

Material

The slide to be stained for CDX2 comprised:

1. Appendix, 2. Pancreas, 3. Tonsil, 4. Lung adenocarcinoma, 5-6. Colon adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CDX2 staining as optimal included:

- A strong, distinct nuclear staining reaction of virtually all epithelial cells in the appendix
- An at least weak to moderate and distinct nuclear staining reaction of virtually all duct epithelial cells in the pancreas
- A strong, distinct nuclear staining reaction of virtually all neoplastic cells in the colon adenocarcinoma, tissue core no. 6.
- An at least weak to moderate nuclear staining reaction of the majority of the neoplastic cells in the colon adenocarcinoma, tissue core no. 5
- No staining reaction in the lung adenocarcinoma and tonsil*.

A weak to moderate cytoplasmic reaction in cells with strong nuclear staining was accepted.

* In tonsil, few lymphocytes showed a weak nuclear staining reaction, which was accepted.



Participation

Number of laboratories registered for CDX2, run 48	291
Number of laboratories returning slides	268 (92%)

Results

268 laboratories participated in this assessment. 214 (80%) 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:

- Too low concentration of the primary antibody.
- Use of less sensitive detection systems.
- Poor performance of mAb clone DAK-CDX2 on the Ventana BenchMark platform.
- Less successful primary antibody.

Performance history

This was the fifth NordiQC assessment of CDX2. The pass rate increased compared to the previous run as shown in table 2 and a consistent improvement has been observed in the 3 latest assessments.

Table 2. **Proportion of sufficient results for CDX2 in the five NordiQC runs performed**

	Run 22 2008	Run 27 2009	Run 33 2011	Run 38 2013	Run 48 2016
Participants, n=	56	93	148	200	268
Sufficient results	64%	46%	51%	73%	80%

Conclusion

The mAb clone **DAK-CDX2** and the rmAb clones **EPR2764Y** and **EP25** can all be used to obtain optimal staining for CDX2. The RTU systems from Dako, Leica and Ventana provided a higher proportion of optimal and sufficient results compared to laboratory developed assays for CDX2.

The pass rate for CDX2 has been improved from 46% in run 27 (2009) to 80% in this run, which in part can be related to reduced use of the less successful clones CDX-88 and AMT28.

Pancreas is an appropriate positive tissue control for CDX2: A moderate, distinct nuclear reaction in the majority of the duct epithelial cells in the pancreas must be seen. Appendix and colon cannot be recommended as primary positive tissue control, due to the high level of CDX2 expression.

Table 1. **Antibodies and assessment marks for CDX2, run 48**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone AMT28	2	Leica/Novocastra	0	0	0	2	-	-
mAb clone CDX2-88	2	Biocare	0	0	1	3	-	-
	2	Biogenex						
mAb clone DAK-CDX2	31	Agilent/Dako	6	9	7	9	48%	57%
rmAb clone EPR2764Y	31	Cell Marque	28	14	7	3	81%	81%
	5	Thermo/Neomarkers						
	4	Immunologic						
	4	Zytomed						
	2	Monosan						
	2	Zeta Corporation						
	1	A.Menarini						
	1	Abcam						
1	Nordic Biosite							
1	Thermo/Pierce							
Ready-To-Use antibodies								
mAb clone BC39 API3184	1	Biocare	0	0	0	1	-	-
mAb clone CDX2-88 PM226	1	Biocare	0	1	0	0	-	-
mAb clone CDX2-88 AM392	1	Biogenex	0	1	0	0	-	-
mAb DAK-CDX2 IR080/IS080	34	Agilent/Dako	18	10	5	1	82%	93%
mAb DAK-CDX2 GA080	26	Agilent/Dako	16	4	3	3	77%	100%
rmAb clone EP25 RMPD059	1	Diagnostic Biosystems	0	0	1	0	-	-
rmAb clone EP25 PA0375	7	Leica/Novocastra	4	3	0	0	100%	100%
rmAb clone EP25 MAD-000645QD	3	Master Diagnostica	0	3	0	0	-	-
rmAb clone EPR2764Y RMA-0631	1	Maixin	1	0	0	0	-	-
mAb clone EPR2764Y RM-2116-R7	1	Thermo/Neomarkers	0	0	1	0	-	-
rmAb clone EPR2764Y 760-4380/ 235R*	103	Ventana/Cell Marque	81	15	5	2	93%	96%
Total	268		154	60	30	24	-	
Proportion			58%	22%	11%	9%	80%	

1) Proportion of sufficient stains (optimal or good).

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

* Products merged due to imprecise antibody selection at the NordiQC homepage for protocol submission.

Detailed analysis of CDX2, Run 48

The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies

mAb clone **DAK-CDX2**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/13)*, Cell Conditioning 1 (CC1, Ventana) (1/7), BERS2 (Leica) (1/4) or Tris-EDTA pH 9 (2/4) as retrieval buffer. The mAb was diluted in the range of 1:10-1:50. Using these protocol settings, 12 of 21 (57%) laboratories produced a sufficient staining result (optimal or good).

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

rmAb clone **EPR2764Y**: Protocols with optimal results were typically based on HIER using CC1 (Ventana) (20/30), TRS pH 9 (3-in-1) (Dako) (1/4), BERS2 (Leica) (4/11) or Tris-EDTA pH 9 (3/4) as retrieval buffer. The rmAb was typically diluted in the range of 1:50-1:1,000. Using these protocol settings, 38 of 47

(81%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for CDX2 for the two most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	Dako		Ventana		Leica	
	Autostainer / Omnis		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 DAK-CDX2	2/13** (15%)	-	1/6 (17%)	-	1/4	-
rmAb clone EPR2764Y	1/4	-	20/28 (71%)	-	3/9 (33%)	0/1

* 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 **DAK-CDX2**, product no. **IR080/IS080**, 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 97-99°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 25 of 27 (93%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-CDX2**, product no. **GA080**, Dako, Omnis:

Protocols with optimal results were typically based on HIER using TRS pH 9 (efficient heating time 24-30 min. at 97°C), 15-30 min. incubation of the primary Ab and Envision FLEX+ (GV800+GV821) as detection system. Using these protocol settings, 17 of 17 (100%) laboratories produced a sufficient staining result.

rmAb clone **EP25** product no. **PA0375**, Leica, Bond III/MAX:

Protocols with optimal results were based on HIER using BERS2 (Bond Leica) (efficient heating time 20 min. at 99-100°C), 15-20 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 7 of 7 (100%) produced a sufficient staining result.

rmAb clone **EPR2764Y**, product no. **RMA-0631**, Maixin, manual staining:

One protocol with an optimal result was based on HIER using Tris-EDTA pH9 (Waterbath), 60 min. (RT) incubation of the primary Ab and KIT-0038 (Maixin) as detection system.

rmAb clone **EPR2764Y**, product no. **760-4380/ 235R***, Ventana/Cell Marque, BenchMark GX/XT/Ultra:

Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 24-90 min.) and 16-60 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) +/- amplification kit were used as detection systems. Using these protocol settings, 93 of 97 (96%) laboratories produced a sufficient staining result.

* Products merged due to imprecise antibody selection at the NordiQC homepage for protocol submission.

Comments

In this assessment and in concordance with the previous NordiQC runs for CDX2, the prevalent feature of an insufficient staining was a too weak or completely false negative staining reaction of cells expected to be demonstrated. Virtually all laboratories were able to demonstrate CDX2 in high-level CDX2 expressing cells in the appendix and the colon adenocarcinoma tissue core no. 6, whereas low-level CDX2 expressing cells in the colon adenocarcinoma tissue core no. 5 and the epithelial cells of the pancreatic ducts could only be demonstrated with a sensitive Ab and an optimally calibrated protocol. This staining pattern was seen in 93% of the insufficient results (50 of 54 laboratories). Too weak staining in combination with excessive background staining, impaired morphology or excessive counterstaining characterized the remaining insufficient results.

33% (89 of 268) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for CDX2. The mAb clone DAK-CDX2 and rmAb clone EPR2764Y were the two most widely used Abs and both could be used to obtain an optimal staining result. rmAb clone EPR2764Y was found most successful as 81% of the laboratories using this clone produced a sufficient staining result and 54% were assessed as optimal. Efficient HIER in an alkaline buffer and careful calibration of the primary Ab were the two most central parameters for optimal results. Choice of detection system was of less importance. Both 2-step and 3-step multimer / polymer based detection systems could be used to obtain optimal staining results, providing the titre of the primary Ab was adjusted. As listed in table 3, optimal results could be obtained on the main IHC systems from Dako, Leica and Ventana. For four of the ten protocols with insufficient marks, the main problematic parameter was too low titre of the primary Ab, whereas no single problematic parameter could be identified for the remaining procedures. In-house experiences generated

at NordiQC, Aalborg University Hospital, revealed fast deterioration of the clone/Ab in diluted working solutions primarily stored at room temperature. This has been documented to occur after 1-2 weeks storage and will only be identified when using correct positive tissue controls (see controls). Also mAb clone DAK-CDX2 could be used within a LD assay to produce an optimal result. However, the clone was less successful compared to rmAb clone EPR2764Y as a reduced proportion of sufficient and optimal results was observed, despite the use of similar protocol settings for the two Abs. HIER in alkaline buffer and a relatively concentrated format of the mAb (range of 1:10 – 50) in combination with a 3-step polymer based detection system were the main requirements for optimal results. Too low titre of the primary Ab and less successful performance for the mAb on Ventana BenchMark were the main causes for insufficient results. In the two latest NordiQC assessments for CDX2, only 1 of 14 protocols based on mAb clone DAK-CDX2 within a LD assay performed on BenchMark provided an optimal result. 12 protocols gave insufficient results on the Ventana system despite applying comparable sufficient protocol settings from other IHC systems.

Ready-To-Use (RTU) antibodies were used by 67% (179 of 268) of the laboratories. Optimal results could be obtained with the RTU systems from the three main IHC system providers, Dako, Leica and Ventana.

The Ventana RTU system based on the rmAb clone EPR2764, 760-4380 was in this assessment the most widely used assay and gave an overall pass rate of 93%. Optimal results could be obtained both by the vendor recommended protocol settings (32 min. incubation of the primary Ab, HIER in CC1 for 64 min. and UltraView as detection kit) and by laboratory modifications of the protocol adjusting incubation time of the primary Ab, HIER time and choice of detection system. Use of OptiView as detection system was the most frequent and successful modification observed. 45 laboratories used OptiView of which 40 obtained an optimal result. The insufficient results were typically caused by reduced HIER and incubation time of the primary Ab in combination with UltraView as detection system.

The Dako RTU system IR080/IS080 for Autostainer based on the mAb DAK-CDX2 provided a pass rate of 82%. Both vendor recommended (20 min. incubation of the primary Ab, HIER in TRS High for 20 min. and EnVision FLEX as detection kit) and laboratory modified protocol settings could produce optimal results. Reduced HIER time and/or incubation time of the primary Ab. Were the main reasons for insufficient results.

The Dako RTU system GA080 for Omnis based on mAb clone DAK-CDX2 provided a slightly reduced overall pass rate of 77% compared to the Dako RTU system IR080/IS080. However, if applying vendor recommended protocol settings (25 min. incubation of the primary Ab, HIER in TRS High for 30 min. and EnVision FLEX+ as detection kit) a pass rate of 100% was seen. Especially, omission of linker was less successful as none of 5 protocols based on this modification generated optimal results (4 were assessed as insufficient).

The Leica RTU system PA0375 based on the rmAb clone EP25 for Bond gave a pass rate of 100%. Optimal results could be obtained both by the vendor recommended protocol settings (15 min. incubation of the primary Ab, HIER in ER2 for 20 min. and Refine as detection kit) and by slightly laboratory modified protocol settings prolonging the incubation time of the primary Ab.

This was the fifth NordiQC assessment of CDX2. Consistent improvement of the pass rate has been obtained in the four latest assessments. The pass rate improved from 46% in 2009 to 80% in the current run. The primary reason for this improvement seems closely related to the extended use of robust Abs as clones EPR2764Y, EP25 and DAK-CDX2 at the expense of the "old" clones AMT28 and CDX2-88. In the current run, 97% (259 of 268) used the clones EPR2764Y, EP25 and DAK-CDX2 while only 3% used AMT28 and CDX2-88. In run 27, 2009, 26% (24 of 93) used the clones EPR2764Y, EP25 and DAK-CDX2, while 73% (68 of 93) used mAb clones AMT28 or CDX2-88.

The access to and extended use of optimally calibrated RTU systems for CDX2 on all the three main IHC systems from Dako, Leica and Ventana has influenced the increase in pass rate in a positive direction. In run 27, 22% of the participants (20 of 93) used one of the above-mentioned RTU systems. Compared to this run, 63% (170 of 268) of the participants used the same RTU systems with a total pass rate of 89%. In comparison, the pass rate for LD assays based on same clones was 69% (57 of 83 laboratories).

Controls

In this assessment and as observed in the previous runs for CDX2, pancreas is recommended as positive tissue control. Virtually all ductal epithelial cells must show an at least weak to moderate and distinct nuclear staining reaction. Appendix and colon cannot be recommended as primary positive tissue controls, since the epithelial cells express high levels of CDX2 and cannot be used to evaluate whether the protocol applied has sufficient analytical sensitivity.

Tonsil can be used as negative tissue control for CDX2. In order to monitor the specificity, no nuclear or cytoplasmic staining must be seen in endothelial cells and smooth muscle cells. The vast majority of lymphocytes should be negative, although weak nuclear staining reaction may be observed in a few lymphocytes as seen in this assessment. The recommendations of the mentioned tissue controls for IHC are concordant with the guidelines published by the International Ad Hoc Expert Committee¹.

¹Torlakovic EE, Nielsen S, Francis G, Garratt J, Gilks B, Goldsmith JD, Hornick JL, Hyjek E, Ibrahim M, Miller K, Petcu E, Swanson PE, Zhou X, Taylor CR, Vyberg M. Standardization of positive controls in diagnostic immunohistochemistry: recommendations from the International Ad Hoc Expert Committee. *Appl Immunohistochem Mol Morphol*. 2015 Jan;23(1):1-18. doi: 10.1097/PAI.0000000000000163. Review. PubMed PMID: 25474126.



Fig. 1a
Optimal CDX2 staining of the appendix using the rmAb clone EPR2764Y as Ready-To-Use format, 760-4380 Ventana, using HIER for 64 min. in CC1, OptiView as detection system and performed on the BenchMark ULTRA, Ventana.
A strong nuclear staining reaction is seen in virtually all the epithelial cells. In the cytoplasmic compartment a weak staining reaction is seen. No background staining is seen.
Also compare with Figs. 2a – 4a – same protocol.

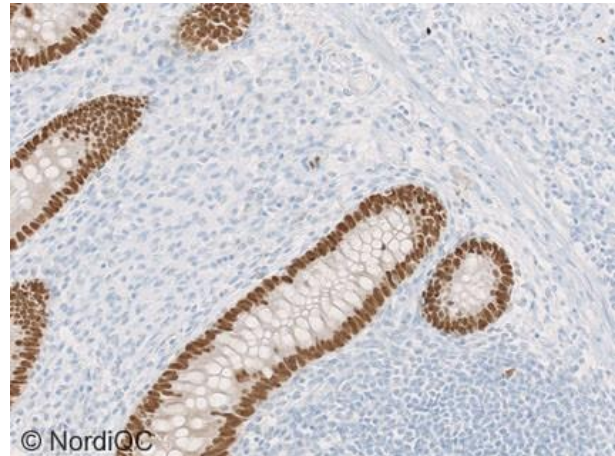


Fig. 1b
CDX2 staining of the appendix using an insufficient protocol based on the rmAb clone EPR2764Y as concentrate within a laboratory developed assay providing a too low analytical sensitivity. A too low titre of the primary Antibody and the use of 2-step multimer system, UltraView Ventana, were the main causes for the insufficient result, which especially is seen in Figs. 2b and 4b – same protocol.
In appendix – same field as Fig. 1a, a moderate nuclear staining reaction is seen in virtually all the epithelial cells.
As observed in the last CDX2 assessment, appendix cannot be recommended as positive tissue control for CDX2 due to the high level of CDX2 expression.

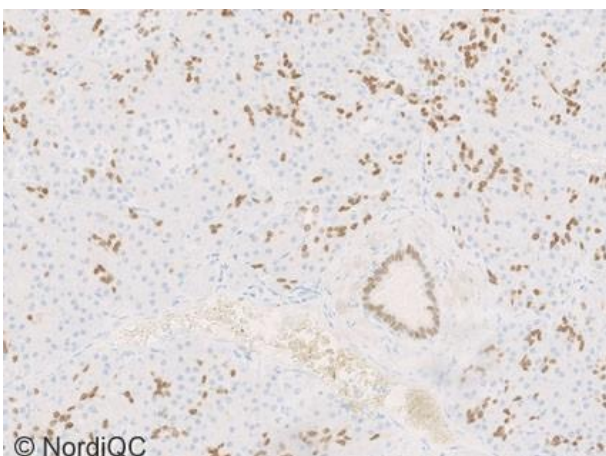


Fig. 2a
Optimal CDX2 staining of the normal pancreas using same protocol as in Fig. 1a.
A moderate to strong nuclear staining reaction is seen in virtually all the ductal epithelial cells.
A high signal-to-noise ratio is observed.

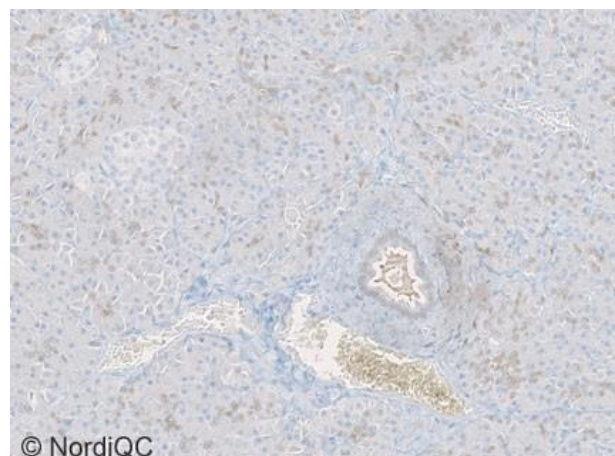


Fig. 2b
Insufficient CDX2 staining of the normal pancreas using same protocol as in Fig. 1b.
Only a faint nuclear staining reaction is seen in the ductal epithelial cells - same field as in Fig. 2a.
Also compare with Figs. 3b and 4b – same protocol.

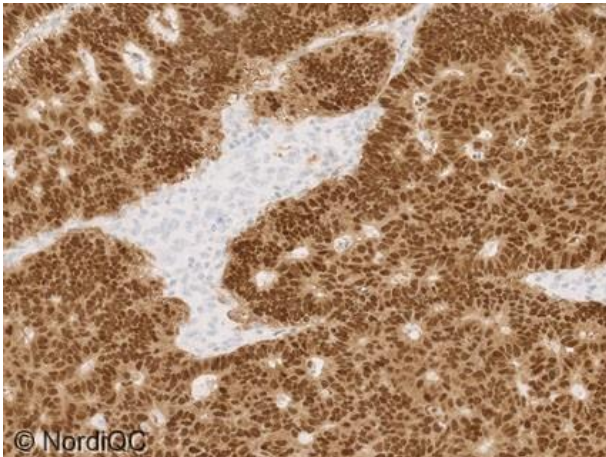


Fig. 3a
Optimal CDX2 staining of the colon adenocarcinoma, tissue core no. 6 using same protocol as in Figs. 1a and 2a.
Virtually all neoplastic cells show a strong nuclear staining reaction. A weak to moderate cytoplasmic staining is seen in the neoplastic cells, while the stromal compartment is unstained.

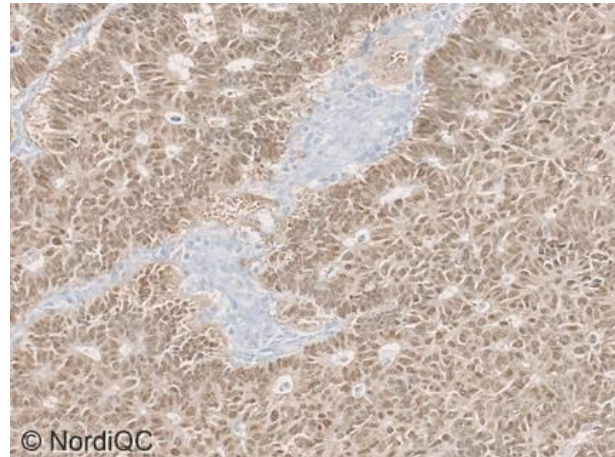


Fig. 3b
CDX2 staining of the colon adenocarcinoma, tissue core no. 6 using same protocol as in Figs. 1b and 2b. - same field as in Fig. 3a.
The vast majority of the neoplastic cells are demonstrated. The intensity is reduced compared to the level expected.

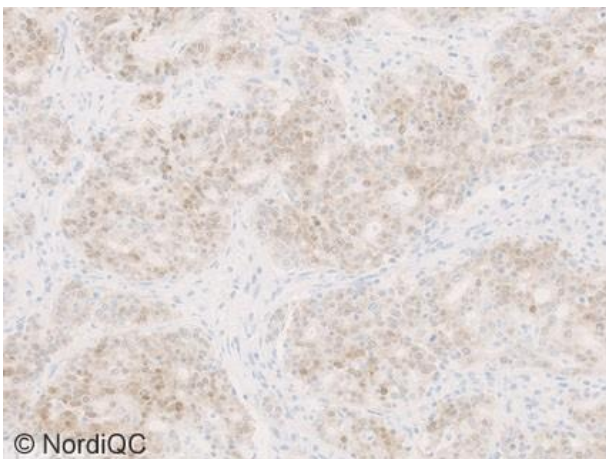


Fig. 4a
Optimal CDX2 staining of the colon adenocarcinoma, tissue core no. 5 using same protocol as in Figs. 1a - 3a.
The majority of the neoplastic cells show a weak to moderate nuclear staining reaction.

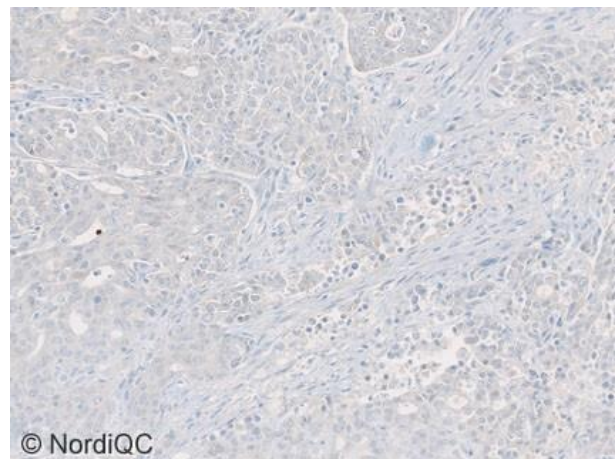


Fig. 4b
Insufficient CDX2 staining of the colon adenocarcinoma, tissue core no. 5 using same protocol as in Figs. 1b - 3b.
All neoplastic cells are false negative.

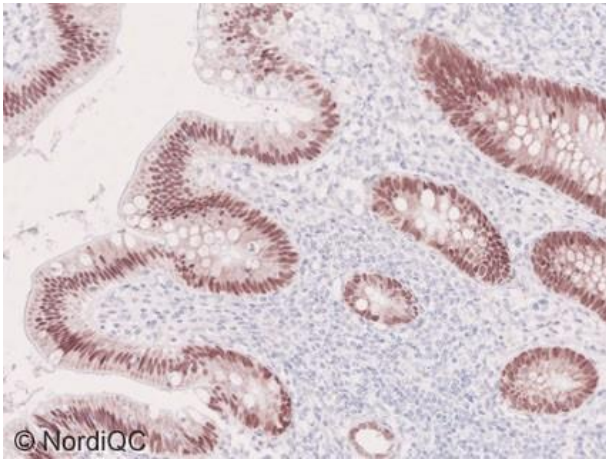


Fig. 5a
 CDX2 staining of the appendix using an insufficient protocol.
 based on the mAb clone CDX-88 as concentrate within a laboratory developed assay providing a too low analytical sensitivity. mAb clone CDX-88 has in the NordiQC CDX2 assessment shown an inferior performance compared to e.g. rmAb clone EPR2764Y and mAb DAK-CDX2. Typically a too low sensitivity for mAb clone CDX-88 is observed.
 Also compare with Fig. 5b – same protocol.

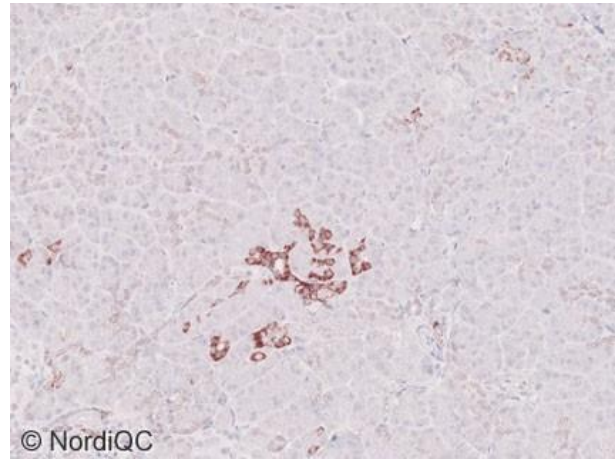


Fig. 5b
 Insufficient CDX2 staining of the normal pancreas using same protocol as in Fig. 5a.
 No nuclear staining reaction is seen in the ductal epithelial cells and only an aberrant cytoplasmic staining reaction is observed.
 The protocol was based on HIER in an alkaline buffer and a biotin based detection kit providing a too low analytical sensitivity and simultaneously a false positive staining caused by endogenous biotin.

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