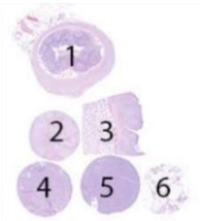
Assessment Run 52 2018

Calretinin (CR)

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



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

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 tumour 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 52	299
Number of laboratories returning slides	288 (96%)

Results
288 laboratories participated in this assessment. 19 laboratories used an inappropriate Ab (Chromogranin A). Of the remaining 269 laboratories, 72% 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:

- Less successful performance of the mAb DAK-Calret 1 on the Dako Omnis and Ventana BenchMark systems.
- Less successful performance of the pAb 18-0211 on the Ventana BenchMark system.
- 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 sixth NordiQC assessment of CR. A minor decrease in the pass rate was seen compared to run 45 in 2015.

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

	Run 17 2006	Run 19 2007	Run 23 2008	Run 33 2011	Run 45 2015	Run 52 2018
Participants, n=	82	87	111	173	210	269
Sufficient results	56%	56%	80%	76%	73%	72%

Conclusion
Optimal staining results could be obtained with the mAbs clones **2E7**, **5A5, CAL6, DAK-Calret 1** and **MX027**, the rmAb clones **BSR235**, **SP13** and **SP65** and the pAb **18-0211**. In general, the laboratory developed (LD) assays had a significantly lower proportion of sufficient results (good or optimal) than the Ready-To-Use (RTU) systems. With the exception of pAb 18-02011, the highest proportion of optimal results was obtained performing HIER in an alkaline buffer for at least 20 min. (or at least 36 min. for Ventana BenchMark users). The concentration of the primary antibodies must be carefully calibrated. Important system related issues were observed: The mAb clone DAK-Calret 1 had a very low pass rate (proportion or sufficient results) on both the Dako Omnis and Ventana Benchmark systems (8% and 0%

respectively) and all 6 laboratories using the pAb 18-0211 on the Ventana Benchmark system failed to produce sufficient results.
The rmAb clone SP65 based Ventana Benchmark RTU system, the mAb clone DAK-Calret 1 based Dako Autostainer RTU system and the mAb CAL6 based Leica Bond RTU system all provided a high proportion of sufficient results. Using the recommended protocol settings, the rmAb clone SP65 RTU system was the most successful assay with an impressive overall pass rate of 100% with 95% being optimal. This was in strong contrast to the very low overall pass rate of 10% for the "non-SP65" based LD assays seen on the Ventana Benchmark system. Used on the recommended platform/system, the mAb clone DAK-Calret 1 RTU system was also a very successful assay with an overall pass rate of 94% with 40% being optimal. In contrast, used on the Dako Omnis system, the pass rate was only 11% with none being optimal, emphasizing that RTU assays should only be used on the platform that they are develop for.

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 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.

Table 1. **Antibodies and assessment marks for CR, run 52**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **2E7**	1	Immunologic	1	0	0	0	-	-
mAb clone **5A5**	3 1	Leica/Novocastra Monosan	1	1	2	0	-	-
mAb clone **CAL6**	7	Leica/Novocastra	1	3	0	3	57%	-
mAb clone **DAK-Calret 1**	34	Dako/Agilent	9	8	8	9	50%	81%
rmAb clone **BSR235**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **SP13**	3 2 2 2	Cell Marque Immunologic Spring Bioscience Thermo Scientific	1	3	4	1	44%	-
pAb **18-0211**	12	Invitrogen/Thermo	3	3	4	2	50%	100%
pAb, **232A**	2	Cell Marque	0	0	2	0	-	-
pAb **61-0006**	1	Genemed	0	0	1	0	-	-
pAb, **CP092C**	1	Biocare	0	0	1	0	-	-
pAb **RBK003**	1	Zytomed Systems	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **CAL6 PA0346**	14	Leica/Novocastra	1	11	2	0	86%	92%
mAb clone **CAL6 PA0346**[3]	1	Leica/Novocastra	1	0	0	0	-	-
mAb clone **DAK-Calret 1 IS/IR627**	35	Dako/Agilent	14	19	2	0	94%	97%
mAb clone **DAK-Calret 1 IS/IR627**[4]	20	Dako/Agilent	0	4	11	5	20%	-
mAb clone **MX027 MAB-0716**	1	Maixin	1	0	0	0	-	-
rmAb **SP13 232R**	1	Cell Marque	0	0	1	0	-	-
rmAb **SP13 MAD-000315QD**	1	Master Diagnostica	0	0	1	0	-	-
rmAb **SP13 RMPD010**	1	Diagnostic Biosystems	0	1	0	0	-	-
rmAb clone **SP65 790-4467**	118	Ventana/Roche	86	20	10	2	90%	96%
pAb **232A-78**	2	Cell Marque	0	0	2	0	-	-

pAb **8223-C010**	1	Sakura Finetek	0	1	0	0	-	-
Unknown RTU Ab	1		0	0	1	0	-	-
Total	269		120	74	52	23	-	
Proportion			45%	27%	19%	9%	72%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the Leica full-automated system (BOND III/MAX) but used by a laboratory on the Intellipath platform (Biocare).
4) RTU system developed for the Agilent/Dako semi-automatic system (Autostainer) but used by laboratories on different platforms (e.g. Leica BOND III/Max or Dako Omnis).

Detailed analysis of CR, Run 52
The following protocol parameters were central to optimal staining:

Concentrated Antibodies
mAb clone **2E7**: One lab used the mAb clone 2E7 and achieved an optimal result. The protocol was based on HIER in Target Retrieval Solution High pH (Dako/Agilent). The mAb was diluted 1:25 and incubated for 27 min. at 32°C and visualized with a 3-step polymer conjugate system (EnVision Flex+, Dako/Agilent) on the Dako Omnis instrument.

mAb clone **5A5**: One protocol with an optimal result was based on HIER in Bond™ Epitope Retrieval Solution 2 (BERS2, Leica). The mAb was diluted 1:100 and incubated for 25 min. at room temperature and visualized with a 3-step polymer conjugate system (Bond Refine Detection, Leica). Using similar protocol settings, 2 of 2 (100%) laboratories produced a sufficient staining result.

mAb clone **CAL6**: One protocol with an optimal result was based on HIER in TRS, High pH (Dako). The mAb was diluted 1:15 and incubated for 30 min. at 32°C and visualized with a 3-step polymer conjugate system (EnVision Flex, Dako/Agilent) on the Dako Omnis instrument.

mAb clone **DAK-Calret 1**: Protocols with optimal results were all based on HIER using BESR2 (Leica) (5/8)*, TRS High pH (3-in-1) (Dako/Agilent) (3/9) or Tris-EDTA/EGTA pH 9 (1/3). The mAb was diluted in the range of 1:20-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 13 of 16 (81%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

rmAb clone **BSR235**: One lab used the rmAb clone BSR235 and achieved an optimal result. The protocol was based on HIER in Tris-EDTA/EGTA pH 9. The rmAb was diluted 1:150 and incubated for 30 min. at room temperature and visualized with a 2-step polymer conjugate system (HRP-Polymer Anti-Rabbit, Nordic Biosite) on the Thermo Autostainer instrument.

rmAb clone **SP13**: One protocol with an optimal result was based on HIER in citrate pH 6,7. The rmAb was diluted 1:10 and incubated for 30 min. at room temperature and visualized with a 3-step polymer conjugate system (PowerVision, Immunologic) on the Tecan Freedom Evo instrument.

pAb **18-0211**: Protocols with optimal results were all based on HIER using TRS High pH (3-in-1) (Dako/Agilent) (1/3), Bond™ Epitope Retrieval Solution 1 (BERS1, Leica) (1/1) or TRS Low pH (3-in-1) (Dako/Agilent) (1/1) as retrieval buffer. The pAb was typically diluted in the range of 1:50-1:150 depending on the total sensitivity of the protocol employed. Using these protocol settings, 5 of 5 (100%) laboratories produced a sufficient staining result (optimal or good).

Table 3. **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**	-	-	1/2 **	-	0/1	-	0/2	0/1
mAb clone **DAK-Calret 1**	3/10 (30%)	-	0/6	-	0/6	-	5/7 (71%)	0/1
rmAb clone **SP13**	-	-	-	-	0/4	-	-	-
pAb **18-0211**	1/2	1/1	-	-	0/6	-	0/1	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/Novocastra, Leica Bond Max/Bond III:
One protocol with an optimal result was based on HIER using BERS2 (Leica) (efficient heating time 20 min. at 100°C), 30 min. incubation of the primary Ab and Bond Polymer Refine Detection (Leica DS9800) as detection system. Using similar protocol settings, 11 of 12 (92%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-Calret 1** product no. **IS/IR627**, Dako/Agilent, Dako Autostainer Link/Classic:
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 10-20 min. at 95-97°C), 20-45 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (Dako/Agilent, K8000/K8002/K8012) as detection system. Using these protocol settings, 32 of 33 (97%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **SP65** product no. **790-4467**, Ventana/Roche, Ventana Benchmark GX/XT/Ultra:
Protocols with optimal results were all based on HIER in Cell Conditioning 1 (CC1, Ventana) (efficient heating time for typically 16-64 min. at 90-100°C), 8-60 min. incubation at 36°C or room temperature of the primary Ab and UltraView (760-500, Ventana) or OptiView (760-700, Ventana) with or without amplification as detection system. Using these protocol settings 100 of 104 (96 %) laboratories produced a sufficient staining result (optimal or good).

Table 4 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 specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for CR for the most commonly used RTU IHC systems**

RTU systems	**Recommendedprotocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Leica BOND mAb clone **CAL6 PA0346**	80% (4/5)	0% (0/5)	89% (8/9)	11% (1/9)
Dako AS mAb clone **DAK-Calret 1 IS/IR627**	89% (17/19)	26% (4/19)	100% (16/16)	56% (9/16)
VMS Ultra/XT rmAb clone **SP65 790-4467**	100% (19/19)	95% (18/19)	88% (87/99)	69% (68/99)

* 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 >25%, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this assessment and in concordance with the previous NordiQC 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 95% of the insufficient results (70 of 74 laboratories). The remaining 5% 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 in the neoplastic cells of the mesothelioma in tissue core no. 3, whereas the demonstration of CR in low-level antigen expressing cells as 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 were more challenging and only seen with appropriate protocol settings (see Fig. 1 - 7).

27% (73 of 269) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for CR. The mAb clone DAK-Calret 1, and the pAb 18-0211 were the most widely used antibodies (see Table 1). Both antibodies could be used to obtain an optimal staining result, but the pass rate was not impressive. For LD assays the pass rate for both mAb clone DAK-Calret 1 and pAb 18-0211 was only 50% (17 of 34 and 6 of 12, respectively). Data focusing on the four main IHC systems (see Table 3) showed that the proportion of optimal results using the mAb clone DAK-Calret 1 as a concentrate was significantly lower on the Ventana BenchMark and Dako Omnis systems compared to the Dako Autostainer and Leica Bond systems. On the Ventana BenchMark and Dako Omnis systems, none (0 of 12) were evaluated as optimal. On the Dako Autostainer and Leica Bond systems, the proportion of optimal results was 30% (3 of 10) and 71% (5 of 7), respectively (see Table 3). The reason for this difference is unclear, but similar findings have previously been observed with several other antibodies on the Dako Omnis and Ventana BenchMark systems. It has been suggested that – for some antibodies – on the fully automated systems with integrated high temperature washing (32°C on the Omnis and 36°C on the BenchMark), it is more difficult to optimize LD assays than on "room temperature systems" (e.g. Dako Autostainer and Leica Bond). Possibly, efficient washing at elevated temperatures (32°C to 36°C) can reverse binding of antibodies to their epitopes, especially if the antibody affinity is relatively low. Whether this is the case with the mAb clone DAK-Calret 1 is so far uncertain. Similar "system dependent" results were observed with the pAb 18-0211. On the Ventana BenchMark system the proportion of sufficient results (optimal or good) was 0% (0 of 6) whereas on non-Ventana BenchMark and non-Dako Omnis systems the proportion of sufficient results was 100% (6 of 6). LD assays based on rmAb clone SP13 was likewise challenging on the Ventana BenchMark system. None were evaluated as optimal (0 of 4) (see Table 3). Summarizing the data from the Ventana Benchmark system showed that establishing LD assays for CR was very challenging on that system with the concentrated Abs available. In total, 20 LD assays for CR based on either the mAb clones DAK-Calret 1 (6), 5A5 (1), CAL6 (1), the rmAb clone SP13 (4), or pAbs from various vendors (8) were assessed and only 10% (2 of 20) achieved sufficient results with none being optimal.

73% (196 of 269) of the laboratories used Abs in Ready-To-Use (RTU) formats. This was a significant increase compared to the previous CR assessment in 2015, where 55% of the laboratories used the RTU format. The most widely used RTU systems for CR were the rmAb clone SP65 based **790-4467** from Ventana intended for use on the Ventana BenchMark system, mAb clone DAK-Calret 1 based **IS/IR627** from Dako/Agilent intended for use on the Dako Autostainer system and mAb clone CALC6 based **PA0346** from Leica intended for use on the Leica Bond system. **790-4467** was used by 118 laboratories and had a high proportion of both sufficient and optimal results (see Table 4). Surprisingly, only 19 laboratories (16%) followed the recommended protocol settings, but they all achieved a sufficient staining result and 18 (95%) were evaluated as optimal. The remaining 99 laboratories modified the protocol settings and 88% (87 of 99) achieved a sufficient staining result with 69% (68 of 99) optimal. These data indicate that the rmAb clone SP68 is a very robust antibody that tolerate a wide variety of protocol settings and still achieve sufficient or optimal results. However, data also showed that there were limits to this protocol tolerance. 11 laboratories using a modified protocol achieved insufficient results. In most cases (7 of 11) these laboratories used extremely short HIER time in CC1, 4 or 8 min. compared to the recommended 36 min. in CC1. Some laboratories combined the ultra-short HIER with a highly sensitive tyramide signal amplified (TSA) detection system (OptiView +amp), but the TSA-system failed to compensate for the insufficient HIER (see Fig. 5 - 7).
The mAb clone DAK-Calret 1 based **IS/IR627** from Dako/Agilent intended for use on the Dako Autostainer system was used by 55 laboratories. 35 of the laboratories used IS/IR627 on the Dako Autostainer system. 54% (19 of 35) of the laboratories followed the recommended protocol settings and 89% (17 of 19) achieved sufficient staining results with 26% (4 of 19) being optimal. 16 laboratories modified the protocol settings bringing the proportion of sufficient results up to 100% (16 of 16) and the proportion of optimal results up to 56% (9 of 16). This improvement in proportion of both sufficient and optimal result was closely related to the fact that 8 of these 16 laboratories used a 3-step polymer

conjugate system (EnVision Flex+) instead of the recommended 2-step polymer conjugate system (EnVision Flex). The IS/IR627 RTU system was developed for the Dako Autostainer system but frequently used on other systems. In the current assessment, 18 laboratories used IS/IR627 on the Dako Omnis, but with little success. The proportion of sufficient results was only 11% (2 of 18) compared to 94% (33 of 35) on the Dako Autostainer system, emphasizing that RTU Abs should be used only on the system that they are develop for (see Fig. 1 - 4). Used on other systems, the RTU Ab must be considered as a LD assay, thus requiring a thorough technical calibration and diagnostic validation.
The mAb clone CALC6 based **PA0346** from Leica intended for use on the Leica Bond system was used in 15 laboratories. 14 laboratories used the PA0346 on the Leica Bond system. 36% (5 of 14) of the laboratories followed the recommended protocol and 80% (4 of 5) achieved sufficient staining results but with none being optimal. 9 laboratories modified the protocol settings bringing the proportion of sufficient results up to 89% (8 of 9) and the proportion of optimal results up to 11% (1 of 9). These minor improvements in results were primarily related to prolonged incubation time of the RTU Ab. The recommended protocol is based on an incubation time of 15 min. of the RTU Ab, whereas the average incubation time in the modified protocols was 26 min. Although PA0346 had an overall high pass rate (good or optimal) of 86% (12 of 14) the proportion of optimal were surprisingly low with only 8% (1 of 14) being optimal. The only protocol achieving an optimal result was based on a modified protocol using prolonged incubation time (30 min.) of the RTU Ab.

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 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.

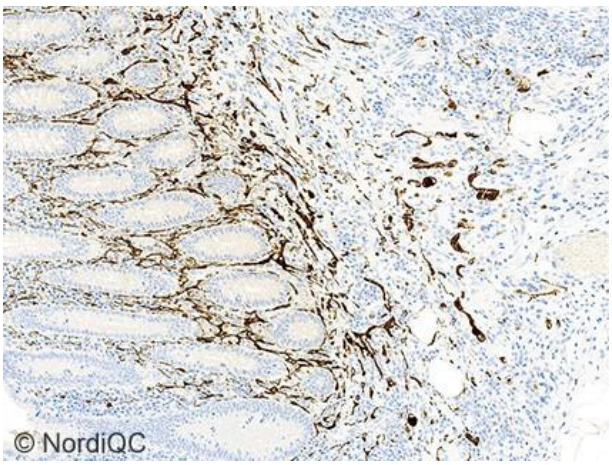


Fig. 1a
Optimal CR staining of the appendix using the mAb clone DAK-Calret 1 in a RTU format (Dako/Agilent, IS/IR627) and with an incubation time of 30 min. after HIER in an alkaline buffer (TRS pH 9, Dako). Staining was performed on the **Dako Autostainer** system using a 3-step polymer system (EnVision 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.

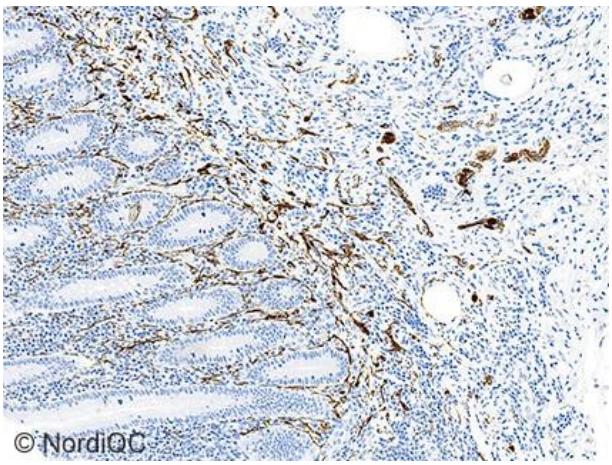


Fig. 1b
Insufficient CR staining of the appendix using the mAb clone DAK-Calret 1 in a RTU format (Dako/Agilent, IS/IR627) with similar protocol settings as in Fig. 1a, but on the **Dako Omnis** system instead of the recommended Dako Autostainer system as in Fig. 1a. Although the same RTU product and similar protocol settings were used, the CR staining on the Dako Omnis system 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 - 4b – same protocol.

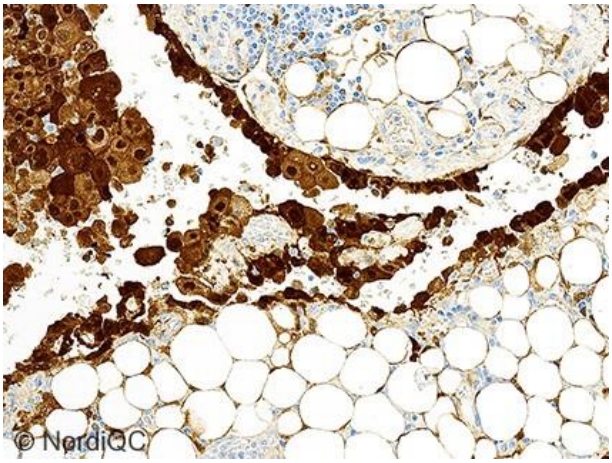


Fig. 2a
Optimal CR staining of the mesothelioma in tissue core no. 6 using the same protocol as in Fig. 1a. A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. A moderate, distinct cytoplasmic and nuclear staining of the majority of fat cells is also seen. Compare with Fig. 2b.

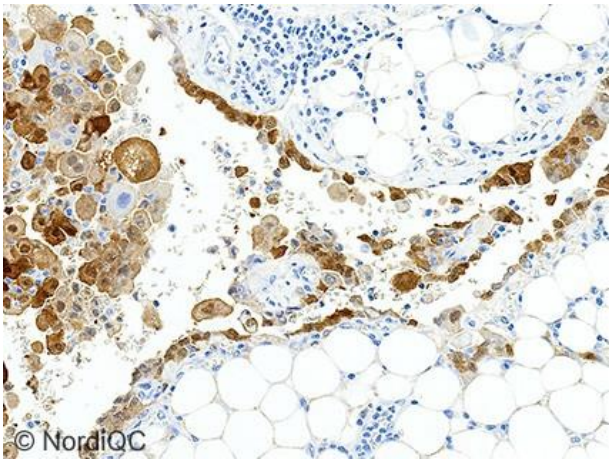


Fig. 2b
Insufficient CR staining of the mesothelioma in tissue core no. 6 using the same protocol as in Fig. 1b. Only a moderate, distinct cytoplasmic and nuclear staining of the majority of the tumour cells is seen. Fat cells are false negative. Compare with Fig. 2a - same field.

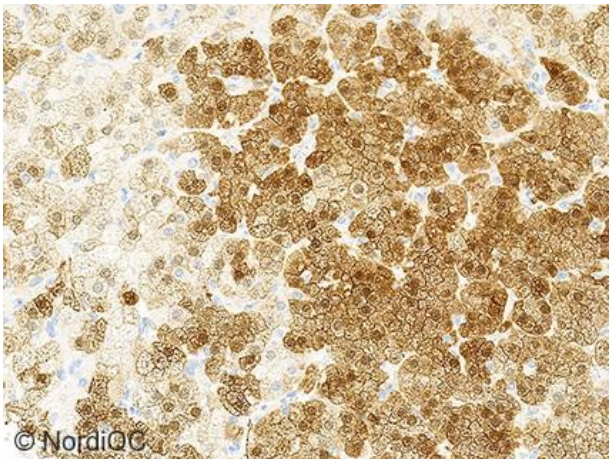


Fig. 3a
Optimal CR staining of the adrenal gland ("low-level expressor") using the same protocol as in Fig. 1a and 2a. A moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells is seen. Compare with Fig. 3b.

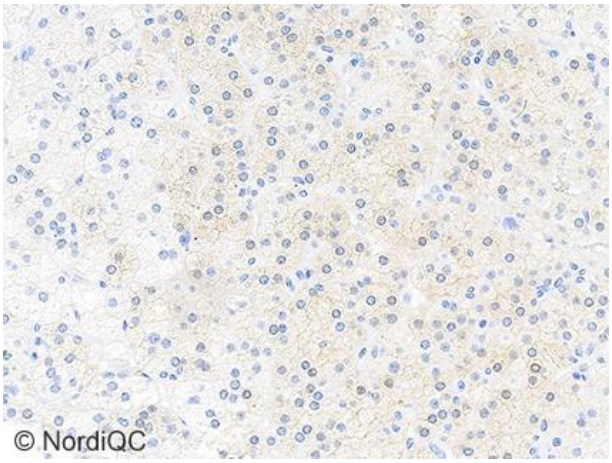


Fig. 3b
Insufficient CR staining of the adrenal gland ("low-level expressor") using the same protocol as in Fig. 1b and 2b. No staining of the cortical epithelial cells is seen. Compare with Fig. 3a – same field.

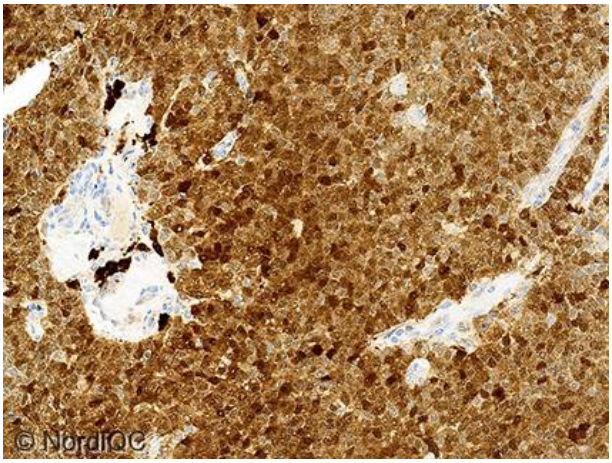


Fig. 4a
Optimal CR staining of the granulosa cell tumour using the same protocol as in Fig. 1a - 3a. A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. Compare with Fig. 4b.

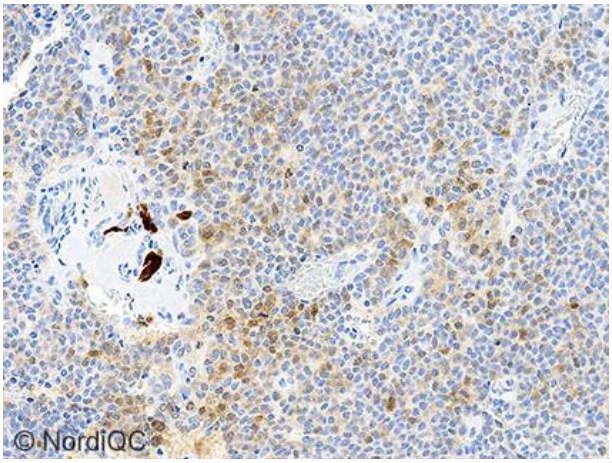


Fig. 4b
Insufficient CR staining of the granulosa cell tumour using the same protocol as in Fig. 1b - 3b. A very weak staining reaction is seen in a minor fraction of the tumour cells. The vast majority of tumour cells are false negative. Compare with Fig. 4a - same field.

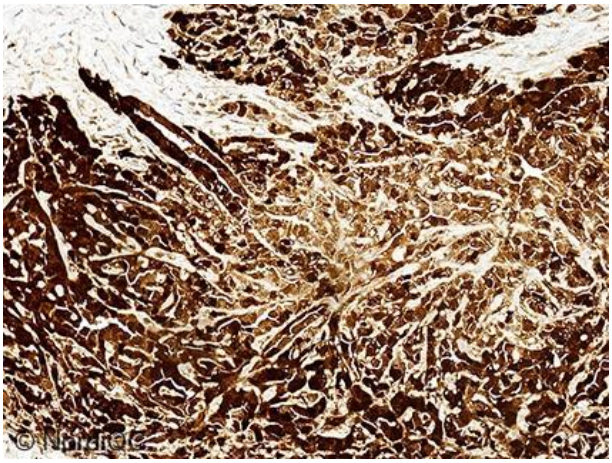


Fig. 5a
Optimal CR staining of the mesothelioma in tissue core no. 3 ("high-level expressor") using the rmAb clone SP65 in a RTU format (Ventana 790-4467) and with an incubation time of 16 min. after HIER in CC1 for 32 min. Staining was performed on the Ventana Benchmark Ultra using a 3-step multimer system (OptiView). A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. Compare with Fig. 5b. Also compare with Figs. 6a – 7a – same protocol.

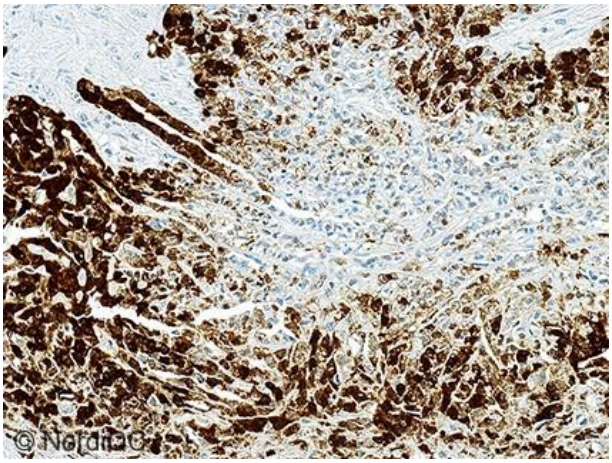


Fig. 5b
Insufficient CR staining of the mesothelioma in tissue core no. 3 ("high-level expressor") using the same protocol on a Ventana Benchmark instrument as in Fig. 5a. but with 2 important modifications. HIER in CC1 was reduced to only 4 min. in combination with the use of OptiView with tyramide based amplification. This results in a change in staining pattern with larger areas of the tumour being virtually negative whereas other areas are intensely positive. Compare with Fig. 5a - same field. Also compare with Figs. 6b and 7b – same protocol.

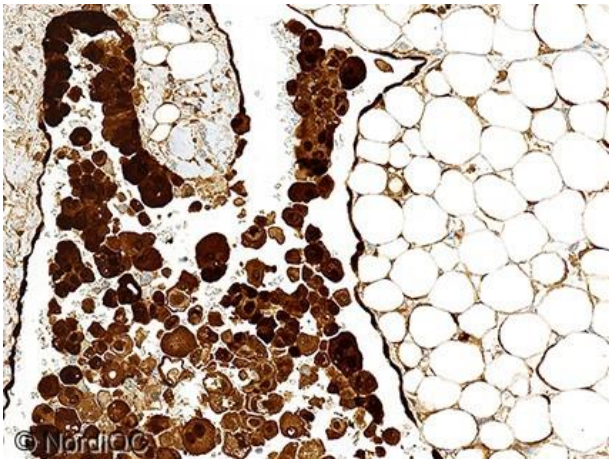


Fig. 6a
Optimal CR staining of the mesothelioma in tissue core no. 6 using the same protocol as in Fig. 5a. A strong, distinct cytoplasmic and nuclear staining of virtually all the tumour cells is seen. A moderate, distinct cytoplasmic and nuclear staining of the majority of fat cells is also seen. Compare with Fig. 6b.

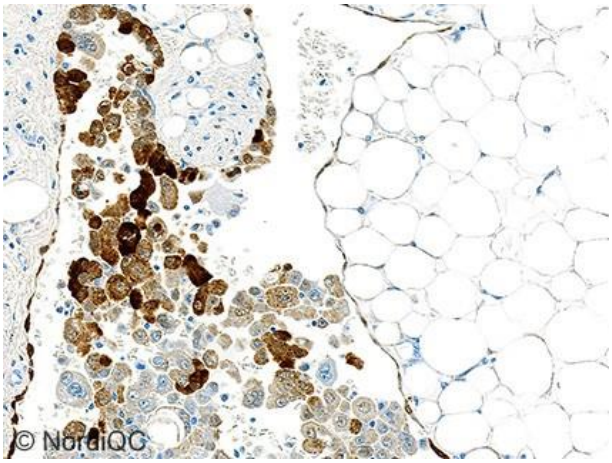


Fig. 6b
Insufficient CR staining of the mesothelioma in tissue core no. 6 using the same protocol as in Fig. 5b. Only a weak to moderate, distinct cytoplasmic and nuclear staining in about 50% of the tumour cells is seen. The rest of the tumour cells and all the fat cells are false negative. Compare with Fig. 6a - same field.

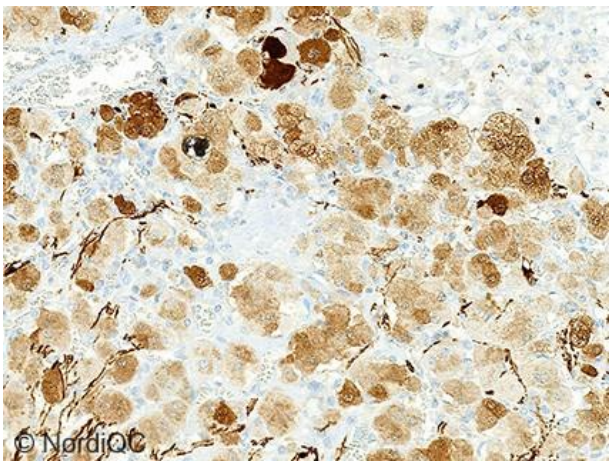


Fig. 7a
Optimal CR staining of the adrenal gland ("low-level expressor") using the same protocol as in Fig. 5a and 6a. A moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells is seen. Compare with Fig. 7b.

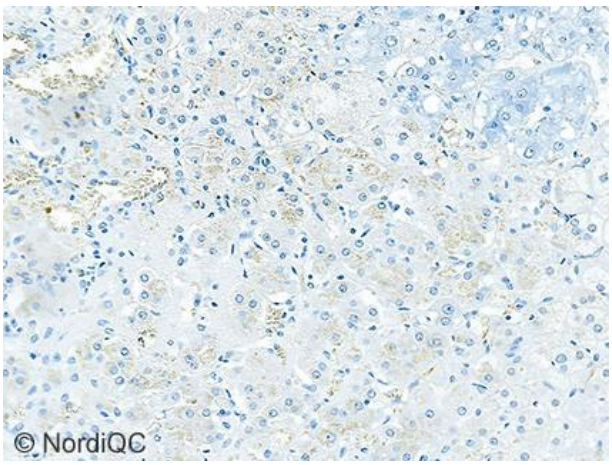


Fig. 7b
Insufficient CR staining of the adrenal gland ("low-level expressor") using the same protocol as in Fig. 5b and 6b. No staining of the cortical epithelial cells is seen. Compare with Fig. 7a – same field.

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