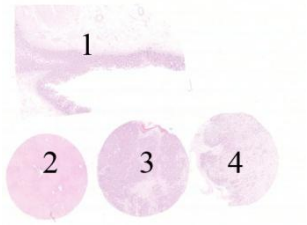
E-cadherin (ECAD)
Assessment Run B16 2013



The slide to be stained for ECAD comprised:

1. Colon, 2. Liver, 3. Ductal breast carcinoma, 4. Lobular breast carcinoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing an ECAD staining as optimal were:

- A strong, distinct membranous staining reaction of virtually all the columnar epithelial cells in the colon.
- A strong, distinct membranous staining reaction of the epithelial cells of the bile ducts and at least a weak to moderate membranous staining reaction of the hepatocytes in the liver.
- A moderate to strong, distinct membranous staining reaction of virtually all neoplastic cells of the breast ductal carcinoma.
- No staining reaction or at maximum a focal membranous staining reaction of the neoplastic cells of the breast lobular carcinoma.

271 laboratories participated in this assessment. 82 % achieved a sufficient mark (optimal or good). Antibodies (Abs) used and assessment marks are summarized in table 1.

Table 1. **Antobodies and assessment marks for ECAD run 39**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **36**	1	BD Biosciences	0	0	1	0	-	-
mAb clone **36B5**	16 1	Leica/Novocastra Vector	2	13	2	0	88 %	100 %
mAb clone **4A2C7**	5	Invitrogen/Zymed	0	3	2	0	60 %	-
mAb clone **BSH38**	1	Nordic Biosite	0	1	0	0	-	-
mAb clone **ECH-6**	1	Zytomed	1	0	0	0	-	-
mAb clone **HECD-1**	9 2 1 1	Invitrogen/Zymed Immunologic Abcam Biocare	7	6	0	0	100 %	100 %
mAb clone **NCH-38**	90 5	Dako Thermo/NeoMarkers	59	30	6	0	94 %	94 %
mAb clone **SPM471**	1	Thermo/NeoMarkers	0	1	0	0	-	-
rmAb clone **EP6**	2	Epitomics	0	2	0	0	-	-
rmAb clone **EP700Y**	6 1 1 1 1	Cell Marque Biocare Bio SB Thermo/NeoMarkers Zytomed	0	7	1	2	70 %	-
Unknown	1	Unknown	1	0	0	0	-	-
Ready-To-Use Antibodies								
mAb clone **36** **790-4497**	51	Ventana	6	12	32	1	35 %	38 %
mAb clone **36B5** **PA0387**	10	Leica	0	10	0	0	100 %	-
mAb clone **NCH-38** **IR/IS059**	44	Dako	40	4	0	0	100 %	100 %

mAb clone **NCH-38 GA059**	1	Dako	1	0	0	0	-	-
rmAb clone **EP700Y 760-4440**	16	Ventana/Cell Marque	0	15	1	0	94 %	-
rmAb clone **EP700Y 246R-1x**	1	Cell Marque	0	1	0	0	-	-
rmAb clone **EP700Y MAD-000051QD**	1	Master Diagnostica	0	1	0	0	-	-
Total	271		117	106	45	3	-	
Proportion			43 %	39 %	17 %	1 %	82 %	

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

Detailed analysis of ECAD, Run B16

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **36B5**: The two protocols with optimal results were both based on HIER using Cell Conditioning 1 (CC1; Ventana) (1/3)* or Tris-EDTA/EGTA pH 9 (1/3) as retrieval buffer. The mAb was diluted 1:50. Using these protocol settings 2 of 2 (100 %) laboratories produced a sufficient staining.
* (number of optimal results/number of laboratories using this buffer)

mAb clone **ECH-6**: The protocol with an optimal result was based on HIER using CC1, standard (BenchMark, Ventana) and a dilution of 1:100.

mAb clone **HECD-1**: Protocols with optimal result were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/2), TRS pH 9 (Dako) (1/1), TRS low pH 6.1, (3-in-1) (Dako) (1/1), BERS2 (Leica) (2/2) or Tris-EDTA/EGTA pH 9 (2/5) as retrieval buffer. The mAb was typically diluted in the range of 1:200-1:1.000 depending on the total sensitivity of the protocol employed. Using these protocol settings 10 of 10 (100 %) laboratories produced a sufficient staining.

mAb clone **NCH-38**: Protocols with optimal result were all based on HIER using TRS pH 9, (3-in-1) (Dako) (17/17), TRS pH 9 (Dako) (8/10), CC1 (BenchMark, Ventana) (27/45), BERS1 (Leica) (1/2), PT module buffer 2 pH 8 (Thermo) (2/2), Tris-EDTA/EGTA pH 9 (3/7) or Citrate pH 6 (1/3) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings 79 of 84 (94 %) laboratories produced a sufficient staining.

Table 2. **Optimal results for E-cadherin using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **NCH-38**	96 % 23/24**	-	59 % 23/39	0 % 0/1	0 % 0/4	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 Antibodies
mAb clone **36** (prod. no. 790-4497, Ventana): Protocols with optimal results were all based on HIER using mild or standard Cell Conditioning 1, 12-32 min. incubation of the primary Ab and iView (760-091) UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings 16 of 42 (38 %) laboratories produced a sufficient staining.

mAb clone **NCH-38** (product.no. IS/IR059, Dako): Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min at 95-99°C), 20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 43 of 43 (100 %) laboratories produced a sufficient staining.

mAb clone **NCH-38** (product no. GA059, Dako, Omnis): The protocol with an optimal result was based on HIER in TRS pH 9 (3-in-1) (efficient heating time 30 min. at 97°C), 25 min. incubation of the primary Ab and EnVision FLEX+ (GV800) as detection system.

The most frequent causes of insufficient stainings were:

- Less successful primary antibodies
- Too low concentration of the primary antibody
- Insufficient HIER - too short efficient heating time.

In this assessment the prevalent feature of an insufficient staining was an aberrant nuclear staining reaction of the vast majority of neoplastic cells of the lobular carcinoma. This feature was only seen for the mAb clone 36, whereas all other 9 antibodies used in this assessment towards ECAD gave a complete negative nuclear staining reaction in this tumour. The nuclear staining reaction was seen both for the concentrated format (BD) and the ready-to-use (RTU) format (Ventana). The aberrant nuclear staining reaction was seen in 34 of 52 protocols based on the mAb clone 36 and from the protocols submitted, neither protocol settings (HIER, incubation time or detection system) nor lot numbers of the primary Ab could be related to the aberrant nuclear staining reaction.

Nuclear staining reaction for ECAD in various neoplasias has been described in the literature, but only when using the mA clone 36. No conclusive data and explanations for the translocation and nuclear staining have been generated. In this assessment, the nuclear staining reaction of the lobular carcinoma was evaluated as insufficient (borderline) as the aberrant staining pattern in diagnostic settings would require a retest primarily to confirm the absence of the membranous staining of the lobular breast carcinoma and secondary to exclude contamination of any other primary antibody.
The remaining insufficient results were typically characterized by a too weak or false negative staining reaction of the structures expected to be demonstrated, typically caused by insufficient HIER and/or a too low concentration of the primary Ab.

The mAb clones NCH-38 and HECD-1 gave by far the highest proportion of sufficient and optimal results. The most widely used antibody, mAb clone NCH-38, applied as a concentrate could produce an optimal result on all the three main IHC platforms (Ventana, Dako and Leica), see table 2. However, the mAb clone NCH-38 gave the highest proportion of optimal results on the Dako Autostainer platform.

The RTU system based on the mAb clone NCH-38 (Dako) gave a pass rate of 100 % of which 90 % were assessed as optimal. The RTU system based on the mAb clone 36B5 (Leica) also gave a pass rate of 100 %, but none were assessed as optimal. The reason was a combination of a less distinct membranous staining of structures expected to be demonstrated and a weak but consistent background staining.
The RTU system based on the rmAb clone EP700Y (Ventana) gave an overall pass rate of 94 %, but excessive background staining compromised the interpretation and no optimal results were obtained. This pattern was also seen for the corresponding concentrated format.

Controls
Both colon and liver can be used as tissue controls for ECAD. In this test, however liver was found to be slightly more informative for appropriate calibration of the protocol.
All hepatocytes must show an at least weak to moderate distinct, predominantly membranous staining reaction. Epithelial cells of bile ducts must be moderately to strongly stained. No staining should be seen in lymphocytes, endothelial cells and smooth muscle cells.
Colon was less useful for calibration of protocols settings to demonstrate low-level antigen expressing cells and neoplasias, since columnar epithelial cells showed a high level of ECAD.

This was the 2nd NordiQC assessment of ECAD. A small increase in the pass rate was seen compared to run B5 (2008), se table 3.

Table 3. **Proportion of sufficient results for ECAD in two NordiQC runs**

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

Conclusion
The mAb clones HECD-1 and NCH-38 were in this assessment the most successful antibodies for demonstration of ECAD.
The mAb clone NCH-38 as concentrate gave an optimal result on all the 3 main IHC systems (Dako, Ventana and Leica). HIER, preferable in an alkaline buffer and appropriate calibration of the primary antibody is mandatory for optimal performance.
Liver is recommendable as tissue control for ECAD. Virtually all hepatocytes must show an at least weak to moderate distinct predominantly membranous staining reaction, while epithelial cells of the bile ducts must show a moderate to strong membranous staining reaction. No staining should be seen in lymphocytes, endothelial cells and smooth muscle cells.

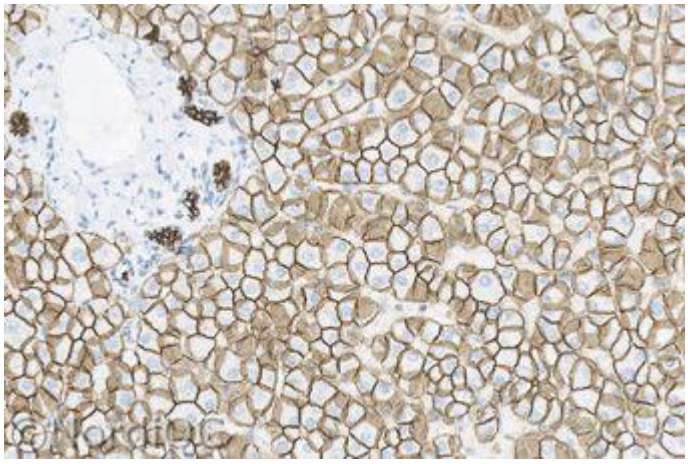


Fig. 1a.
Optimal ECAD staining of the liver using the mAb clone NCH-38 as RTU format, Dako. Virtually all the hepatocytes show a distinct, moderate and predominantly membranous staining reaction, while the epithelial cells of the bile ducts show a strong staining reaction. Also compare with Figs. 2a - 4a, same protocol.

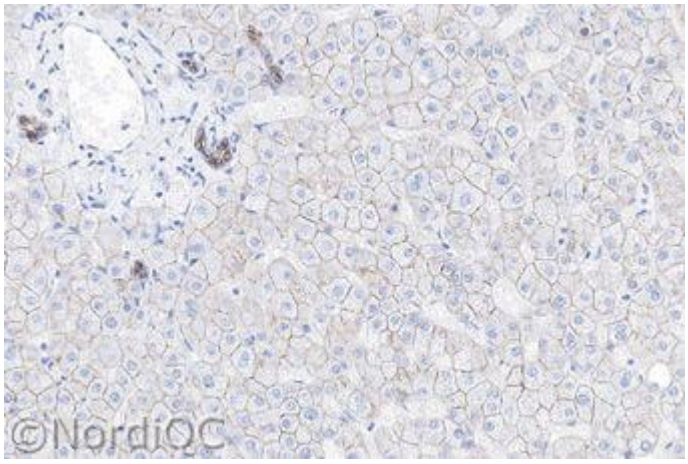


Fig. 1b.
Insufficient ECAD staining of the liver using the mAb clone NCH-38 with protocol settings giving a too low sensitivity - same field as in Fig. 1a. The hepatocytes show a weak and equivocal staining reaction and only epithelial cells of the bile ducts with a high level of ECAD expression are distinctively demonstrated. Also compare with Fig. 2b, same protocol.

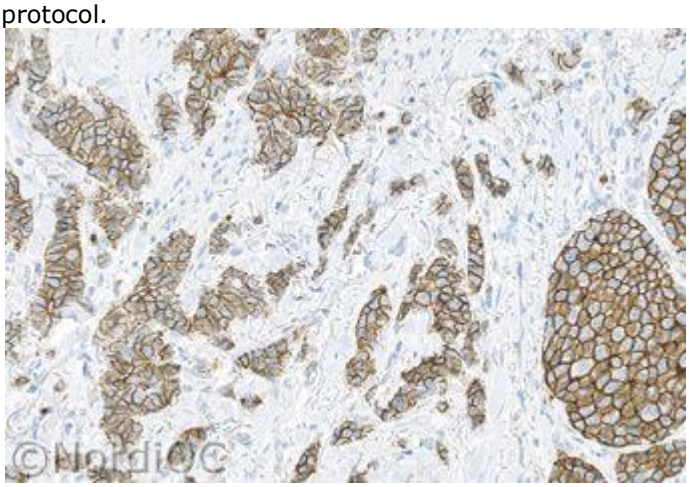


Fig. 2a.
Optimal ECAD staining of the breast ductal carcinoma using same protocol as in Fig. 1a. The majority of the neoplastic cells show a distinct, moderate to strong and predominantly membranous staining reaction. No background staining is seen.

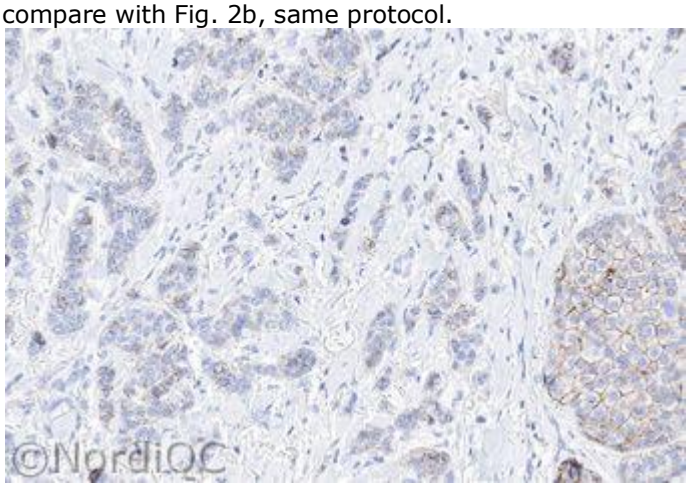


Fig. 2b.
Insufficient ECAD staining of the breast ductal carcinoma using same protocol as in Fig. 1b - same field as in Fig. 2a. Only scattered neoplastic cells show a weak membranous staining reaction, while the vast majority are false negative.

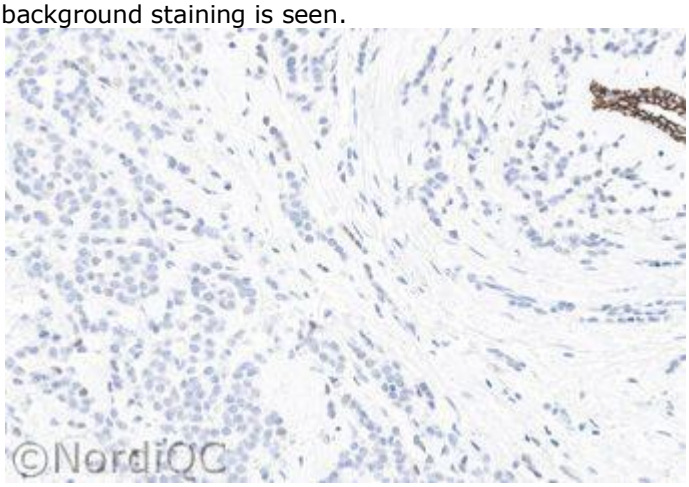


Fig 3a.
Optimal ECAD staining of the breast lobular carcinoma using same protocol as in Figs. 1a & 2a. The neoplastic cells are all unstained and only the ductal epithelial cells of the entrapped benign glands show a strong, distinct membranous staining reaction.

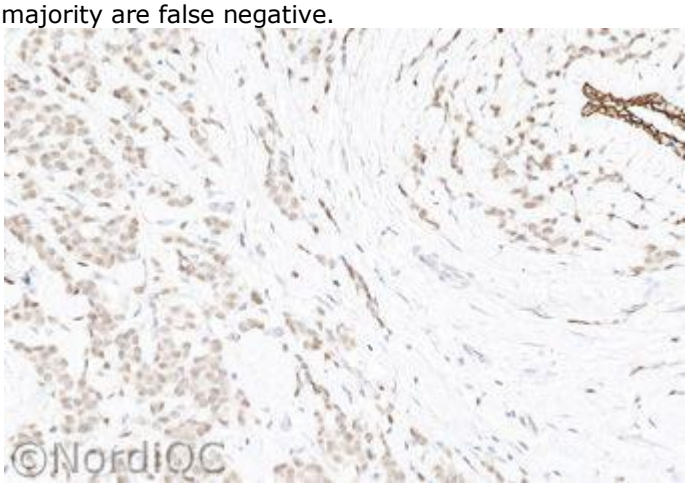


Fig. 3b.
Staining ECAD of the breast lobular carcinoma using the mAb clone 36 - same field as in Fig. 3a. No membraneous staining but an aberrant nuclear staining in all the neoplastic cells is present. This pattern was only seen for the mAb clone 36 (both as concentrate, BD, and RTU, Ventana), evaluated as insufficient

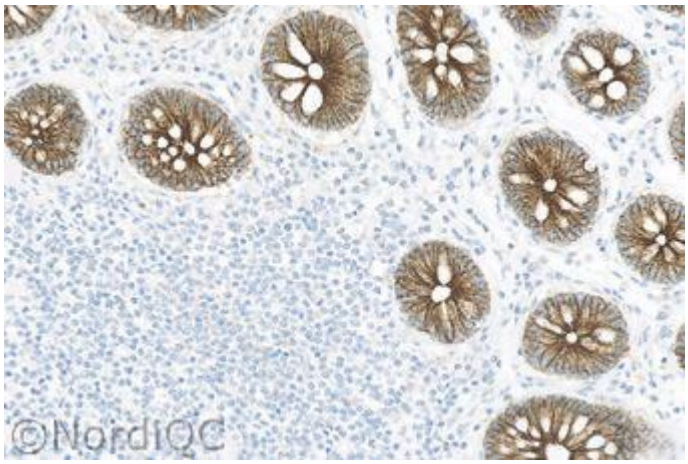


Fig. 4a.
Optimal ECAD staining of the colon using same protocol as in Figs. 1a - 3a. The epithelial cells are distinctively demonstrated and no background staining is seen.

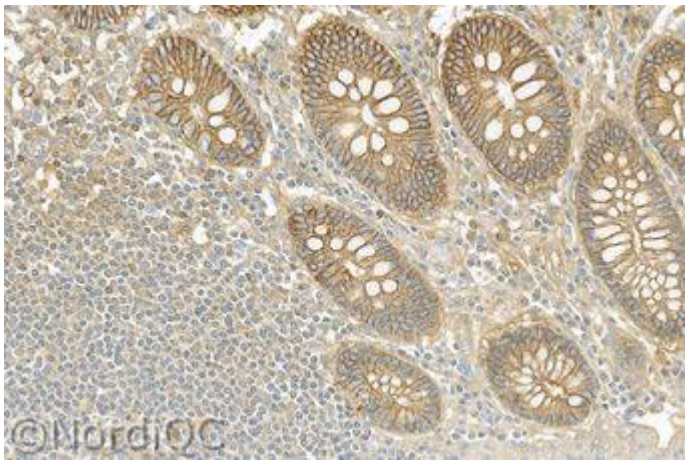


Fig. 4b.
ECAD staining of the colon using the rmAb clone EP70Y. The signal-to-noise ratio is reduced compared to the level expected as a general background staining is seen and at the same time the epithelial cells show a low staining intensity. The staining was assessed as sufficient (good) as the overall result could be evaluated in all the five tissues included in the material circulated. The staining pattern was typically seen for the rmAb clone EP700Y both as concentrate and as RTU format (Ventana / Cell Marque).

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