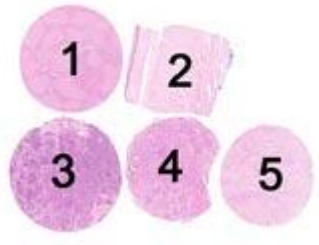
Assessment Run 36 2012

Cytokeratin, pan- (CK-PAN)

Material

The slide to be stained for CK-PAN comprised of:



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

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 virtually all the bile ductal epithelial cells and at least a moderate cytoplasmic staining reaction with membrane accentuation of the hepatocytes.
- A strong, distinct cytoplasmic staining 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 staining reaction in the majority of the neoplastic cells of the lung adenocarcinoma.
- An at least moderate, distinct cytoplasmic staining reaction in the majority of the neoplastic cells of the renal cell carcinoma and the small cell lung carcinoma.

204 laboratories participated in this assessment. Two labs used an inappropriate antibody. Of the remaining 202 labs, 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 36**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone cocktail **AE1/AE3**	73	Dako	32	19	8	14	70 %	70 %
	14	Thermo/NeoMarkers	2	5	3	4	50 %	100 %
	7	Leica/Novocastra	0	1	1	5	14 %	-
	2	Biocare	0	1	0	1	-	-
	2	Cell Marque	1	1	0	0	-	-
	2	Chemicon	0	1	1	0	-	-
	1	Biogenex	0	1	0	0	-	-
	1	ID Labs	0	1	0	0	-	-
	1	Progen	0	0	0	1	-	-
	1	Zytomed	0	1	0	0	-	-
mAb clone cocktail **AE1/AE3 + 5D3**	5	Biocare	2	3	0	0	100 %	100 %
mAb clone cocktail **AE1/AE3 + DC10**	1	Leica/Novocastra (home-made cocktail)	0	1	0	0	-	-
mAb clone **KL1**	5 1	Beckman Coulter AbD Serotec	3	2	0	1	83 %	100 %
mAb clone **Lu-5**	1 1	Immunologic BMA Biomedicals	0	0	0	2	-	-
mAb **MNF116**	13 1	Dako Abcam	0	4	3	7	29%	-
mAb cocktail **MNF116+DC10+ AE1/AE3+CAM5.2**	1	Dako/BD (home-made coctail)	1	0	0	0	-	-
mAb clone **OSCAR**	1	Covance	1	0	0	0	-	-
mAb clone cocktail **PAN CK Ab-2**	1	Thermo/NeoMarkers	0	0	1	0	-	-

Ready-To-Use Abs								
mAb clone cocktail **AE1/AE3 IR/IS053**	25	Dako	15	9	0	1	96 %	100 %
mAb clone cocktail **AE1/AE3 313M-17/18**	2	Cell Marque	1	1	0	0	-	-
mAb clone cocktail **AE1/AE3 PA0909**	2	Leica	0	0	0	2	-	-
mAb clone cocktail **AE1/AE3 N1590**	1	Dako	0	1	0	0	-	-
mAb clone cocktail **AE1/AE3 MAD-001000QC**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone cocktail **AE1/AE3 RTU-AE1AE3**	1	Novocastra	0	0	1	0	-	-
mAb clone cocktail **AE1/AE3+5D3 PM162AA**	1	Biocare	1	0	0	0	-	-
mAbcloe cocktail **AE1/AE3, Ks13.1 E020**	1	Linaris	0	0	0	1	-	-
mAb clone cocktail **AE1/AE3 & PCK26 760-2135/2595**	33	Ventana	17	2	2	12	58 %	100 %
Total	202		76	54	21	51	-	-
Proportion			38 %	27 %	10 %	25 %	65 %	-

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

Detailed analysis of PAN-CK, Run 36
Following protocol parameters were central to obtain an optimal staining:

Concentrated Abs
mAb clone cocktail **AE1/AE3** (Dako): The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution pH 9 (TRS pH 9; Dako) 3-in-1 (8/15)*, TRS pH 9 (Dako) (3/6), Cell Conditioning 1 (CC1; Ventana) (16/31) or Tris-EDTA/EGTA pH 9 (5/10) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings 47 of 61 (77 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this reagent)

mAb clone cocktail **AE1/AE3** (Thermo/NeoMarkers): The two protocols giving an optimal result were both based on HIER using either TRS pH 9, 3-in-1 (Dako) (1/1) or Bond Epitope Retrieval Solution 2 (BERS 2; Leica) (1/3) as the retrieval buffer. The mAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 2 of 2 (100 %) laboratories produced an optimal staining.

mAb clone cocktail **AE1/AE3** (Cell Marque): The protocol giving an optimal result was based on HIER using CC1 (Ventana). The mAb was diluted 1:50.

mAb clone cocktail **AE1/AE3+5D3** (Biocare): The two protocols giving an optimal result were both based on HIER using either TRS pH 9 (3-in-1) (Dako) (1/1) or BERS 2 (Leica) (1/1) as retrieval buffer.
The mAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 2 of 2 (100 %) laboratories produced an optimal staining.

mAb clone **KL1** (Beckman Coulter/Serotec): The protocols giving an optimal result were all based on HIER using either BERS 2 (Bond, Leica) (1/1) or CC1 (Ventana) (2/2) as retrieval buffer.
The mAb was diluted in the range of 1:15-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 of 3 (100 %) laboratories produced an optimal staining.

mAb clone **OSCAR** (Covance): The protocol giving an optimal result was based on HIER using CC1 (Ventana). The mAb was diluted 1:100.

Ready-To-Use Abs
mAb clone cocktail **AE1/AE3** (prod. no. IS/IR053, Dako): The protocols giving an optimal result were typically based on HIER in PT-Link (heating time for 10-20 min at 95-97°C) using TRS pH 9, 3-in-1 (Dako) or TRS pH 9 (Dako) as HIER buffer, 20 min incubation of the primary Ab and EnVision Flex or EnVision Flex+ (K8000/K8002) as detection system. Using these protocol settings 21 of 21 (100 %) laboratories produced a sufficient staining.

mAb clone cocktail **AE1/AE3 & PCK26** (prod. no. 760-2513, Ventana): The protocols giving an optimal result were typically based on a combined pre-treatment using HIER (mild CC1) followed by Protease 3 (4 min), 8-32 min incubation of the primary Ab and UltraView (760-500, Ventana) or OptiView (760-700, Ventana) as the detection system. Using these protocol settings 18 of 18 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient staining were:

- Inappropriate epitope retrieval - e.g., proteolysis as single pre-treatment for mAb clone cocktail AE1/AE3
- Impaired morphology due to excessive proteolysis for the mAb clone cocktail AE1/AE3/PCK26
- Too low concentration of primary antibody
- Less successful primary antibodies.

In concordance with previous NordiQC CK-PAN assessments, the prominent feature of an insufficient staining was a too weak or false negative staining reaction of cells and structures expected to be demonstrated. The majority of laboratories were able to stain CK in columnar epithelial cells of the bile ducts and neoplastic cells of the lung adenocarcinoma, whereas the demonstration of CK in neoplastic cells of the renal cell carcinoma, small cell lung carcinoma and hepatocytes was more difficult and only seen when using protocols with a high sensitivity and appropriate protocol settings. The pass rate was highly influenced by the choice of 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 five NordiQC CK-PAN assessments, summarized in table 2.

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

Pass rates for run 15, 20, 24, 30 & 36								
	Total		**HIER**		**Proteolysis**		**HIER + proteolysis**	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
MAb AE1/AE3	423	294 (70 %)	392	290 (74 %)	31	4 (13 %)	0	-
MAb MNF116	67	29 (43 %)	29	9 (31 %)	35	20 (57 %)	3	2 (67 %)
MAb KL1	39	26 (67 %)	39	26 (67 %)	0	-	0	-
MAb AE1/AE3/PCK26	72	33 (46 %)	8	2 (25 %)	25	0	39	31 (80 %)
MAb AE1/AE3/5D3	22	21 (95 %)	21	21 (100 %)	1	0	0	-

These data clearly indicate 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 was 70%, but only 13% when protease was used as pre-treatment. For another commonly used antibody cocktail, AE1/AE3/PCK26, epitope retrieval using HIER in CC1 (Ventana) followed by proteolysis, provided a pass rate of 80%, compared to 25% and 0% with either HIER or proteolysis, respectively, as single retrieval method.
In this context it has to be stressed, that the CK-PAN data sheets from many vendors give imprecise and misleading guidelines of epitope retrieval and protocol setup: The general protocol recommended by Dako, the most used vendor for the mAb clone cocktail AE1/AE3, is based on proteolytic pre-treatment when used as a concentrate, while HIER is recommended when the clone cocktail is used in a RTU format!
For the mAb AE1/AE3 clone cocktail, Thermo Scientific recommends protelytic pretreatment when using UltraVision LP as detection kit, but HIER when UltraVision Quanto is used with this Ab!
For the most widely used Ready-To-use product, the mAb clone cocktail AE1/AE3/PCK26 from Ventana, the recommended protocol is based on Protease 1 for 4 min, which consistently has produced insufficient stains, see table 2. For this product, HIER in mild CC1, followed by proteolysis in Protease 3 for 4-8 min. was much more robust (this observation has also been published by Ventana in the customer newsletter Bioperspectives in 2007). In this assessment, it was observed that a combined retrieval based on Protease 1 and mild CC1 impaired the

morphology due to excessive proteolysis. HIER based on short CC1 and Protease 3 produced a false negative staining result.

It was also observed that the mAb clone cocktail AE1/AE3 from different vendors gave deviating staining patterns, even though identical protocol settings were applied. All protocols (6 out of 6), based on HIER and the mAb clone cocktail AE1/AE3 concentrate from Leica/Novocastra gave false negative staining results and the overall staining reaction resembled the pattern seen by the use of proteolysis. If the mAb clone AE3 is used in combination with proteolytic pre-treatment or too low concentration the Ab, demonstration of the primary CK-LMW type 8 is difficult, compromising the sensitivity, giving false negative reactions in cells with low expression of CK, such as liver cells. The aberrant staining pattern of the Leica/Novocastra Ab might be caused by a too low concentration of the clone AE3 in the final product.

Controls
As seen in the previous NordiQC assessments of CK-PAN, liver and esophagus in combination are recommendable as positive 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 the squamous epithelial cells must show a strong distinct cytoplasmic staining due to the expression of HMW cytokeratins.

Effect of external quality assessment
This was the 6th assessment of CK-PAN in NordiQC (Table 3) The overall pass rate have remained almost constant in the last 4 runs performed.

Table 3. **Proportion of sufficient results for CK-PAN in the 6 NordiQC runs performed**

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

A significant 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 assessment run 30, 2010. For laboratories participating for the first time the pass rate was 40 % (19 out of 47 laboratories), whereas the pass rate was 72 % (111 out of 155 laboratories) for the laboratories participating in both runs.

Conclusion
The mAb clone cocktails **AE1/AE3, AE1/AE3/5D3** and **AE1/AE3/PCK26**, and the mAb clone **KL1** are all recommendable antibodies and 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 positive control tissues irrespective of the antibody applied. The vast majority of the hepatocytes must show a distinct cytoplasmic staining reaction with a membrane enhancement, while virtually all the squamous epithelial cells of the esophagus must show a strong cytoplasmic staining reaction.

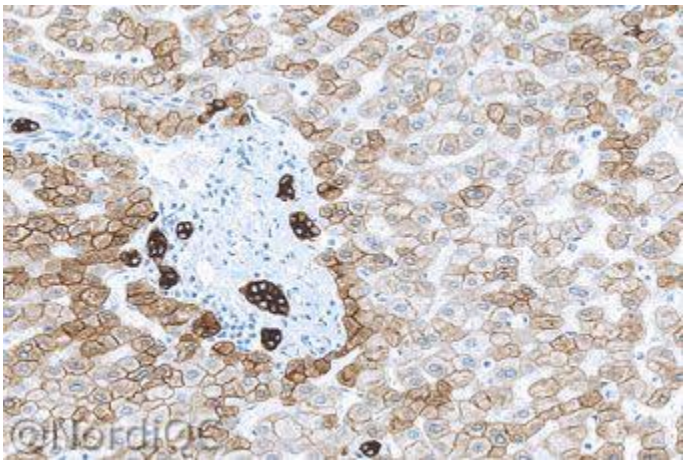


Fig. 1a
Optimal CK-PAN staining of the liver using the mAb clone cocktail AE1/AE3/PCK26, Ventana with a combined epitope retrieval method based on HIER in mild CC1 followed by Protease 3. The majority of the hepatocytes show a distinct, moderate to strong staining reaction with membrane enhancement, while the columnar epithelial cells of the bile ducts show a strong cytoplasmic staining reaction. Compare with Figs. 2a - 4a, same protocol.

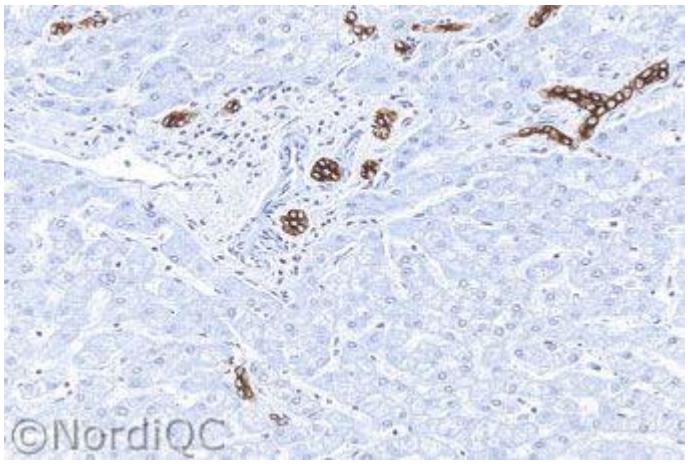


Fig. 1b
Insufficient CK-PAN staining of the liver, using the mAb clone cocktail AE1/AE3/PCK26, Ventana with proteolytic pre-treatment (Protease 1) as single retrieval method. Only the epithelial cells of the bile ducts are demonstrated, while the hepatocytes are unstained. Compare with Figs. 2b - 3b, same protocol.

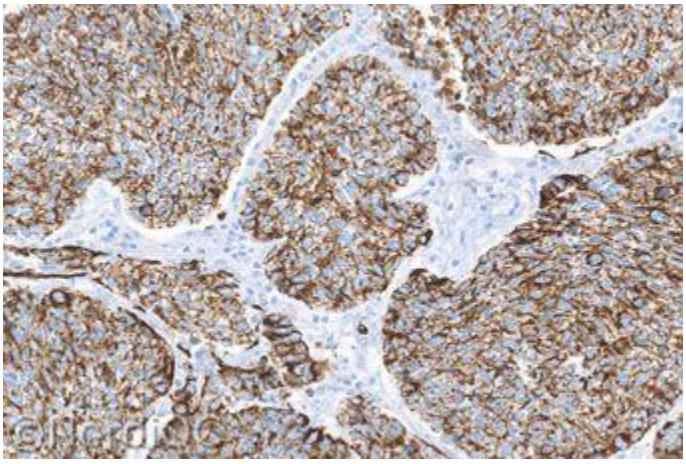


Fig. 2a
Optimal CK-PAN staining of the small cell lung carcinoma using same protocol as in Figs. 1a, 3a and 4a.
The majority of the neoplastic cells show a moderate to strong and distinct dot-like cytoplasmic staining reaction. No background staining is seen.

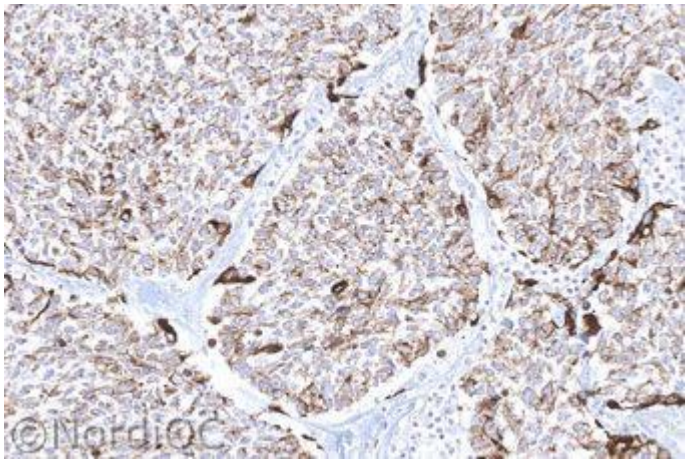


Fig. 2b
Staining for CK-PAN of the small cell lung carcinoma using same insufficient protocol as in Fig. 1b.
The intensity of the neoplastic cells demonstrated is reduced compared to the result shown in Fig. 2a. Remnants of the normal epithelial cells show a strong staining reaction. However, also compare with Fig. 3b., same protocol.

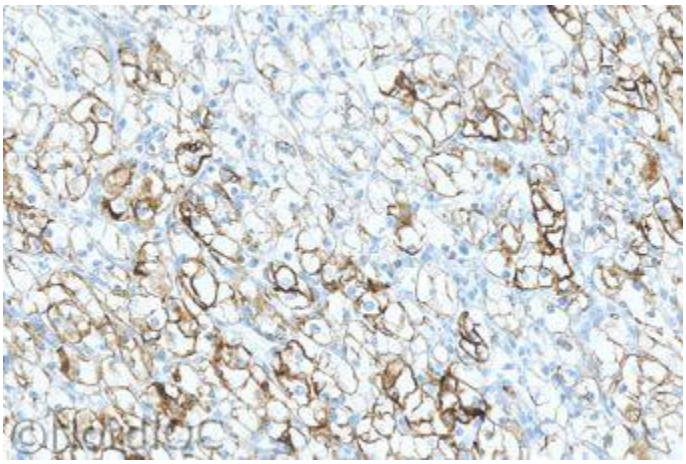


Fig. 3a
Optimal CK-PAN staining of the renal cell carcinoma using same protocol as in Figs. 1a, 2a and 4a. The majority of the neoplastic cells show a weak to moderate and distinct predominantly membranous staining reaction.

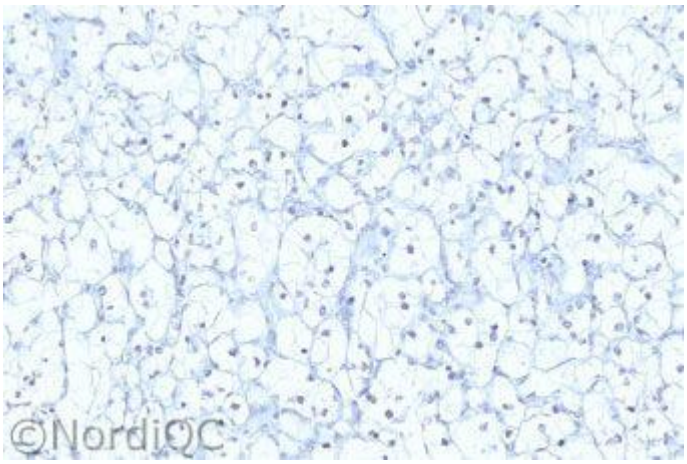


Fig. 3b.I
nsufficient CK-PAN staining of the renal cell carcinoma using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a. No staining reaction is seen.

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 show a strong distinct cytoplasmic staining reaction. No background staining is seen.

Fig. 4b
Insuffcient staining for CK-PAN using the mAb clone cocktail AE1/AE3/PCK26, Ventana with HIER in standard CC1 and Protease 1. Due to the excessive epitope retrieval the morphology is significantly impaired and the interpretation is compromised.

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