Assessment Run 33 2011

Cytokeratin, low molecular weight (CK-LMW)

Material
The slide to be stained for CK-LMW comprised:

1. Appendix, 2. Liver, 3. Esophagus, 4. Renal clear cell carcinoma,
5. Breast ductal carcinoma, 6. Small cell lung carcinoma,
7. Hepatocellular carcinoma.

All tissues were fixed in 10% neutral buffered formalin for 24 - 48 hours.

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

- A strong, distinct cytoplasmic reaction of virtually all the appendiceal columnar epithelial cells, the bile ductal epithelial cells and at least a weak to moderate, predominantly membranous staining reaction of the large majority of the hepatocytes.
- A moderate to strong, distinct cytoplasmic staining reaction of the majority of the neoplastic cells of the breast ductal carcinoma, the renal cell carcinoma and the hepatocellular carcinoma.
- An at least weak to moderate cytoplasmic and dot-like staining reaction in the majority of the neoplastic cells of the small cell lung carcinoma.
- A weak to moderate staining of the basal cells and scattered intermediate cells, when using an Ab reacting with CK8.

164 laboratories participated in this assessment. 23 labs used an inappropriate Ab like CK-Pan, CK7 or CK20. Out of the remaining 141 labs 64 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for CK-LMW, run 33**

Concentrated Abs	Reactivity	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5D3**	CK 8/18	19 5 2 1 1 1 1	Leica/Novocastra Thermo/NeoMarkers Biocare BioGenex Monosan Santa Cruz Vector	9	5	11	5	47 %	100 %
mAb clone **35ßH11**	CK 8	1 1	Dako DBS	0	0	1	1	-	-
mAb clones **B22.1** & **B23.1**	CK 8/18	3	Cell Marque	1	0	2	0	-	-
mAb clone **C51**	CK 18*	5	Invitrogen/Zymed	3	2	0	0	100 %	-
mAb clone **CAM 5.2**	CK 8 (7)	28	Becton Dickenson	0	12	10	6	43 %	-
mAb clone **DC10**	CK 18	23 5 1 1 1 1 1	Dako Novocastra BioGenex DBS ID Labs Thermo/NeoMarkers Zymed	20	11	2	0	94 %	96 %
mAb clone **TS1**	CK 8	6 2 1	Leica/Novocastra Thermo/NeoMarkers Gene Company	6	1	1	1	78 %	100 %
mAb clone **TS1** + mAb clone **DC10**	CK 8/18	1	Homemade cocktail: Thermo/NeoMarkers	1	0	0	0	-	-
rmAb clone **EP17**	CK 8	2	Epitomics	1	1	0	0	-	-
rmAb clone **EP1628Y**	CK 8	1	Epitomics	1	0	0	0	-	-

Ready-To-Use Abs									
mAb clone **5D3** **PA0067**	CK 8/18	2	Leica/Novocastra	1	0	0	1	-	-
mAb clone **5D3** **PM056**	CK 8/18	1	Biocare	0	0	1	0	-	-
mAb clone **35ßH11 760-2637**	CK 8	4	Ventana/Cell Marque	0	0	0	4	-	-
mAb clone **35ßH11 N1560**	CK 8	4	Dako	0	0	0	4	-	-
mAb clones **B22.1** & **B23.1 760-4344**	CK 8/18	7	Ventana/Cell Marque	2	5	0	0	100 %	100 %
mAb clone **CAM 5.2 84.005-11-08**	CK 8 (7)	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **DC10 IR618**	CK 18	8	Dako	6	2	0	0	100 %	100 %
Total		141		51	40	28	22	-	
Proportion				36 %	28 %	20 %	16 %	64 %	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below.
* Claimed by Invitrogen/Zymed to be CK8.

The following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs
mAb clone **5D3**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution pH 9 (3-in-1) (TRS;Dako) (3/4)*, TRS pH 9 (Dako) (2/5), Bond Epitope Retrieval Solution 2 (BERS2; Bond, Leica) (2/3), Tris-EDTA/EGTA pH 9 (1/2) or EDTA/EGTA pH 8 (1/1) as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 11 out of 11 (100 %) laboratories produced a sufficient staining (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mAb clones **B22.1** & **B23.1**: The protocol giving an optimal result was based on a combined pre-treatment by using HIER in Cell Conditioning 1 (CC1; BenchMark, Ventana) followed by enzymatic pre-treatment in Protease 3 (Ventana) a dilution of 1:200 of the primay Ab and UltraView + amplification as detection kit.

mAb clone **C51**: The protocols giving an optimal result were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (1/1) or TRS pH 9 (Dako) (2/2) as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 out of 3 (100 %) laboratories produced an optimal staining.

mAb clone **DC10**: The protocols giving an optimal result were mostly based on HIER using either CC1 (BenchMark, Ventana) (7/10), BERS2 (Bond, Leica) (3/3), TRS pH 9 (3-in-1) (Dako) (5/8), TRS pH 9 (Dako) (1/3) or Tris-EDTA/EGTA pH 9 (3/6) as the retrieval buffer. The mAb was typically diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 23 out of 24 (96 %) laboratories produced a sufficient staining (optimal or good).
1 laboratory used a combined pre-treatment by using HIER in Cell Conditioning 1 (CC1; BenchMark, Ventana) followed by enzymatic pre-treatment in Protease 2 (Ventana).

mAb clone **TS1**: The protocols giving an optimal result were mostly based on HIER using either BERS2 (Bond, Leica) (4/4) or TRS pH 9 (3-in-1) (Dako) (1/2) as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 out of 6 (100 %) laboratories produced a sufficient staining (optimal or good).
1 laboratory used a combined pre-treatment by using HIER in Cell Conditioning 1 (CC1; BenchMark, Ventana) followed by enzymatic pre-treatment in Protease 3 (Ventana).

rmAb clone **EP17**: The protocol giving an optimal result was based on HIER using CC1 (BenchMark, Ventana)(1/2) as the retrieval buffer. The mAb was diluted 1:100.

rmAb clone **EP1628Y**: The protocol giving an optimal result was based on HIER using TRS pH 9 (3-in-1) (Dako) (1/1) as the retrieval buffer. The mAb was diluted 1:400.

Ready-To-Use Abs
mAb clone **5D3** (product.no. PA0067, Leica/Novocastra): The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 1 (Bond, Leica), an incubation time of 15 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system. Using these protocol settings 2 out of 2 laboratories produced an optimal staining.

mAb clones **B22.1** & **B23.1** (prod. no. 760-4344, Ventana/Cell Marque): One of two protocols giving an optimal result was based on HIER using Cell Conditioning 1 (short), an incubation time of 36 min in the primary Ab and OptiView (760-700) as the detection system. The other protocol used a combined pre-treatment using HIER in CC1 (mild) and Protease 2 for 4 min, an incubation time of 32 min in the primary Ab and iView (760-091) as the detection system.

mAb clone **D10** (prod. no. IR618, Dako): The protocols giving an optimal result were based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 as HIER buffer, heating time 10-30 min at 95-97°C, an incubation time of 20 min in the primary Ab and EnVision Flex/Flex+ (K8000/K8002) as the detection system. Using these protocol settings 8 out of 8 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient stains were:
- Less successful antibodies (notable all of 10 protocols based on the mAb clone 35BH11 gave an insufficient result)
- Inappropriate epitope retrieval (e.g. enzymatic pre-treatment for the mAb clone 5D3 and TS1)
- Too low concentration of the primary Ab.

In this assessment and in concordance with the previous CK-LMW assessments in NordiQC the prevalent feature of an insufficient staining was a too weak or false negative reaction of the cells expected to be demonstrated. The majority of the laboratories were able to demonstrate CK-LMW in structures with a high antigen expression as the bile duct epithelium and the hepatocellular carcinoma, whereas the demonstration of CK-LMW in structures with a reduced antigen expression such as the small cell lung carcinoma and renal cell carcinoma was more difficult and only seen with appropriate protocol settings.
The choice of the primary Ab had a great impact on the pass rate, as e.g. the proportion of sufficient stains based on the mAb clone DC10 was 95 % compared to 0 %, when the mAb clone 35BH11 was used.
Also the use of epitope retrieval for the individual primary Abs had a significant impact on the pass rate, as seen for e.g. the mAb clone 5D3. If proteolytic pre-treatment was used 8 out of 8 protocols (100 %) were assessed as insufficient due to a general too low sensitivity and also typically causing an impaired morphology due to excessive digestion of the cellular membranes, which especially was seen in the small cell lung carcinoma. If HIER was applied, 15 out of 25 protocols (60 %) were assessed as sufficient, out of which 10 (40 %) were optimal. In this context the vendors' data sheets for the mAb clone 5D3 gives misleading guidelines concerning the epitope retrieval: Thermo Scientific / NeoMarkers and Biocare recommend proteolysis as pre-treatment for the mAb clone 5D3, while Leica / Novocastra recommends HIER for the clone when sold as a Ready-To-Use format prod. no. PA0067, but proteolysis for the concentrated format!
The impact of pass rate related to the choice of the primary Ab and epitope retrieval applied is illustrated in table 2, where the cumulated data for the 5 most widely used clones in the last four assessments for CK-LMW is listed. Note, e.g., the over-all pass rate of 58% for the mAb clone 5D3, compared to 80% when HIER was applied and 10% when protease was used.

Table 2. **The impact of pass rate related to the choice of the primary Ab and epitope retrieval**

Pass rate for run 16, 20, 25 & 33								
	Total		**HIER**		**Prot. pre-treatm.**		**HIER + proteolysis**	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
MAb clone CAM 5.2	95	42 (44 %)	29	9 (31 %)	50	32 (64 %)	6	1 (17 %)
MAb clone DC10	112	105 (94 %)	111	104 (94 %)	0	0	1	1 (100 %)
MAb clone 5D3	66	38 (58 %)	45	36 (80 %)	21	2 (10 %)	0	0
MAb clone 35BH11	48	6 (13 %)	28	4 (14 %)	20	2 (10 %)	0	0
MAb clone C51	26	24 (92 %)	26	24 (92 %)	0	0	0	0

These data clearly indicate that the traditional mAb clones CAM 5.2 and 34BH11 have been less successful in four successive assessments for CK-LMW. The most robust markers for CK-LMW in the runs were the mAb clones C51 and DC10. The literature and vendors' information on the mAb clone C51 give conflicting information about the reactivity of this clone as to whether it is raised towards CK types 8 or CK18. In an analysis previously performed in the NordiQC laboratory, the reaction pattern of the mAb clone C51 was found to be identical with CK18 antibodies like clone DC10 and different from Abs reacting with CK8 as the clone TS1.

Abs against CK 8 and in particular the newly launched rmAb clone EP17 gave in optimal stains a weak to moderate staining in both basal and intermediate squamous epithelial cells of the esophagus. These cells were negative when Abs against CK 18 were used. The rmAb clone EP17 gave by far the strongest and most consistent staining in the cells expected to be demonstrated, compared to all the other clones used.
As observed in the previous assessments of CK-LMW, liver was found to be a reliable positive control, as all laboratories that could demonstrate the membranous reaction in the hepatocytes also could demonstrate CK-LMW in the renal cell carcinoma and the small cell lung carcinoma, which in this run were the most challenging tumours.

This was the 5th assessment of CK-LMW in NordiQC (Table 3) and in the last 3 runs almost same pass rate has been obtained.

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

	Run 9 2003	Run 16 2006	Run 20 2007	Run 25 2009	Run 33 2011
Participants, n=	54	66	74	99	141
Sufficient results	57 %	45 %	67 %	66 %	64 %

In this CK-LMW assessment many new laboratories participated for the first time and for these a general lower pass rate was observed compared to the laboratories also participating in the previous run 25, 2009: For the laboratories participating for the first time the pass rate was 55 % (31 out of 56), whereas the pass rate was 71 % (60 out of 85 laboratories) for the laboratories participating in both runs.

Conclusion
Among the commonly used Abs, the mAb clones DC10 and C51 were the most robust Abs for the demonstration of CK-LMW. A sufficient result for CK-LMW could be obtained by 94 – 100% of the participants when using one of these two markers either as a concentrate properly calibrated or as a RTU format/system. An optimal staining could also be obtained by the mAb clone 5D3, the mAb clone cocktail B22.1 & B23.1 and the newly launched rmAb clone EP17. The epitope retrieval and protocol settings have to be specifically tailored to each of the clones/cocktails.
Liver is an appropriate positive control for CK-LMW: The majority of hepatocytes must show an at least moderate staining with an enhancement along the cell membranes.

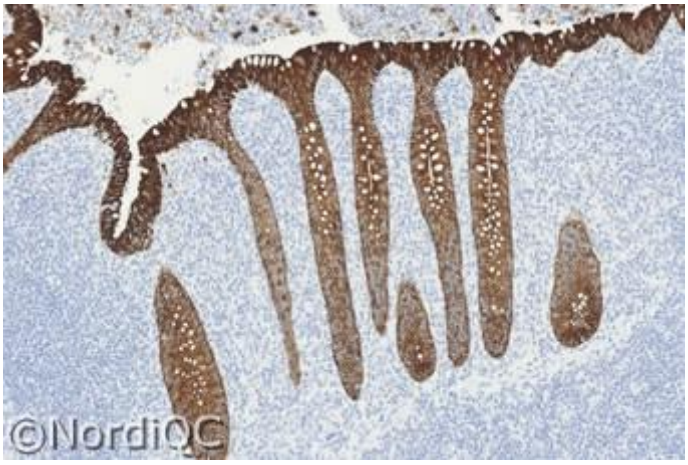

Fig. 1a
Optimal staining for CK LMW of the appendix using the mAb clone 5D3 for CK 8/18 optimally calibrated and with HIER in an alkaline buffer.
Virtually all the columnar epithelial cells show a strong cytoplasmic staining reaction, while no background staining is seen.
Also compare with Figs. 2a - 4a, same protocol.

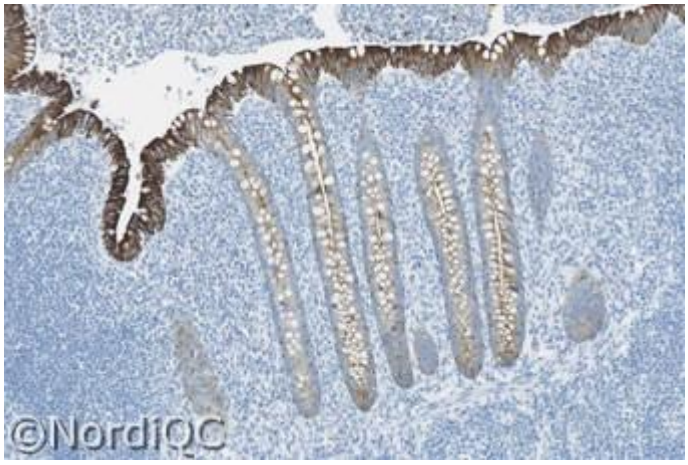

Fig. 1b
Insuffcient staining for CK LMW of the appendix using the mAb clone 5D3 for CK 8/18 with HIER in an alkaline buffer but with a protocol providing a too low sensitivity (Primary Ab too diluted.) – same field as in Fig. 1a.
Only the luminal columnar epithelial cells show a moderate to strong cytoplasmic staining, while virtually no staining is seen in the basal part of the crypts.
Also compare with Figs. 2b - 4b, same protocol.

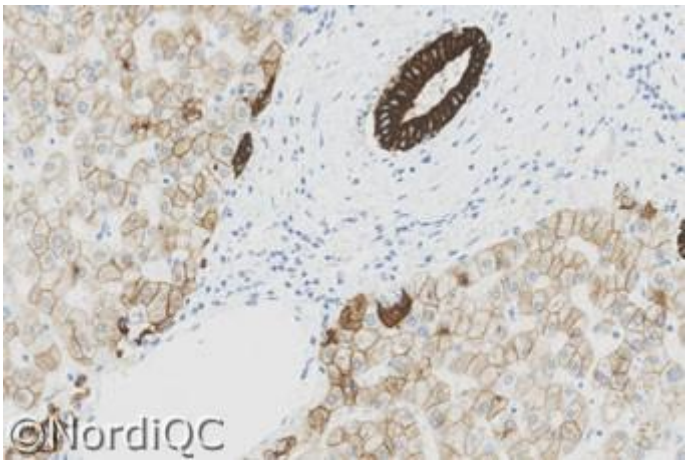

Fig. 2a
Optimal staining for CK LMW of the liver.
The majority of the hepatocytes show a distinct, moderate staining with a membrane enhancement, while the columnar epithelial cells of the bile ducts show a strong cytoplasmic staining.
Same protocol used in Figs. 1a - 4a.

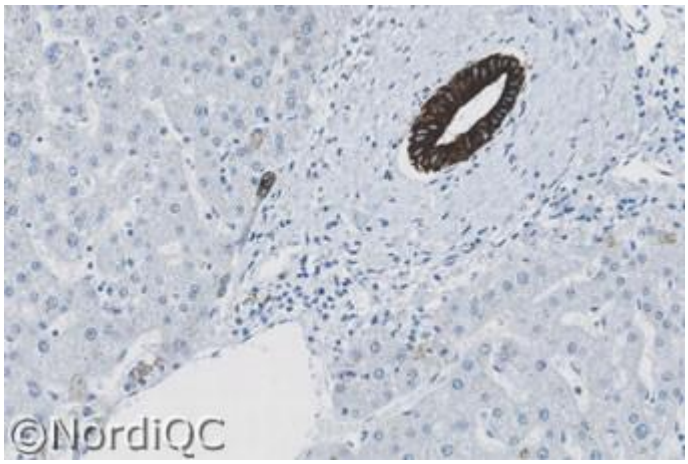

Fig. 2b
Insufficient staining for CK LMW of the liver - same field as in Fig. 2a.
Only the bile duct epithelial cells are demonstrated, while the hepatocytes are almost negative.
Same protocol used in Figs. 1b - 4b.

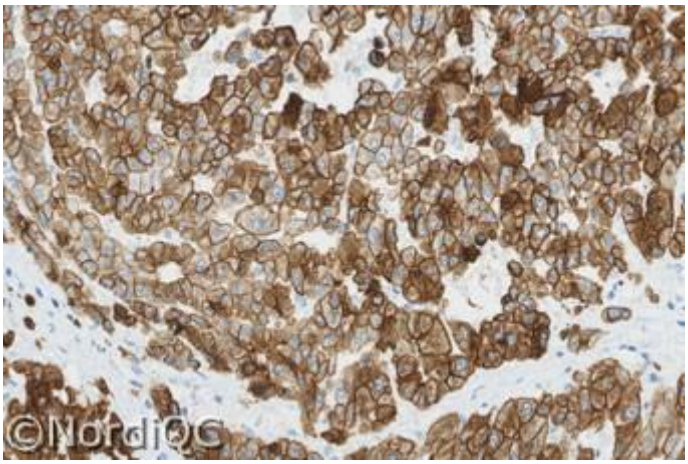

Fig. 3a
Optimal staining for CK-LMW of the hepatocellular carcinoma.
Virtually all the neoplastic cells show a distinct, moderate to strong staining reaction with a membrane enhancement.
Same protocol used in Figs. 1a - 4a.

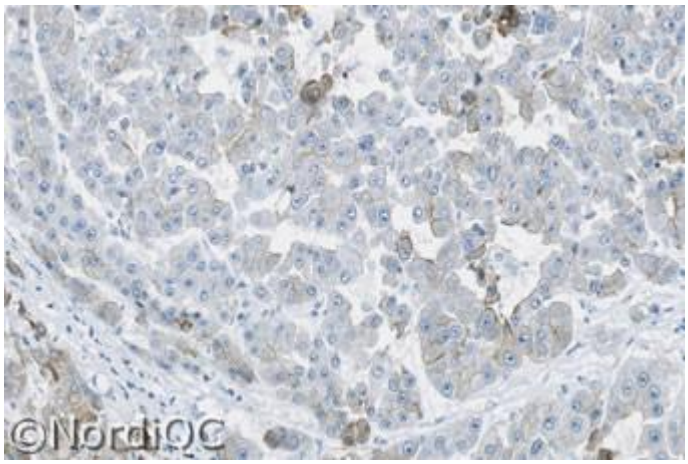

Fig. 3b
Insufficient staining for CK LMW of the hepatocellular carcinoma - same field as in Fig. 3a.
Only scattered neoplastic cells cells show a weak and diffuse staining reaction.
Same protocol used in Figs. 1b - 4b.

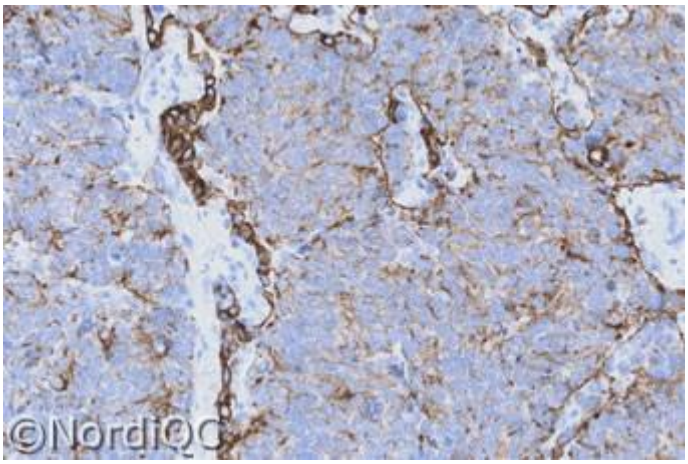

Fig. 4a
Optimal staining for CK-LMW of the small cell lung carcinoma. The majority of the neoplastic cells show a weak to moderate cytoplasmic reaction, while the remnants of the normal lung epithelial cells (at the periphery of the tumor nests) show a strong cytoplasmic reaction.
Same protocol used in Figs. 1a - 4a.

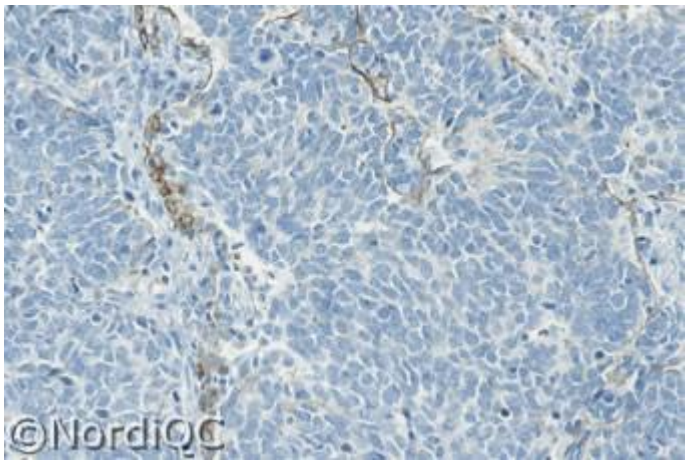

Fig. 4b
Insufficient staining for CK-LMW of the small cell lung carcinoma - same field as in Fig 4a. Only the remnants of the normal lung epithelial cells are demonstrated, while the neoplastic cells are virtually negative.
Same protocol used in Figs. 1b - 4b.

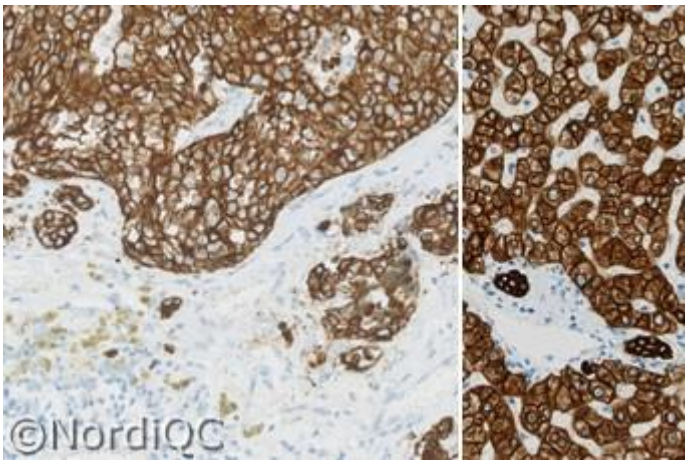

Fig. 5a
Optimal staining for CK-LMW using the rmAb clone EP17 against CK 8.
Left: Virtually all the neoplastic cells of the breast ductal carcinoma show a strong cytoplasmic reaction and no background staining is seen.
Right: The majority of the hepatocytes show a distinct, moderate to strong staining with a membrane enhancement.
Also compare the staining pattern obtained by an Ab against CK 18 - Figs. 5b same fields.

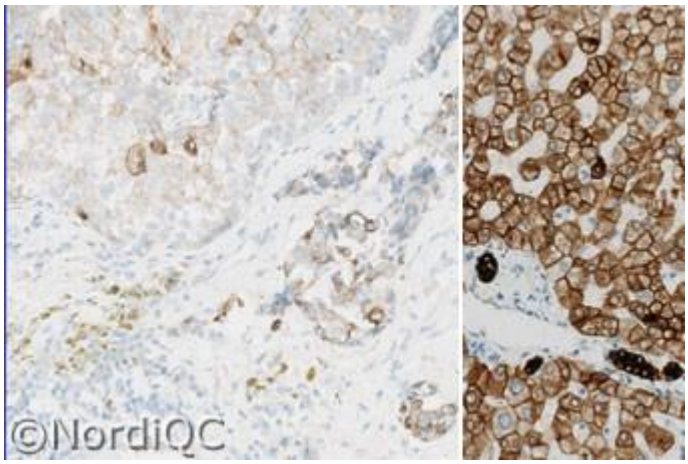

Fig. 5b
Optimal staining for CK-LMW using the mAb clone DC10 against CK 18.
Left: Only scattered neoplastic cells in the breast ductal carcinoma are demonstrated. This staining pattern was seen for all Abs reacting with CK 18 only.
Right: The majority of the hepatocytes show a distinct, moderate staining with a membrane enhancement.
Also compare the staining pattern obtained by an Ab against CK 8 - Figs. 5a.
The obtained staining was assessed as optimal, as the reduced staining in the breast ductal carcinoma most likely was related to the biology of this tumour and an otherwise optimal staining as seen in the Figs. 1a - 4a was obtained.

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