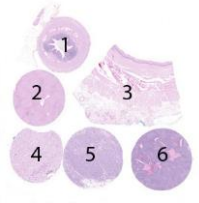
Assessment Run 49 2017

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. Neuroendocrine carcinoma (small cell lung carcinoma)

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CK-LMW staining as optimal included:

- A strong, distinct cytoplasmic staining reaction of virtually all appendiceal columnar epithelial cells and bile duct epithelial cells.
- An at least weak to moderate predominantly membranous staining reaction of virtually all hepatocytes.
- A moderate to strong, distinct cytoplasmic staining reaction of virtually all neoplastic cells in the breast ductal carcinoma.
- An at least weak to moderate cytoplasmic staining reaction in the majority of neoplastic cells in the renal clear cell carcinoma and lung neuroendocrine carcinoma.

Participation

Number of laboratories registered for CK-LMW, run 49	245
Number of laboratories returning slides	229 (94%)

Results
229 laboratories participated in this assessment. 16 submitted slides stained with an inappropriate primary antibody like CK-PAN or CK-HMW. Of the remaining 213 laboratories, 66% achieved a sufficient mark (optimal or good).
Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibodies
- Too low concentration of the primary Ab
- Less successful performance of the mAb clone 5D3 on the Ventana BenchMark platform
- Use of less sensitive detection systems
- Inappropriate epitope retrieval

Performance history
This was the seventh NordiQC assessment of CK-LMW. The pass rate decreased compared to the previous run 38, 2013 as shown in table 2 and is now similar to the level seen from 2007 - 2011.

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

	Run 9 2003	Run 16 2006	Run 20 2007	Run 25 2009	Run 33 2011	Run 38 2013	Run 49 2017
Participants, n=	55	66	74	99	141	161	213
Sufficient results	57%	45%	67%	66%	64%	77%	66%

Conclusion
The mAb clone cocktail **B22.1/B23.1**, rmAb clone cocktail **EP17/30** and rmAb clone **EP17** were the most successful antibodies for immunohistochemical demonstration of CK-LMW.
Using one of these three Abs either within a laboratory developed (LD) assay or as Ready-To-Use (RTU) system a pass rate of 93% was seen, which was significantly higher than the overall pass rate of 66% obtained in this assessment. The consistent and common use of less successful Abs such as mAb 34βH11 and in particular CAM5.2 prevents improvement of the overall pass rate for CK-LMW.
Liver is recommended as primary positive tissue control. Virtually all hepatocytes must show an at least moderate predominantly membranous staining reaction, while the epithelial cells lining the bile ducts must show a strong cytoplasmic staining reaction. No staining should be seen in the connective tissue and lymphocytes in the portal rooms. Appendix cannot be recommended as positive tissue control as the epithelial cells express high-level CK-LMW.

Table 1. **Antibodies and assessment marks for CK-LMW, run 49**

Concentrated antibodies	Reactivity	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **34βH11**	*CK8*	1	In-house	0	0	1	0	-	-
mAb clone **5D3**	*CK8/18*	18 3 3 1 1	Leica/Novocastra Thermo/Neomarkers Biocare Biogenex Vector	3	10	8	5	50%	100%
mAb clones **B22.1/B23.1**	*CK8/18*	6 1 1	Cell Marque Bio SB Immunologic	4	4	0	0	100%	100%
mAb clone **BS83**	*CK18*	1	Nordic Biosite	0	1	0	0	-	-
mAb clone **CAM5.2**	*CK8 (7)*	1	Cell Marque Zytomed	0	2	0	0	-	-
mAb clone **C51**	*CK18*	2 1	Invitrogen/Zymed Biogenex	0	2	1	0	-	-
mAb clone **DC10**	*CK18*	20 3 1 1 1 1 1	Agilent/Dako Thermo/Neomarkers Biogenex Empire Genomics Histolab Immunologic Spring Bioscience	6	15	6	1	75%	90%
mAb clone **K8.8/DC10**	*CK8/18*	1	Thermo/Neomarkers	0	0	1	0	-	-
mAb clone **TS1**	*CK8*	1	Leica/Novocastra	0	0	0	1	-	-
rmAb clone **EP17**	*CK8*	6	Epitomics	6	0	0	0	100%	100%
rmAb clones **EP17/EP30**	*CK8/18*	3	Agilent/Dako	2	1	0	0	-	-
Ready-To-Use antibodies									
mAb clone **35βH11 MAB-0051**	*CK8*	1	Maixin	0	1	0	0	-	-
mAb clone **35βH11 760-2637**	*CK8*	2	Roche/Ventana	0	0	0	2		
mAb clone **35βH11** no product number	*CK8*	1	ZS	0	0	0	1	-	-
mAb clones **5D3 PA0067**	*CK8/18*	8	Leica/Novocastra	2	3	3	0	63%	83%
mAb clone **5D3 PDM070-10MM**	*CK8/18*	2	Diagnostics BioSystems	0	1	1	0	-	-
mAb clones **B22.1/B23.1 760-4344**	*CK8/18*	27	Roche/Ventana	16	7	4	0	85%	96%
mAb clones **B22.1/B23.1 818M-97/98**	*CK8/18*	8	Cell Marque	4	2	0	2	75%	-
mAb clones **B22.1/B23.1 MAD-000589QD**	*CK8/18*	2	Master Diagnostica	1	1	0	0	-	-
mAb clone **CAM5.2 345779**	*CK8 (7)*	29	Becton Dickinson	1	9	8	11	34%	-
mAb clone **CAM5.2 349205**	*CK8 (7)*	6	Becton Dickinson	0	0	4	2	-	-
mAb clone **CAM5.2 790-4555**	*CK8 (7)*	10	Roche/Ventana	3	3	3	1	60%	80%
mAb clone **DC10 IR618/IS618**	*CK18*	12	Agilent/Dako	0	8	4	0	67%	-
mAb clone **DC10 GA618**	*CK18*	9	Agilent/Dako	2	5	2	0	78%	100%
rmAb clones **EP17/EP30 IR094**	*CK8/18*	16	Agilent/Dako	16	0	0	0	100%	100%
Total		213		66	75	46	26	-	
Proportion				31%	35%	22%	12%	66%	

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

Detailed analysis of CK-LMW, run 49
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **5D3**: Protocols with optimal results were based on HIER using Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/7)* as retrieval buffer. The mAb was diluted in the range of 1:100-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings, 5 of 5 (100%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clones **B22.1/B23.1**: Protocols with optimal results were based on HIER using Cell Conditioning 1 (CC1, Ventana) (4/7) 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, 4 of 4 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DC10**: Protocols with optimal results were typically based on HIER using CC1 (Ventana) (4/11) or BERS2 (Leica) (1/4). One participant used a combined enzyme/HIER pre treatment with Protease 2 (Ventana) and CC1 (Ventana) (1/1). The mAb was typically diluted in the range of 1:25-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings, 9 of 10 (90%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP17**: Protocols with optimal results were based on HIER using CC1 (Ventana) (4/4), Target Retrieval Solution, High pH (Dako Omnis) (1/1) or Tris-EDTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:100-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 6 of 6 (100%) laboratories produced an optimal staining.

rmAb clones **EP17/EP30**: Two protocols with optimal results were both based on HIER using CC1 (Ventana) (1/1) or TRS, High pH (Dako Omnis) (1/1) as retrieval buffer. The rmAb was diluted 1:50. Using these protocol settings, 2 of 2 (100%) laboratories produced an optimal staining

Table 3. **Proportion of optimal results for CK-LMW for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer**		**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 **5D3**	0/3**	0/1	0/6	-	3/5 (60%)	-
mAb clone **DC10**	0/3	-	4/7 (57%)	-	-	-

* 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 **5D3** product no. PA0067, Leica/Novocastra, BOND III:
Protocols with optimal results were based on HIER using BERS1 (Bond Leica) (efficient heating time 20 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings 5 of 6 (83%) produced a sufficient staining result (optimal or good).

mAb clones **B22.1/B23.1**, product no. 760-4344 Roche/ Ventana, BenchMark Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 16-64 min.) or a combined pre-treatment using Protease 1 or 3 (efficient time 8-10 min.) after HIER in CC1 (efficient heating time 8-32 min.), 12-32 min. incubation of the primary Ab and UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 22 of 23 (96%) laboratories produced a sufficient staining result.

mAb clone **CAM5.2**, product no.790-4555 Roche / Ventana, BenchMark Ultra:
Protocols with optimal results were based on HIER using Cell Conditioning 1 (efficient heating time 24-32 min.) or a combined pre-treatment using Protease 3 (efficient time 10 min.) after HIER in CC1 (efficient heating time 8 min.), 12-30 min. incubation of the primary Ab and UltraView + amplification kit (760-500+760-080) or OptiView (760-700) as detection system. Using these protocol settings, 4 of 5 (80%) laboratories produced a sufficient staining result.

mAb clone **DC10**, product no. **GA618**, Dako, Dako Omnis:
One protocol with optimal result was based on HIER using TRS pH 9 (efficient heating time 30 min. at 97°C), 12 min. incubation of the primary Ab and EnvisionFLEX (GV800) as detection system. Using this and comparable protocol settings, 6 of 6 (100%) laboratories produced a sufficient staining result.

rmAb clones **EP17/EP30**, product no. IR094, Dako, Autostainer+/Autostainer Link:
Optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-25 min. at 97-98°C), 20-30 min. incubation of the primary Ab and EnVision FLEX (K8000/K8012) as detection systems. Using these protocol settings, 9 of 9 (100%) laboratories produced an optimal staining result.

Table 4. **Comparison of pass rates for vendor recommended and laboratory modified RTU protocols**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Optimal	Sufficient	Optimal	Sufficient
Dako AS48 rmAb EP17/EP30 **IR/IS094**	5/5 (100%)	5/5 (100%)	4/4	4/4
Leica Bond mAb 5D3 **PA0067**	1/5 (20%)	4/5 (80%)	1/3	2/3
VMS Ultra/XT mAb B22.1/B23.1 **760-4344**	0/3	3/3	16/24 (67%)	20/24 (83%)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit
Only protocols performed on the specified vendor IHC stainer are included.

Comments
In this assessment and in concordance with the previous NordiQC CK-LMW assessments, the prevalent feature of an insufficient staining was a too weak or completely false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 96% of the insufficient results (69 of 72 laboratories). The remaining insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction compromising interpretation. Too weak staining was typically characterized by reduced staining reaction both in regard to intensity and proportion of cells expected to be demonstrated. This was in particular observed in the neoplastic cells of the lung neuroendocrine carcinoma, renal clear cell carcinoma and hepatocytes. Virtually all laboratories successfully demonstrated CK-LMW in the majority of neoplastic cells of the breast carcinoma, epithelial cells of appendix and bile ducts which all have high expression level of CK-LMW.

37% (79 of 213) of the laboratories used concentrated Ab format within LD assays for CK-LMW. The mAb clones 5D3 and DC10 were the two most widely used Abs and could both be used to obtain optimal staining results as shown in tables 1 and 3. However, the overall pass rates and proportion of optimal results for these Abs were low. Many precautions should be taken into account to set-up protocols providing optimal results. The mAb clone 5D3 consistently gave insufficient results with protocols performed on BenchMark XT/Ultra (n=7), despite applying comparable protocol settings that resulted in sufficient and optimal marks on the Leica IHC platform. Careful calibration of mAb 5D3, HIER in an alkaline buffer and use of a 3-step polymer detection were the main critical parameters for an optimal performance. In this context, it again has to be mentioned that Leica, the main provider of mAb clone 5D3, gives misleading guidelines concerning the epitope retrieval: For the concentrated format of 5D3, proteolytic pre-treatment is recommended, while the data sheet for the corresponding RTU format PA0067 states HIER must be used.
mAb clone DC10 provided a slightly superior performance compared to mAb clone 5D3 and an overall pass rate of 75% (21 of 28 laboratories). For mAb DC10, it was observed that use of a 3-step polymer / multimer based detection system was essential for optimal results and provided an overall higher proportion of sufficient results compared to 2-step systems. If mAb clone DC10 was used with a 3-step system, a pass rate of 79% (11 of 14 laboratories) was obtained, 43% optimal. In comparison, 2-step systems gave a pass rate of 64% (9 of 14 laboratories), 0% optimal.
Interestingly, three recently launched Abs for CK-LMW, mAb clone cocktail B22.1/B23.1, rmAbs clone cocktail EP17/EP30 and rmAb clone EP17 grouped together provided a pass pate of 100% (17 of 17) of which 71% was optimal. All three Abs, and in particular rmAb clone EP17, seemed to have higher analytical sensitivity for CK-LMW compared to the well-established mAb clones 5D3 and DC10.
Despite that these three Abs only were used by a limited number of laboratories, data indicate, that HIER in alkaline buffer and careful calibration of the primary Ab were the central parameters for optimal staining, whereas neither choice of detection system (2-step or 3-step) nor IHC stainer platform seemed to influence the performance.

RTU antibodies were used by 63% (134 of 213) of the laboratories. The Ventana RTU system for the BenchMark IHC platform based on mAb clone cocktail B22.1/B23.1 (760-4344) was the most widely used RTU system applied by 27 laboratories. An overall pass rate of 85% was seen, 59% optimal. Optimal results could only be obtained by use of laboratory modified protocol settings using UltraView +

amplification or OptiView as detection system. If the protocols were performed accordingly to the recommendations provided by Ventana, using UltraView as detection system, none of three submitted protocols provided optimal results. Laboratory modified protocol settings provided a pass rate of 83%, 67% optimal.

For the Dako RTU system for Autostainer 48 based on rmAb clone cocktail EP17/EP30 (IR094), an overall pass rate of 100% was observed. Optimal results could both be obtained using the protocol recommendations given by Dako and by laboratory modified protocol settings typically adjusting HIER time and/or incubation time of the primary Ab. Seven laboratories used the RTU format on Omnis by protocol settings similar to the Dako recommendations for Autostainer but modified to "Omnis RTU" settings using HIER for 25-30 min. in TRS High pH and 15-20 min. incubation of the primary Ab and polymer conjugate. All seven laboratories obtained an optimal mark.

The RTU format mAb clone CAM5.2, prod. no. 345779/349205, Becton Dickinson (BD) was the most widely used RTU format applied by 35 laboratories. An overall pass rate of 29% was observed and only 3% optimal. Several factors might have caused the disappointing low pass rate. First, it must be emphasized that this RTU format is not developed for a particular IHC system/platform and must be used within a LD assay identifying best practice protocol settings focusing on choice of epitope retrieval method, detection system etc. The protocol that provided an optimal result was based on proteolytic pre-treatment, which is in compliance with previous NordiQC assessments for CK-LMW indicating this epitope retrieval method may be the preferred method for mAb clone CAM5.2, as shown in run 38, 2013 (www.nordiqc.org). However, in this assessment only 3 of 11 protocols based on proteolytic pre-treatment gave a sufficient result and per se the choice of epitope retrieval method did not influence the pass rate in this run. In addition, it was observed that 15 of the 35 laboratories diluted the BD RTU format further, and for this group only 14% obtained a sufficient mark.
mAb clone CAM5.2 has recently been launched as RTU format by Ventana (prod. no. 790-4555). A superior pass rate of 60% was seen for this product compared to the BD format. Still, the proportion of sufficient and in particular optimal results was inferior to the other Ventana RTU system based on mAb clone cocktail B22.1/B23.1.

This was the seventh NordiQC assessment of CK-LMW (see table 2). In this run a significantly lower pass rate was obtained compared to the previous run 38, 2013. A large proportion of new participants and more challenging tissue material circulated may in part explain the reduced pass rate. However, persistent and slightly increased use of less successful Abs in this assessment also seemed to have an impact.
In run 38 (2013), 18% of the laboratories used mAb clone CAM5.2 from BD compared to 21% in this run. As shown in table 1, this Ab provided a significantly inferior performance especially when compared to the mAb clone cocktail B22.1/B23.1, rmAb clone cocktail EP17/30 and rmAb clone EP17.
Using one of these three Abs either within a laboratory developed (LD) assay or as Ready-To-Use (RTU) system a pass rate of 93% (56 of 60 protocols) was seen and 73% optimal. In comparison, the pass rate for CAM5.2, BD was 29% (10 of 35 protocols), 3% optimal.

Controls
In this assessment and as observed in previous NordiQC assessments, liver is recommendable as positive tissue control for CK-LMW. Virtually all hepatocytes must show an at least moderate predominantly membranous staining reaction, while the epithelial cells lining the bile ducts must show strong cytoplasmic staining reaction. No staining should be seen in the connective tissue and lymphocytes in portal rooms.
Appendix cannot be recommended as positive tissue control as the epithelial cells express high-level CK-LMW and thus cannot be used to monitor the consistency of the IHC protocol and the analytical sensitivity to demonstrate CK-LMW in low-level expressing cells and neoplasias.
Basal squamous cells of the esophagus will display a weak to moderate cytoplasmic staining reaction if using Abs towards CK8 as e.g. the rmAb clone EP17.

Fig. 1a
Optimal result for-CK LMW of the appendix using the rmAb clone cocktail EP17/30 for CK 8/18 as RTU format IR094, Dako and performed on the Autostainer Link 48 stainer, Dako.
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.

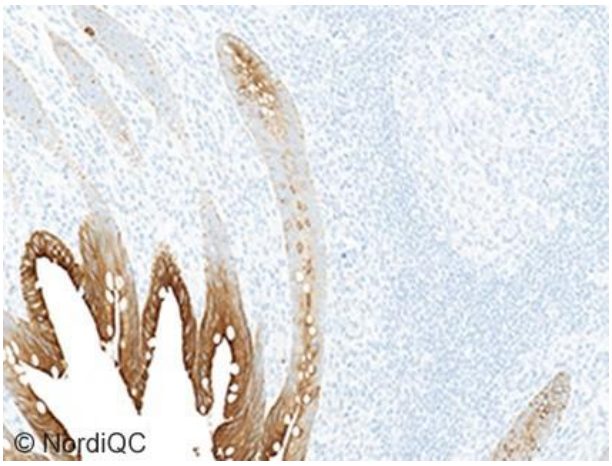


Fig. 1b
Insufficient staining for CK-LMW of the appendix using the mAb clone CAM5.2 for CK 8(7) in combination with HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana).
Only the luminal columnar epithelial cells show a moderate to strong cytoplasmic staining, while virtually no staining is seen in the basal part of the crypts.
Also compare with Figs. 2b - 4b, same protocol.

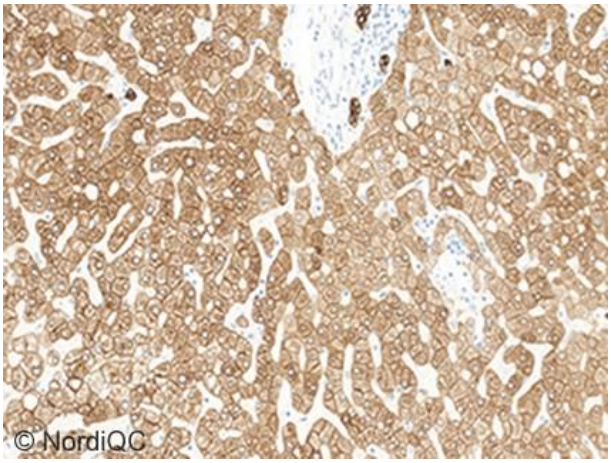


Fig. 2a
Optimal staining for CK-LMW of the liver using the same protocol as in Fig. 1a.
The vast majority of hepatocytes show a distinct, moderate staining reaction with a membrane enhancement, while the columnar epithelial cells of the bile ducts show a strong cytoplasmic staining reaction.
Same protocol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

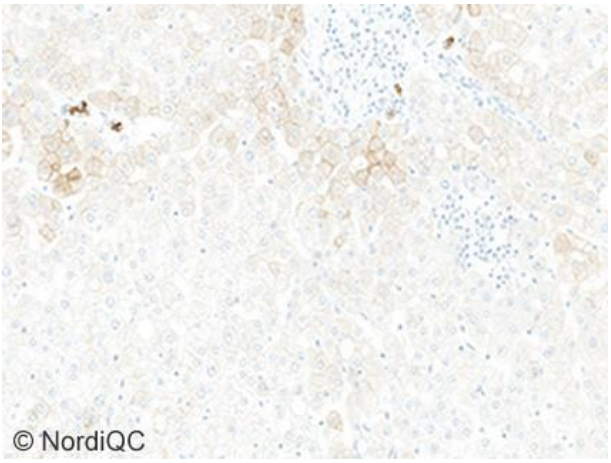


Fig. 2b
Insufficient staining for CK-LMW of the liver using the same protocol as in Fig. 1b - same field as in Fig. 2a.
Only the bile duct epithelial cells are distinctively demonstrated, while the hepatocytes are almost negative.
Same protocol used in Figs. 1b - 4b.

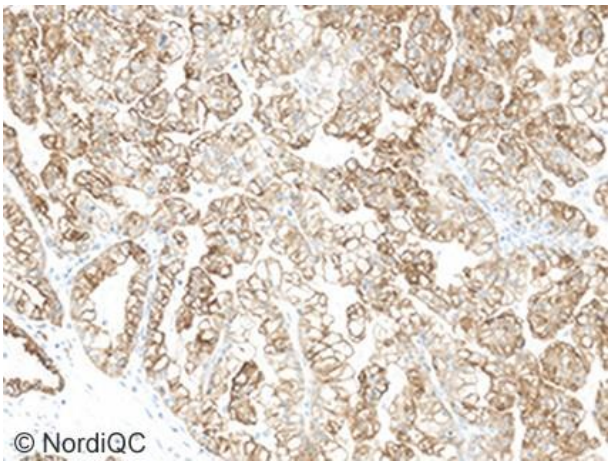


Fig. 3a
Optimal staining for CK-LMW of the renal clear cell carcinoma. Virtually all the neoplastic cells show a distinct, moderate to strong staining reaction.
No background staining is seen.
Same protocol used in Figs. 1a - 4a.

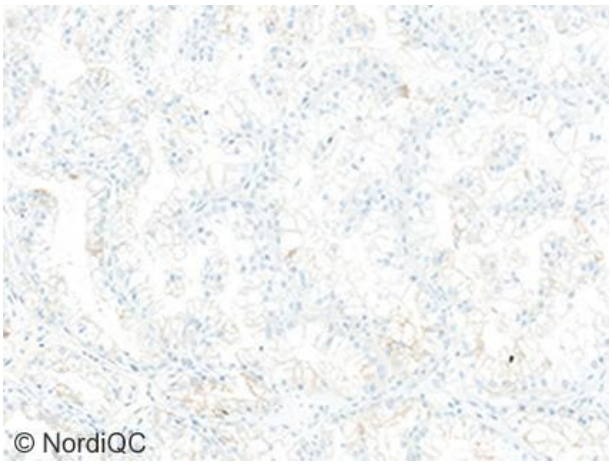


Fig. 3b
Insufficient staining for CK-LMW of the renal clear cell carcinoma - same field as in Fig. 3a.
Only scattered neoplastic cells show a weak and equivocal staining reaction.
Same protocol used in Figs. 1b - 4b.

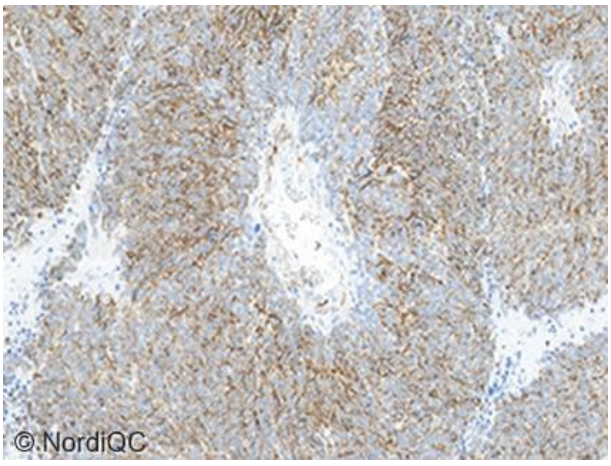


Fig. 4a
Optimal staining for CK-LMW of the SCLC.
The majority of neoplastic cells show a weak to moderate and distinct staining reaction. A dot-like cytoplasmic staining reaction is observed.
No background staining is seen.
Same protocol used in Figs. 1a - 4a.

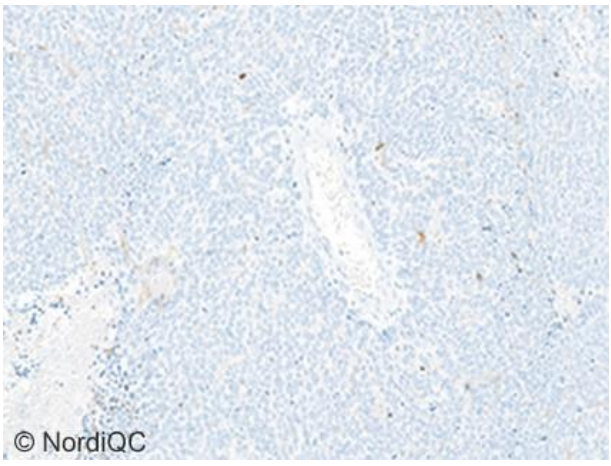


Fig. 4b
Insufficient staining for CK-LMW of SCLC - same field as in Fig. 4a.
Virtually all neoplastic cells are false negative.
Same protocol used in Figs. 1b - 4b.

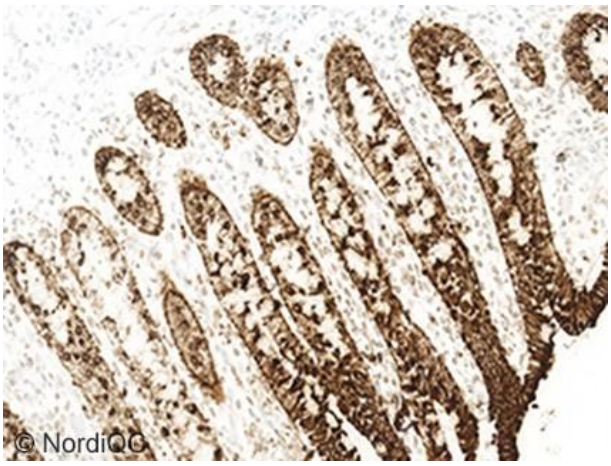


Fig. 5a
Insufficient staining for CK-LMW using the mAb clone cocktail B22.1+B22.3 for CK8/18 as Ready-To-Use format (760-4344, Ventana/Roche), with HIER in CC1 followed by proteolytic pre-treatment and OptiView with amplification as detection system. Neither proteolysis nor amplification is recommended by the vendor.
An impaired morphology and poor signal-to-noise ratio is seen.
Compare with the optimal result in Fig. 1a.
Also see Fig. 5b, same protocol.

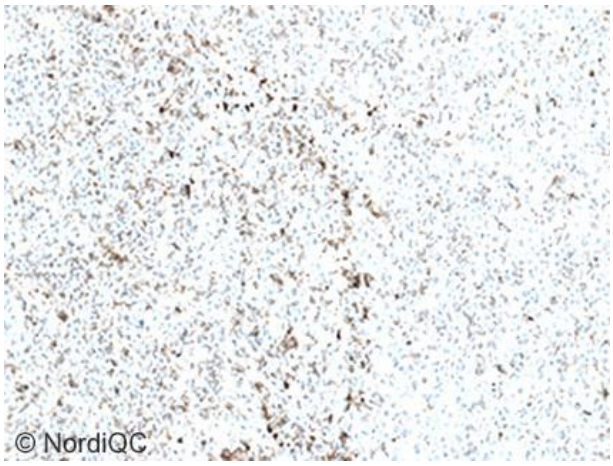


Fig. 5b
Insufficient staining for CK-LMW of the SCLC using same protocol as in Fig. 5a.
The fragile membranes of the neoplastic cells are digested and an aberrant nuclear staining staining reaction is seen.
This off-label (not vendor recommended) use of the RTU product provided both an impaired morphology and reduced analytical sensitivity.

SN/LE/MV/RR 20.03.2017