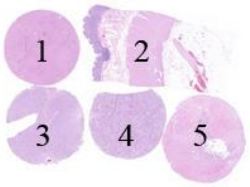
Assessment Run 53 2018

E-Cadherin (ECAD)

Table 4 updated 30.08.2021

Material

The slide to be stained for ECAD comprised:



1. Liver, 2. Colon, 3-4. Ductal breast carcinomas, 5. Lobular breast carcinoma. All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing an ECAD staining as optimal included:

- A strong, distinct membranous staining reaction of the epithelial cells of the bile ducts and an at least moderate membranous staining reaction of the hepatocytes in the liver.
- A strong, distinct membranous staining reaction of virtually all the columnar epithelial cells in the colon.
- A moderate to strong, distinct membranous staining reaction of virtually all neoplastic cells of the breast ductal carcinomas.
- No staining reaction or at maximum a focal and weak membranous staining reaction of the neoplastic cells of the breast lobular carcinoma.
- No staining reaction of stromal cells e.g. lymphocytes and plasma cells in lamina propria of the colon mucosa.

Participation

Number of laboratories registered for ECAD, run 53	307
Number of laboratories returning slides	298 (97%)

Results

298 laboratories participated in this assessment. Of these, 264 (89%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary Ab
- Inefficient heat induced epitope retrieval (HIER) e.g., use of acidic buffer or too short HIER time
- Poor signal-to-noise ratio or false positive staining reaction of assays based on the rabbit monoclonal Ab (rmAb) EP700Y
- Unexplained technical issues

Performance history

This was the third NordiQC assessment of ECAD. An increase in pass rate was seen compared to run B16, 2013 (see Table 2).

Table 2. **Proportion of sufficient results for ECAD in the three NordiQC runs performed**

	Run B5 2008	Run B16 2013	Run 53 2018
Participants, n=	94	271	298
Sufficient results	75 %	82 %	89%

Conclusion

The mouse monoclonal Ab (mAb) clones **NCH-38, 36, 36B5, 4A2C7, DBM 15.49, ECH-6, HECD-1 and GM016** could all be used to obtain optimal staining results for ECAD. Irrespective of the clone applied, efficient HIER, preferable in an alkaline buffer, and careful calibration of the primary Ab were the most important prerequisites for an optimal staining result. The most common Ready-To-Use (RTU) systems (IS/IR/GA059 and 790-4497) based on the mAb clones NCH-38 (Dako) and 36 (Ventana), respectively, provided the highest proportion of sufficient and optimal results. The RTU system GA059 (Omnis) was very robust, as all protocols (31 of 31) gave optimal results. Assays based on the rmAb clone EP700Y, both as concentrated (Conc) formats and RTU systems, were challenged by poor signal-to-noise ratio or false positive staining (e.g. plasma cells) hampering interpretation of the specific signal for ECAD.
Liver and colon are recommended as positive and negative tissue controls: Epithelium of the bile ducts in the liver must show a strong, distinct membranous staining reaction whereas hepatocytes must display an at least moderate membranous staining intensity. In the colon, epithelium must be strongly stained, and no reaction should be seen in stromal cells (e.g. plasma cells or smooth muscle cells).

Table 1. **Antibodies and assessment marks for ECAD, run 53**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **NCH-38**	82 1 1	Agilent/Dako Immunologics Thermo S./Neomarkers	57	22	4	1	94%	98%
mAb clone **36**	1 1	BD Biosciences Biogenex	0	1	0	1	-	-
mAb clone **36B5**	13	Leica/Novocastra	2	10	1	0	92%	100%
mAb clone **4A2C7**	4	Life Tech./Invitrogen	2	2	0	0	-	-
mAb clone **BS38**	1	Nordic Biosite	0	1	0	0	-	-
mAb clone **DBM15.49**	1	Diagnostic BioSystems	1	0	0	0	-	-
mAb clone **ECH-6**	2	Zytomed Systems	1	0	1	0	-	-
mAb clone **HECD-1**	9 1	Life Tech./Invitrogen Takara Bio Inc.	4	5	0	1	90%	100%
mAb clone **GM016**	1	Genemed	1	0	0	0	-	-
mAb clone **SPM471**	1	Thermo S./Neomarkers	0	0	1	0	-	-
rmAb **EP700Y**	5	Cell Marque	0	4	1	0	-	-
rmAb **EP6**	1	Zeta Corporation	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **36 790-4497**	68	Roche/Ventana	54	11	3	0	96%	100%
mAb clone **GM016 8229-C010**	2	Sakura Finetek	2	0	0	0	-	-
mAb clone **NCH-38 GA059**	31	Agilent/Dako	31	0	0	0	100%	100%
mAb clone **NCH-38 GA059**[3]	6	Agilent/Dako	5	1	0	0	-	-
mAb clone **NCH-38 IS/IR059**	27	Agilent/Dako	26	1	0	0	100%	100%
mAb clone **NCH-38 IS/IR059**[3]	6	Agilent/Dako	4	2	0	0	-	-
mAb clone **MX020 MAB-0738**	1	Maixin	0	1	0	0	-	-
mAb clone **BS38 MAD-000643QD**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone **HECD-1 MAD-000761QD**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone **35B5 PA0387**	6	Leica/Novocastra	0	6	0	0	-	-
rmAb clone **EP700Y 760-4440**	17	Roche/Ventana	0	2	15	0	13%	-
rmAb clone **EP700Y 246R-18**	6	Cell Marque	0	1	5	0	-	-
mAb clone **EP6 API3012**	1	Biocare Medical	0	1	0	0	-	-
Total	298		192	72	31	3	-	
Proportion			65%	24%	10%	1%	89%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of ECAD, Run 53
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **NCH-38**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using Bond Epitope Retrieval Solution 2 (BERS2; Leica) (6/6)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (12/16) or Cell Conditioning 1 (CC1; Ventana) (39/52) as 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, 49 of 50 (98%) laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **36B5**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako) (1/2) or CC1 (Ventana) (1/3) as retrieval buffer. The mAb was diluted in the range of 1:25-1:30 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

mAb clone **4A2C7**: Protocols with optimal results were based on HIER using CC1 (Ventana) (1/3) or Cell Conditioning 2 (CC2; Ventana) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:100-1:4,000 depending on the total sensitivity of the protocol employed.

mAb clone **DBM15.49**: One protocol with an optimal result was based on HIER using Montage EDTA antigen retrieval solution (Diagnostic BioSystems). The mAb was diluted 1:100 and Montage PolyVue Plus Auto Detection System (Diagnostic BioSystems) was applied as detection system.

rmAb clone **ECH-6**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer. The mAb was diluted 1:600 and OptiView (Ventana) was applied as detection system.

rmAb clone **HECD-1**: Protocols with optimal results were all based on HIER using BERS2 (Leica) (3/5) or TRS pH 9 (3-in-1) (Dako) (1/1). The mAb was typically 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.

rmAb clone **GM016**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer. The mAb was diluted 1:150 and UltraView (Ventana) was applied as detection system.

Table 3. **Proportion of optimal results for ECAD for the most commonly used antibody as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark 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 **NCH-38**	8/10** (80%)	-	1/1	-	32/42 (76%)	-	6/6 (100%)	0/2

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

Ready-To-Use antibodies and corresponding systems
mAb clone **36**, product no. **790-4497**, Ventana, BenchMark XT/Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min. at 95-100°C), 12-32 min. incubation of the primary Ab. and UltraView (760-500) or OptiView (760-700) as detection systems. Using these protocol settings 43 of 43 (100%) laboratories produced a sufficient staining result.

mAb clone **GM016**, product no. **8229-C010**, Sakura Finetek, Tissue-Tek Genie:
Protocols with optimal results were based on HIER using Tissue-Tek Genie High pH Antigen Retrieval Solution (Sakura Finetek) (efficient heating time 45 min. at 98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro DAB kit (8826-K250) as detection system.

mAb clone **NCH-38**, product no. **IS/IR069**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-99°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 25 of 25 (100%) laboratories produced a sufficient staining result.
mAb clone **NCH-38**, product no. **GA059**, Dako, OMNIS:

Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (efficient heating time 30 min. at 97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX+ (GV800/GV823+GV821) as detection system. Using these protocol settings, 24 of 24 (100%) laboratories produced an optimal staining.

mAb clone **BS38**, product no. **MAD-000643QD**, Master Diagnostica, MD-Stainer:
One protocol with an optimal result was based on HIER using a TRIS-EDTA/EGTA pH9 based buffer (Master Diagnostica) (efficient heating time 20 min. at 100°C), 40 min. incubation of the primary Ab and Master polymer plus (MAD-000230QP) as detection system.

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

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb NCH-38 **IS/IR059**	100% (10/10)	100% (10/10)	100% (13/13)	100% (13/13)
Dako Omnis mAb NCH-38 **GA059**	100% (21/21)	100% (21/21)	(3/3)	(3/3)
VMS Ultra/XT/GX mAb 36 **790-4497**	100% (11/11)	72% (8/11)	95% (54/57)	81% (46/57)

* 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 were included.

Comments
In this third NordiQC assessment for ECAD, the prevalent features of an insufficient staining result were characterized either by a generally too weak/false negative staining reaction of the cells expected to be demonstrated or poor-signal-to noise ratio caused primarily by the use of the rmAb clone EP700Y. Too weak or false negative staining result was observed in 21% of the insufficient results (7 of 34). Virtually all laboratories were able to demonstrate ECAD in high-level antigen expressing cells, e.g., normal columnar cells of the colon and epithelial cells of the bile ducts in the liver. However, the assays must be carefully calibrated according to low-level antigen expressing cells as normal hepatocytes showing an at least moderate distinct membranous staining intensity. Otherwise, and from a diagnostic point of view, it may be difficult to discriminate between ductal breast carcinoma displaying a weak or focal membranous staining reaction (the neoplastic cells should normally show a strong, complete and distinct membranous staining intensity) from lobular breast carcinoma in which the neoplastic cells lack or often also displays a weak membranous staining reaction.
Poor-signal-to noise ratio or false positive staining result was observed in 71% of the insufficient results (24 of 34). In general, the use of the rmAb clone EP700Y, either with a laboratory developed (LD) or RTU assay, provided an aberrant cytoplasmic or a false positive membranous staining reaction of stromal cells e.g. plasma cells in lamina propria of the colon mucosa (see Fig. 5b).
In the remaining 9% of the insufficient results, poor signal-to-noise ratio in combination with a too weak staining reaction for ECAD was seen.

42% (125 of 298) of the laboratories used a LD-assay for detection of ECAD. The mAb clone NCH-38 was by far the most commonly used primary Ab within a LD-assay and provided a pass rate of 94% (79 of 84) of which 68% (57 of 84) were assessed as optimal (see Table 1). All protocols assessed as optimal used HIER in an alkaline buffer, dilution range of the primary Ab between 1:10-1:200 and a 2- or 3-step multimer/polymer detection system. As shown in Table 3, the mAb clone NCH-38 provided high proportion of optimal results (range 76- 100%) on all main IHC platforms from the three major vendors (Dako, Ventana and Leica). The main causes for insufficient results were use of HIER in acidic buffer and too diluted primary Ab.

The mAb clones 36B5 and HECD-1, used within a LD-assay, both provided a high pass rate of 92% (12 of 13) and 90% (9 of 10), respectively. However, the proportion of optimal results was low applying the mAb 36B5 and in line with the observation described in the report run B16 (2013). Less distinct membranous staining reaction of cellular structures expected to be demonstrated together with background staining, was the main causes for the overall decrease in performance (optimal results). No technical parameters could be identified, separating protocols with optimal performance from protocols with a lower score (good or borderline).

58% (173 of 298) of the laboratories used a RTU system for detection of ECAD. In this assessment, the RTU systems IS/IR/GA059 (Dako) and 790-4497 (Ventana) based on the mAb clones NCH-38 and 36,

respectively, provided high pass rate and proportion of optimal results (see Table 1). For the RTU system GA059 on the Omnis, all protocols (31 of 31) were assessed as optimal. Both vendor and laboratory modified protocol settings (typically adjusting HIER, incubation time of the primary Ab and/or choice of detection system) could be used to obtain optimal result (see Table 4).

The RTU system 790-4497 based on the mAb clone 36 (Ventana), provided a pass rate of 96% (65 of 68) and 79% (54 of 68) were assessed as optimal. In comparison to run B16 (2013), this is a major improvement both in pass rate but also in proportion in optimal result. As described in the previous assessment report for ECAD, a significant proportion of protocols based on the mAb clone 36 gave an aberrant nuclear staining reaction of the included lobular breast carcinoma, and thus, accounted for the overall low performance. In this assessment, this aberrant nuclear staining was not seen in any of neoplasias (ductal and lobular breast carcinomas). The different reaction patterns observed between these two runs is unclear but could be related to the nature of the included lobular breast carcinomas. The main causes for an insufficient result were either too weak staining or excessive cytoplasmic staining reaction of the neoplastic cells hampering interpretation of the specific signal. As shown in Table 4, laboratories modifying their protocol settings, providing an overall pass rate of 95% of which 81% were assessed as optimal, laboratories applying only minor adjustments to the vendor recommended protocol settings (≤ 25%, see Table 4 - typically adjusting incubation time in primary Ab or HIER time), 100% (11 of 11) produced a sufficient result of which 72% (8 of 11) were assessed as optimal. This indicates that the mAb 36 is robust and can provide optimal staining reaction across a wide spectrum of protocols parameters.

The RTU system 760-4440 based on the rmAb clone EP700Y (Ventana), provided a low pass rate of 13% (2 of 17) and none was assessed as optimal. Poor signal-to-noise ratio or false positive staining (e.g. weak membranous staining of plasma cells in lamina propria mucosa of the colon) were the prevalent features of an insufficient result. The same problem has been observed in run B16 (2013). It is strongly recommended for participants using this assay to change to a more specific primary Ab (see above). Surprisingly, the lobular breast carcinoma provided in most cases the expected optimal reaction pattern and from a diagnostic/clinical point of view, the assay could be used to distinguish between ductal and lobular breast carcinomas. However, false positive staining and the excessive background staining in cellular structures expected to be negative (all other cores assessed), accounted for the overall poor performance (see Fig. 5b-6b).

This was the third assessment of ECAD in NordiQC (see Table 2). An increase in pass rate was obtained compared to the latest run B16, 2013. The extended use of robust assays, both as Conc formats and RTU systems tailored to a specific IHC platform, accounted for the overall increase in sufficient results. Grouped together, the most common RTU system from Dako and Ventana, provided a pass rate of 98% (123 of 126) and 90% (111 of 123) were assessed as optimal. Irrespective of clone applied, HIER, preferable in an alkaline buffer, and carefully calibration of the primary Ab in relation to the expected antigen level of the recommended control material (see below), were the most important technical parameters for an optimal performance provided that a robust and specific clone has been selected.

Controls

Liver and colon are recommended as positive and negative tissue controls for ECAD. In liver, the protocol must be calibrated to provide an at least moderate, distinct membranous staining reaction of the hepatocytes. Epithelium of the bile ducts should display a strong and distinct membranous staining intensity.

In the colon, only epithelium should display a strong membranous staining reaction. No staining reaction must be seen in stromal cells such as lymphocytes, plasma cells, smooth muscle cells or endothelial cells.

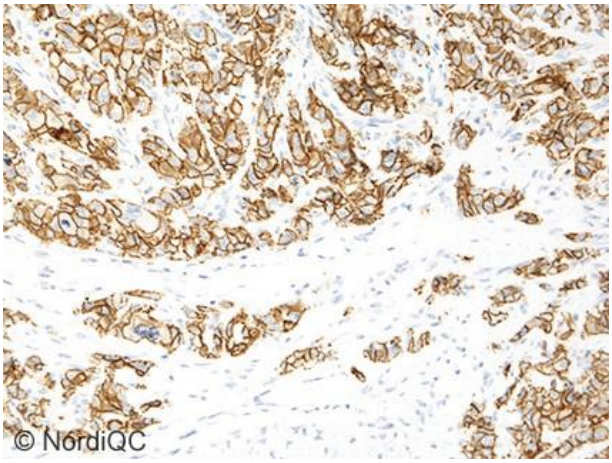


Fig. 1a (x200)
Optimal ECAD staining of the ductal breast carcinoma (tissue core no. 3) using the RTU system (GA059/Omnis, Dako) based on the mAb clone NCH-38, HIER in TRS (3-1) pH 9 (Dako) and Flex+ (Dako) as detection system. Virtually all neoplastic cells show a strong and distinct membranous staining reaction. Same protocol used in Figs. 2a - 3a.

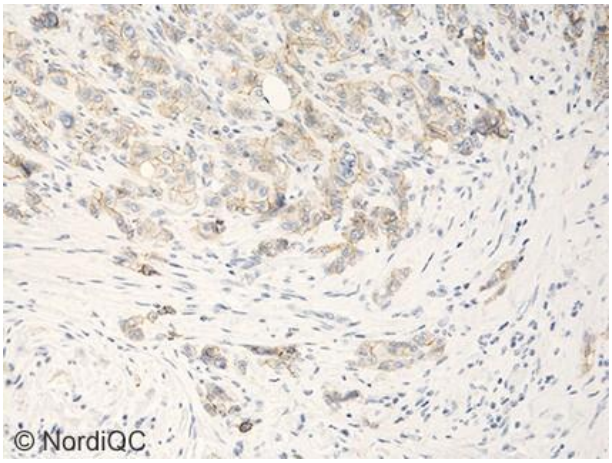


Fig. 1b (x200)
Insufficient staining of ECAD in the ductal breast carcinoma (tissue core no. 3) using the mAb clone NCH-38 within a LD assay with too diluted Ab and Flex+ (Dako) as the detection system – same field as in Fig. 1a. The neoplastic cells display too weak or false negative membranous staining reaction. Importantly, assays must be calibrated according to the intensity seen in hepatocytes in Fig. 2a. Otherwise, neoplastic cells of ductal breast carcinomas may be misinterpreted as lobular breast carcinomas due to the weak or focal membranous staining intensity as seen in this example (compare with Figs. 1a - 4b). Same protocol used in Figs. 2b - 3b.

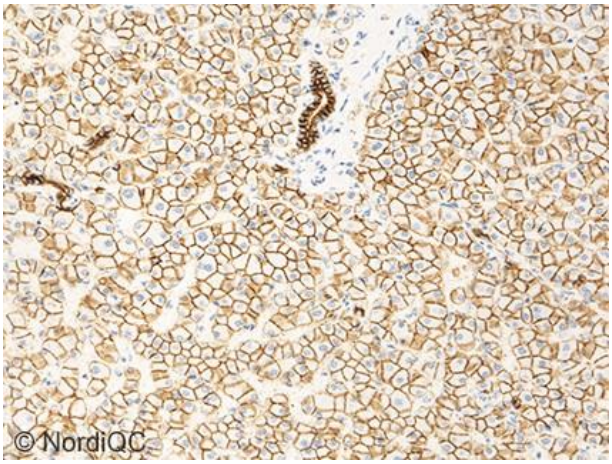


Fig. 2a (x200)
Optimal staining of ECAD in the liver using same protocol as in Fig. 1a. The bile ducts show a strong staining intensity and the hepatocytes display a moderate, distinct membranous staining reaction.

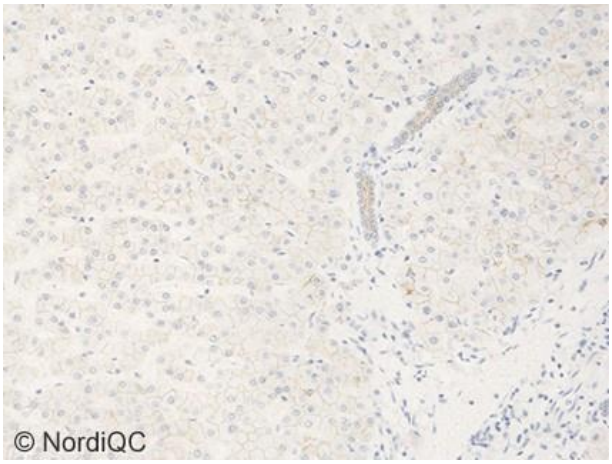


Fig. 2b (x200)
Insufficient staining of ECAD in the liver using same protocol as in Fig. 1b. The staining intensity is reduced, and the large majority of hepatocytes are false negative.

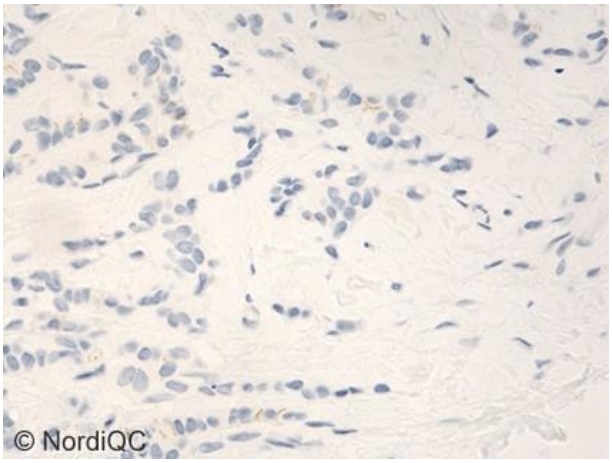


Fig. 3a (x400)
Optimal ECAD staining of the lobular breast carcinoma using same protocol as in Figs. 1a and 2a. The majority of neoplastic cells are negative while a minor fraction of the tumour cells show a partial membranous, faint to weak staining reaction.

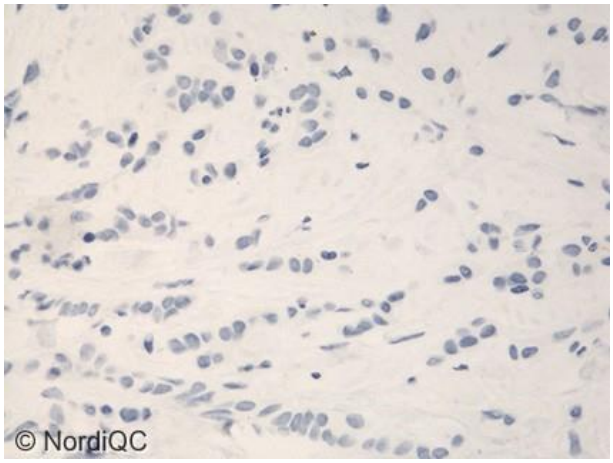


Fig. 3b (x400)
ECAD staining of the lobular breast carcinoma using same protocol as in Figs. 1b and 2b. Although the neoplastic cells display similar reaction pattern as in Fig. 3a, the staining is too weak in other cores (see explanation in Fig. 1b).

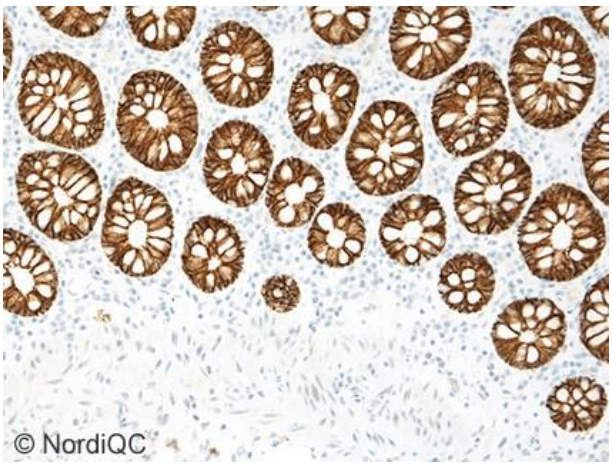


Fig. 4a (x200)
Optimal ECAD staining of the colon. The protocol was based on the mAb clone 36 as RTU format (790-4497/Ventana), HIER in CC1 (Ventana) and OptiView (Ventana) as detection system. The columnar epithelial cells display a strong membranous staining reaction while the stromal cells are negative. The protocol provided the same reaction patterns as seen in the ductal and lobular breast carcinomas (Figs. 1a and 3a) and of hepatocytes in the liver (Fig. 2a) - data not shown.

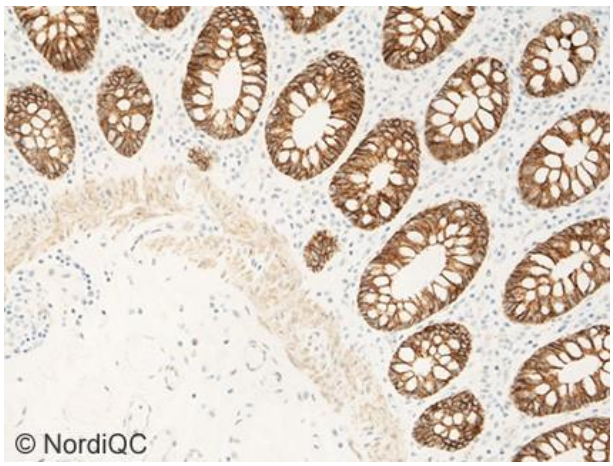


Fig. 4b (x200)
Insufficient ECAD staining of the colon using a protocol providing too low sensitivity and poor signal-to-noise ratio. The protocol was based on same the RTU format with similar protocol settings as in Fig. 4a, but with minor modifications (shorter HIER time and longer incubation time in primary Ab). The smooth muscle cells of lamina muscularis mucosae show an aberrant cytoplasmic staining reaction. The epithelium displays slightly weaker intensity compared to the result shown in Fig. 4a. Also, the neoplastic cells of ductal breast carcinomas and hepatocytes in the liver showed too weak membranous staining intensity - data not shown (see similar expression pattern in Fig. 1b and 2b). There is no obvious explanation for the overall lower performance of the assay applied in Fig. 4b, other than the modifications mentioned above or that the primary Abs of both assays were based on two different lot numbers.

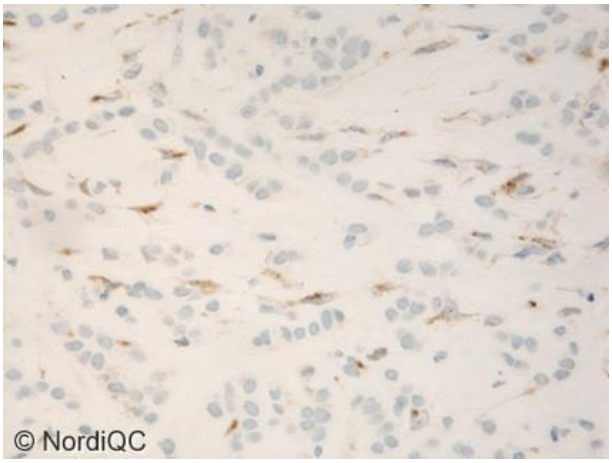


Fig. 5a . (x400)
Insufficient ECAD staining of the lobular breast carcinoma applying the same protocol as in Fig. 4b. Interpretation of the specimen is hampered due to an aberrant cytoplasmic staining of stromal cells intermingling between the neoplastic cells. Microscopy require high power magnification for identification of morphological details, assuring that the neoplastic cells are negative. Compare with optimal result in Fig. 3a.

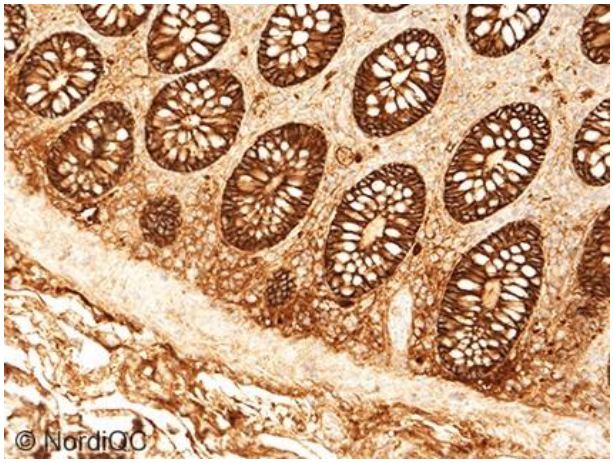


Fig. 5b (x400)
Insufficient ECAD staining of the colon using a protocol providing poor signal-to-noise ratio and false positive staining. The RTU format (246R-18/Cell Marque) based on rmAb EP700Y was applied on a Benchmark Ultra (Ventana), HIER performed in CC1 (Ventana) and UltraView with amplification (Ventana) used as detection system – same protocol used in Fig. 6a and 6b. Strong background staining is seen and virtually all stromal cells (e.g. lymphocytes and plasma cells) in lamina propria mucosa display a weak to strong false positive membranous staining reaction. Although weaker in most cases, assays based on this primary Ab typically gave this aberrant staining pattern despite laboratories applying several different protocol settings (e.g. low sensitive detection systems and/or shorter incubation time in primary Ab).

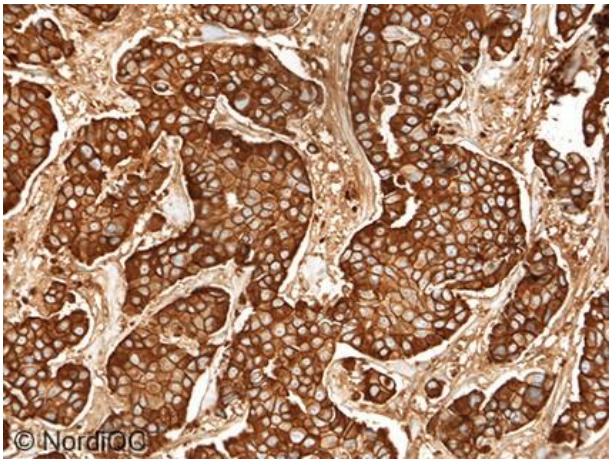


Fig. 6a (x200)
Insufficient ECAD staining of the ductal breast carcinoma (tissue core no. 4) using same protocol as in Fig. 5b. Interpretation of the specific and distinct membranous staining reaction for ECAD is difficult due to too strong cytoplasmic staining reaction of the neoplastic cells in combination with excessive background staining of stromal cells, providing poor signal-to-noise ratio.

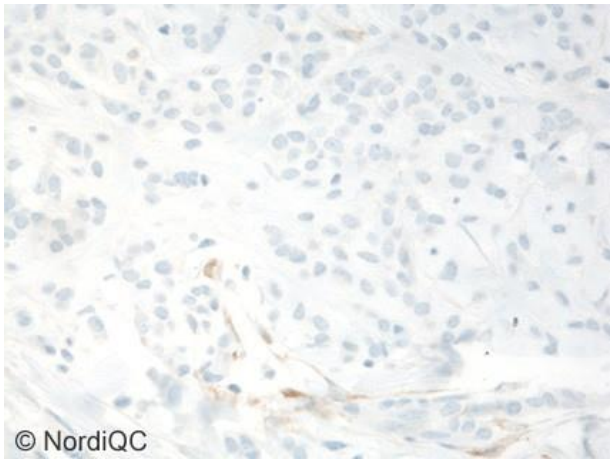


Fig. 6a (x400)
Insufficient ECAD staining of the lobular breast carcinoma using same protocol as in Fig. 5b and 6a. Although the reaction pattern is comparable to optimal staining as seen in Fig. 3 a. (except for a weak aberrant cytoplasmic staining of endothelial cells), the protocol provided poor signal-to-noise ratio or false positive staining of cellular structures expected to be negative (see Fig. 5b and 6a).

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