This is illustrated in table 2 where the cumulated data for the most widely used clones in the last four assessments for CK-Pan is listed relating the pass rate for the clone to the epitope retrieval method. E.g. the over-all pass rate for AE1/AE3 was 69%, but 74% when HIER was applied and 12% when protease was used.

Table 2. **Cumulated data for selected CK-PAN clones**

Pass rates for run 15, 20, 24 & 30								
	Total		**HIER**		**Proteolysis**		**HIER + proteolysis**	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
MAb AE1/AE3	294	203 (69%)	269	200 (74%)	25	3 (12%)	0	-
MAb MNF116	53	25 (46%)	31	7 (23%)	30	18 (60%)	2	2 (100%)
MAb KL1	33	21 (64%)	33	21 (64%)	0	0	0	-
MAb AE1/AE3/PCK26	39	12 (31%)	6	1 (17%)	20	0	13	12 (92%)
MAb AE1/AE3/5D3	16	15 (94%)	15	15 (100%)	1	0	0	-
MAb Ab2	10	6 (60%)	5	4 (80%)	5	2 (40%)	0	-
MAb 5D3/LP34	8	2 (25%)	7	2 (29%)	1	0	0	-

From this table is seems that the most robust marker for CK-Pan is the mAb clone cocktail AE1/AE3/5D3 used with HIER as all of 15 protocols based on this combination resulted in a sufficient staining result.
For the mAb cocktail AE1/AE3/PCK26 (Ready-To-Use format Ventana) 12/13 protocols (92%) based on a combined epitope retrieval of proteolysis and HIER gave a sufficient staining result (whereas 0/20 protocols based on proteolysis gave a sufficient staining result, all false negative). If HIER was performed as single pre-treatment an aberrant strong cytoplasmic staining reaction in virtually all smooth muscle cells was seen. Applying the combined pre-treatment using proteolysis and HIER, the staining reaction in the smooth muscle cells was significantly reduced and still giving an optimal result similar to the result obtained by using AE1/AE3, AE1/AE3/5D3 and KL1 with HIER.

As seen in the previous assessments of CK-Pan, liver and esophagus combined is recommendable as positive controls for CK-Pan: It is crucial that the majority of the hepatocytes (expressing only a limited amount of the primary low molecular weight CK types 8 and 18) show at least a moderate staining reaction. In the esophagus virtually all the squamous epithelial cells expressing the high molecular weight cytokeratins must show at least a moderate distinct cytoplasmic staining. In this context it has to be emphasized that the mAb clone KL1 only has a weak affinity for the primary high molecular weight CK types 5 & 14, which is expressed in the basal squamous epithelial cells and thus only show a weak staining even in protocols giving an otherwise optimal staining using the mAb clone KL1. Due to the weak staining reaction of the basal squamous epithelial cells in the esophagus, the Merkel cells was easily recognized using the mAb clone KL1 as these cells showed a strong cytoplasmic staining due to the CK types CK8, 18 and 19.

This was the 5th assessment of CK-Pan in NordiQC, and a slight but constant increase in the proportion of sufficient results has been obtained as as shown in table 3:

Table 3. **Proportion of sufficient results for CK-PAN in the five NordiQC runs performed**

	Run 8 2003	Run 15 2005	Run 20 2007	Run 24 2008	Run 30 2010
Participants, n=	72	85	103	123	168
Sufficient results	53 %	58 %	62 %	60 %	65 %

Conclusion

The mAb clone cocktails **AE1/AE3, AE1/AE3/5D3** and **AE1/AE3/PCK26**, and mAb clone **KL1** can all be used to obtain an optimal staining for CK-Pan. The epitope retrieval and protocol settings have to be specifically tailored to each of the clones/cocktails. Liver and esophagus combined are appropriate control tissues irrespective of the clone/cocktail applied: Almost all hepatocytes must show a distinct cytoplasmic staining with membrane enhancement, while virtually all the squamous epithelial cells of the esophagus must show at least a moderate cytoplasmic staining.

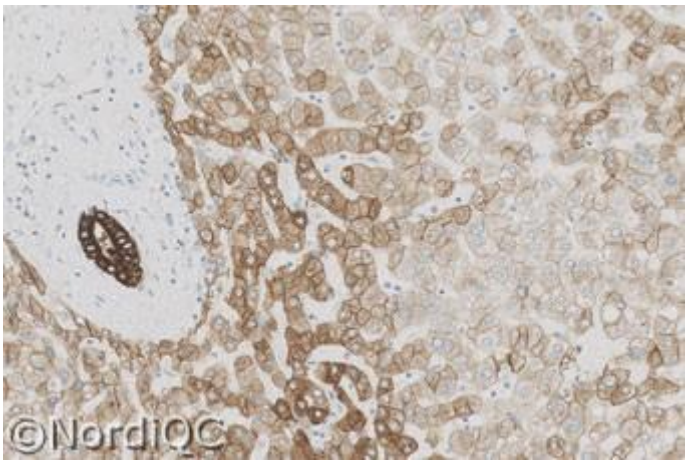

Fig. 1a
Optimal staining for CK-Pan of the liver based on HIER and the mAb clone cocktail AE1/AE3. The majority of the hepatocytes show a distinct, moderate to strong staining with membrane enhancement, while the columnar epithelial cells of the bile duct show a strong cytoplasmic staining. Compare with Figs. 2a-4a, same protocol.

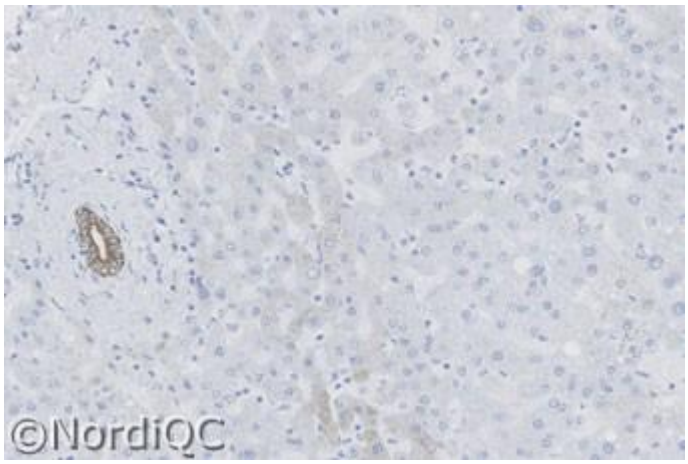

Fig. 1b
Insufficient CK-Pan staining of the liver, using an efficient HIER and Ab clone KL1 but applying the Ab in a too low concentration - same field as in Fig. 1a. Only the epithelial cells of the bile duct are demonstrated, while the hepatocytes are unstained. Compare with Figs. 2b-4b, same protocol.

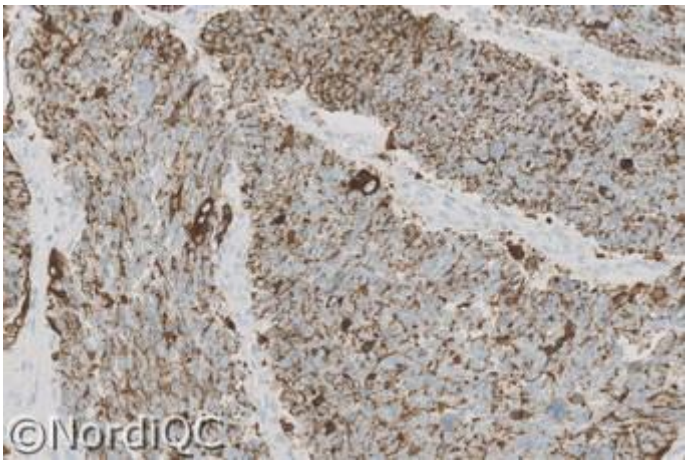

Fig. 2a
Optimal CK-pan staining of the small cell lung carcinoma using same protocol as in Figs. 1a, 3a and 4a.
The majority of the neoplastic cells show a moderate, distinct dot-like cytoplasmic staining.

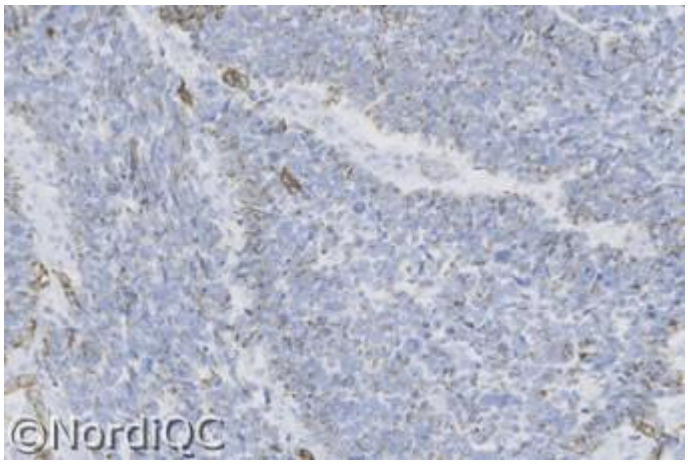

Fig. 2b
Insufficient CK-Pan staining of the small cell lung carcinoma using same protocol as in Figs. 1b, 3b and 4b - same field as in Fig. 2a. Only scattered neoplastic cells show a weak staining reaction. Also compare with Figs. 3b. & 4b., same protocol.

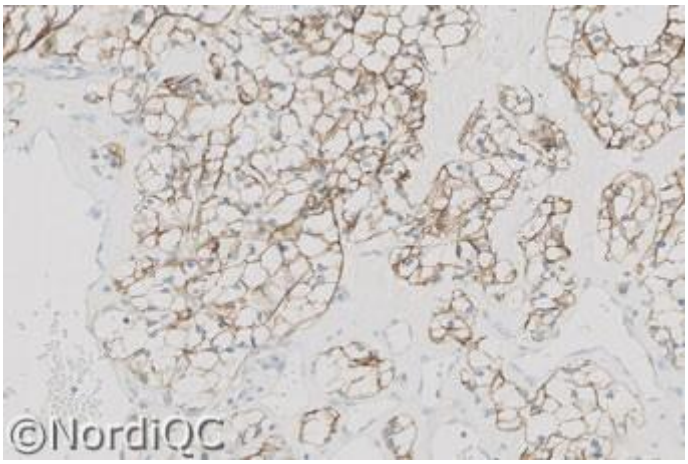

Fig. 3a
Optimal CK-Pan staining of the renal cell carcinoma using same protocol as in Figs. 1a, 2a and 4a. The majority of the neoplastic cells show a distinct predominantly membranous staining.

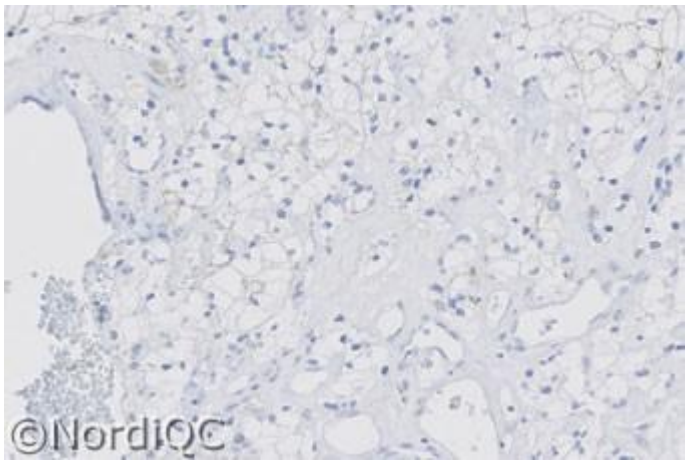

Fig. 3b
Insufficient CK-Pan staining of the renal cell carcinoma using same protocol as in Figs. 1b, 2b and 4b – same field as in Fig. 3a. No staining reaction is seen.

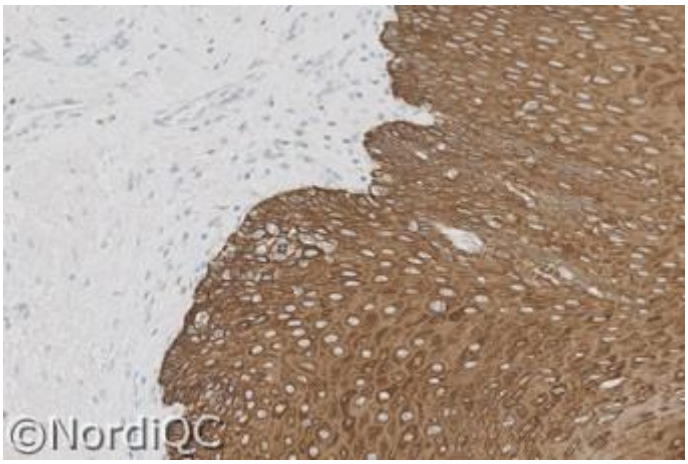

Fig. 4a
Optimal CK-Pan staining of the esophagus using same protocol as in Figs. 1a-3a. All the squamous epithelial cells throughout the entire epithelial layer are stained.

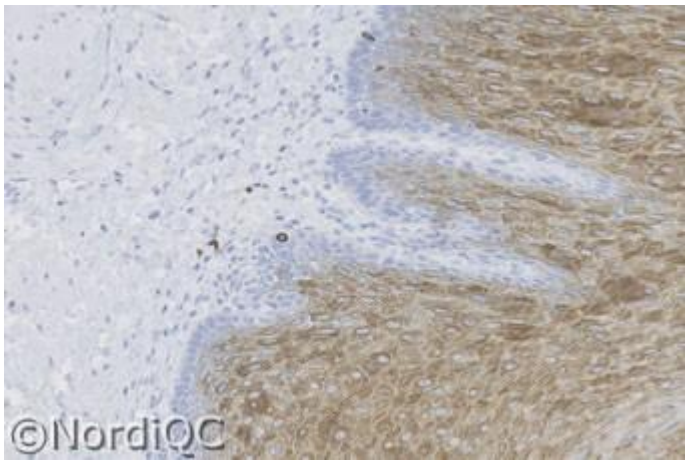

Fig. 4b
CK-Pan staining of the esophagus same insufficient protocol as in Figs. 1b-3b, same field as in Fig. 4a. The intermediate and superficial squamous epithelial cells only show a weak to moderate staining, while the basal cells are negative. Scattered Merkel cells in the basal layer show a strong cytoplasmic staining.

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