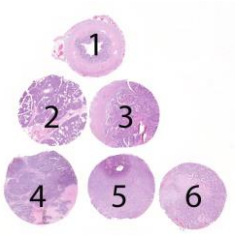
Assessment Run 46 2016

Chromogranin A (CGA)

Recommended CGA protocols Recommended CGA control tissue

Material

The slide to be stained for CGA comprised:
1. Appendix, 2. Pancreas, 3. Colon adenocarcinoma, 4. Small cell lung carcinoma, 5. Pancreatic neuroendocrine tumour, 6. Thyroid medullary carcinoma



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CGA staining as optimal included:

- A strong and distinct cytoplasmic staining reaction of neuroendocrine cells in the appendiceal mucosa and islets of pancreas.
- An at least weak to moderate, distinct granular cytoplasmic staining reaction of normal ganglion cells and axons in the nerve plexus of appendix.
- At least a moderate, distinct cytoplasmic reaction of virtually all neoplastic cells in the pancreatic neuroendocrine carcinoma and the medullary thyroid carcinoma.
- An at least weak, distinct granular cytoplasmic staining reaction of the vast majority of neoplastic cells in the small cell lung carcinoma.
- No staining reaction of the appendiceal columnar epithelial cells, pancreatic exocrine cells and neoplastic cells in the colon adenocarcinoma.

Participation

Number of laboratories registered for CGA, run 46	262
Number of laboratories returning slides	242 (92%)

Results

242 laboratories participated in this assessment. 162 (67%) 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:
- Less successful primary antibody
- Too low concentration of the primary antibody
- Omission of HIER
- Insufficient HIER – too short efficient heating time

Performance history

This was the sixth NordiQC assessment of CGA. The pass rate decreased slightly compared to the previous run as shown in table 2.

Table 2. **Proportion of sufficient results for CGA in the six NordiQC runs performed**

	Run 9 2003	Run 13 2005	Run 18 2006	Run 22 2008	Run 31 2011	Run 46 2016
Participants, n=	74	88	94	117	170	242
Sufficient results	39%	64%	70%	61%	75%	67%

The reduced pass rate in this run may in part be explained by a large proportion of new participants and new and more challenging tissue material circulated. However, also increased use of less successful Abs in this assessment seemed to have an impact. In run 31, 2011 12% of the laboratories used mAb clones 5H7 or DAK-A3, compared to 19% in this run. As shown in table 1, these two Abs provided an inferior performance compared to e.g mAb LK2H10.

Conclusion

The mAb clone **LK2H10** was the most successful Ab for the demonstration of CGA. As concentrated format within a laboratory developed assay, optimal results were obtained on all three main IHC platforms (Dako, Leica and Ventana) and a high pass rate was observed in general. The widely used mAb clone DAK-A3 provided a low pass rate, concordant to the results observed in previous CGA assessments.
HIER was mandatory for an optimal result. In this context it has to be stressed that the data sheets for mAb clone LK2H10 from the vast majority of vendors still provide misleading information for this clone recommending a protocol omitting HIER.

Normal appendix is recommendable as positive and negative tissue controls: Ganglion cells and axons in the peripheral nerves must show an at least weak to moderate distinct granular staining reaction, while smooth muscle cells and columnar epithelial cells should be negative. Neuroendocrine cells will show an intense staining reaction.

Table 1. **Antibodies and assessment marks for CGA, run 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5H7**	4	Leica/Novocastra	0	0	3	1	-	-
mAb clone **DAK-A3**	36	Dako/Agilent	0	2	17	17	6%	-
mAb clone **LK2H10**	22 18 6 3 2 2 1 1 1 1 1	Thermo/Neomarkers Cell Marque Immulologic Biogenex Millipore Zytomed Abcam A.Menarini Diagnostic Biosystems Europroxima Monosan Unknown	24	31	0	4	93%	98%
mAb clone **PHE5**	1	Unknown	0	0	1	0	-	-
mAb clones **LK2H10+PHE5**	6 5	Thermo/Neomarkers Biocare	3	8	0	0	100%	100%
rmAb clone **EP38**	1	Epitomics	0	1	0	0	-	-
rmAb clone **SP12**	1 1	Master Diagnostica Thermo/NeoMarkers	0	0	0	2	-	-
pAb **A0430***	38	Dako/Agilent	8	17	8	5	66%	-
pAb **NB120-17064**	1	Novus Biologicals	0	1	0	0	-	-
pAb **RB-9003**	1	Thermo/NeoMarkers	0	1	0	0		
Ready-To-Use antibodies								
mAb clone **5H7 PA0430**	6	Leica/Novocastra	0	0	2	4	-	-
mAb clone **LK2H10 760-2519**	69	Ventana/Roche	27	28	6	8	80%	96%
mAb clone **LK2H10 E001**	3	Linaris	0	3	0	0	-	-
mAb **LK2H10 AM126-5M**	1	Biogenex	0	0	1	0	-	-
mAb **LK2H10 238M-90**	1	Cell Marque	1	0	0	0	-	-
mAb clone **LK2H10 MAD-000616QD**	2	Master Diagnostica	1	1	0	0	-	-
mAb clones **LK2H10+PHE5 PM010**	2	Biocare	1	1	0	0	-	-
mAb clones **LK2H10+PHE5 BSB5345**	1	Bio SB	0	1	0	0	-	-
mAb clones **LK2H10+PHE5 MAB-0202**	1	Maixin	1	0	0	0		
pAb **IR502***	2	Dako	0	1	1	0		
Total	242		66	96	39	41	-	
Proportion			27%	40%	16%	17%	67%	

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

Detailed analysis of CGA, Run 46
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **LK2H10**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (5/15)*, TRS pH 9 (Dako) (4/10), TRS pH 6.1 (Dako) (2/6), Cell Conditioning 1 (CC1, Ventana) (7/12), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/5) or BERS1 (Leica)(3/3) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:1,000. Using these protocol settings, 47 of 48 (98%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clones **LK2H10+PHE5**: Protocols with optimal results were based on HIER using TRS pH 9 (Dako) (1/1) or Cell Conditioning 1 (CC1, Ventana) (2/5) as retrieval buffer. The mAb was diluted in the range of 1:250-1:400. Using these protocol settings, 4 of 4 (100%) laboratories produced a sufficient staining result.

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

Concentrated antibodies	**Dako Autostainer / Omnis**		**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 **LK2H10**	9/16** (56%)	0/1	11/31 (36%)	-	6/8 (75%)	2/2

* 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 **LK2H10**, product no. **760-2519**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 16-64 min.) and 8-32 min. incubation of the primary Ab. UltraView (760-500) or OptiView (760-700) were used as detection systems. Using these protocol settings, 47 of 49 (96%) laboratories produced a sufficient staining result.

mAb clones **LK2H10+PHE5**, product no. **MAB-0202**, Maixin, manual staining:
One protocol with an optimal result was based on HIER using Citrate pH6 (Pressure Cooker), 60 min. (RT) incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

Comments
In this assessment and in concordance with the previous NordiQC assessments of CGA, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells and structures expected to be demonstrated. This pattern was observed in 94% of the insufficient results (75 of 80). Virtually all laboratories were able to demonstrate CGA in high level antigen expressing structures such as neoplastic cells of the medullary thyroid carcinoma and normal neuroendocrine cells in the appendix and pancreatic Langerhans islets. The demonstration of CGA in low expressing structures as neoplastic cells of the SCLC and peripheral nerves was more challenging and required a carefully calibrated protocol.

The mAb clone LK2H10 was the most widely used antibody for demonstration of CGA and provided optimal results on all three main IHC platforms from Dako, Leica and Ventana, respectively (see table 3). Used as a concentrate within a laboratory developed (LD) assay, mAb clone LK2H10 gave an overall pass rate of 93% (55 of 59) of which 41% were optimal (see table 1). The two main prerequisites for sufficient staining were use of HIER and careful calibration of the titre of the primary Ab, whereas use of alkaline HIER buffers versus non-alkaline HIER buffers and 3-step versus 2-step detection systems were of less importance. However it was observed that use of 3-step polymer/multimer based detection systems seemed to provide higher proportions of optimal results compared to 2-step polymer/multimer based systems.
The mAb clone cocktail LK2H10+PHE5 provided a pass rate of 100% (11 of 11) within a LD assay and all 3 optimal results were based on efficient HIER in an alkaline buffer.

mab clone DAK-A3 was used by 36 participants and provided significantly inferior performance compared to mAb clone LK2H10. Despite similar protocol settings, a disappointing pass rate of 6% (2 of 36) was seen for mAb clone DAK-A3 none of which were optimal. For mAb clone DAK-A3 the insufficient results typically were characterized by a reduced staining intensity and proportion of cells demonstrated. Overall, a too low sensitivity/affinity of the clone seemed to cause the inferior performance. Compared to the previous runs for CGA, an increased use of mAb clone DAK-A3 was observed at the expense of the pAb

A0430 (which has been withdrawn from the market). In run 31 mAb clone DAK-A3 was used by 9% (16 of 170) of the laboratories compared to 15% (36 of 242) in this run, whereas pAb A0430 were used by 31% (53 of 170) in run 31 and 16% (38 of 242) in this run.

The RTU system from Ventana based on the mAb clone LK2H10, prod. No. 760-2519 gave a high proportion of sufficient and optimal results as shown in table 1. Optimal and sufficient results could only be obtained by laboratory modified protocol settings using HIER as pre-treatment, whereas no sufficient results were obtained using the vendor recommended protocol settings from Ventana stating that no HIER should be applied. 5 of 5 protocols performed accordingly to the official recommendation, thus all were assessed as insufficient due to a false negative staining result.
It was observed that a significant higher proportion of optimal results were obtained by performing HIER 16-64 min. in CC1 and use of OptiView as detection system compared to the use of UltraView. With UltraView 23% (7 of 30) of the results were optimal, compared to 85% (17 of 20) if OptiView was used as detection system. Use of short HIER for 8 min. in CC1 was found less successful as all 4 protocols based on this setting gave an insufficient result.

In this assessment the mAb clone 5H7 (Leica/Novocastra) showed an inferior performance both as concentrate and RTU format, as all 10 protocols based on this clone gave insufficient results characterized by a too weak staining reaction. The protocol settings applied for the mAb clone 5H7 were similar to the laboratories producing optimal staining results with the mAb clone LK2H10. Neither laboratory validated protocols nor protocols based on the guidelines from Leica gave a sufficient result for the RTU format PA0430 of mAb clone 5H7.

Controls
In concordance to previous assessments for CGA, appendix is recommendable as positive and negative tissue control for CGA: An at least weak to moderate distinct granular staining must seen in the axons and ganglion cells of the peripheral nerves. A strong staining must be seen in the neuroendocrine cells in the appendiceal mucosa and a diffusion of the staining has to be accepted in the vicinity of these cells. The epithelial cells in the mucosa and the smooth muscle cells should be negative.
It has to be stressed that pancreas cannot be used as positive tissue control as recommended by many vendors. Endocrine cells in the pancreatic islets have a high level of CGA expression, which is not relevant compared to the reduced and limited level expressed in many neuroendocrine tumours and consequently a false negative result in these tumors can occur despite a positive staining reaction in pancreas is seen.

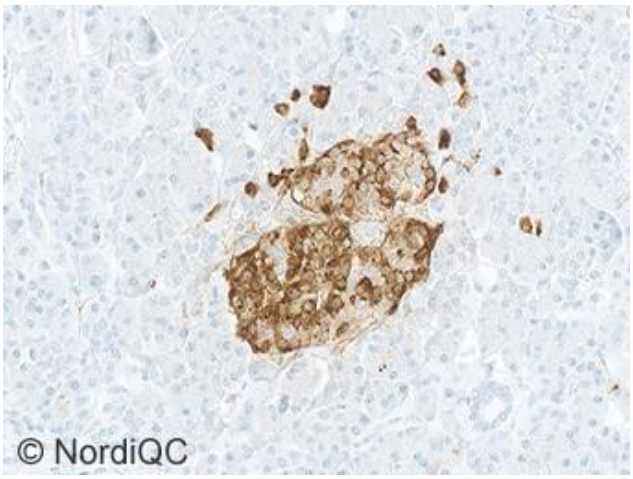


Fig. 1a
Optimal CGA staining of the pancreas using the mAb clone LK2H10 as Ready-To-Use format 760-2519, Ventana, by a laboratory modified protocol using HIER in CC1 and a 3-step multimer based detection system (OptiView).
The vast majority of endocrine islet cells show a moderate to strong and distinct cytoplasmic staining reaction and a high signal-to-noise ratio is observed.
Also compare with Figs. 2a - 5a – same protocol.

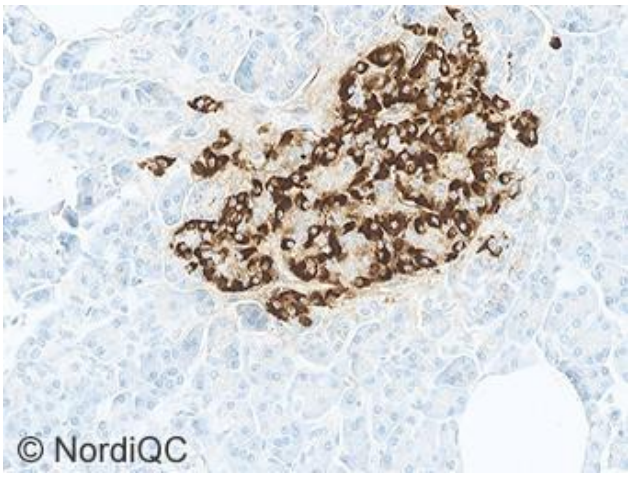


Fig. 1b
CGA staining of the pancreas using an insufficient protocol giving a too low sensitivity.
The protocol was based on the same mAb clone LK2H10, Ready-To-Use format 760-2519, Ventana omitting HIER as recommended in the package insert for the product. OptiView was used as detection system.
Also compare with Figs. 2b - 4b – same protocol.

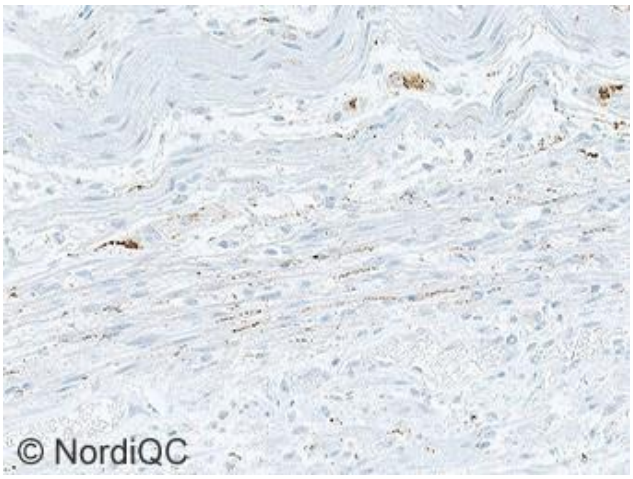


Fig. 2a
Optimal CGA staining of the appendix using same protocol as in Fig. 1a.
A moderate and distinct granular cytoplasmic staining reaction of normal ganglion cells and axons in the nerve plexus is seen.
No background staining is seen.
Also compare with Figs. 3a - 5a – same protocol.

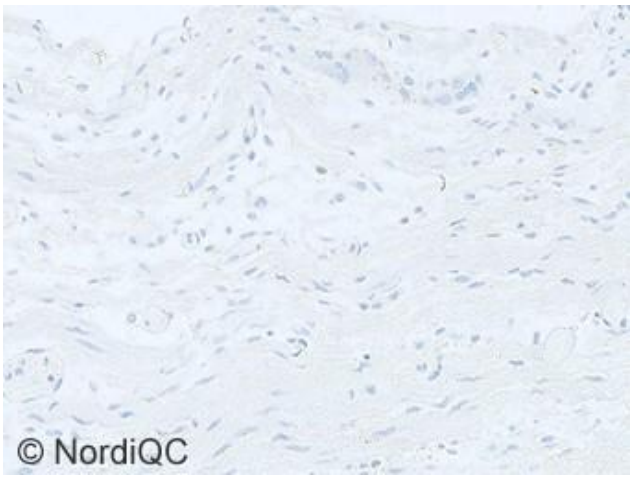


Fig. 2b
Insufficient CGA staining of the appendix using same protocol as in Fig. 1b – same field as in Fig. 2a.
No staining reaction of ganglion cells and axons can be identified.
Also compare with Figs. 3b and 4b – same protocol.

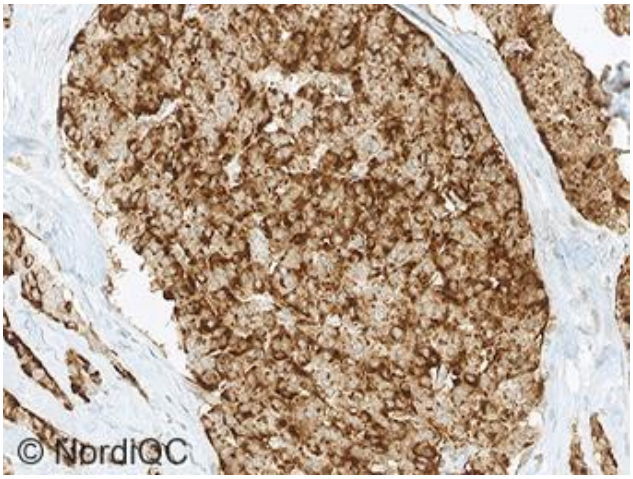


Fig. 3a
Optimal CGA staining of the pancreatic neuroendocrine carcinoma using same protocol as in Figs. 1a. and 2a. Virtually all the neoplastic cells show a strong and distinct staining reaction. No background staining is seen.

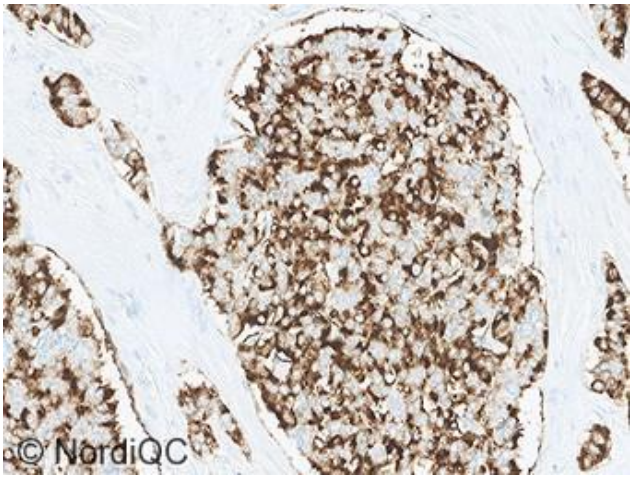


Fig. 3b
Staining for CGA of the pancreatic neuroendocrine carcinoma using same insufficient protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The vast majority of the neoplastic cells are demonstrated. However also compare with Fig. 4b – same protocol.

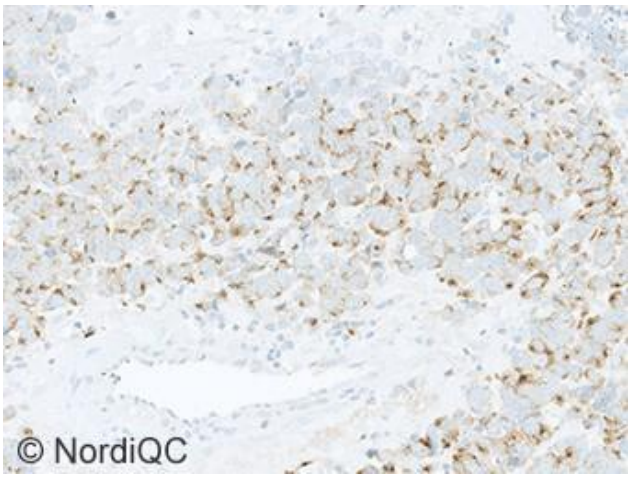


Fig. 4a
Optimal CGA staining of the SCLC using same protocol as in Figs. 1a – 3a.
Virtually all the neoplastic cells show a strong and distinct cytoplasmic staining reaction with a dot-like accentuation. No background staining is seen.

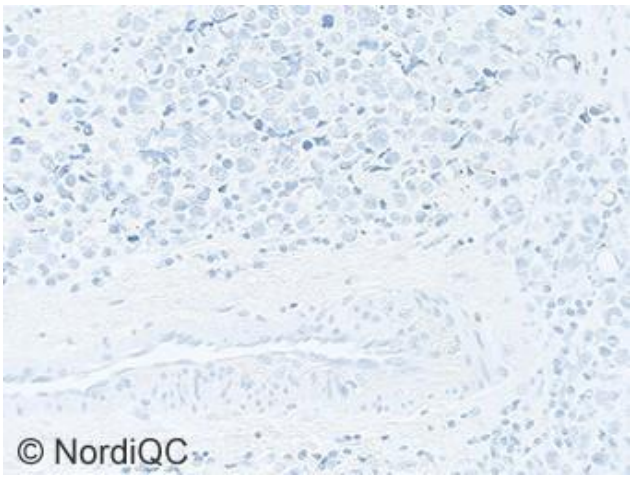


Fig. 4b
Insufficient CGA staining of the SCLC using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
Only scattered neoplastic cells show a weak and diffuse cytoplasmic staining reaction.

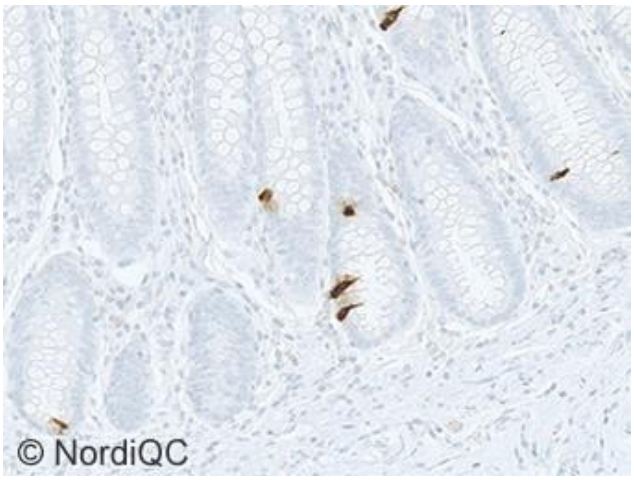


Fig. 5a
Optimal CGA staining of the appendix mucosa using same protocol as in Figs. 1a – 4a.
The neuroendocrine cells show an intense staining reaction. A weak diffusion of the signal is seen in the close vicinity of the positive cells, whereas all other epithelial cells are negative.
Appendix is highly recommended as positive and negative tissue control and comprises structures with clinical relevant levels of CGA ranging from low (nerves) to high (neuroendocrine cells) and epithelial and muscle cells being negative.
In this context it has to be stressed that pancreas cannot be used as positive tissue control as recommended by many vendors. Endocrine cells in the pancreatic islets have a high level of CGA expression, which is not relevant compared to the reduced and limited level expressed in many neuroendocrine tumours.
Consequently a false negative result in these tumors can occur, despite a positive staining reaction in pancreas is seen. This is shown in Figs. 1b – 4b.

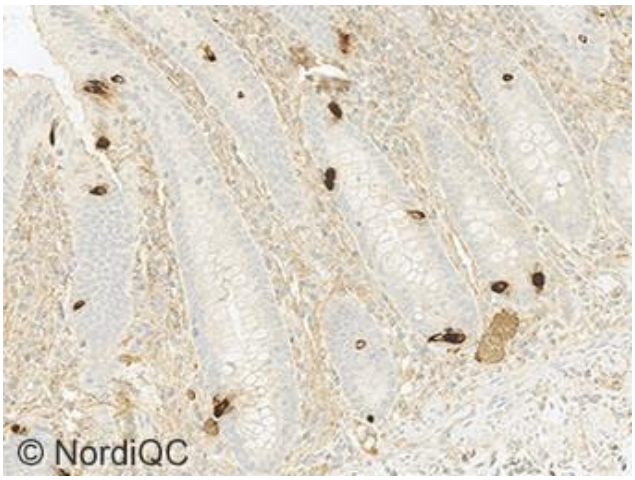


Fig. 5b
Insufficient CGA staining of the appendix mucosa using a protocol not calibrated appropriately.
The protocol was based on efficient HIER in an alkaline buffer and a 2-step polymer based detection system and the mAb clone DAK-A3 as a concentrate by a laboratory developed (LD) assay.
An aberrant diffuse background staining and general cytoplasmic staining of epithelial cells is observed compromising the interpretation.
mAb clone DAK-A3 in general provided a significant inferior performance compared to e.g. mAb clone LK2H10.
For mAb DAK-A3 a pass rate of 6% was seen (2 of 36 participants) and 0% optimal.

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