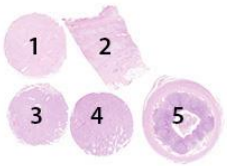
Assessment Run 64 2022

Calretinin (CR)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CR, typically identifying sex cord-stromal tumours and mesotheliomas in the characterization of tumours of unknown origin. Relevant clinical tissues, both normal and neoplastic, were selected displaying a broad spectrum of antigen densities for CR (see below).

Material
The slide to be stained for **CR** comprised:



1. Mesothelioma, 2. Adrenal gland, 3. Granulosa cell tumour, 4. Lung adenocarcinoma, 5. Appendix.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CR staining as optimal included:

- A moderate to strong, distinct cytoplasmic and nuclear staining reaction of peripheral nerves (ganglion cells and axons) in the appendix.
- A strong, distinct cytoplasmic and nuclear staining reaction of subtypes of macrophages in the appendix and macrophages intermingling between the neoplastic cells of the lung adenocarcinoma.
- A strong, distinct cytoplasmic and nuclear staining reaction of all neoplastic cells in the mesothelioma.
- An at least weak to moderate, distinct cytoplasmic and nuclear staining reaction of the majority of cortical epithelial cells in the adrenal gland and adipocytes (including appendix).
- A moderate to strong, distinct cytoplasmic and nuclear staining reaction of virtually all neoplastic cells in the granulosa cell tumour.
- No staining reaction of neoplastic cells of the lung adenocarcinoma and of the columnar epithelial cells of the appendix.

Participation

Number of laboratories registered for CR, run 64	372
Number of laboratories returning slides	350 (94%)

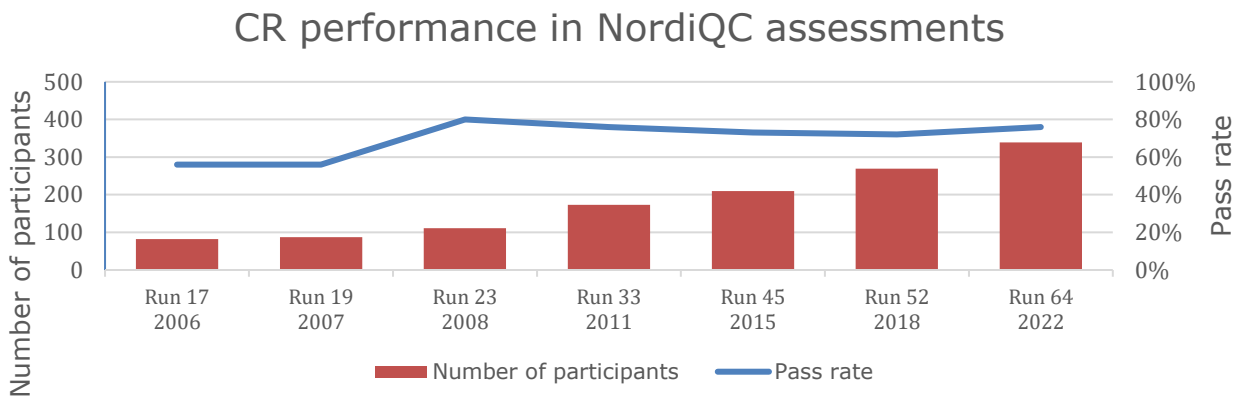
Results
At the date of assessment, 94% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

350 laboratories participated in this assessment. 10 laboratories used an inappropriate antibody (Ab) (Chromogranin A) and one laboratory stained erroneously circulated CR slide with Desmin. Of the remaining 339 laboratories, 76% achieved a sufficient mark. Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Less successful performance of the mAb DAK-Calret 1 on the Omnis (Dako/Agilent) and BenchMark (Ventana/Roche) platforms.
- Use of HIER in citric based buffers.
- Too short efficient HIER time.
- Too low concentration of the primary Ab.
- Use of detection systems with a low sensitivity.

Performance history
This was the seventh NordiQC assessment of CR. The pass rate has been on a consistent level during the latest four runs (see Graph 1).

Graph 1. **Proportion of sufficient results for CR in the seven NordiQC runs performed**



Conclusion
Optimal staining results could be obtained with the mAbs clones **5A5, CAL6, DAK-Calret 1, C5G4** and **IHC523**, the rmAb clones **BSR235**, **SP13, SP65,** and **RM324** and the pAbs **18-0211 and 61-0006**. Irrespective of the clone/antibody applied, efficient HIER in an alkaline buffer, careful calibration of the primary antibody and use of a 3-step multimer/polymer detection system were the most important requirements for an optimal staining result. Performance of the mAb clone DAK-Calret 1, both as Ready-To-Use (RTU) formats and concentrates, was influenced by the chosen platform and gave a low pass rate on both the Omnis (Dako/Agilent) and Benchmark (Ventana/Roche) systems, 40% and 25%, respectively. The number of laboratories using a RTU format increased from 73% (Run 52) to 80% in this assessment. The RTU system PA0346 (Leica Biosystems) based on the mAb clone CAL6, and using recommended protocol settings, was the most successful assay with an overall pass rate of 100% with 50% being optimal. The RTU system 790-4467 (Ventana/Roche) based on the rmAb clone SP65 also provided a relative high proportion of sufficient and optimal results, but virtually none (2/175) of the participants followed the basic recommendations provided by the vendor. Among the three major vendors, the RTU system IR627 (Dako/Agilent) based on the mAb clone DAK-Calret 1 (Autostainer) provided the lowest overall pass rate.

Adrenal gland and appendix are recommendable positive and negative tissue controls for CR. The majority of the cortical epithelial cells in the adrenal gland must show an at least weak to moderate, distinct cytoplasmic and nuclear staining reaction. In appendix, columnar epithelial cells and smooth muscle cells must be negative, whereas peripheral nerves (ganglion cells and axons) and macrophages should display a moderate to strong, distinct cytoplasmic and nuclear staining reaction. Furthermore, adipocytes in the submucosa of the appendix could serve as an additional low expressor tissue control.

Table 1. **Antibodies and assessment marks for CR, run 64**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **2E7**	1	BioGenex	0	0	1	0	-	-
mAb clone **5A5**	1	Monosan	1	0	0	0	-	-
mAb clone **ZM85**	1	Zeta Corporation	0	1	0	0	-	-
mAb clone **CAL6**	19	Leica Biosystems	12	4	1	2	84%	63%
mAb clone **DAK-Calret 1**	25 1	Dako/Agilent Thermo Scientific	6	12	6	2	69%	23%
rmAb clone **BSR235**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **SP13**	1 1 1 1 1 1	Cell Marque Zytomed Systems Abcam Epredia Diagnostic Biosystems Zeta Corporation	0	2	2	2	33%	-
pAb **18-0211**	6 1	Invitrogen/Thermo S. Zymed	5	1	1	0	86%	71%
pAb **232A**	1	Cell Marque	0	0	0	1	-	-
pAb **61-0006**	1	Genemed	1	0	0	0	-	-
pAb, **CP092C**	1	Biocare Medical	0	1	0	0	-	-
pAb **RBK003**	1	Zytomed Systems	0	1	0	0	-	-
pAb **CR7696**	1	Swant	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **CAL6 PA0346[3]**	8	Leica Biosystems	4	4	0	0	100%	50%
mAb clone **CAL6 PA0346[4]**	10	Leica Biosystems	3	3	3	1	60%	30%
mAb clone **DAK-Calret 1 IS/IR627[3]**	16	Dako/Agilent	3	5	7	1	50%	19%
mAb clone **DAK-Calret 1 IS/IR627[4]**	43	Dako/Agilent	5	15	11	12	47%	12%
mAb clone **C5G4 CCM-0222**	1	Celnovte Biotechnology	1	0	0	0	-	-
mAb clone **IHC523 IHC523**	1	GenomeMe	1	0	0	0	-	-
rmAb **SP13 232R**	4	Cell Marque	2	0	1	1	-	-
rmAb **SP13 MAD-000315QD**	1	Master Diagnostica	0	0	1	0	-	-
rmAb **BSR235 MAD-000784QD**	2	Master Diagnostica	0	0	1	1	-	-
rmAb **RM324 8522-C010**	2	Sakura Finetek	2	0	0	0	-	-
rmAb clone **SP65 790-4467[3]**	2	Ventana/Roche	2	0	0	0	-	-
rmAb clone **SP65 790-4467[4]**	177	Ventana/Roche	120	38	18	1	89%	68%
pAb **232A**	2	Cell Marque	0	0	1	1	-	-
pAb **IP092**	1	Biocare Medical	0	0	1	0	-	-
pAb **HAP134**	1	PathnSitu	0	1	0	0	-	-
pAb **08-1211**	1	Invitrogen/Thermo S.	0	0	1	0	-	-
Total	339		169	88	56	26	-	
Proportion			50%	26%	16%	8%	76%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Detailed analysis of CR, Run 64
The following protocol parameters were central to optimal staining:

Concentrated Antibodies
mAb clone **DAK-Calret 1**: Protocols with optimal results were all based on Heat Induced Epitope Retrieval (HIER) using Bond Epitope Retrieval Solution 2 (BESR2, Leica Biosystems) (3/6)*, Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/2) or Target Retrieval Solution (TRS) High pH (3-in-1) (Dako/Agilent) (2/11). The mAb was typically diluted in the range of 1:40-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 10 of 11 (91%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mAb clone **CAL6**: Protocols with optimal results were based on HIER using TRS High pH (3-in-1) (10/10) or BERS2 (2/6). The mAb was typically diluted in the range of 1:15-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings, 11 of 13 (85%) laboratories obtained a sufficient result.

mAb clone **5A5**: One protocol with an optimal result was based on HIER in BERS2. The mAb was diluted 1:50 and Bond Refine (Leica Biosystems) was used as detection system.

rmAb clone **BSR235**: One protocol with an optimal result was based on HIER in Tris-EDTA/EGTA pH 9. The rmAb was diluted 1:100 and HRP-Polymer Anti-Rabbit (Zytomed systems) was used as detection system.

pAb **18-0211**: Protocols with optimal results were based on HIER using TRS High pH (3-in-1) (3/3), BERS2 (1/1) or Cell Conditioning 1 (CC1, Ventana/Roche) (1/3) as retrieval buffer. The pAb was diluted in the range of 1:50-1:150 depending on the total sensitivity of the protocol employed. Using these protocol settings, 6 of 7 (86%) laboratories produced a sufficient staining result.

pAb **61-0006**: One protocol with an optimal result was based on HIER in Tris-EDTA/EGTA pH 9. The pAb was diluted 1:500 and GTVision (Gene Tech) was used as detection system.

Table 2. **Proportion of optimal results for CR for the most commonly used antibodies as concentrates on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark GX / 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 **CAL6**	-	-	10/10 ** (100%)	-	-	-	1/1	-
mAb clone **DAK-Calret 1**	1/1	-	0/4	-	0/2	-	2/4	1/2
pAb **18-0211**	1/1	-	2/2	-	1/3	-	1/1	-

* 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 **CAL6** product no. **PA0346**, Leica Biosystems, Leica Bond Max/Bond III:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 20 min. at 100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 12 of 12 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-Calret 1** product no. **IS/IR627**, Dako/Agilent, Autostainer Link/Classic:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (heating time 10-20 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 13 of 21 (62%) laboratories produced a sufficient staining result.

rmAb clone **SP65** product no. **790-4467**, Ventana/Roche, Ventana Benchmark XT/Ultra:
Protocols with optimal results were typically based on HIER in CC1 (efficient heating time for 16-64 min. at 94-100°C), 16-60 min. (36°C) incubation of the primary Ab and UltraView (760-500) or OptiView (760-700, Ventana/Roche) as detection system. Using these protocol settings, 126 of 135 (93%) laboratories produced a sufficient staining result.

rmAb clone **RM324**, product no. **8522-C010**, Sakura FineTek, Tissue-Tek Genie Advanced:
Protocols with optimal results were based on HIER using Tissue-Tek Genie High pH Antigen Retrieval Solution (efficient heating time 45 min. at 98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro Detection Kit (8826-K250) as detection system.

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 according 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 CR for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Leica BOND mAb CAL6 **PA0346**	100% (8/8)	50% (4/8)	63% (5/8)	25% (2/8)
Dako AS mAb DAK-Calret 1 **IR/IS627**	50% (8/16)	19% (3/16)	75% (6/8)	38% (3/8)
VMS Ultra/XT rmAb SP65 **790-4467**	(2/2)	(2/2)	89% (154/173)	67% (116/173)

* 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 are integrated.

Comments
In this assessment and in concordance with the previous CR assessments, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 88% (72/82) of the insufficient results. The remaining 12% of insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction compromising interpretation.
The majority of the participating laboratories were able to demonstrate CR in high-level antigen expressing cells, such as peripheral nerves and the neoplastic cells of the mesothelioma, whereas the demonstration of CR in low-level antigen expressing cells as cortical epithelial cells of the adrenal gland, adipocytes (all specimens) and neoplastic cells of the granulosa cell tumour were more challenging and only obtainable with appropriate protocol settings.

The mAb clones CAL6, DAK-Calret 1 and the rmAb clone SP65 were the most widely used antibodies for demonstration of CR and applied by 89% (301/339) of the laboratories (see Table 1).
Used as concentrated format within laboratory developed (LD) assays, the mAb clone CAL6 provided 84% (16/19) sufficient results of which 63% (12/19) were assessed as optimal. As shown in Table 2, the mAb clone CAL6 gave superior results on the Omnis platform – all (10/10) results were assessed as optimal. The protocols were based on efficient HIER in TRS pH High (3-in-1), the average dilution factor was 1:21 (range 1:15-1:50) and all laboratories applied the sensitive Envision FLEX+ as detection system. In comparison and using mAb clone CAL6 as LD assay on the BOND platforms, only 25% (2/8) of the protocols provided an optimal result, mainly related to the use of too diluted primary Ab with an average dilution factor of 1:130 (range 1:40-1:300) and/or use of the less efficient citric based HIER Buffer BERS1.
LD assays based on the mAb clone DAK-Calret 1 provided 69% (18/26) sufficient results of which 23% (6/26) were assessed as optimal. As described in the previous report (Run 52, 2018), the performance of the mAb clone DAK-Calret 1 is depending on the chosen platform and in particular challenging to optimize on the fully automated platforms as Omnis and Benchmark (see Table 2). Grouped together and applying the mAb clone DAK-Calret 1 on the above-mentioned platforms, none (0/11) of the protocols were assessed as optimal, and the overall pass rate on the two platforms was only 45% (5/11). This observation should encourage laboratories to substitute DAK-Calret 1 with alternative clones providing optimal results on these platforms as e.g., CAL6 or SP65 for Omnis and BenchMark, respectively. In contrast, and using similar protocol settings on the semi-automated Autostainer platform, the overall pass rate was 100% (13/13) and proportion of optimal results increased significantly to 46% (6/13). As outlined in the previous report, the reason for this difference in performance on the respective platforms is unclear, but "low affinity" Abs might be difficult to optimize on the fully automated platforms with integrated high temperature wash system (32°C on the Omnis and 36°C on the BenchMark) - reversing antibody binding to the epitope.

The following was observed for less commonly used concentrated formats within LD assays: The pAb 18-0211 could provide optimal results on all four main IHC systems (see Table 2). Although the antibody has proven to be challenging on the BenchMark platform with a pass rate of 0% (0/6) in Run 52, the pass rate in this assessment, and on the same platform, was 66% (2/3) of which one protocol was assessed as

optimal. Likewise, to the other Abs listed for CR giving optimal results, efficient HIER in CC1 (64 min. at 95 °C) and a relative high working concentration of the primary Ab (1:50) seems to be the most important prerequisites for optimal performance on the BenchMark.
Laboratories applying the rmAb clone SP13 as concentrate could not produce an optimal result (see Table 1) despite using protocol settings providing high technical sensitivity as efficient HIER in an alkaline buffer, high concentration of the primary Ab and a sensitive 3-step multimer/polymer based detection system.
For the pAb 232A (Cell Marque) as concentrate (and RTU product), an aberrant "Mouse Acites Golgi (MAG)" like reaction pattern was observed. In particular, the epithelial cells of the appendix displayed these deviant staining deposits. The protocols were assessed as insufficient (false positive).

In total, 80% (272/339) of the laboratories used a RTU format for demonstration of CR. The most commonly used RTU format was the rmAb clone SP65 applied by 66% (179/272) of the participants (see Table 1).
As shown in Table 3, and applying vendor recommended protocol settings (VRPS), the RTU systems PA0346 (Leica Biosystems), IR/IS627 (Dako/Agilent) and 790-4467 (Ventana/Roche) based on the mAb clones CAL6, DAK-Calret 1 and the rmAb clone SP65, respectively, could all produce optimal results. However, only 1% of the laboratories used the Ventana/Roche RTU system 790-4467 according to VRPS which is a significant decrease from 16% in the previous run.
This observation is difficult to elucidate upon, but it has been noted by NordiQC and associated reference laboratories, that the performance of the RTU format 790-4467 based on the rmAb clone SP65 seems to have declined recently and might explain why laboratories are optimizing the assays and shifting towards using LMPS. No deviation in performance between lot numbers could be identified and completely identical protocol settings could either provide an insufficient or an optimal result e.g., as observed for the use of lot H00188, HIER in CC1 for 36-64 min. at 95-99°C, incubation time in primary Ab for 16-32 min. at 36°C and use of UltraView as the detection system. Thus, the problem could be of a more generic nature which for most laboratories are difficult to solve/unravel. In this context, it must be mentioned that there is a degree of data uncertainty for lot numbers used, as this might be changed from the time of data entry for protocol submission to the time for the actual performance of the IHC staining. We highly emphasize that participants ensure reporting the correct and actual lot number used for staining.
The most important parameter affecting the overall performance of the assays was the choice of the detection system and using the "optimal protocol settings" as described for the RTU system 790-4467 (Ready-To-Use antibodies and corresponding systems), laboratories using UltraView had a pass rate of 88% (68/77) of which 65% (50/77) were optimal, whereas protocols based on OptiView provided a pass rate of 100% (63/63) with 78% (49/63) optimal results. The latter is in line with the data observed for the RTU system 790-4497 in the recent run 52, also giving a pass rate of 100%, 95% being optimal.

The Dako/Agilent RTU system IR/IS627 based on mAb clone DAK-Calret 1, intended for use on the Autostainer, was used by 24 laboratories (see Table 3). Both VRPS and LMPS could be used to obtain optimal results, but the proportion of sufficient and optimal results was significantly increased applying modifications to the basic recommendations from the vendor. Modifications providing improvements were typically based on the use of the 3-step polymer conjugate system EnVision FLEX+ instead of the recommended 2-step polymer conjugate system EnVision FLEX and similar observations were also seen in run 52. In this run, VRPS gave a declined pass rate of 50% compared to 94% in the previous run. No obvious parameters could explain for this discrepancy other than many new participants are attending the NordiQC Quality Assurance Program (see Graph 1). In addition, data must be analyzed with caution due to relatively few observations.
The IR/IS627 RTU format was frequently used on non-validated platforms and in the current assessment, 33 laboratories used this format on the Omnis, Dako/Agilent. As for the concentrated format based on the same antibody, the RTU format also gave inferior results on this particular platform, providing an overall pass rate of only 36% (12/33), emphasizing that RTU formats should only be used within a system and caution must be exercised when migrating to other platforms. Therefore, and for laboratories wishing to use an off-label RTU format, the process with focus on intended use require thorough technical calibration and analytical/diagnostic validation. In this specific case, it seems difficult to obtain the appropriate level of analytical sensitivity with migration of the RTU format IR/IS627 to the Omnis, which now has been revealed in two consecutive runs for CR.
It was observed that the RTU system IR/IS627 (Autostainer) in a few cases provided an aberrant desmin or alpha smooth muscle actin like reaction pattern (see fig. 6a) in addition to the expected CR result. The reaction could be related to contamination of the Ab (either at the laboratory or by the vendor) or a technical issue as drying out during the staining process. The reaction was observed for lot 20084558, but only in 22% of the protocols based on this lot. If observed repeatable, laboratories should contact vendor to elucidate on this issue.

The Leica Biosystems RTU system PA0346 based on the mAb clone CAL6 and for laboratories following VRPS, the pass rate was 100% (8/8) with 50% (4/8) optimal results (see Table 3). Modification of the protocol settings could also provide sufficient and optimal results, but performance declined primarily due to the use of HIER in the less efficient citric based buffer BERS1 compared to the recommended high pH

buffer BERS2. Three laboratories used BERS1, and only 33% (1/3) of the protocols were assessed as sufficient – none being optimal.

This was the seventh NordiQC assessment of CR and the pass rate is almost at the same level compared to the previous three runs (see Graph 1). The stagnation or lack of progress in pass rates, can partly be explained by the increasing numbers of new participants from run to run. Virtually all assays in this assessment (89%), both RTU formats and concentrates, were either based on the mAb clones CAL6, DAK-Calret 1 or the rmAb clone SP65. The overall pass rate for these assays were 76% (236/301). The prevalent feature of an insufficient staining result was use of the mAb clone DAK-Calret 1 on either the Omnis or Benchmark platforms. Grouped together, only 39% (17/44) obtained a sufficient mark. Importantly, protocols must stain according to the expected antigen levels, and both adrenal gland and appendix are essential and critical assay performance controls assisting to monitor the required level of the analytical sensitivity and specificity of the assay (see below).

Controls
Adrenal gland and appendix are recommendable positive and negative tissue controls for CR.
Adrenal gland will serve as a "low-level expressor" (LE) positive tissue control, in which an at least weak to moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells must be seen. Appendix serves both as negative tissue and "high-level expressor" (HE) positive tissue control. Columnar epithelial cells and smooth muscle cells should be negative, while moderate to strong, distinct cytoplasmic and nuclear staining of the peripheral nerves (ganglion cells and axons) and macrophages should be seen. Furthermore, adipocytes in the submucosa of the appendix could serve as an additional LE positive tissue control.

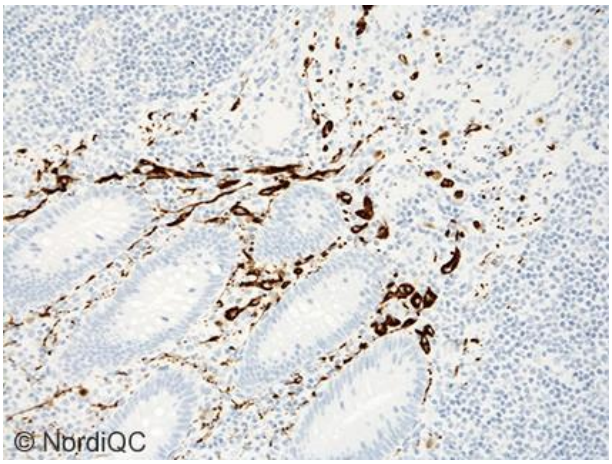


Fig. 1a (x200)
Optimal CR staining of the appendix using the mAb clone **CAL6** as concentrate on the **Dako/Agilent Omnis**, efficient HIER in TRS pH 9 (3-in-1), incubation time in primary Ab (1:30) for 30 min. and EnVision FLEX+ as the detection system. A strong, distinct cytoplasmic and nuclear staining reaction of the peripheral nerves is seen. No reaction is seen in the columnar epithelial cells (same protocol used in Figs. 2a - 5a).

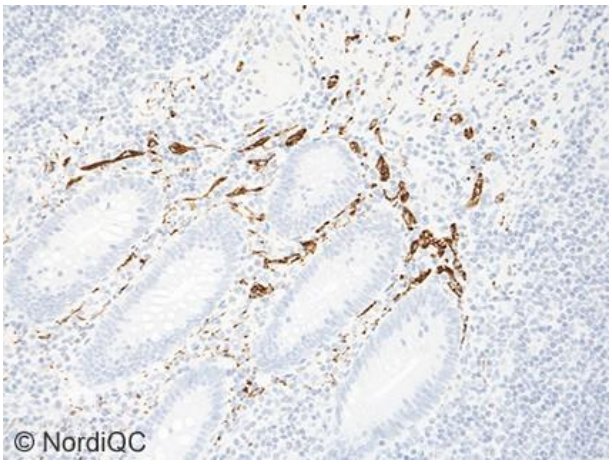


Fig. 1b (x200)
Insufficient CR staining of the appendix using the mAb clone **DAK-Calret 1** as concentrate on the **Dako/Agilent Omnis** with almost identical protocol settings as in Fig. 1a, based on efficient HIER in TRS pH 9 (3-in-1), incubation time in primary Ab (1:25) for 20 min. and EnVision FLEX+ as the detection system (same protocol used in Figs. 2b – 5b). Although the peripheral nerves display a moderate to strong intensity, the protocol is challenged due to the use of DAK-Calret 1 on the Omnis, and thus, peripheral nerves give a false impression (too easy to stain) of the analytical sensitivity of the protocol applied. Compare with Fig. 1a – 6b.

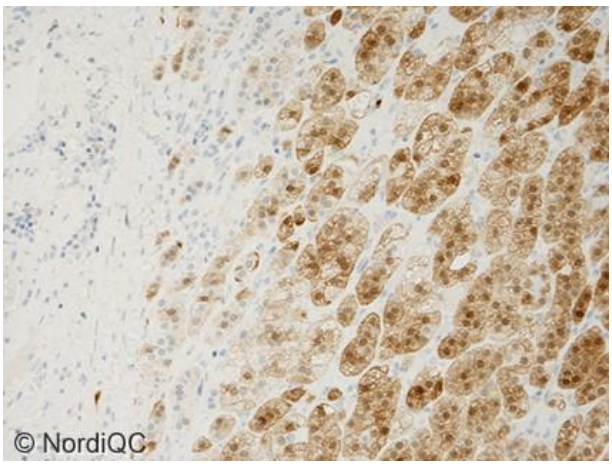


Fig. 2a (x200)
Optimal CR staining of the adrenal gland (low-level expressor) using the same protocol as in Fig. 1a. A weak to moderate, but distinct cytoplasmic and nuclear staining reaction of the majority of the cortical epithelial cells is seen. Compare with Fig. 2b.

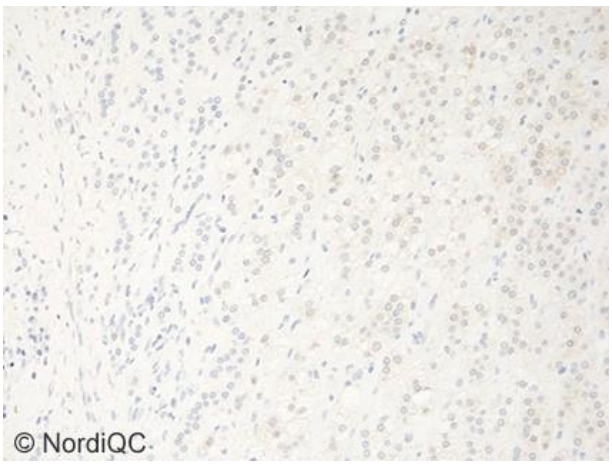


Fig. 2b (x200)
Insufficient CR staining of the adrenal gland (low-level expressor) the same protocol as in Fig. 1b. The cortical epithelial cells are false negative or only show a barely visible cytoplasmic staining reaction. Compare with Fig. 2a - same field.

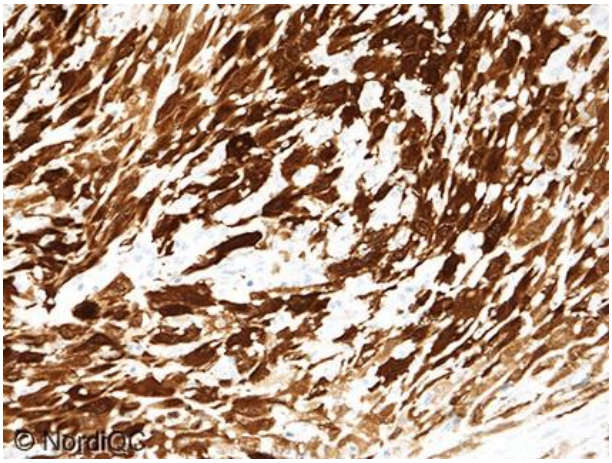


Fig. 3a (x200)
Optimal CR staining of the mesothelioma using the same protocol as in Figs. 1a - 2a. All neoplastic cells display a strong, distinct cytoplasmic and nuclear staining reaction. Compare with Fig. 3b.

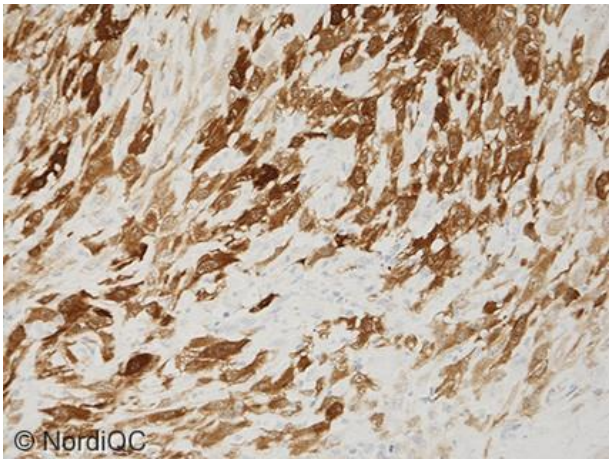
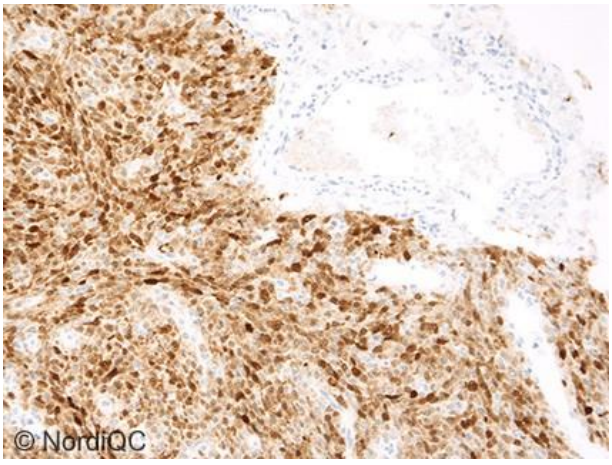


Fig. 3b (x200)
CR staining of the mesothelioma using the same protocol as in Figs. 1b - 2b. The intensity of the staining reaction is reduced. Compare with Fig. 3a – same field.



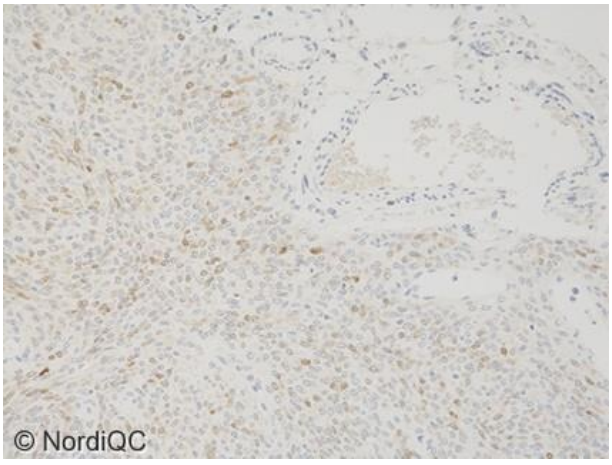


Fig. 4a (x200)
Optimal CR staining of the granulosa cell tumour using the same protocol as in Fig. 1a - 3a. Virtually all the neoplastic cells show an at least weak to moderate, but distinct cytoplasmic and nuclear staining reaction. Compare with Fig. 4b.

Fig. 4b (x200)
Insufficient CR staining of the granulosa cell tumour using the same protocol as in Figs. 1b - 3b. A too weak, predominantly nuclear staining reaction in a reduced fraction of the neoplastic cells is seen. Also, a significant proportion of the neoplastic cells are false negative. Users of the mAb clone DAK-Calret 1 on the Omnis (but also on the BenchMark platform), were in general struggling with appropriate analytical sensitivity of the assays and should substitute the clone DAK-Calret 1 with e.g., clone CAL6 or SP65. Compare with Fig. 4a - same field.

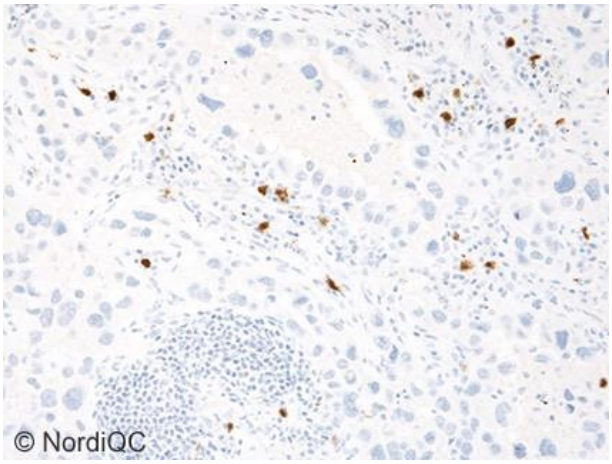


Fig. 5a (x200)
Optimal CR staining of the lung adenocarcinoma using the same protocol as in Figs. 1a - 4a. The neoplastic cells are as expected negative, whereas dispersed macrophages display a strong, but distinct cytoplasmic and nuclear staining reaction. Compare with Fig. 5b.

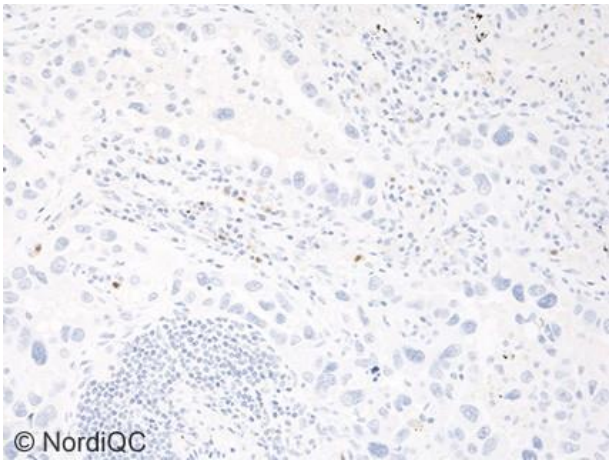


Fig. 5b (x200)
Insufficient CR staining of the lung adenocarcinoma using the same protocol as in Figs. 1b – 4b. Although the neoplastic cells are negative as expected, the protocol overall provided too low analytical sensitivity and here the macrophages are only faintly demonstrated (see explanation above in Fig. 4b). Compare with Fig. 5a - same field.

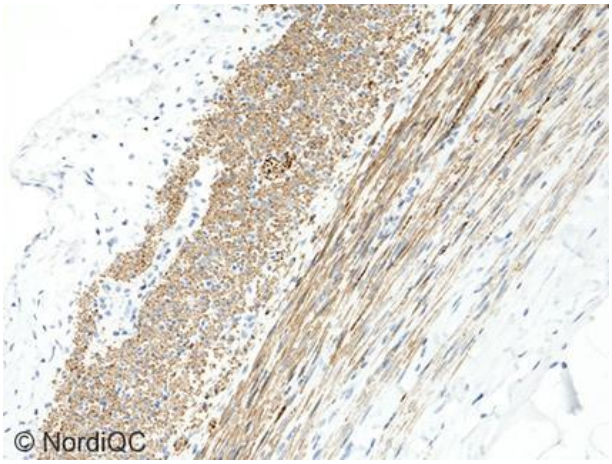


Fig. 6a (x200)
Insufficient CR staining of the appendix using the Dako/Agilent RTU format IR/IS627 (lot. 20084558) based on the mAb clone DAK-Calret 1 on the Autostainer. The peripheral nerves (true positive) are difficult to identify due to false positive staining reaction of the smooth muscle cells in lamina propria muscularis. The causes for this aberrant staining reaction could be contamination of the primary Ab (e.g., with smooth muscle actin antibody) or technical issues e.g., drying out of the sections during the staining process.

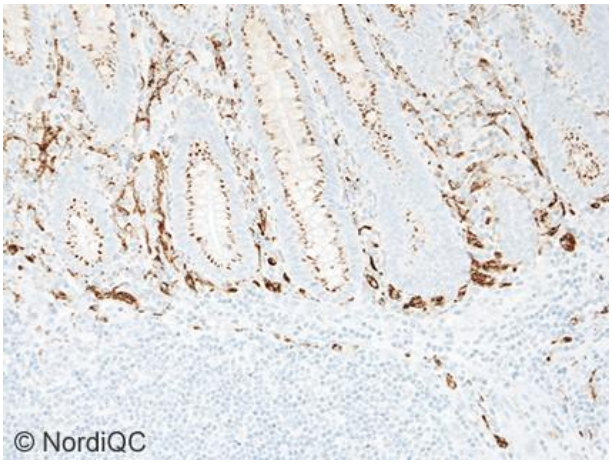


Fig. 6b (x200)
Insufficient CR staining of the appendix using a protocol based on the pAb 232. The epithelial cells display an aberrant MAG like staining reaction and all protocols, both based on concentrates and RTU formats of this Ab, were false positive.

MB/RR/LE/SN 25.03.2022