

### Material

The slide to be stained for CEA comprised:

1. Appendix, 2. Liver, 3-4. Colon adenocarcinoma, 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 majority of the columnar epithelial cells of the appendix with an enhancement of the glycocalyx.
- A moderate to strong predominantly cytoplasmic staining reaction in virtually all the neoplastic cells in the colon adenocarcinoma no. 3 and in the majority of the neoplastic cells in the colon adenocarcinoma no. 4.
- An at least weak to moderate predominantly cytoplasmic staining reaction focally of the neoplastic cells in the urothelial carcinoma.
- No staining in any other cells. Especially no staining reaction of non-specific cross-reacting antigen (NCA = CEACAM6) in leukocytes and biliary glycoprotein (BGP = CEACAM1) in bile canaliculi.



210 laboratories participated in this assessment. 20 laboratories used a polyclonal antibody to CEA cross reacting with NCA and BGP and hence considered inappropriate and not assessed.

Of the remaining 190 participants, 59 % achieved a sufficient mark.

Antibodies (Abs) used and marks are summarized in table 1.

Table 1. **Antibodies and assessment marks for CEA, run 37**

Concentrated Antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. <sup>1</sup>	Suff. OPS <sup>2</sup>
mAb clone <b>12-140-10</b>	6	Leica/Novocastra	0	0	1	5	-	-
mAb clone <b>CEA31</b>	1	Cell Marque	1	0	0	0	-	-
mAb <b>COL-1</b>	9	Thermo/Neomarkers	13	4	4	0	81 %	100 %
	4	Biocare						
	4	Invitrogen/Zyomed						
	3	Immunologic						
	1	Zytomed						
mAb <b>II-7</b>	89	Dako	12	42	33	2	61 %	93 %
mAb <b>PARLAM 4</b>	1	BioScience Products AG	0	0	1	0	-	-
rmAb <b>EP216</b>	1	Epitomics	0	1	0	0	-	-
Ready-To-Use Antibodies								
mAb clone <b>B01-94-11-M AM009</b>	1	Biogenex	0	0	1	0	-	-
mAb clone <b>CEA31 760-4594</b>	12	Ventana/Cell Marque	9	2	1	0	92 %	92 %
mAb clone <b>CEA31 236M</b>	1	Cell Marque	1	0	0	0	-	-
mAb clone <b>CEA31 ZM-0062</b>	1	Zhongshan	0	0	1	0	-	-
mAb clone <b>COL-1 PM058</b>	1	Biocare	0	1	0	0	-	-

mAb clone <b>II-7 IR/IS622</b>	33	Dako	1	19	13	0	61 %	80 %
rmAb clone <b>II-7 N1586</b>	2	Dako	0	2	0	0	-	-
mAb clone <b>II-7 PA0004</b>	4	Leica	1	3	0	0	-	-
mAb clone <b>TF3H8-1 760-2507</b>	16	Ventana	0	0	0	16	-	-
Total	190		38	74	55	23	-	
Proportion			20 %	39 %	29 %	12 %	59 %	

1) Proportion of sufficient stains (optimal or good)

2) Proportion of sufficient stains with optimal protocol settings only, see below.

### Detailed analysis of CEA, Run 37

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

#### Concentrated Abs

mAb clone **CEA31**: The protocol with an optimal result was based on heat induced epitope retrieval (HIER) using CC1 (BenchMark, Ventana) as the retrieval buffer. The mAb was diluted 1:400.

mAb clone **COL-1**: Protocols with optimal results were all based on HIER using either Target Retrieval Solution pH 9 (3-in-1) (TRS pH 9;Dako) (1/1)\*, Cell Conditioning 1 (CC1; Ventana) (8/9), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (1/1), BERS 1 (Leica) (1/2), Tris-EDTA/EGTA pH 9 (1/3) or Citrate pH 6 (1/2) as the retrieval buffer. 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 14 of 14 (100 %) laboratories produced a sufficient staining (optimal or good).

\* (number of optimal results/number of laboratories using this buffer)

mAb clone **II-7**: Protocols with optimal results were all based on HIER using either TRS pH 9, 3-in-1 (Dako) (3/17), TRS pH 9 (Dako) (2/8), BERS 2 (Leica) (3/6), Tris-EDTA/EGTA pH 9 (3/10) or EDTA/EGTA pH8 (1/1) as the retrieval buffer. The mAb was typically diluted in the range of 1:30-1:320 depending on the total sensitivity of the protocol employed. Using these protocol settings 25 of 27 (93 %) laboratories produced a sufficient staining (optimal or good).

In table 2, the overall proportion of optimal staining results using the two most frequently used concentrated Abs on the three most commonly used IHC stainer platforms is summarized. Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.

Table 2. **Optimal results for CEA using concentrated antibodies on the 3 main IHC systems\***

Concentrated antibodies	Dako		Ventana		Leica	
	Autostainer Link / Classic		BenchMark XT / Ultra		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 <b>II-7</b>	20 % 5/25*	-	0 % 0/35	0 % 0/1	50 % 3/6	0 % 0/2
mAb clone <b>COL-1</b>	100 % 1/1	-	89 % 8/9	-	100 % 1/1	50 % 1/2

\* 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 Abs (RTU)

mAb clone **CEA31** (prod. no. 760-4594, Ventana/Cell Marque): Protocols with optimal results were typically based on HIER using mild or standard Cell Conditioning 1, 16-40 min incubation of the primary Ab and UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings 11 of 12 (92 %) laboratories produced an optimal staining.

mAb **II-7** (product.no. IR/IS622, Dako): The protocol with optimal results was based on HIER in PT-Link using TRS pH 9 (3-in-1) (heating time 10 min. at 97°C), 20 min incubation of the primary Ab and EnVision FLEX+ (K8002) as detection system. Using these protocol settings 4 of 5 (80 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **II-7** (product. no. PA0004, Leica/Novocastra): The protocol with optimal result was based on HIER using BERS 2 (Bond, Leica), 15 min of primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 3 of 3 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient stainings were:

- Less successful primary Ab – e.g. all 22 protocols based on the mAb clones TF3H8-1 and 12-140-10 gave insufficient results
- Inappropriate retrieval - Omission of HIER or use of proteolysis
- Less successful performance of the mAb clone II-7 on the BenchMark IHC platform, Ventana
- Too low concentration of the primary Ab

In this assessment and concordant to the previous NordiQC assessments of CEA (run 12 2004, run 27 2009) the prevalent features of an insufficient staining were either a generally too weak staining reaction or a false positive staining reaction.

A too weak staining reaction was seen in 69 % of the insufficient results (54 out of 78 laboratories). Virtually all laboratories were able to demonstrate CEA in the colon adenocarcinoma no. 3, whereas the colon adenocarcinoma no. 4 and in particular the urothelial carcinoma were much more challenging and required an optimally calibrated protocol.

The mAb clones II-7, CEA31 and COL-1 could all be used to obtain an optimal staining, although the clones CEA31 and COL-1 provided a higher proportion of optimal results, see table 1. This was seen for both the concentrated formats and the RTU systems based on these clones.

Optimal results could be obtained for the mAb clone II-7 when applied on either the Dako Autostainer system or the Leica Bond system, whereas no optimal results were seen on the Ventana BenchMark system, despite similar protocol settings were used on the three IHC platforms (see table 2).

In general, the mAb clone II-7 showed a significant inferior performance on the Ventana BenchMark system compared to other IHC platforms. If the mAb clone II-7 was applied in the range of 1:30-320, using HIER in an alkaline buffer CC1 pH 8.5 and performed on the BenchMark platform a sufficient result was obtained by 18 out of 35 laboratories (51 %). In contrast 23 out of 25 protocols (92 %) based on identical settings (Ab titre and HIER in an alkaline buffer) and performed on the Dako Autostainer system were assessed as sufficient and of these 5 were assessed as optimal (20 %).

Irrespective of the clone or IHC platform used, inappropriate retrieval, as omission of HIER (n=9 laboratories) or use of proteolytic pre-treatment (n=3 laboratories), gave insufficient results due to weak staining reactions.

A false positive reaction was seen in 28 % of the insufficient results (22 out of 78 laboratories). This was related to the following primary Ab clones: The mAb clone 12-140-10 giving a cross reaction with non-specific cross-reacting antigen, (NCA; CEACAM6) in leucocytes and the mAb clones PARLAM 4 and TF3H8-1 giving a cross reaction with both NCA and biliary glycoprotein (BGP; CEACAM1). 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 control for CEA.

In the appendix the vast majority of the epithelial cells must show an at least weak to moderate cytoplasmic staining reaction. If only the glycocalyx is demonstrated, an inadequate staining in neoplasias with low CEA expression is seen (e.g. as observed in the urothelial carcinoma in this assessment).

Liver is recommended as negative control. Bile canaliculi and leucocytes must be negative to verify the specificity of the primary Ab and no cross reaction to BGP or NCA is present. Hepatocytes must be negative to verify a high signal-to-noise ratio and staining due to endogenous biotin or use of a poorly calibrated primary Ab is seen.

### Effect of external quality assessment

This was the 3rd assessment of CEA in NordiQC and a consistent decrease in the pass rate has been seen during these 3 runs as listed in table 3.

Table 3. **Proportion of sufficient results for CEA in the three NordiQC runs performed**

	Run 12 2004	Run 27 2009	Run 37 2013
Participants, n=	60	123	190
Sufficient results	86 %	75 %	59 %

Several parameters contribute to the low pass rate. The consistent use and commercial availability of Abs with a false positive staining (n=23 laboratories) and use of inappropriate retrieval settings (n=12 laboratories) had significant impact, as all these protocols were assessed as insufficient.

In this run for CEA a difference in the pass rates was observed for the laboratories participating in the assessment for the first time compared to the laboratories also participating in the latest assessment, run 27 2009. For the laboratories participating for the first time the pass rate was 49 % (36 of 74 laboratories), whereas the pass rate was 66 % (76 of 116 laboratories) for the laboratories participating in both runs.

The tailored recommendations given to the laboratories obtaining an insufficient mark did have a slightly positive impact. In run 27, 28 laboratories were given a tailored recommendation and subsequently submitted a staining in this run. 20 laboratories followed the recommendation of which 12 (60 %) improved to a sufficient result, while 8 did not change their protocol and none of these obtained a sufficient result. Recommendations given to laboratories using the mAb clone II-7 on the Ventana BenchMark platform were less successful.

Conclusive the relatively low pass rate in this run mainly was caused by the increased number of new participants, the extended use of inappropriate protocol settings but also in part of less successful tailored recommendations.

### Conclusion

The mAb clones **II-7**, **CEA31** and **COL-1** can all be recommended for the demonstration of CEA. The performance of the Abs seems to be influenced by the IHC stainer platform. The mAb clone II-7 gave an inferior staining performance on the Ventana BenchMark platform compared to other platforms.

Irrespective of the clone HIER preferable in an alkaline buffer is mandatory for an optimal staining result. Appendix and liver are the recommended controls for CEA. In the appendix the vast majority of the columnar epithelial cells must show an at least weak to moderate intra-cytoplasmic staining reaction. In the liver no staining must be seen.

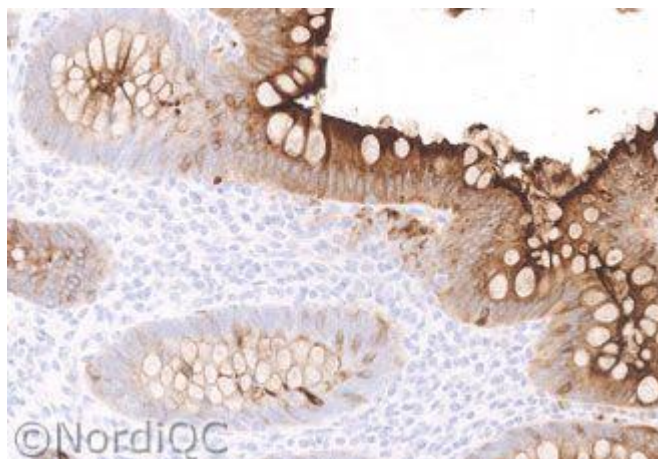


Fig 1a  
Optimal CEA staining of the appendix using the mAb clone CEA31 optimally calibrated and with HIER. A weak to moderate staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction. Also compare with Figs. 2a – 4a, same protocol.

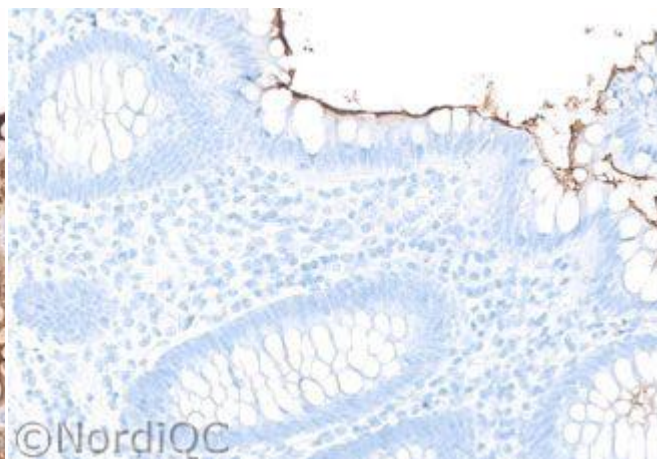
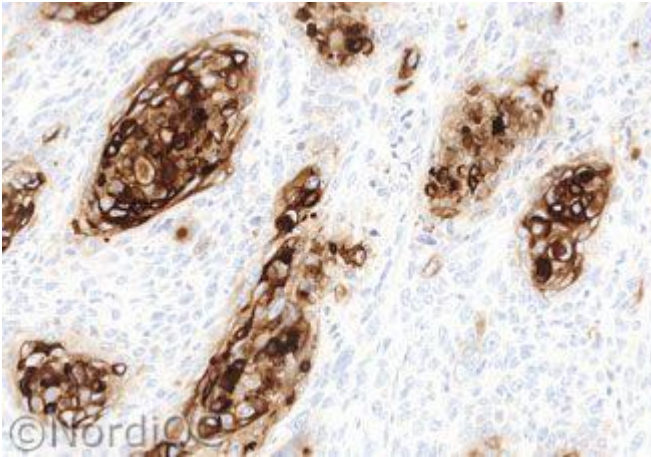
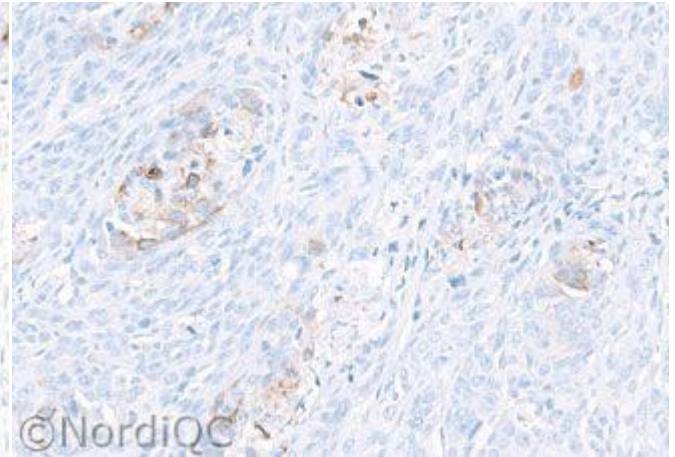


Fig 1b  
Insufficient CEA staining of the appendix using the mAb clone II-7 with a less successful protocol – insufficient HIER and too diluted. Only the glycocalyx is distinctively demonstrated, while the cytoplasmic compartment of the epithelial cells is unstained - same field as in Fig. 1a. Also compare with Figs. 2b & 3b, same protocol.

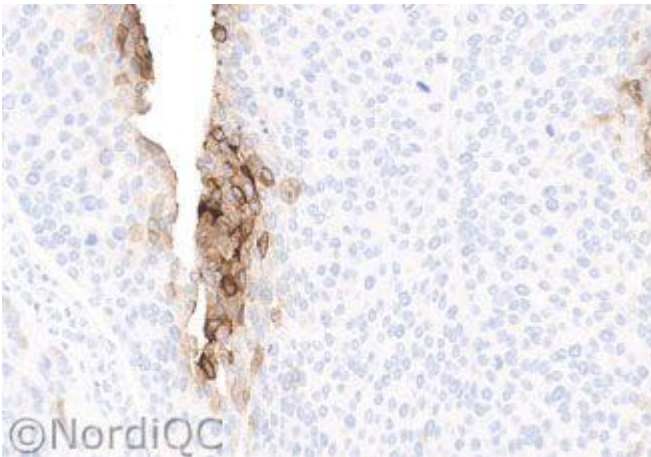




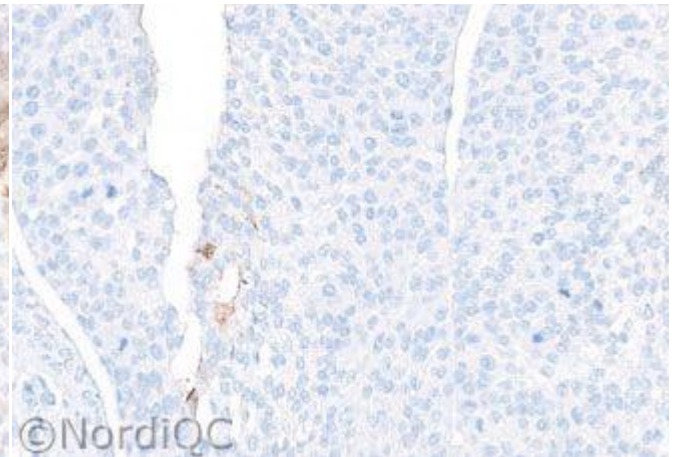
**Fig 2a**  
Optimal CEA staining of the colon adenocarcinoma, tissue core no. 4 using same protocol as in Fig. 1a. Virtually all the neoplastic cells show a strong and distinct cytoplasmic staining reaction. No background staining is seen.



**Fig 2b**  
Insufficient CEA staining of the colon adenocarcinoma, tissue core no. 4 using same protocol as in Fig. 1b. – same field as in Fig. 2a. The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 2a.



**Fig 3a**  
Optimal CEA staining of the urothelial carcinoma using same protocol as in Figs. 1a & 2a. Focally the neoplastic cells show a strong and distinct staining reaction. No background staining is seen.



**Fig 3b**  
Insufficient CEA staining of the urothelial carcinoma using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a. Only dispersed neoplastic cells show a weak and equivocal staining reaction.

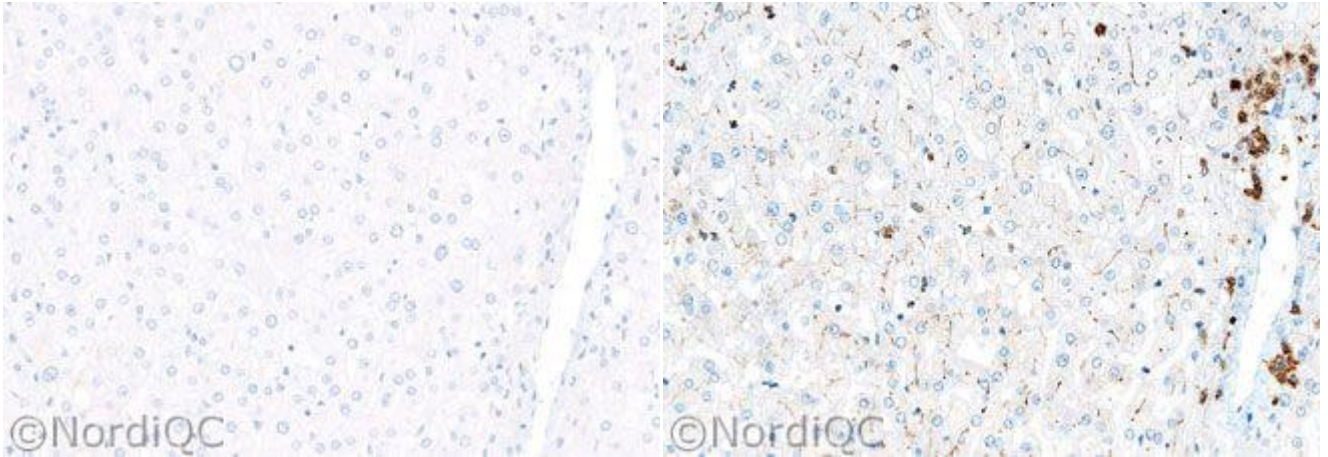


Fig 4a  
Optimal CEA staining of the liver using same protocol as in Figs. 1a - 3a based on the mAb clone CEA31. No staining reaction is seen in the Kupffer cells, leucocytes and the bile canaliculi.  
No background staining is seen.

Fig 4b  
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. 4a.

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