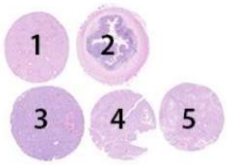
Assessment Run 54 2018

Carcinoembryonic antigen (CEA)

Material
The slide to be stained for CEA comprised:

1. Liver, 2. Appendix, 3. Colon adenocarcinoma, 4-5. Urothelial carcinoma



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CEA staining as optimal included:

- An at least weak to moderate cytoplasmic staining reaction of the vast majority of columnar epithelial cells in the appendix with enhancement of the glycocalyx.
- A moderate to strong predominantly cytoplasmic staining reaction of virtually all neoplastic cells in the colon adenocarcinoma, tissue core no. 3, and the majority of neoplastic cells in the urothelial carcinoma, tissue core no. 5.
- An at least weak to moderate predominantly cytoplasmic staining reaction focally of the neoplastic cells in the urothelial carcinoma, tissue core no. 4.
- No staining in any other cells. Polyclonal antibodies, which always give a staining reaction of non-specific cross-reacting antigen (NCA = CEACAM6) in leukocytes and biliary glycoprotein (BGP = CEACAM1) in bile canaliculi, were considered inappropriate.

Participation

Number of laboratories registered for CEA, run 54	307
Number of laboratories returning slides	287 (94%)
Number of laboratories returning slides using appropriate antibodies*	272 (89%)

*15 laboratories used an inappropriate polyclonal antibody cross reacting with NCA and BGP.

Results
272 laboratories participated in this assessment. 171 (63%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reaction were:

- Less successful primary Ab – all protocols (n=11) based on the mAb clone TF3H8-1 gave insufficient results
- Less successful performance of the mAb clone II-7 (on all platforms, but especially on the Ventana BenchMark and Dako Omnis platforms)
- Inappropriate retrieval - omission of retrieval or use of proteolysis
- Too low concentration of the primary Ab
- Use of less sensitive detection systems.

Performance history
This was the fifth NordiQC assessment of CEA. A significant increase in the pass rate was observed compared to the latest run (Run 47 in 2016) as listed in Table 2. This seems to be related to an increased used of the mAbs clones CEA31 and COL-1.

Table 2. **Proportion of sufficient results for CEA in the four NordiQC runs performed**

	Run 12 2004	Run 27 2009	Run 37 2013	Run 47 2016	Run 54 2018
Participants, n=	60	123	190	255	272
Sufficient results	86%	75%	59%	42%	63%

Conclusion
The mAb clones **CEA31** and **COL-1** can both be recommended for demonstrating CEA, irrespective of IHC stainer platform. The proportion of sufficient results were 92% and 93%, respectively. The mAb clone **II-7** was less successful. Although optimal staining results could be obtained, this clone showed significantly lower analytical sensitivity compared to mAb clone CEA31 and COL-1. This was especially evident on the Ventana BenchMark and Dako Omnis platforms. Irrespective of the clone applied, heat induced epitope

retrieval (HIER), preferably in an alkaline buffer, was mandatory for optimal staining results. Careful calibration of the primary antibody and use of sensitive 3-step multimer/polymer detection systems were also important prerequisites for an optimal staining result. The most widely used RTU system based on mAb clone CEA31 (Ventana, 760-4594) was very successful with 88% obtaining sufficient results. In contrast, only 10% of the Dako Omnis users obtained sufficient results using the mAb clone II-7 based RTU GA622. The mAb clone TF3H8-1 (Ventana, 760-2507) cross reacts with NCA and BGP and should not be used to demonstrate CEA. Appendix and liver are the recommended control tissues for CEA. In appendix, the vast majority of columnar epithelial cells must show an at least weak to moderate intra-cytoplasmic staining reaction. No staining should be seen in liver.

Table 1. **Antibodies and assessment marks for CEA, run 54**

Concentrated Antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb **BS33**	1	Nordic Biosite	0	0	1	0	-	-
mAb clone **CEA31**	24	Cell Marque	15	7	2	0	92%	100%
mAb **COL-1**	9 7 4 4 1 1 1 1 1	BioCare Thermo/Neomarkers Immunologic Invitrogen/Zymed Diagnostic BioSystems Genemed GeneTex Leica Zytomed	16	11	1	1	93%	100%
mAb **II-7**	48	Dako/Agilent	4	10	9	25	29%	-
Ready-To-Use Antibodies								
mAb clone **CEA31 760-4594**	90	Ventana/Cell Marque	57	22	4	7	88%	94%
mAb clone **CEA31 760-4594** [3]	1	Ventana/Cell Marque	0	1	0	0	-	-
mAb clone **CEA31 236M**	5	Cell Marque	3	2	0	0	-	-
mAb clone **COL-1 MAD-002095QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **COL-1 PM058**	1	Biocare	1	0	0	0	-	-
mAb clone **COL-1 PA0848**	2	Leica	0	1	0	0	-	-
mAb clone **COL-1 Kit-0008**	2	Maixin	1	0	1	0	-	-
mAb clone **COL-1 8225**	1	Sakura	1	0	0	0	-	-
mAb clone **II-7 GA622**	21	Dako/Agilent	0	2	11	8	10%	-
mAb clone **II-7 GA622** [4]	2	Dako/Agilent	0	0	1	1	-	-
mAb clone **II-7 IR/IS622**	24	Dako/Agilent	1	13	4	6	58%	90%
mAb clone **II-7 IR/IS622** [5]	4	Dako/Agilent	1	0	1	2	-	-
mAb clone **II-7 PA0004**	5	Leica	0	1	3	1	-	-

mAb clone **TF3H8-1 760-2507**	11	Ventana/Roche	0	0	9	2	0%	-
Unknown clone	1	Leica	0	0	1	0	-	-
Total	272		100	71	48	53	-	
Proportion			37%	26%	18%	19%	63%	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the Ventana Benchmark system, but used on the Leica Bond platform.
4) RTU system developed for the Dako/Agilent full-automatic system (Dako Omnis), but used by laboratories on different platforms (e.g. Ventana Benchmark and Dako Autostainer).
5) RTU system developed for the Dako/Agilent semi-automatic system (Dako Autostainer), but used by laboratories on different platforms (e.g. Ventana Benchmark and Leica Bond).

Detailed analysis of CEA, Run 54
The following protocol parameters were central to obtain optimal staining:

Concentrated Antibodies
mAb clone **CEA31**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Cell Conditioning 1 (CC1, Ventana) (8/12)*, Target Retrieval Solution (TRS) pH 9 (Dako) (4/6), TRS pH 9 (3-in-1) (Dako) (2/2) or Cell Conditioning 2 (CC2, Ventana) (1/1). The mAb was typically diluted in the range of 1:100-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings, 19 of 19 (100%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **COL-1**: Protocols with optimal results were all based on HIER using either CC1 (Ventana) (9/14), TRS pH 9 (Dako) (3/3), Citrate pH 6 (2/2), TRS pH 6 (3-in-1) (Dako) (1/2) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/3). The mAb was typically diluted in the range of 1:100-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 21 of 21 (100%) laboratories produced a sufficient staining.

mAb clone **II-7**: Protocols with optimal results were all based on HIER using either Bond Epitope Retrieval Solution 1 (BERS1, Leica) (3/6) or BERS 2 (Leica) (1/10) as 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, 4 of 5 (80%) laboratories produced a sufficient staining.

Table 3. **Proportion of optimal results for CEA for the most commonly used antibodies as concentrates on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark GX / XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	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 **CEA31**	2/2**	-	4/5 80%	-	8/11 73%	1/1	-	-
mAb clone **COL-1**	-	1/2	2/2	0/1	9/11 (82%)	-	1/3	-
mAb clone **II-7**	0/5 (0%)	-	0/4	-	0/7 (0%)	-	1/2	3/3

* 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 Abs (RTU)
mAb clone **CEA31** product no. **760-4594**, Ventana/Cell Marque, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 16-64 min.), 12-32 min. incubation of the primary Ab and UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 78 of 83 (94%) laboratories produced a sufficient staining.

mAb clone **COL-1**, product no. **Kit-0008**, Maixin, manual:
One protocol with an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 (Waterbath) for 20 min., 60 min. incubation of the primary Ab and MaxVision III DAB (Maixin) as detection system.

mAb clone **COL-1,** product no. **8225**, Sakura Finetek, Genie:
One protocol with an optimal result was based on 30 min. HIER using Sakura Finetek Tissue-Tek Genie High pH Antigen Retrieval Buffer, 30 min. incubation of the primary Ab and Tissue-Tek PRO DAB Detection Kit (8826-K250) as detection system.

mAb clone **COL-1**, product no. **PM058**, Biocare, IntelliPath:
One protocol with an optimal result was based on HIER using Diva Decloaker (BioCare) in a pressure cooker 15 min. at 110°C and MACH4 (BioCare, M4U534) as detection system.

mAb clone **II-7** product no. **IR/IS622**, Dako/Agilent, Dako Autostainer and Dako Autostainer Link:
One protocol with an optimal result was based on HIER in PT-Link using TRS High pH (3-in-1) (efficient heating time 20 min.), 20 min. incubation of the primary Ab and EnVision Flex+ (3-step protocol with 15 min. in linker) as detection system. Using similar protocol settings, 9 of 10 (90%) laboratories produced a sufficient staining.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for CEA for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS mAb clone **CEA31, 760-4594**	83% (10/12)	25% (3/12)	88% (69/78)	69% (54/78)
Dako AS mAb clone **II-7, IR/IS622**	43% (3/7)	0% (0/7)	65% (11/17)	6% (1/17)
Dako OMNIS mAb clone **II-7, GA622**	13% (2/15)	0% (0/15)	0% (0/6)	0% (0/6)

* 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 integrated.

Comments
In this assessment and in concordance with the previous NordiQC assessments of CEA, the prevalent features of an insufficient staining were either a generally too weak or false negative staining reaction of the cells and structures expected to be demonstrated or a false positive staining reaction. Too weak or false negative staining reaction was seen in 84% of the insufficient results (85 of 101 laboratories). False positive staining was seen in 14 laboratories (14%) and 2 laboratories failed because of technical staining issues. Virtually all laboratories were able to demonstrate CEA in the colon adenocarcinoma, tissue core no. 3, whereas the urothelial carcinoma, tissue core no. 5, and in particular the urothelial carcinoma, tissue core no. 4, were much more challenging and required an optimally calibrated protocol. Compared to the previous CEA assessment (Run 47 in 2016) a significant increase in the pass rate was seen. The reason could be an increased number of participants using the mAbs clones CEA31 and COL-1. Both clones (CEA31 and COL-1) have repeatedly shown a very high analytical sensitivity in the previous assessments. The proportion of laboratories using the mAbs clones CEA31 or COL-1 increased from 36% (91 of 255) in 2016 to 57% (156 of 272) in the current run.

38% (102 of 272) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for CEA. The mAb clones II-7, CEA31 and COL-1 were the three most widely used. They could all be used to obtain an optimal staining, but the clones CEA31 and COL-1 provided a significantly higher proportion of optimal and sufficient results, see Table 1. The mAb clones CEA31 and COL-1 could produce optimal staining on virtually all the main stainer platforms (see Table 3) especially on the challenging tissue materials (e.g. tissue core 4 and 5), and in concordance with the previous NordiQC CEA assessment in 2016 (Run 47) they clearly demonstrated a higher analytical sensitivity compared to mAb clone II-7 (see Fig. 1a – 4b). Only 4 of 48 (8%) laboratories using mAb clone II-7 produced optimal staining, whereas 55% (16 of 29) and 63% (15 of 24) produced optimal staining when using mAb clone COL-1 and CEA31, respectively. The four LD assays based on mAb clone II-7 giving optimal staining where all based on HIER and a sensitive 3-step polymer detection system performed on the Leica Bond platform. Despite similar protocol settings (range of Ab titre, 3-step multimer/polymer and HIER), no optimal results were

seen with mAb clone II-7 on any of the other main platforms (Dako Autostainer, Dako Omnis or Ventana BenchMark).

RTU antibodies was used in 62% (170 of 272) of the laboratories. A high proportion of optimal results was obtained with the RTU systems based on the mAb clone CEA31 and the mAb clone COL-1. As seen in the previous CEA assessment (run 37, 2013 and run 47 in 2016), the most successful and robust assay for CEA was obtained with the RTU system based on the mAb clone CEA31 from Ventana/Cell Marque (760-4594). The pass rate was 88% (79 of 90 laboratories) and 63% were assessed as optimal (57 of 90 laboratories). In contrast, the widely used RTU system based on the mAb clone II-7 from Dako (IR/IS622 for the Dako Autostainer and GA622 for the Dako Omnis) again performed poorly, especially on the Dako Omnis. Using the GA622 RTU on the Dako Omnis, only 10% of the laboratories had a sufficient staining (2 of 21) and none were assessed as optimal. 58% (14 of 24) of the laboratories using the IR/IS622 RTU on the Dako Autostainer obtained a sufficient staining reaction but only 4% (1 of 24) was optimal. Assessment data showed that laboratories modifying the protocol settings for the IR/IS622 RTU performed better than the laboratories following the recommended protocol settings (see Table 4). The proportion of sufficient results increased from 43% (3 of 7) to 65% (11 of 17) when the protocol settings were modified. If the modifications included the use of a 3-step Envision Flex detection system instead of the recommended 2-step Envision Flex the proportion of sufficient results increased further to 92% (11 of 12).

In concordance with previous assessments, HIER was required in order to obtain sufficient results. Irrespective of the clone (RTU or LD-assay) or IHC platform used, inappropriate retrieval, as omission of HIER (n=7 laboratories) or use of proteolytic pre-treatment (n=1 laboratory), gave insufficient results due to weak staining reactions (see Fig. 5a-6b). The majority of optimal results (92 of 100) was obtained with the use of HIER in an alkaline buffer, but 8 laboratories obtained optimal results using various low pH buffers. Common for these laboratories was the use of carefully calibrated protocols, all based on sensitive 3-step polymer/multimer detection systems.

False positive reaction was seen in 14% of the insufficient results (14 of 101 laboratories). In 11 of these cases, this was related to the use of mAb clone TF3H8-1 giving a cross reaction with both NCA (CEACAM6) and biliary glycoprotein (BGP = CEACAM1) (see Fig. 7a and 7b). All slides showing this positive reaction in either leukocytes and/or bile canaliculi were assessed as insufficient.

Controls
Appendix, in combination with liver, is the recommended positive and negative tissue controls for CEA. In the appendix the vast majority of epithelial cells must show an at least weak to moderate cytoplasmic staining reaction. If only the glycocalyx is demonstrated, inadequate staining in neoplasias with low CEA expression is seen (as observed in the urothelial carcinoma, tissue core no. 4, in this assessment – see Figs. 1, 4, 5 and 6). Liver is recommended as negative tissue control. Bile canaliculi and leucocytes must be negative with no cross reaction to BGP or NCA to verify the specificity of the primary Ab.

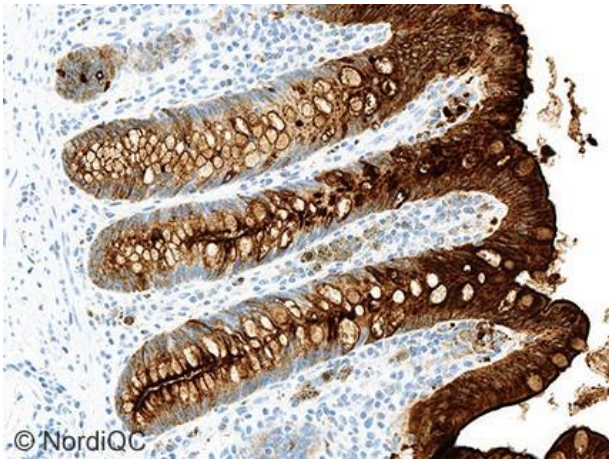


Fig. 1a (x200)
Optimal CEA staining of the appendix using the **mAb clone CEA31** in a RTU format (Ventana, 760-4594) with an incubation time of 16 min. after HIER in CC1 for 32 min. Staining was performed on the Ventana Benchmark Ultra using a 3-step multimer system (OptiView). A moderate to strong staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction. No background staining is seen. Also compare with Figs. 2a – 4a, same protocol.

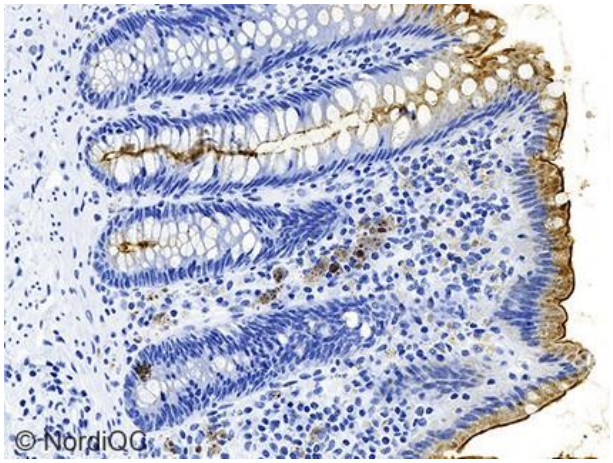


Fig. 1b (x200)
Insufficient CEA staining of the appendix using the **mAb clone II-7** in a RTU format (Dako, GA622) with an incubation time of 25 min. after HIER in an alkaline buffer (TRS pH 9, Dako). Staining was performed on the Dako Omnis using a 3-step polymer system (EnVision Flex+). In spite of using the vendors recommended protocol settings, the "clone II-7"-protocol only demonstrates the glycocalyx distinctively, while the cytoplasmic compartment in the vast majority of epithelial cells is unstained or only very faintly stained - same field as in Fig. 1a. Also compare with Figs. 2b - 4b, same protocol.

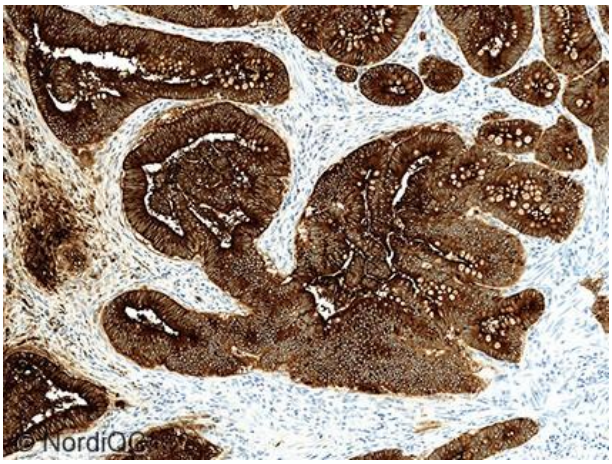


Fig. 2a (x100)
Optimal CEA staining of the colon adenocarcinoma, tissue core no. 3, with high level CEA expression using same protocol as in Fig. 1a. All the neoplastic cells show a strong and distinct cytoplasmic staining reaction. Weak background staining in the vicinity of the neoplastic cells, due to diffusion of antigen, is seen and accepted.

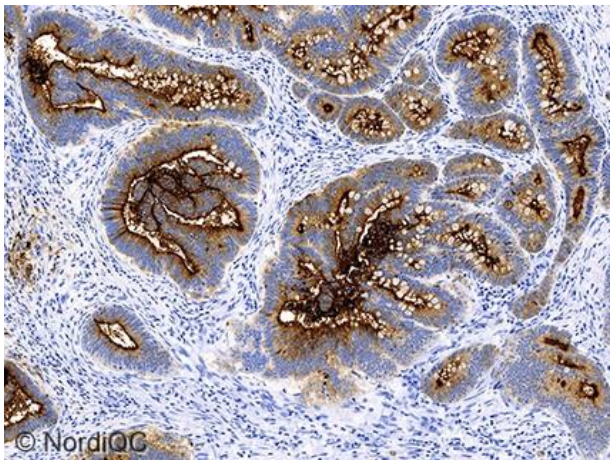


Fig. 2b (x100)
CEA staining of the colon adenocarcinoma, tissue core no. 3, with high level CEA expression using the same insufficient protocol as in Fig. 1b. – same field as in Fig. 2a. The intensity of the neoplastic cells demonstrated is reduced compared to the level expected and obtained in Fig. 2a.

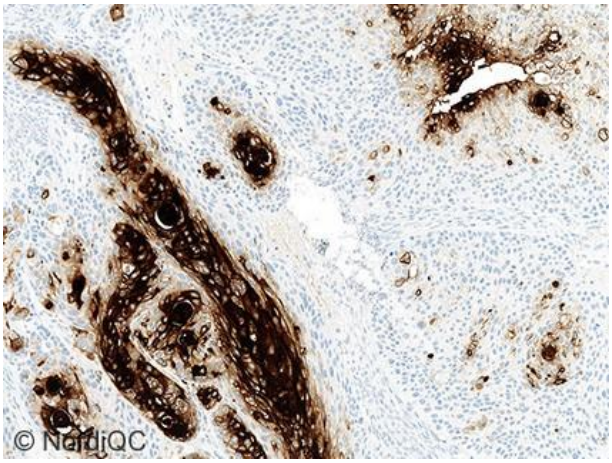


Fig. 3a (x100)
Optimal CEA staining of the urothelial carcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a. A large proportion of the neoplastic cells show a strong and distinct staining reaction. No background staining is seen.

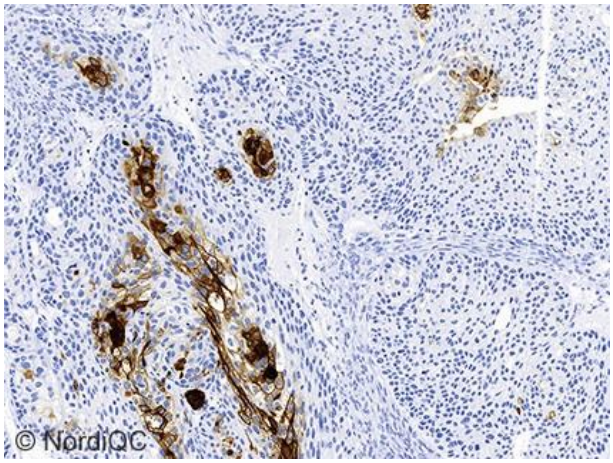


Fig. 3b (x100)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 5, using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a. The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 3a.

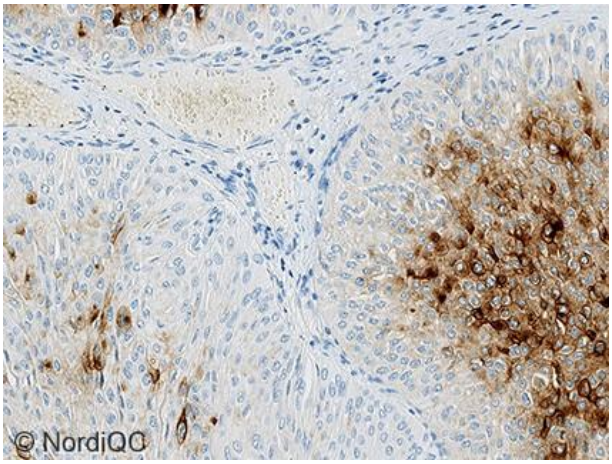


Fig. 4a (x200)
Optimal CEA staining of the urothelial carcinoma, tissue core no. 4, with low level CEA expression using same protocol as in Figs. 1a - 3a. Focally the neoplastic cells show a moderate to strong and distinct staining reaction. No background staining is seen.

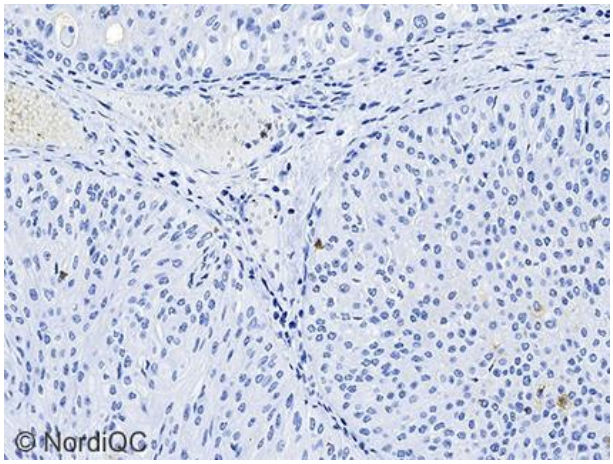


Fig. 4b (x200)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 4, with low level CEA expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. The neoplastic cells show no staining reaction and a false negative result in this tumour is seen.

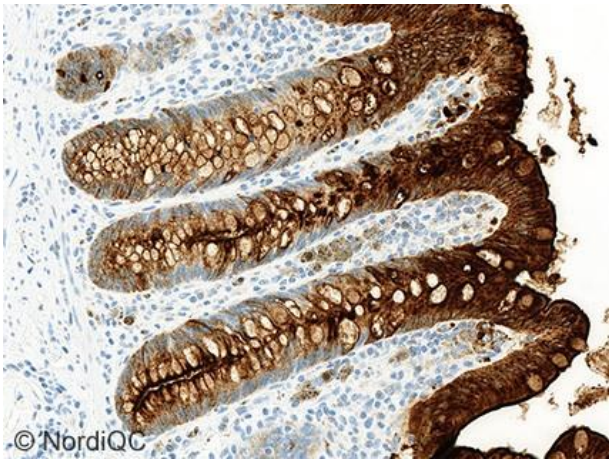


Fig. 5a (x200) same as Fig. 1a
Optimal CEA staining of the appendix using the **mAb clone CEA31** RTU (Ventana, 760-4594) with an incubation time of 16 min. after HIER in CC1 for 32 min. Staining was performed on the Ventana Benchmark Ultra using a 3-step multimer system (OptiView). A weak to strong staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction. No background staining is seen. Compare also to Fig 6a, same protocol.

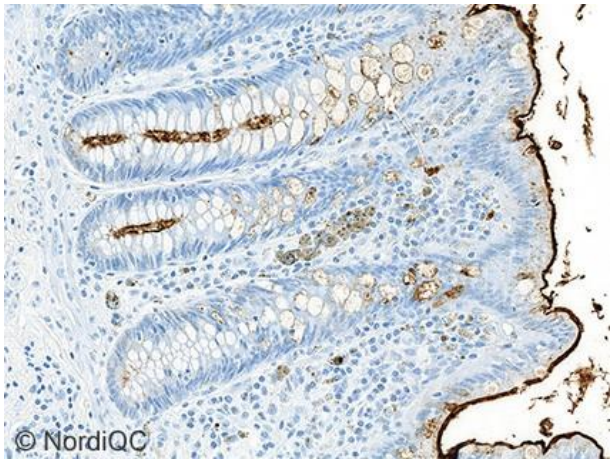


Fig. 5b (x200)
Insufficient CEA staining of the appendix using the mAb clone CEA31 with similar protocol settings as used in Fig 5a. Only difference was the **omission of HIER**. The lack of HIER results in a drastic reduction in staining intensity. Only the glycocalyx is distinctively demonstrated, whereas the cytoplasmic compartment of the epithelial cells is unstained - same field as in Fig. 5a. Compare also to Fig 6b, same protocol.

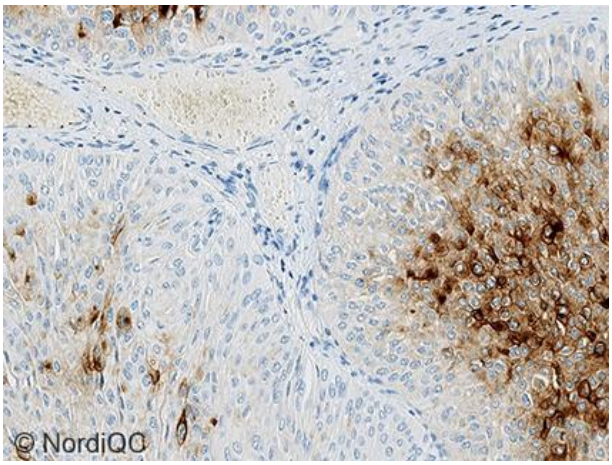


Fig. 6a (x200) same as Fig. 4a
Optimal CEA staining of the urothelial carcinoma, tissue core no. 4, with low level CEA expression using same protocol as in Fig. 5a. Focally the neoplastic cells show a moderate to strong and distinct staining reaction. No background staining is seen.

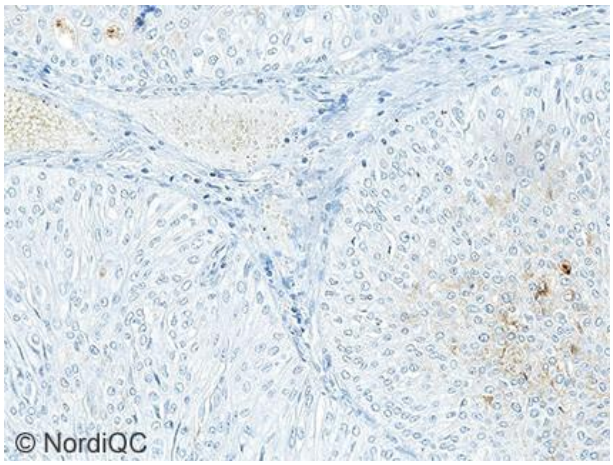


Fig. 6b (x200)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 4, with low level CEA expression using same protocol as in Fig. 5b – same field as in Fig. 6a. The neoplastic cells show no or only very faint staining reaction and a false negative result in this tumour is seen.

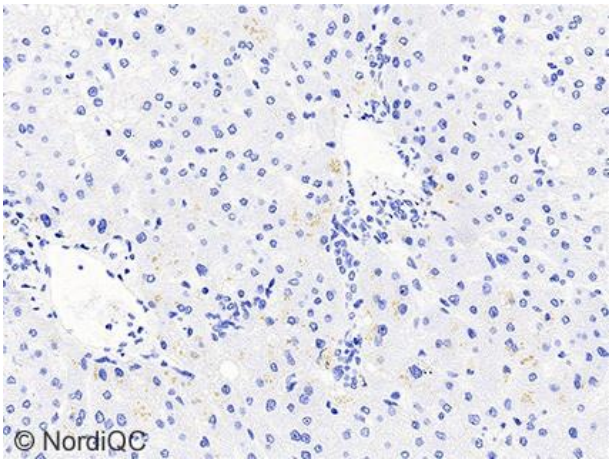


Fig. 7a (x200)
Optimal CEA staining of the liver using a similar protocol as in Figs. 5a and 6a based on the **mAb clone CEA31**. No staining reaction is seen in the Kupffer cells, leucocytes and the bile canaliculi. No background staining is seen.

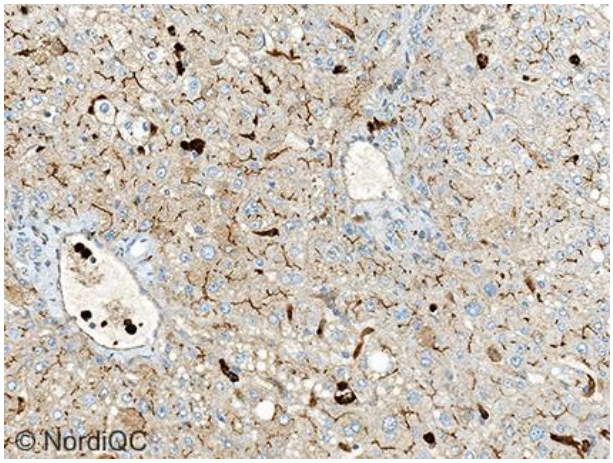


Fig. 7b (x200)
Insufficient CEA staining of the liver using the **mAb clone TF3H8-1.** Both the Kupffer cells, leucocytes and bile canaliculi are stained due to a cross reaction of the Ab to NCA (CEACAM6) and BGP (CEACAM1) – same field as in Fig. 7a.

ON/LE/MV/RR 23.11.2018