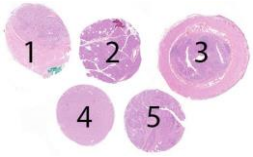
Assessment Run 67 2023

Chromogranin A (CGA)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CGA, typically used in the diagnostic work-up of neuroendocrine tumors. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen
densities for CGA (see below).

Material

The slide to be stained for CGA comprised:



1. Colon adenocarcinoma, 2. Pancreas, 3. Appendix, 4. Neuroendocrine tumour (Colon neuroendorine tumour (NET)), 5. Small cell lung carcinoma (SCLC)

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.
- An at least moderate to strong, distinct cytoplasmic reaction of virtually all neoplastic cells in the neuroendocrine tumour.
- 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 67	392
Number of laboratories returning slides	367 (94%)

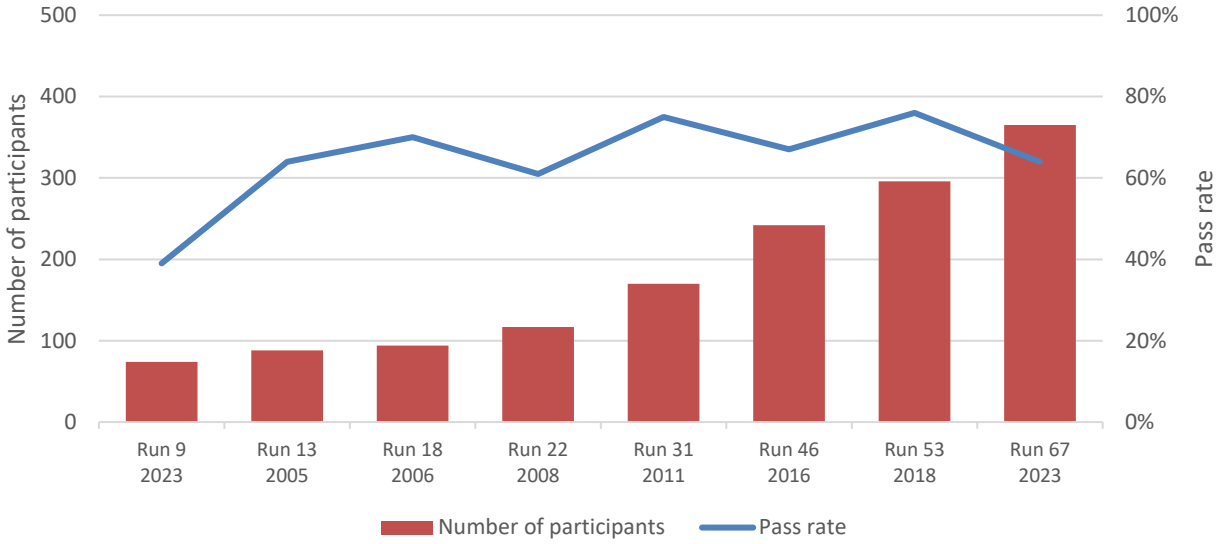
All slides returned after the assessment were assessed and received advice if the result being insufficient, but the data were not included in this report.

Results
367 laboratories participated in this assessment. 2 laboratories used an inappropriate antibody. They were not included in the analysis. Of the remaining 365 laboratories 235 (64%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Less successful primary Ab
- Use of a 2-step detection system
- Insufficient HIER

Performance history
This was the eighth NordiQC assessment of CGA. The pass rate decreased to 64% in this assessment compared to the previous run 53 as shown in Graph 1, but on par to the mean pass rate of 65% obtained in the eight NordiQC assessments performed.
No obvious reason for the decline in this run compared to run 53 could be found. The combination of increased no. of new participants and the circulated material potentially being more challenging requiring an overall increased level of analytical and diagnostic sensitivity of the protocols compared to the previous run 53 could be causing the drop in the pass rate.

Graph 1**. Proportion of sufficient results for CGA in the eight NordiQC run performed**



Conclusion
The mAb clone **LK2H10** was the most successful Ab for the demonstration of CGA. As concentrated (conc.) format within a laboratory developed assay, optimal results were obtained on all four main stainer platforms if HIER was performed in an alkaline buffer with a 3-step detection system. The corresponding and widely used Ready-To-Use system (44% of all participants) from Ventana/Roche based on mAb clone LK2H10 gave an overall pass rate of 87%. The mAb clones DAK-A3 and 5H7 gave significant inferior performances, despite similar protocol settings as mAb LK2H10. The two clones were in total used by 16% of the participants providing an unacceptable pass rate of 3%, no optimal results.

Controls
In concordance with previous assessments for CGA, appendix is recommendable as positive and negative tissue control: An at least weak to moderate distinct granular staining must be seen in the axons and ganglion cells of the peripheral nerves. Neuroendocrine cells in the appendiceal mucosa should display a strong staining and diffusion of the staining in the vicinity of these cells has to be accepted. Columnar epithelial cells and smooth muscle cells should be negative.
In this context it must be stressed that pancreas cannot be used as positive tissue control even though recommended by some vendors. Endocrine cells in the pancreatic islets have a high level of CGA expression, which cannot reliable be used as control of sufficient sensitivity of the protocol. The low-level and limited expression of CGA in many neuroendocrine tumours and carcinomas can consequently lead to a false negative staining result in these tumours despite positive staining reaction in pancreas.

Table 1. **Antibodies and assessment marks for CGA, run 67**

Concentrated antibodies	N	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **5H7**	5	Leica Biosystems	0	1	0	4	20%	0%
mAb clone **DAK-A3**	38	Dako/Agilent	0	1	12	25	3%	0%
mAb clone **LK2H10**	1 2 54 2 2 4 1 1 1 1 1 13 4	Biogenex Bio SB Cell Marque Diagnostic Biosystems Immunologic Invitrogen Linaris Millipore Monosan Nordic BioSite Progen Biotechnik GmbH Thermo Fisher Scientific Zytomed Systems GmbH	26	33	25	3	68%	30%
mAb clone **ZM12**	1	Zeta Corporation	0	0	1	0	-	-
mAb clones **LK2H10+PHE5**	7 1 6 1	Biocare Medical Invitrogen NeoMarkers Thermo Fisher Scientific	3	8	4	0	73%	27%
mAb clone **IHC544**	1	GenomeMe	0	1	0	0	-	-
rmAb clone **SP12**	1 1	Diagnostic Biosystems Thermo Fisher Scientific	0	1	0	1	-	-
rmAb clone **BP6129**	1	Biolynx Biotechnology	0	0	1	0	-	-
pAb **A0430***	3	Dako/Agilent	0	1	2	0	-	-
pAb **ab15160**	1	abcam	0	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **5H7 PA0430/PA0515 (VRPS)[3]**	8	Leica Biosystems	0	0	7	1	0%	0%
mAb clone **5H7 PA0430/PA0515 (LMPS)[4]**	7	Leica Biosystems	0	0	7	0	0%	0%
mAb clone **LK2H10 760-2519 (VRPS)[3]**	15	Ventana/Roche	7	6	2	0	87%	47%
mAb clone **LK2H10 760-2519 (LMPS)[4]**	147	Ventana/Roche	73	55	17	2	87%	50%
mAb clone **LK2H10 BMS018**	2	Zytomed Systems GmbH	1	0	0	1	-	-
mAb **LK2H10 AM126**	1	Biogenex	0	1	0	0	-	-
mAb **LK2H10 238M-90/98**	8	Cell Marque	1	5	2	0	75%	13%
mAb clone **LK2H10 MAD-000616QD**	3	Master Diagnostica	0	1	1	1	-	-
mAb clone **LK2H10 8286-C010**	2	Sakura Finetek	0	0	2	0	-	-
mAb clone **LK2H10 PDM067**	1	Diagnostic Biosystems	1	0	0	0	-	-
mAb clone **LK2H10 Unknown**	1	Unknown	0	0	1	0	-	-
mAb clones **LK2H10 BFM-0052**	1	Bioin Biotechnology	0	1	0	0	-	-
mAb clones **LK2H10+PHE5 PM010 AA**	6	Biocare Medical	2	2	2	0	67%	33%

mAb clone **317F1D8 PA069**	1	Abcarta	0	1	0	0	-	-
mAb clone **C1E8 CCM-0852**	1	Celnovte	0	0	1	0	-	-
mAb clone **CGA/413+CHGA/777 +CHGA/798 AMA51-5M**	1	Biogenex	0	0	0	1	-	-
mAb clone **MX018, MAB0707**	1	Fuzhou Maixin	0	0	1	0	-	-
rmAb clone **EP38 01.09.70.03.56.01**	1	Zybio	0	1	0	0	-	-
Ab clone **DGR067 DGR067**	1	Shanghai DG Diagnology	1	0	0	0	-	-
pAb **IR502***	1	Dako/Agilent	0	0	1	0	-	-
pAb **412751**	1	Nichirei Bioscience	0	0	1	0	-	-
Total	365		116	119	91	39		
Proportion			32%	33%	25%	11%	64%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).
*discontinued products

Detailed analysis of CGA, Run 67
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **LK2H10**: Protocols with optimal results were typically based on Heat Induced Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) High pH (Dako/Agilent) (10/42)*, Cell Conditioning 1 (CC1, Ventana/Roche) (12/26), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (2/5) or Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/7) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:1,000. Using these protocol settings, 54 of 80 (68%) 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 CC1 (Ventana/Roche) (1/5), BERS2 (Leica Biosystems) (1/3) or BERS1 (Leica Biosystems) (1/3) as retrieval buffer. The mAb was diluted in the range of 1:100-1:800. Using these protocol settings, 7 of 9 (78%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for CGA for the most commonly used antibody concentrate on the four main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT/Ultra**		**Leica Biosystems 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	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **LK2H10**	2/11 (18%)	0/1	8/28 (29%)	0/2	12/25 (48%)	-	2/5 (40%)	1/6 (17%)
mAb clones **LK2H10+PHE5**	-	-	-	-	1/4	-	1/3	1/2

* 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/Roche, BenchMark GX/XT/Ultra:
Optimal protocols using UltraView (760-500) as detection system were typically based on HIER using CC1 (efficient heating time 20-64 min.) and 20-36 min. incubation of the primary Ab.
Optimal protocols using OptiView (760-700) as detection system were typically based on HIER using CC1 (efficient heating time 16-64 min.) and 4-32 min. incubation of the primary Ab.
Using these protocol settings, 137 of 146 (94%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **Proportion of sufficient and optimal results for CGA for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS GX/XT/Ultra mAb LK2H10 **760-2519**	13/15 (87%)	7/15 (47%)	127/145 (88%)	73/145 (50%)

* 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, detection kit – only protocols performed
on the specified vendor IHC stainer integrated.

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 88% of the insufficient results (115 of 130). Virtually all laboratories were able to demonstrate CGA in high-level antigen expressing structures such as neoplastic cells of the neuroendocrine tumour and normal neuroendocrine cells in the appendix and pancreatic Langerhans islets. Demonstration of CGA in low-level expressing structures as neoplastic cells of the SCLC and peripheral nerves in the appendix 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 four main IHC platforms from Dako/Agilent, Leica Biosystems and Ventana/Roche, respectively (see Table 2). Used as a conc within a laboratory developed (LD) assay, mAb clone LK2H10 gave a significantly reduced pass rate of 68%, 30% optimal compared to the previous assessment run 53, with a pass rate of 91%, 67% were optimal. The main prerequisites for sufficient staining were use of HIER in an alkaline buffer, careful calibration of the titre of the primary Ab and a 3-step detection system. 61% (53 of 87) of the laboratories used a 3-step detection system, giving a pass rate of 94% (50 of 53), 47% optimal (n=25). If using a 2-step detection system, a significantly lower pass rate of 26% (9 of 34) was obtained, one optimal.

The mAb clone cocktail LK2H10+PHE5 provided a pass rate of 73% (11 of 15) within a LD assay of which 27% were optimal (see Table 1). As for mAb clone LK2H10, the main prerequisites for sufficient staining were use of HIER in an alkaline buffer, careful calibration of the titre of the primary Ab and a 3-step detection system. If using a 3-step detection system a pass rate of 100% was obtained (10 of 10), 30% optimal, whereas 2-step detection system obtained a pass rate of 20% (1 of 5), no optimal.

mAb clone DAK-A3 was used by 38 participants and provided a significantly inferior performance compared to mAb clone LK2H10. Despite similar protocol settings, a disappointing pass rate of 3% (1 of 38) was seen. Insufficient results were typically characterized by a reduced staining intensity and proportion of cells demonstrated. Overall, too low analytical sensitivity/affinity of this clone seemed to cause the inferior performance. The observation and results were concordant to the data seen in runs 46 and 53 and laboratories using this clone should consider change to other Ab as mAb clone LK2H10 and recalibrate and validate the IHC assay.

The RTU system from Ventana/Roche based on the mAb clone LK2H10 (760-2519) gave a high proportion of sufficient and optimal results as shown in Table 1. Optimal and sufficient results could be obtained both by using laboratory modified protocol settings and by the recommended protocol settings from Ventana (see Table 3). The vast majority of laboratories modified the protocol. The most common modifications were prolonged HIER and/or incubation time of primary Ab.
It was observed that a significant higher proportion of optimal results were obtained by use of OptiView as detection system compared to the use of UltraView. With UltraView 29% (22 of 77) of the results were optimal, of which four used UltraView amplification, compared to 70% (58 of 83) if OptiView was used.

In this assessment the mAb clone 5H7 (Leica Biosystems) showed an inferior performance both as conc. and RTU format, as only a 5% (1 of 20) pass rate was obtained. Insufficient results were characterized by a too weak or false negative staining reaction. The protocol settings applied for the mAb clone 5H7

were typically based on HIER using a non-alkaline buffer. Two laboratories used HIER in an alkaline buffer, however, the results were borderline.
The observation and results for mAb clone 5H7 were concordant to the data seen in runs 46 and 53 and laboratories using this clone should consider change to other Ab as mAb clone LK2H10 and recalibrate and validate the IHC assay.

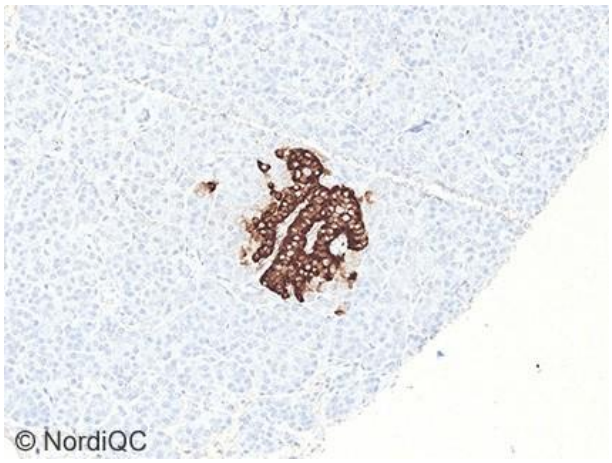


Fig. 1a
Optimal CGA staining of the pancreas using the mAb clone LK2H10 as RTU format (Ventana/Roche, 760-2519) using a modified protocol with HIER at high pH for 32 min., 32 min. incubation of the primary Ab and a 3-step multimer based detection system performed on BenchMark Ultra.
The vast majority of endocrine islet cells show a strong and distinct cytoplasmic staining reaction.
Also compare with Figs. 2a - 5a – same protocol.

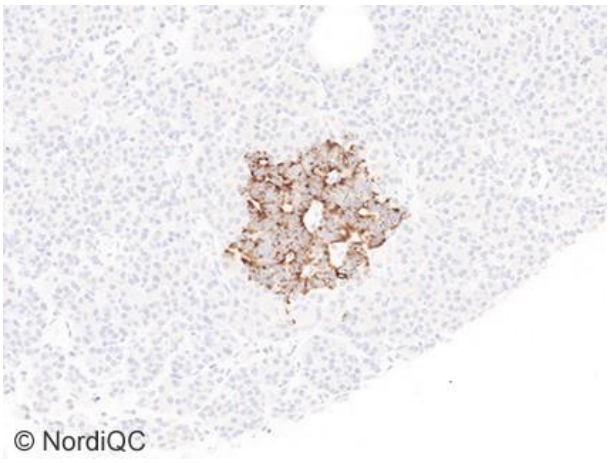


Fig. 1b
CGA staining of the pancreas using an insufficient protocol with overall too low analytical sensitivity.
The protocol was based on the mAb clone DAK-A3, using similar protocol settings as Fig. 1a with HIER at high pH and a 3-step multimer based detection system performed on BenchMark Ultra, Ventana/Roche.
Also compare with Figs. 2b - 4b – same protocol.

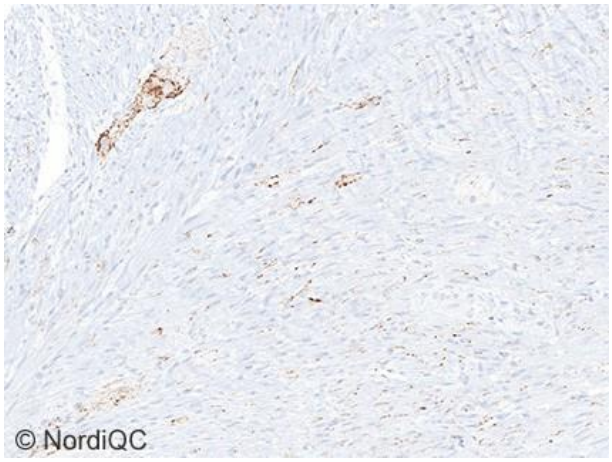


Fig. 2a
Optimal CGA staining of the appendix using same protocol as in Fig. 1a. A moderate and distinct granular cytoplasmic staining reaction of normal ganglion cells and axons in the nerve plexus is seen. No background staining is seen. Also compare with Figs. 3a - 5a – same protocol.

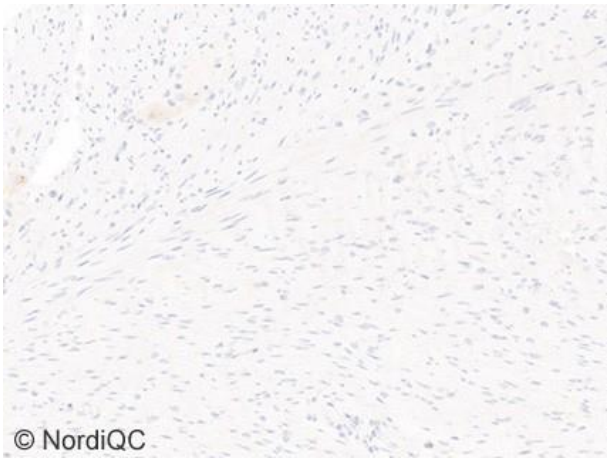


Fig. 2b
Insufficient CGA staining of the appendix using same protocol as in Fig. 1b – same field as in Fig. 2a.
Virtual all ganglion cells and axons are negative.
Also compare with Figs. 3b and 4b – same protocol.

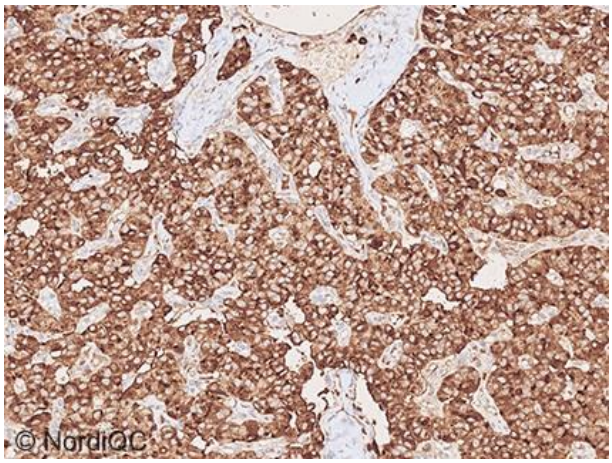


Fig. 3a
Optimal CGA staining of the neuroendocrine tumor using same protocol as in Figs. 1a and 2a.
Virtually all the neoplastic cells show a strong and distinct staining reaction.

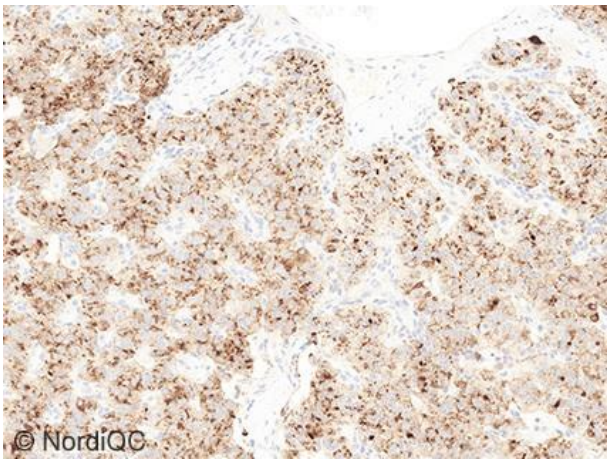


Fig. 3b
CGA staining of the neuroendocrine tumor using the same insufficient protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
A significant reduced staining intensity is seen
Also compare with Fig. 4b – same protocol.

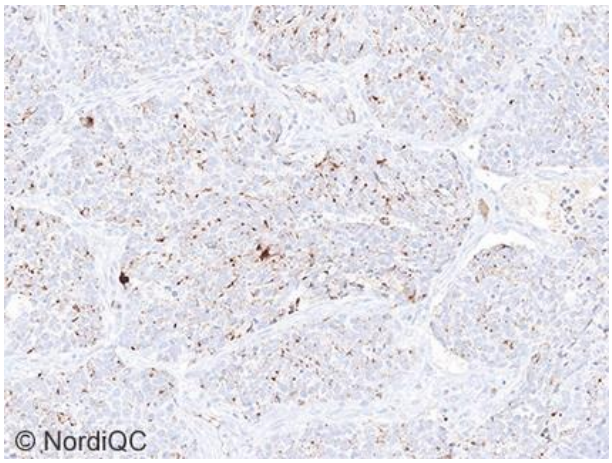


Fig. 4a
Optimal CGA staining of the SCLC using same protocol as in Figs. 1a – 3a.
The majority of the neoplastic cells show a weak to moderate dot-like accentuation. No background staining is seen.

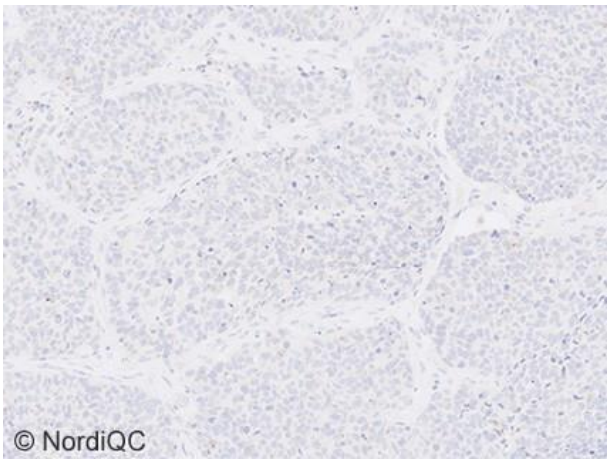


Fig. 4b
Insufficient CGA staining of the SCLC using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
Only scattered neoplastic cells show a weak and diffuse granular staining reaction.

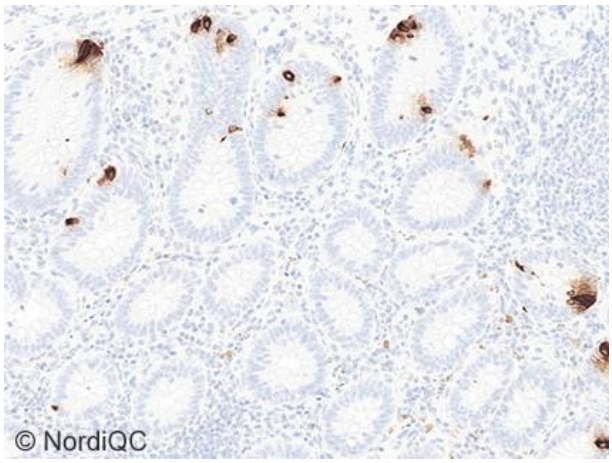


Fig. 5a
Optimal CGA staining of the appendix mucosa using same protocol as in Figs. 1a – 4a.
The neuroendocrine cells show an intense staining reaction. A weak diffusion of the signal is seen in the close vicinity of the positive cells, whereas all other epithelial cells are negative.

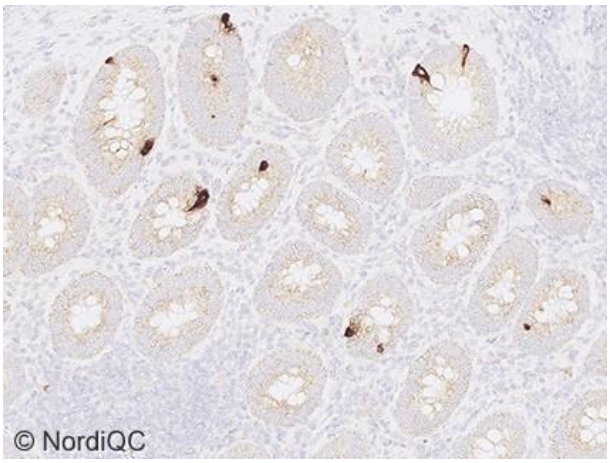


Fig. 5b
Insufficient CGA staining of the appendix mucosa using a protocol not calibrated appropriately.
The protocol was based on the mAb clone DAK-A3, using HIER at high pH, a 3-step polymer-based detection system and performed on Autostainer Link 48, Dako/Agilent.
An aberrant cytoplasmic staining of epithelial cells is observed compromising the interpretation. Compare with optimal result in Fig. 5a.

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