Assessment Run 38 2013

Cytokeratin, High Molecular Weight (CK-HMW)

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

1. Prostate hyperplasia, 2. Esophagus, 3. Liver,
4. Prostate intraepithelial neoplasia (PIN) / prostate adenocarcinoma,
5. Breast ductal carcinoma, 6. Lung squamous cell carcinoma.

All tissues were fixed in 10% neutral buffered formalin.

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

- A strong and distinct cytoplasmic staining reaction of all squamous epithelial cells of the esophagus throughout all the cell layers*.
- A strong and distinct cytoplasmic staining reaction of the majority of the basal cells of the prostate hyperplastic glands and the PIN lesions.
- A moderate to strong cytoplasmic staining reaction of the majority of the neoplastic cells of the lung squamous cell carcinoma.
- No staining of the neoplastic cells of the breast ductal carcinoma and of the epithelial cells of the bile ducts of the liver.

* Antibodies against CK14 only demonstrate the basal squamous epithelial cells.

213 laboratories participated in this assessment. 6 participants used an inappropriate antibody like CK-PAN, CK13 and CK19. Of the remaining 207 laboratories 45 % achieved a sufficient mark (optimal or good). Antibodies (Abs) used and marks are summarized in table 1.

Table 1. **Antibodies and assessment marks for CK-HMW, run 38**

Concentrated Antibodies	Reactivity	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **34BE12**	CK 1, 5, 10, 14, (19)*	51 2 2 1 1 1 1 1 1	Dako Leica/Novocastra Thermo/Neomarkers Abcam Biocare Bio SB Cell Marque Enzo Gene Tech	0	6	54	1	10 %	-
mAb clone **BS42**	unknown	1	Nordic Biosite	0	0	1	0	-	-
mAb clone **D5/16B4**	CK 5, 6	28 2 1 1	Dako Cell Marque Genemed Zymed	15	13	4	0	88 %	100 %
mAb clone **DE-SQ**	CK 13, 14, 15, 16	1	Thermo/Neomarkers	0	0	1	0	-	-
mAb clone **LL002**	CK 14	6 1 1	Leica/Novocastra AbD Serotec Thermo/Neomarkers	5	1	2	0	75 %	83 %
mAb clone **XM26**	CK 5	23 1	Leica/Novocastra DBS	19	5	0	0	100 %	100 %
mAb clone cocktail **XM26+LL002**	CK 5, 14	2 2	DBS Zytomed	1	2	1	0	-	-
mAb clone cocktail **Y4A3+XM26+**	p63, CK 5, 14	1	Zytomed	0	1	0	0	-	-

LL002									
mAb clone **34BE12+** rmAb clone **EP1601Y**	CK 1, 5, 10, 14, (19)* + CK 5	1	Homemade cocktail: Dako/Cell Marque	0	0	1	0	-	-
mAb clone **XM26+** mAb clone **LL002**	CK 5, 14	1	Homemade cocktail: Leica/Novocastra/ Cell Marque	0	1	0	0	-	-
Ready-To-Use Abs									
mAb clone **34BE12 IR051**	CK 1, 5, 10, 14, (19)*	24	Dako	0	0	24	0	0 %	0 %
mAb clone **34BE12 790-4373**	CK 1, 5, 10, 14, (19)*	17	Ventana	0	2	15	0	12 %	-
mAb clone **34BE12 PM127**	CK 1, 5, 10, 14, (19)*	1	Biocare	0	0	1	0	-	-
mAb clone **34BE12 PA0134**	CK 1, 5, 10, 14, (19)*	2	Leica/Novocastra	0	0	1	1	-	-
mAb clone **34BE12 MON-RTU1072**	CK 1, 5, 10, 14, (19)*	1	Monosan	0	1	0	0	-	-
mAb clone **D5/16B4 IS/IR780**	CK 5, 6	9	Dako	3	4	2	0	78 %	78 %
mAb clone **D5/16B4 790-4554**	CK 5, 6	7	Ventana	3	2	2	0	71 %	100 %
mAb clone **D5/16B4 356M-18**	CK 5, 6	2	Cell Marque	1	1	0	0	-	-
mAb clone **D5/16B4 MS-1814-R7**	CK 5, 6	1	Thermo/Neomarkers	1	0	0	0	-	-
mAb clone **LL002 760-4251**	CK 14	1	Ventana/Cell Marque	1	0	0	0	-	-
mAb clone **XM26 PA0468**	CK 5	3	Leica/Novocastra	3	0	0	0	-	-
mAb clone cocktail **34BE12+4A4**	CK 1, 5, 10, 14, (19)* + p63	1	Ventana	0	0	1	0	-	-
rmAb/mAb clone cocktail **EP1601Y+LL002 905H-08**	CK 5, 14	1	Cell Marque	1	0	0	0	-	-
rmAb clone **SP53 760-4805**	CK 14	1	Ventana	1	0	0	0	-	-
pAb **MAD-000122QD**	CK 5	1	Master Diagnostica	0	0	1	0	-	-
Ab **Unknown**	-	1	Unknown	1	0	0	0	-	-
Total	207			55	39	111	2	94	
Proportion				26 %	19 %	54 %	1 %	45 %	

1) Proportion of sufficient stains (optimal or good),
2) Proportion of sufficient stains with optimal protocol settings only, see below.
* Apart from reacting with CK types 1, 10, 5 and 14, the Ab also reacts with an unknown CK type, possibly a denaturated CK19.

The following protocol parameters were central to obtain optimal staining:

Concentrated Antibodies
mAb clone **D5/16B4**: Protocols with optimal results were all based on heat induced epitope retrieval

(HIER) using either Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/5)*, Cell Conditioning 1 (CC1;BenchMark, Ventana) (11/16), Tris-EDTA/EGTA pH 9 (2/5) or EDTA/EGTA pH 8 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:20-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 27 of 27 (100 %) laboratories produced a sufficient staining (optimal or good). 1 laboratory used a combined pre-treatment using HIER in CC1 (BenchMark, Ventana) and proteolysis in Protease 3 (Benchmark, Ventana). Using this protocol a titre of 1:50 was applied.
* (number of optimal results/number of laboratories using this buffer)

mAb clone **LL002**: Protocols with optimal results were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (1/1), CC1 (Benchmark, Ventana) (2/4), Bond Epitope Retrieval Solution 2 (BERS2; Bond, Leica) (1/2) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 5 of 6 (83 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **XM26**: Protocols with optimal results were all based on HIER using TRS pH 9, 3-in-1 (Dako) (5/6), TRS pH 9 (Dako) (2/2), CC1 (Benchmark, Ventana) (5/7), BERS1 (Leica) (2/2), BERS2 (Leica) (3/5), Diva Decloaker pH 6.2 (Biocare) (1/2) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was diluted 1:25-1:200. Using these protocol settings 22 of 22 (100 %) laboratories produced a sufficient staining (optimal or good). 2 laboratories used a combined pre-treatment using HIER in CC1 (BenchMark, Ventana) and proteolysis in Protease 3 (Benchmark, Ventana). The mAb was diluted in the range 1:50-1:100. Both laboratories produced an optimal staining.

mAb clone cocktail **XM26+LL002**: The protocol with an optimal result was based HIER using Tris-EDTA/EGTA pH 9 (1/2) as the retrieval buffer. The antibody was diluted 1:100. Using these protocol settings 1 of 1 (100 %) laboratories produced an optimal staining.

Table 2 summarizes the overall proportion of optimal staining results using the most frequently used concentrated Abs and IHC stainer platforms.

Table 2. Optimal results for CK-HMW using concentrated antibodies 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 clones **D5/16B4**	22 % 1/5**	-	69 % 11/16	-	0 % 0/2	0 % 0/1
mAb clone **XM26**	88 % 7/8**	-	71 % 5/7	-	60 % 3/5	100 % 2/2

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use Antibodies

mAb clone **D5/16B4** (product.no. IS/IR780, Dako): 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 97°C), 20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 7 of 9 (78 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **D5/16B4** (prod. no. 790-4554, Ventana): Two of the three protocols with optimal results were based on HIER using standard CC1, 16-32 min. incubation of the primary Ab and UltraView (760-500) + amplification kit or OptiView(760-700) as detection system. One protocol was based on a combined pre-treatment using HIER in mild CC1 and proteolysis in Protease 3 (Ventana), 16 min. incubation of the primary Ab and OptiView (760-700) as detection system. Using these protocol settings 3 of 3 (100 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **LL002** (prod. no. 760-4251, Ventana/Cell Marque): The protocol with an optimal result was based on HIER using standard CC1, 32 min. incubation of the primary Ab and UltraView (760-500) as detection system. Using these protocol settings 1 of 1 laboratory produced an optimal staining.

mAb clone **XM26** (prod. no. PA0368, Leica): The protocols with optimal results were based on HIER using BERS 2 (Bond, Leica), 15 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 3 of 3 (100 %) laboratories produced an optimal staining.

rmAb clone **SP53** (prod. no. 760-4805, Ventana): The protocol giving an optimal result was based on HIER using mild CC1, 16 min. incubation of the primary Ab and OptiView (760-700) as detection system. Using these protocol settings 1 of 1 laboratory produced an optimal staining.

The most frequent causes of insufficient stainings were:

- Less successful performance of the primary Ab (97 of 107 protocols based on the mAb clone 34BE12 gave an insufficient staining, typically false positive)
- Too low concentration of the primary Ab
- Insufficient HIER - too short efficient HIER time and/or use of Citrate pH 6 as HIER buffer

In this assessment and in concordance with the two previous NordiQC assessment for CK-HMW, run 32 2011 and run B6 2008, the prevalent feature of an insufficient result was an aberrant false positive staining. The false positive staining was characterized by a distinct cytoplasmic staining reaction of the neoplastic cells of the breast ductal carcinoma and of the epithelial cells of the bile ducts in the liver. This pattern was seen in 84 % of the insufficient results (n=95/113) and was only seen when the mAb clone 34BE12 was used. According to the datasheets from different vendors, the mAb clone 34BE12 reacts with the CK-HMW types 1, 5, 10 & 14, but when the Ab is applied with HIER and by sensitive protocol settings, a cross-reaction with an unidentified CK-LMW subtype, possibly denaturated CK19 giving a false positive staining in epithelial cells not expressing CK-HMW, was seen. If the sensitivity was reduced in order to reduce the aberrant staining reaction with CK-LMW, a too weak staining for CK-HMW was seen.
The cross-reaction was reduced but not completely eliminated by using proteolytic pre-treatment or a combined pre-treatment using both HIER and proteolysis. If proteolysis was used, the morphology frequently was impaired due to excessive digestion of the cytoplasmic compartment of the neoplastic cells in the breast carcinoma. The cross-reaction of the mAb clone 34BE12 was not seen in the prostate epithelial cells. Thus, the mAb clone 34BE12 can be used for the demonstration of CK-HMW in the basal cells prostate specimens, but due to the above mentioned cross-reaction to CK-LMW in e.g. breast epithelial cells/breast carcinoma it cannot be recommended as a general marker for CK-HMW.

The mAb clone XM26 against CK5 gave by far the highest proportion of sufficient and optimal results. In total 27 of 27 protocols based on this clone either as a concentrate (n=24) or as a Ready-To-Use system (Leica, n=3) gave a sufficient result out of which 22 (82 %) gave an optimal result. As seen in table 2 an optimal result could be obtained on all the three main IHC platforms from Ventana, Dako and Leica, when the Ab was applied as a concentrate.
Other Abs as the mAb clone D5/16B4 for CK5/6, mAb clone LL002 against CK5/6 and the rmAb clone SP53 also gave a high proportion of sufficient and optimal results.
In total 84 of 95 protocols (88 %) based on one of these above mentioned clones either as a single Ab or mixed in a cocktail gave a sufficient staining result. All of these clones could give an optimal staining providing HIER, preferable in an alkaline buffer, was applied. The insufficient results based on one of the above mentioned clones were all characterized by a too weak or false negative staining of the structures expected to be demonstrated, typically caused by insufficient HIER and/or a too low concentration of the primary Ab.

Controls
Esophagus is recommended as positive tissue control for CK-HMW. Virtually all the squamous epithelial cells must show a moderate to strong cytolasmic staining reaction in all cell layers. The most superficial cells may show a reduced staining reaction. For Abs against CK14, mainly basal cells will be demonstrated and dispersed intermediate squamous epithelial cells will only show a weak staining reaction.
Liver is recommended as negative tissue control for CK-HMW. No staining must be seen in the hepatocytes and epithelial cells of the bile ducts. Tonsil can be used as an alternative to esophagus, while appendix can be used instead of liver.

Performance history
This was the 5th assessment of CK-HMW in NordiQC. An increase in pass rate was seen, as listed in table 3.

Table 3. **Proportion of sufficient results for CK-HMW in five NordiQC runs**

	Run 12 2004	**Run 16 2006**	**Run B6 2008**	**Run 32 2011**	**Run 38 2013**
Participants, n=	73	87	97	163	207
Sufficient results	77 %	88 %	24 %	23 %	45 %

However, the overall pass rate is still very low, which is related to the persistent use of the mAb clone 34BE12 as a general marker for CK-HMW. If the mAb clone 34BE12 was excluded from the assessment a pass rate of 88 % was seen.

Conclusion

The mAb clones **XM26**, **D5/16B4**, **LL002** and the rmAb clone **SP53** could all be used to obtain an optimal staining result. The mAb clone XM26 against CK5 gave the highest proportion of sufficient and optimal results and could be applied on all the 3 main IHC platforms from Ventana, Dako and Leica. HIER, preferable in an alkaline buffer, is mandatory for all clones. The mAb clone 34BE12 can not be used as general marker for CK-HMW due to cross-reaction with CK-LMW.

Esophagus is recommended as positive tissue control. For Abs against CK5 and CK5/6, virtually all the squamous epithelial cells must show a moderate to strong cytoplasmic staining reaction. Liver or appendix must be used as negative tissue controls, in which no staining must be seen.

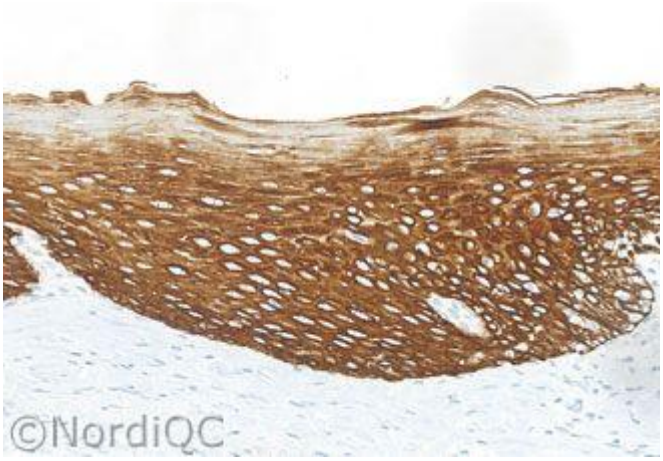


Fig. 1a
Optimal staining for CK-HMW of the esophagus using the mAb clone XM26 against CK5 optimally calibrated and with HIER in an alkaline buffer.
Virtually all the squamous epithelial cells show a distinct, moderate to strong cytoplasmic staining reaction, while no background staining is seen.

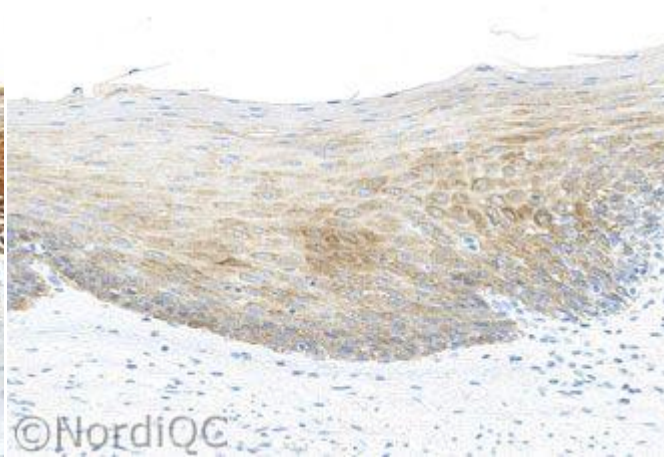


Fig. 1b
Insufficient staining for CK-HMW of the esophgus based on the mAb clone D5/16B against CK5/6 with a protocol giving a too low sensitivity – insufficient HIER - same field as in Fig. 1a.
Both the intensity and proportion of cells demonstrated is significantly reduced compared o the level expected and obtained in Fig. 1a.
Also compare with Figs. 2b & 3b, same protocol.

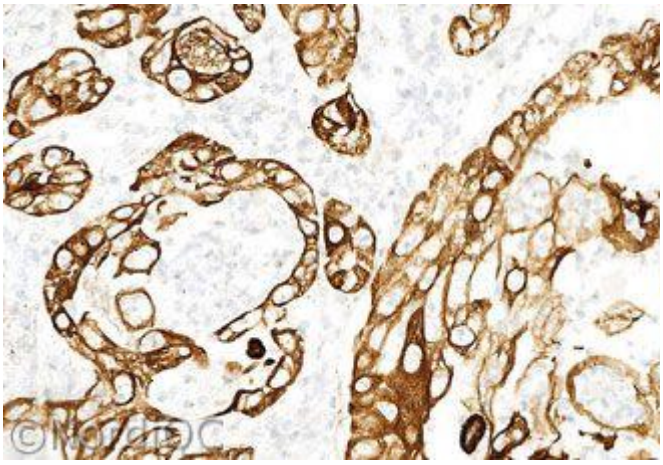


Fig. 2a
Optimal staining for CK-HMW of the lung squamous cell carcinoma using same protocol as in Fig. 1a.
Virtually all the neoplastic cells expressing CK-HMW show a moderate to strong cytoplasmic staining reaction.
No background staining is seen.

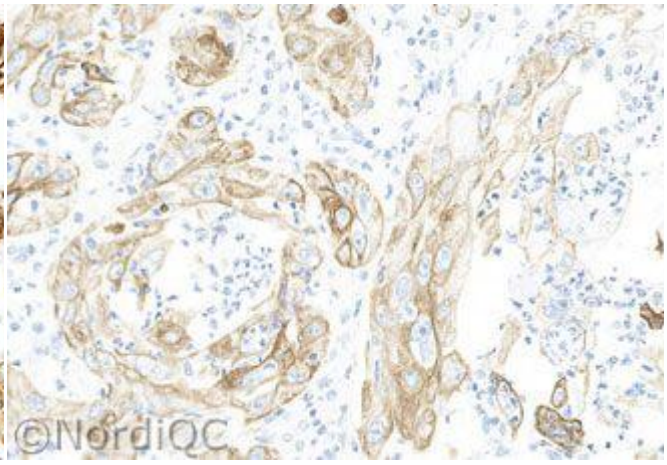


Fig. 2b
Staining for CK-HMW using same insufficient protocol as in Fig. 1a – same field as in Fig. 2a.
The vast majority of the neoplastic cells are demonstrated, but the intensity is significantly reduced compared to the optimal result shown in Fig. 2b.
Also compare with Fig. 3b – same protocol.

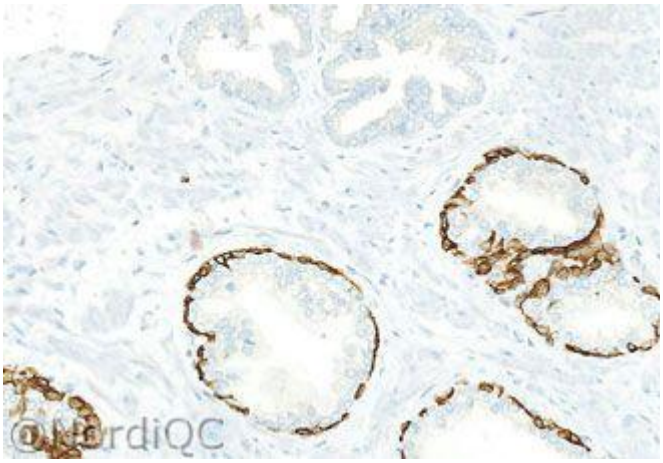


Fig. 3a
Optimal staining for CK-HMW of the prostate PIN lesion using same protocol as in Figs. 1a. & 2a.
Virtually all the basal cells decorating the glands with high grade PIN show a strong cytoplasmic staining reaction, while no staining is seen in the foci with prostate carcinoma – top.
No background staining is seen.

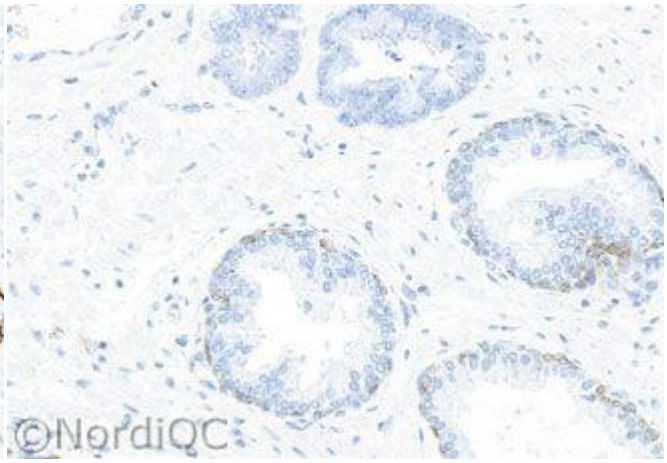


Fig. 3b
Insufficient staining for CK-HMW of the prostate PIN lesion using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The basal cells show a weak and equivocal staining reaction and it is difficult to differentiate between the glands with PIN and the invasive glands.

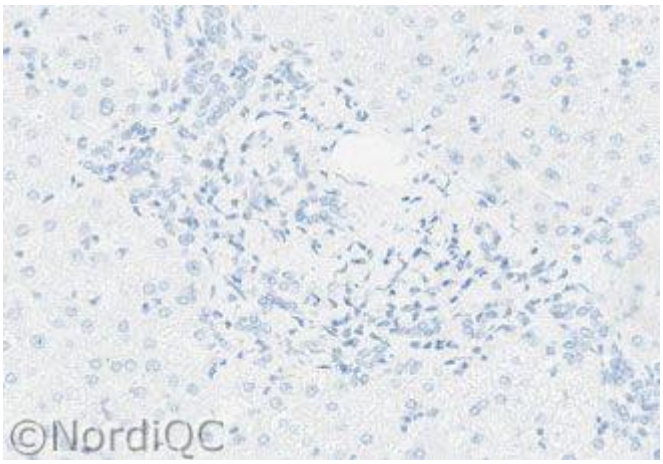


Fig. 4a
Optimal staining for CK-HMW of liver using same protocol as in Figs. 1a. - 3a.
No staining is seen in neither the liver cells nor the epithelial cells of the bile ducts.

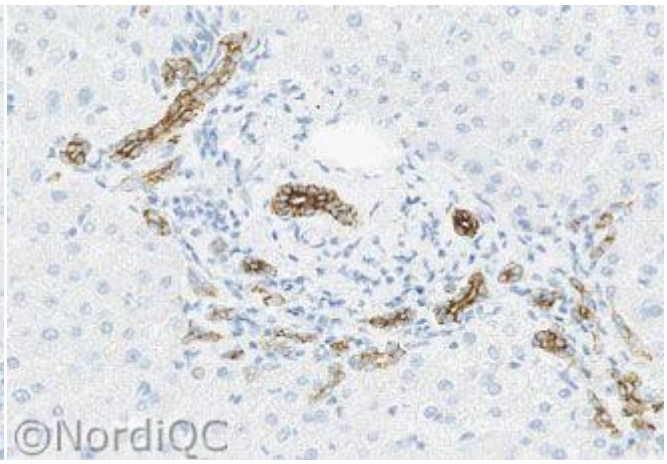
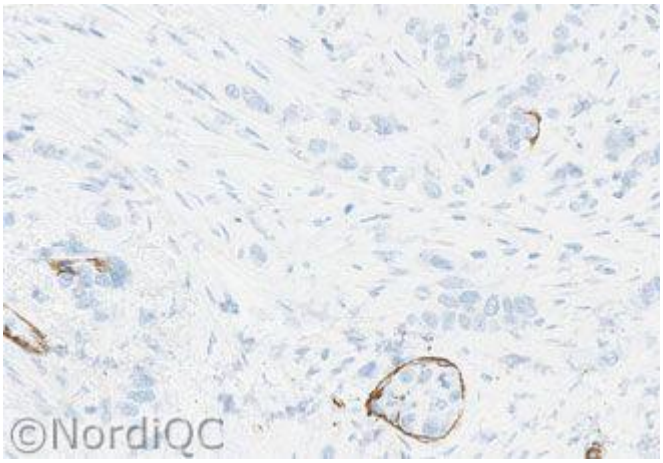
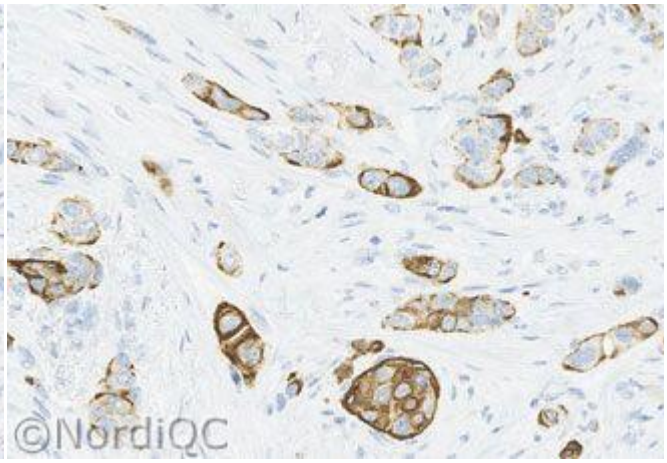


Fig. 4b
Insufficient false positive staining for CK-HMW of the liver using the mAb clone 34BE12 with HIER in an alkaline buffer – same field as in Fig. 4a.
A moderate to strong and aberrant cytoplasmic staining reaction is seen in the epithelial cells of the bile ducts.
The aberrant positive staining reaction most likely is caused by a cross-reaction of the mAb clone 34BE12 with a denatured form of CK19.
The cells do not express CK-HMW.



Optimal staining for CK-HMW of the breast ductal carcinoma using same protocol as in Figs. 1a. - 4a.
The neoplastic cells expressing CK-LMW are negative, while the remnants of entrapped myoepithelial cells expressing the CK-HMW subtypes CK5 & CK14 show a moderate cytoplasmic staining.



Insufficient false positive staining for CK-HMW of the breast ductal carcinoma using same protocol as in Fig. 5b - same field as in Fig. 5a.
A moderate to strong aberrant cytoplasmic staining reaction is seen in the majority of the neoplastic cells. The neoplastic cells did express neither CK5 nor CK14 and the aberrant positive staining reaction is, as described in Fig. 4b, caused by a cross-reaction of the mAb clone 34BE12.

SN/RR/LE 18-6-2013