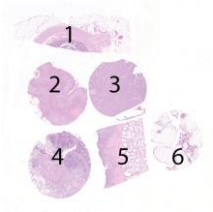
Assessment Run 45 2015

Calretinin (CR)

Material
The slide to be stained for CR comprised:



1. Appendix, 2. Adrenal gland, 3. Granulosa cell tumour, 4. Lung adenocarcinoma, 5-6. Malignant mesotheliomas

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CR staining as optimal included:

- A strong, distinct cytoplasmic and nuclear staining reaction of peripheral nerves (ganglion cells and axons) and macrophages in the appendix.
- An at least weak to moderate, distinct cytoplasmic and nuclear staining reaction of the majority of cortical epithelial cells of the adrenal gland and of fat cells in the tissues included.
- A moderate to strong, distinct cytoplasmic and nuclear staining reaction of the majority of neoplastic cells of the granulosa cell tumor and the two mesotheliomas.
- 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 45	252
Number of laboratories returning slides	217 (86%)

Results
217 laboratories participated in this assessment. 7 laboratories used an inappropriate Ab (Chromogranin A). Of the remaining 210 laboratories, 73% achieved a sufficient mark. Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining were:
- Too low concentration of the primary Ab.
- Use of less sensitive detection systems.
- Less successful performance of the mmAb clone DAK-Calret 1 on the Ventana BenchMark platform.

Performance history
This was the fifth NordiQC assessment of CR. A minor decrease in the pass rate was seen compared to run 33 in 2011 (see table 2).

Table 2: **Proportion of sufficient results for CR in the five NordiQC runs performed**

	Run 17 2006	Run 19 2007	Run 23 2008	Run 33 2011	Run 45 2015
Participants, n=	82	87	111	173	210
Sufficient results	56 %	56 %	80 %	76 %	73 %

Conclusion
The mmAbs clones **2E7**, **5A5**, **CAL6**, **DAK-Calret 1**, and the rmAbs **SP13** and **SP65** are all recommendable Abs for demonstrating CR. Efficient HIER in an alkaline buffer in combination with appropriate calibration of the concentration of the primary Ab and use of a sensitive (preferably 3-step) detection system is mandatory for optimal performance. On the Ventana BenchMark platform, less successful performance of the mmAb clone DAK-Calret 1 was seen. The Ventana Ready-To-Use system based on the rmAb clone SP65 was the most successful assay with an overall pass rate of 94%.
Adrenal gland and appendix are recommendable positive and negative tissue controls for CR.
Adrenal gland will serve as a "low expressor" (LE) positive tissue control, in which an at least weak to moderate, distinct cytoplasmic and nuclear staining reaction of the majority of cortical epithelial cells must be seen. Appendix serve both as a negative and "high expressor" (HE) tissue control. Columnar epithelial cells and smooth muscle cells should be negative, while strong, distinct cytoplasmic and nuclear staining reaction of peripheral nerves (ganglion cells and axons) and macrophages should be seen. Furthermore, fat cells in the submucosa of the appendix could serve as an additional LE positive tissue control.

Table 1: **Antibodies and assessment marks for CR, run 45**

	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mmAb clone **2E7**	1	Immunologic	1	0	0	0	-	-
mmAb clone **5A5**	21 1	Leica/Novocastra Immunologic	3	10	8	1	59%	56%
mmAb clone **CAL6**	6 1	Leica/Novocastra Monosan	4	1	0	2	71%	-
mmAb clone **DAK-Calret 1**	35	Dako	10	13	9	3	66%	87%
rmAb clone **SP13**	3 1 2	Thermo/Neomarkers Spring Bioscience Cell Marque	1	2	2	1	50%	-
pAb **18-0211**	16	Invitrogen/Zymed	2	8	6	0	63%	-
pAb **232A**	5	Cell Marque	0	1	2	2	20%	-
pAb **61-0006**	1	Genemed	0	1	0	0	-	-
pAb **7699/3H**	1	Swant	0	0	0	1	-	-
pAb **RBK003**	1	Zytomed	0	0	1	0	-	-
Ready-To-Use antibodies								
mmAb clone **CAL6 PA0346**	8	Leica/Novocastra	2	3	2	1	63%	-
mmAb clone **DAK-Calret 1 IS/IR627**	38	Dako	9	17	10	2	68%	79%
rmAb **SP13 RMA-0524**	1	Maixin	1	0	0	0	-	-
rmAb **SP13 232R-18**	1	Cell Marque	0	1	0	0	-	-
rmAb **SP13 MAD-000315QD**	1	Master Diagnostica	0	1	0	0	-	-
rmAb clone **SP65 790-4467**	64	Ventana	52	8	2	2	94%	94%
pAb **232A-78**	1	Cell Marque	0	1	0	0	-	-
pAb **PP092**	1	BioCare	0	1	0	0	-	-
Total	210		85	68	42	15	-	
Proportion			41%	32%	20%	7%	73%	

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

Detailed analysis of CR, Run 45
The following protocol parameters were central to obtain optimal staining:

Concentrated Antibodies
mmAb clone **2E7:** One protocol with an optimal result was based on heat induced epitope retrieval (HIER) using Target Retrieval Solution pH 9 (3-in-1) (Dako) (efficient heating time 20 min. at 97°C), 20 min incubation of the primary Ab at dilution 1:400 and EnVision FLEX+ (Dako K8012) as detection system. Only one lab used mmAb clone **2E7**.

mmAb clone **5A5**: Protocols with optimal results were all based on HIER using either Tris-EDTA/EGTA pH 9 (1/2)*, Bond Epitope Retrieval Solution 2 (Leica) (1/4), or Cell Conditioning 1 (Ventana) (1/11). The mmAb was diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 9 of 16 (56%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mmAb, clone **CAL6**: Protocols with optimal results were all based on HIER using either Target Retrieval Solution pH 9 (3-in-1) (Dako) (3/7) or Bond Epitope Retrieval Solution 2 (Leica) (1/4). The mmAb was diluted in the range of 1:15-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings 4 of 4 (100%) laboratories produced a sufficient staining result.

mmAb clone **DAK-Calret 1**: Protocols with optimal results were all based on HIER using either Target Retrieval Solution pH 9 (Dako) (3/11), Target Retrieval Solution pH 9 (3-in-1) (Dako) (2/7), Tris-EDTA/EGTA pH 9 (3/5), Bond Epitope Retrieval Solution 2 (Leica) (1/4), or Borg Decloaker pH 9,5

(BioCare) (1/2) as retrieval buffer. The mmAb was typically diluted in the range of 1:20-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 20 of 23 (87%) laboratories produced a sufficient staining result.

rmAb, clone **SP13:** One protocol with an optimal result was based on HIER using Bond Epitope Retrieval Solution 1 (Leica) (efficient heating time 20 min. at 99°C), 30 min incubation of the primary Ab at dilution 1:10 and Bond Polymer Refine Detection (Leica DS9800) as detection system. Only one lab used these protocol settings.

pAb **18-0211:** Protocols with optimal results were all based on HIER using either Tris-EDTA/EGTA pH 9 (1/2) or Target Retrieval Solution pH 9 (Dako) (1/1). The pAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 of 3 (100%) laboratories produced a sufficient staining result.

Table 3 summarizes the overall proportion of optimal staining results for the most frequently used concentrated antibodies on the three most commonly used IHC stainer platforms.

Table 3: **Proportion of optimal results for CR using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic / 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
mmAb clone **DAK-Calret 1**	5/17 (29%)**	-	0/5 (0%)	-	1/4 (25%)	0/1
mmAb clone **5A5**	0/2	-	1/11 (9%)	-	1/4 (25%)	-
mmAb clone **CAL6**	3/4 (75%)	-	0/2	-	1/1	-
pAb **18-0211**	1/3	-	0/6	-	0/3	0/1

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use (RTU) antibodies and corresponding systems
mmAb clone **CAL6** product no. **PA0346**, Leica/Novocastra, Leica Bond Max/Bond III:
Protocols with optimal results were based on HIER using BERS2 (Bond, Leica) (efficient heating time 20 min. at 99-100°C), 20 min ncubation of the primary Ab and Bond Polymer Refine Detection (Leica DS9800) as detection system. Using these protocol settings 2 of 2 laboratories produced an optimal staining.

mmAb clone **DAK-Calret 1** product no. **IS/IR627**, Dako, Dako Autostainer Link/Classic/Omnis:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1), TRS pH 9 or Tris-EDTA/EGTA pH 9 (heating time 10-30 min at 95-97°C), 20-30 min incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002/K8012) as detection system. Using these protocol settings 23 of 29 (79%) laboratories produced a sufficient staining (optimal or good).

rmAb clone **SP65** product no. **790-4467**, Ventana, Ventana Benchmark XT/Ultra:
Protocols with optimal results were all based on HIER in Cell Conditioning 1 (efficient heating time for 8-64 min at 95-100°C), 4-60 min at 36°C or room temperature of the the primary Ab and UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings 60 of 64 (94 %) laboratories produced a sufficient staining.

Comments
In this assessment and in concordance with the observations in the previous assessments for CR, the prevalent feature of an insufficient result was a general too weak or false negative staining of structures expected to be demonstrated. Too weak or false negative staining reaction was seen in 95% of the insufficient results (54 of 57)

Virtually all the participating laboratories were able to demonstrate CR in peripheral nerves and in the neoplastic cells of the mesothelioma in tissue core no. 5, whereas the demonstration of CR in fat cells, neoplastic cells of the mesothelioma in tissue core no. 6, neoplastic cells of the granulosa cell tumour and in particular the cortical epithelial cells of the adrenal gland was more challenging, and was only seen with appropriate protocol settings.

The mmAb clone DAK-Calret 1 was the most widely used Ab as concentrated format within laboratory developed (LD) assays for CR. 36 laboratories used the mmAb clone DAK-Calret 1 and in concordance with the previous assessments for CR (run 33 2011) the proportion of sufficient results was highly influenced by the choice of the detection system and IHC platform used (se Table 3). If the mmAb clone DAK Calret 1 was used as a concentrate, diluted in the range of 1:20-600, HIER in alkaline buffer as BERS2 or TRS pH 9 and applied with a 2-step polymer based detection system as EnVision FLEX (Dako) or similar, 8 of 14 laboratories obtained a sufficient staining result (55%) out of which 3 (21%) were assessed as optimal. If the same protocol settings were applied with a more sensitive 3-step polymer based detection system e.g., EnVision FLEX+ (Dako) or Bond Refine (Leica), 15 of 16 laboratories produced a sufficient staining result (94%) of which 7 (44%) were optimal. Choice of IHC platform is also very important as DAK-Calret 1 performed poorly on the Ventana BenchMark platform. 6 laboratories performed DAK-Calret 1 protocols on the BenchMark XT or Ultra, and were all assessed as insufficient. In fact, one of the 6 laboratories used the Dako RTU format of DAK-Calret 1 with no success. Although Ventana BenchMark users applied DAK-Calret 1 with similar protocol settings as Dako Autostainer users achieving optimal marks, they consistently failed to produce sufficient results (Fig. 1 – Fig. 4). The reason for this difference is unknown, but might be related to a more efficient washing procedure (at 36°C) on the Ventana BenchMark platform compared to e.g. the Dako Autostainer and Leica Bond platforms.

The mmAb clone 5A5 was used within a LD assay by 22 laboratories and the proportion of sufficient results was – much similar to mmAb clone DAK-Calret 1 – highly influenced by the choice of detection system and the dilution of the primary Ab. In total, 11 non-Ventana BenchMark laboratories used the mmAb clone 5A5. 6 laboratories used 3-step polymer based detection systems (e.g. Bond Refine, Leica) and all obtained sufficient staining results (100%) of which 2 (33%) was assessed as optimal. In contrast, 5 laboratories using 2-step polymer based detection system and only 3 obtained sufficient staining results (60%) and none was assessed as optimal. On the Ventana BenchMark platform 5 laboratories used 2-step multimer based detection system (UltraView, Ventana) and none obtained sufficient staining results (0%). In contrast, 6 laboratories used 3-step multimer based detection systems (UltraView with amplification, or OptiView, Ventana) and 4 of 6 laboratories obtained sufficient staining (67%) of which 1 (17%) was assessed as optimal. Fig. 5a – Fig. 5b shows the importance of choice of detection system and careful calibration of the mmAb clone 5A5.

In this assessment, 55% (115 of 210) of the labs used Ready-To-Use (RTU) antibodies. Optimal results could be obtained with the RTU systems based on the mmAb clone CAL6, the mmAb clone DAK-Calret 1, the rmAb SP13 and the rmAb SP65. In concordance with the previous CR assessment (run 33, 2011), the most successful and robust assay for CR was obtained with the RTU system based on the rmAb SP65 from Ventana. The pass rate was 94% (60 out of 64 laboratories) and 81% were assessed as optimal (52 of 64 laboratories). Using the rmAb clone SP65 according to the recommendations from Ventana (HIER in mild CC1 and 20 min incubation time of primary Ab) strong and consistent staining for CR was seen in all cores. Particular the demonstration of CR in the low level expressing cortical epithelial cells of the adrenal gland was impressing and superior to all the other antibodies used in this assessment (Fig. 6a). The robustness of the SP65 RTU assay becomes evident from the wide range of protocol settings that gives sufficient and even optimal staining results. It was, though, seen that under very extreme protocol settings even the SP65 RTU assay could fail (Fig. 6a – Fig. 6b). The rmAb clone SP65 is only available in a RTU format and only for the Ventana BenchMark platform. In this assessment 65% (64 laboratories of 98) of the Ventana BenchMark participants used the SP65 RTU assay, whereas the remaining 35% used various Abs in concentrated format within a LD assay. A significant difference in pass rate was seen for these two groups. The general pass rate for all the LD assays on the BenchMark platform was 35% (12 laboratories out of 34) out of which only 3% was assessed as optimal (1 of 34 laboratories). In contrast, the pass rate for the SP65 RTU system on the BenchMark platform was 94% (60 of 64 laboratories) and 81% were assessed as optimal (52 of 64 laboratories).

Controls

Adrenal gland and appendix are recommendable positive and negative tissue controls for CR.
Adrenal gland will serve as a "low 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 expressor" (HE) positive tissue control. Columnar epithelial cells and smooth muscle cells should be negative, while strong, distinct cytoplasmic and nuclear staining of the peripheral nerves (ganglion cells and axons) and macrophages should be seen. Furthermore, fat cells in the submucosa of the appendix could serve as an additional LE positive tissue control.

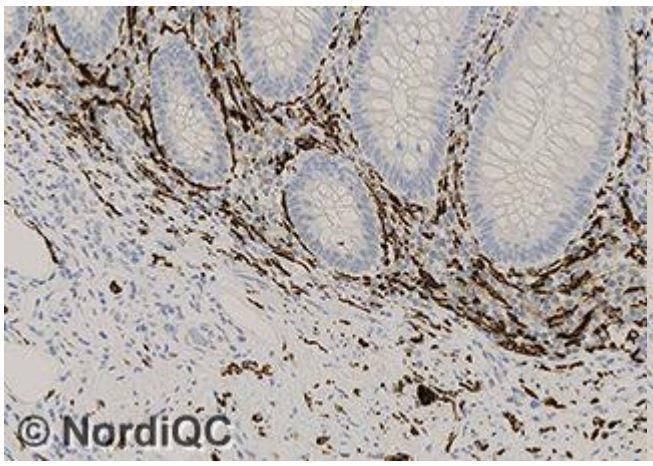


Fig. 1a (x200)
Optimal CR staining of the appendix using the mmAb clone DAK-Calret 1 (Dako) in a RTU format (Dako IR627) and with an incubation time of 20 min. after HIER in an alkaline buffer (TRS pH 9, Dako). Staining was performed on the Dako Autostainer using a 3-step polymer system (EnVision Flex+). A strong, distinct cytoplasmic and nuclear staining of the peripheral nerves is seen. No reaction is seen in the columnar epithelial cells (same protocol used in Figs. 2a - 4a). Compare with Fig. 1b.

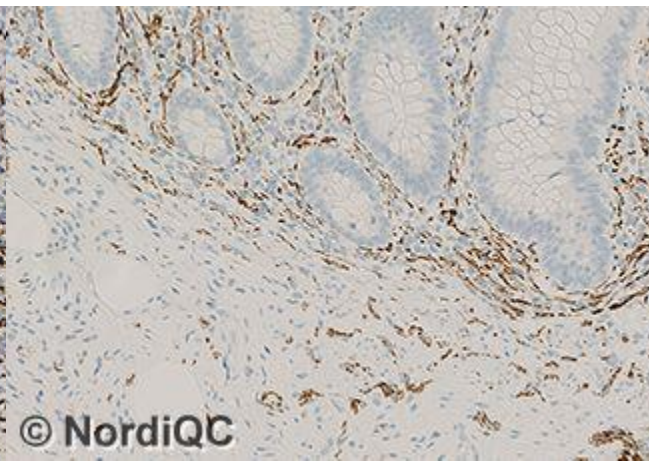


Fig. 1b (x200)
Insufficient CR staining of the appendix using the mmAb clone DAK-Calret 1 (Dako) in a RTU format (Dako IR627) and with an incubation time of 32 min. after HIER in an alkaline buffer (CC1, pH 8,5, Ventana). Staining was performed on the Ventana BenchMark using a 3-step multimer system (UltraView with amplification). Although the same RTU product and similar protocol settings were used, the CR staining on the Ventana BenchMark was significantly weaker. A moderate, distinct cytoplasmic and nuclear staining of the peripheral nerves is seen. Compare with Fig. 1a. – same field. Also compare with Figs. 2b, 3b and 4b – same protocol.

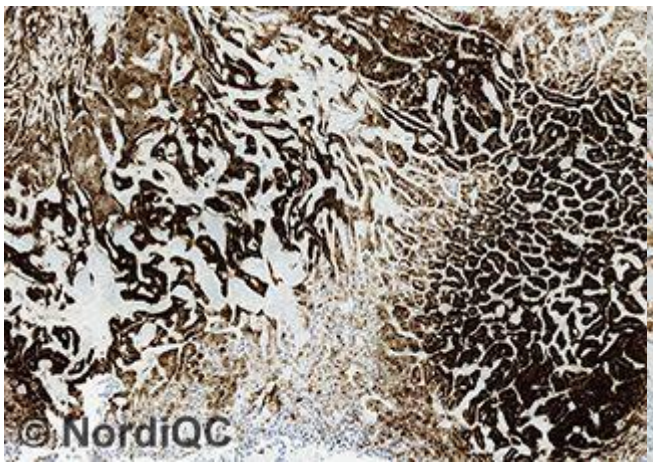


Fig. 2a (x100)
Optimal CR staining of the mesothelioma in core no. 5 ("high expressor") using the same protocol as in Fig. 1a. A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. Compare with Fig. 2b.

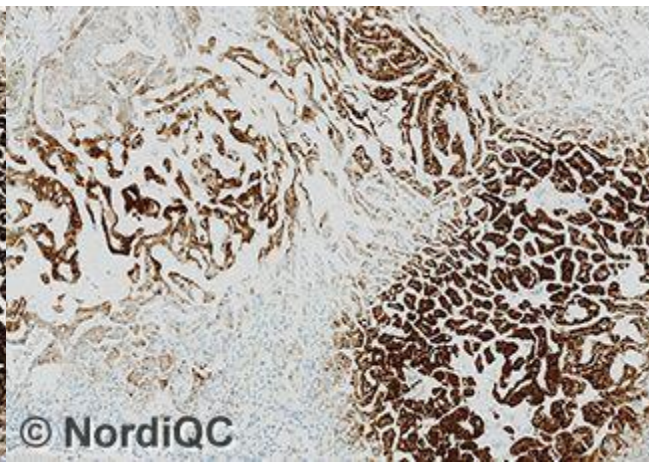


Fig. 2b (x100)
Insufficient CR staining of the mesothelioma in core no. 5 ("high expressor") using the same protocol as in Fig. 1b. Only a moderate, distinct cytoplasmic and nuclear staining of the majority of the tumour cells is seen. Compare with Fig. 2a - same field.

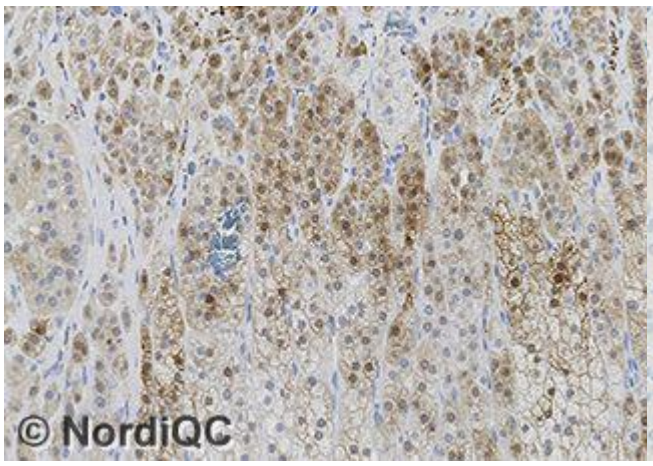


Fig. 3a (x200)
Optimal CR staining of the adrenal gland ("low expressor") using the same protocol as in Fig. 1a and 2a. A moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells is seen. Compare with Fig. 3b.

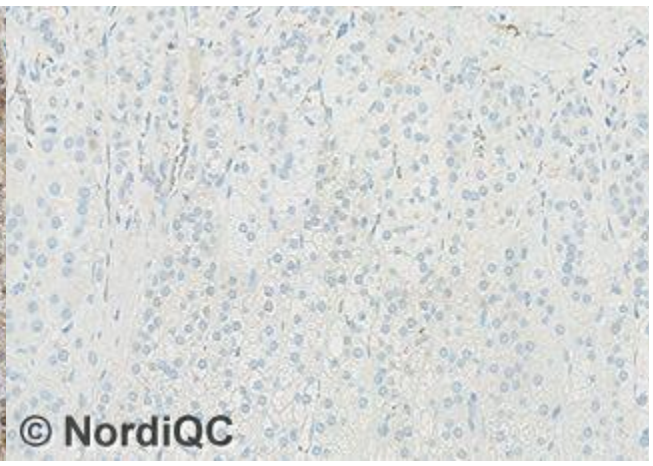


Fig. 3b (x200)
Insufficient CR staining of the adrenal gland ("low expressor") using the same protocol as in Fig. 1b and 2b. No staining of the cortical epithelial cells is seen. Compare with Fig. 3a – same field.

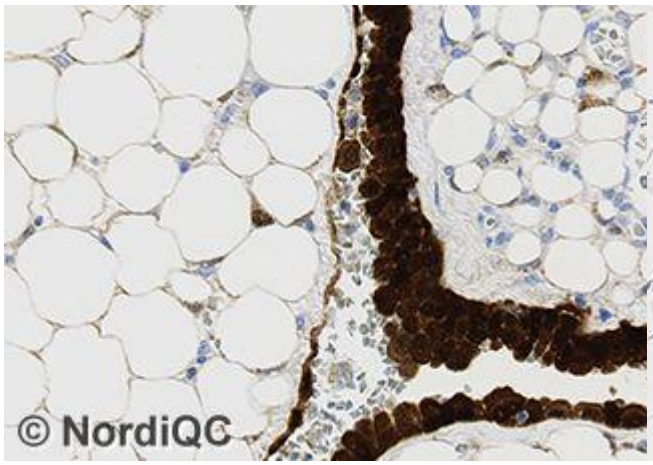


Fig. 4a (x400)
Optimal CR staining of the mesothelioma in core no. 6 ("medium expressor") using the same protocol as in Fig. 1a, 2a and 3a. A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. In addition to the positive tumour cells a weak to moderate, distinct cytoplasmic and nuclear staining is seen in virtually all the fat cells. Compare with Fig. 4b.

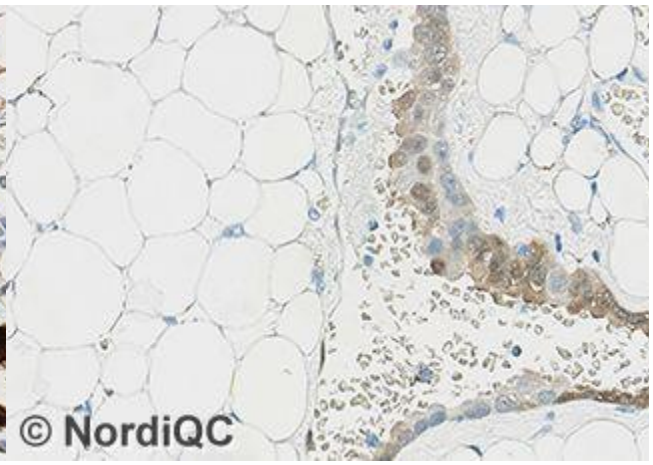


Fig. 4b (x400)
Insufficient CR staining of the mesothelioma in core no. 6 ("medium expressor") using the same protocol as in Fig. 1b, 2b and 3b. The majority of the tumour cells are virtually negative and no staining is seen in the fat cells. Compare with Fig. 4a – same field.

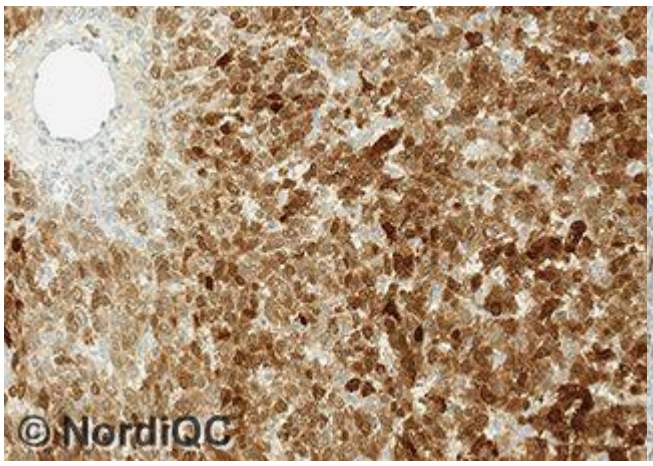


Fig. 5a (x200)
Optimal CR staining of the ganulosa cell tumour using the mmAb clone 5A5 (Leica) in a 1:25 dilution and with an incubation time of 32 min. after HIER in an alkaline buffer (CC1, Ventana). Staining was performed on the Ventana BenchMark using a 3-step multimer system (OptiView, Ventana). A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. Compare with Fig. 5b.

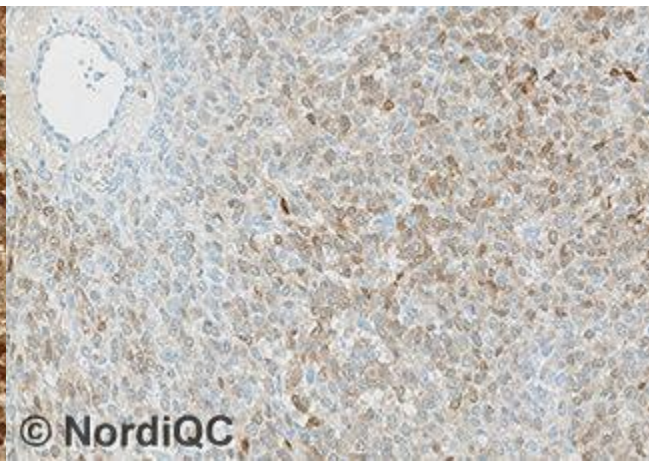


Fig. 5b (x200)
Insufficient CR staining of the ganulosa cell tumour using the mmAb clone 5A5 (Leica) in a 1:40 dilution and with an incubation time of 32 min. after HIER in an alkaline buffer (CC1, Ventana). Staining was performed on the Ventana BenchMark using a 2-step multimer system (UltraView, Ventana). The combination of a 2-step multimer system and a reduced titer of the primary Ab results in a too weak staining reaction of the tumour cells. Compare with Fig. 5a - same field.

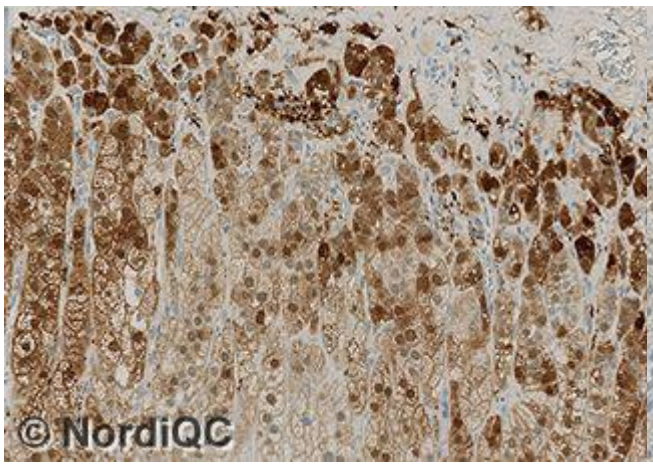


Fig. 6a (x200)
Optimal CR staining of the adrenal gland using the rmAb clone SP65 (Ventana) in a RTU format (790-4467) and with an incubation time of 16 min. after HIER in CC1 for 16 min. Staining was performed on the Ventana BenchMark using a 3-step multimer system (OptiView, Ventana). A moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells is seen. Compare with Fig. 6b.

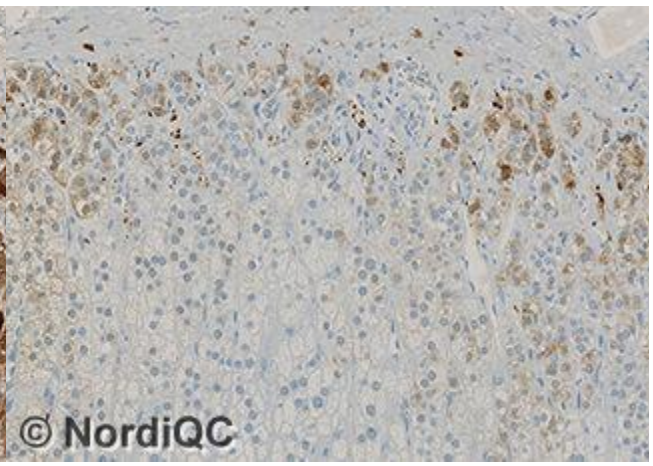


Fig. 6b (x200)
Insufficient CR staining of the adrenal gland using the rmAb clone SP65 (Ventana) in a RTU format (790-4467) and with an incubation time of 4 min. after HIER in CC1 for 8 min. Staining was performed on the Ventana BenchMark using a 3-step multimer system (OptiView, Ventana). The combination of a short HIER protocol and a very short incubation of the primary Ab results in a too weak staining reaction of the cortical epithelial cells. Compare with Fig. 6a – same field.

ON/SN/LE/MV/RR 11.12.2015