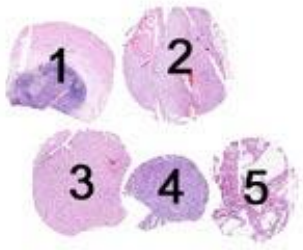
Assessment Run 33 2011
Calretinin (CR)

Material

The slide to be stained for CR comprised:

1. Appendix, 2. Adrenal gland, 3. Kidney, 4. Breast ductal carcinoma,
5. 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 of the peripheral nerves (ganglion cells and axons) and the macrophages in the appendix.
- An at least weak to moderate, distinct cytoplasmic and nuclear staining of the majority of the cortical epithelial cells of the adrenal gland and of the fat cells in the tissues included.
- A moderate to strong, distinct cytoplasmic and nuclear staining of the majority of the neoplastic cells of the mesothelioma.
- A negative or only focal staining of the epithelial cells of the kidney.
- A negative or only focal staining of the neoplastic cells of the ductal breast carcinoma.

180 laboratories participated in this assessment. 7 laboratories used an inappropriate Ab like Chromogranin A (all these correctly sent in their protocol for CR!). Out of the remaining 173 laboratories, 76 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for CR, run 33**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **2E7**	3	Immunologic	0	2	0	1	-	-
mAb clone **5A5**	28 4 1	Leica/Novocastra Thermo/Neomarkers Monosan	8	14	10	1	67 %	74 %
mAb clone **DAK-Calret 1**	41 1	Dako Thermo/Neomarkers	11	17	11	3	67 %	83 %
rmAb clone **SP13**	3 2 1	Thermo/Neomarkers Spring Master Diagnistica	0	2	2	2	33 %	-
pAb **18-0211**	18	Invitrogen/Zymed	10	6	2	0	89 %	100 %
pAb **CP092**	2	Biocare	0	2	0	0	-	-
pAb **E1174**	1	Spring	0	1	0	0	-	-
pAb **ILM 7696**	4	Immunologic	0	2	1	1	-	-
pAb **RBK003**	1	Zytomed	0	1	0	0	-	-
Ready-To-Use Abs								
mAb clone **CAL6 PA0346**	3	Leica/Novocastra	2	1	0	0	-	-
mAb clone **DAK-Calret 1 IS/IR627**	26	Dako	7	11	6	2	69 %	78 %
rmAb clone **SP65 790-4467**	28	Ventana	24	4	0	0	100 %	100 %
pAb **232A-77/78**	2	Cell Marque	0	2	0	0	-	-
pAb **760-2700**	3	Ventana/Cell Marque	0	3	0	0	-	-
pAb **ZA0026**	1	Zhongshan	0	1	0	0	-	-

Total	173		62	69	32	10	-	
Proportion			36 %	40 %	18 %	6 %	76 %	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below
3) Highly platform dependent, see explanation below.

The following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs
mAb clone **5A5**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Bond Epitope Retrieval Solution 2 (BERS2; Bond, Leica) (5/8)*, Cell Conditioning 1 (CC1; BenchMark, Ventana) (1/15)* or Tris-EDTA/EGTA pH 9 (2/4)* as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 14 out of 19 (74 %) laboratories produced a sufficient staining (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mAb clone **DAK-Calret 1**: The protocols giving an optimal result were all based on HIER using either Target Retrieval Solution pH 9 (3-in-1) (TRS; Dako) (4/11), BERS2 (Bond, Leica) (3/3), Borg Decloaker pH 9.5 (Biocare) (1/1) or Tris-EDTA/EGTA pH 9 (3/10) as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 20 out of 24 (83 %) laboratories produced a sufficient staining.

pAb **18-0211**: The protocols giving an optimal result were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (2/2), TRS pH 9 (Dako) (1/3), BERS2 (Bond, Leica) (4/5), CC1 (BenchMark, Ventana) (1/5), Tris-EDTA/EGTA pH 9 (1/1) or Citrate pH 6 (1/2) as the retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 15 out of 15 (100 %) laboratories produced a sufficient staining.

Ready-To-Use Abs
mAb clone **CAL6** (product.no. PA0346, Leica/Novocastra): The protocols giving an optimal result were both based on HIER using BERS2 (Bond, Leica), an incubation time of 15-20 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system. Using these protocol settings 2 out of 2 laboratories produced an optimal staining.

mAb clone **DAK-Calret 1** (prod. no. IS/IR627, Dako): The protocols giving an optimal result were based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (heating time 10-30 min at 95-97°C) and an incubation time of 20 min in the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as the detection system. Using these protocol settings 14 out of 18 (78 %) laboratories produced a sufficient staining.(optimal or good).

rmAb clone **SP65** (prod. no. 790-4467, Ventana): The protocols giving an optimal result were all based on HIER using short, mild or standard Cell Conditioning 1, an incubation time of 16-48 min in the primary Ab and UltraView (760-500) or OptiView (760-700) as the detection system. Using these protocol settings 27 out of 27 (100 %) laboratories produced a sufficient staining.

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

In this assessment and in concordance with the observations in the previous assessments for CR the prevalent feature of an insufficient staining was a general too weak or false negative staining of the structures expected to be demonstrated. Virtually all the participating laboratories were able to demonstrate CR in the peripheral nerves and in the macrophages, whereas the demonstration of CR in the fat cells, the neoplastic cells of the mesothelioma and in particular the cortical epithelial cells of the adrenal gland was more challenging and was only seen with appropriate protocol settings.
For the most widely used mAb clone DAK-Calret 1 the proportion of sufficient results was e.g. highly influenced by the choice of the detection system and the IHC platform used. If the mAb clone DAK Calret 1 was used as a concentrate, diluted in the range of 1:50-300, HIER in an alkaline buffer as BERS2, or TRS pH 9 and applied with a 2-step polymer based detection system as EnVision FLEX (Dako), 11 out of 22 laboratories obtained a sufficient staining result (50%) out of which 1 (5%) was 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), 14 out of 14 laboratories produced a sufficient staining result (100%) of which 9 (64%) were optimal. A significant difference in the overall performance for DAK-Calret 1 was also related to the IHC platform applied, as

6 out of 6 (100 %) protocols performed on the fully automated platform BenchMark XT or Ultra, Ventana were assessed as insufficient. In contrast, 4 out of 4 (100 %) protocols performed on a similar fully automated platform as the Bond-max or Bond III (Leica) were assessed as sufficient, out of which 3 (75 %) were optimal.

The most successful and robust assay for CR in this assessment was obtained by the Ready-To-Use (RTU) system based on the newly launched rmAb clone SP65 from Ventana giving a pass rate of 100 % out of which 86 % (24 out of 28 laboratories) were optimal. The RTU format of the clone SP65 could be used in a wide range of protocol settings and still give a sufficient, even optimal staining. It was, though, observed that a weak background staining occurred when the RTU format was used by very sensitive protocol settings as e.g. HIER in standard CC1 (64 min) and 32 min. incubation in the primary Ab.
Using the rmAb clone SP65 according to the recommendations from Ventana (HIER in mild CC1 and 20 min incubation time) a very strong and consistent staining for CR was seen and in particular the demonstration of CR in the cortical epithelial cells of the adrenal gland was superior to all other Abs used in this assessment.

The adrenal cortex was found to be a critical staining quality indicator for CR: An at least weak to moderate cytoplasmic and nuclear staining must be seen in the majority of the cortical epithelial cells. However, this reaction pattern can only reliably be identified when a non-biotin based detection system is used, as the adrenal cortical cells are rich in endogenous biotin. Hence, a false positive cytoplasmic reaction can mimic the specific reaction excluding the use of adrenal cortex as a reliable control.
This was the 4th assessment of CR in NordiQC (Table 2), and a slight decrease in the proportion of sufficient results was seen compared to the previous run 23.

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

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

In this assessment many new laboratories participated for the first time. However, virtually the same pass rate was observed for the laboratories participating in the CR assessment for the first time compared to the laboratories also participating in the previous run 23, 2008: For the laboratories participating for the first time the pass rate was 77 % (58 out of 75), while the pass rate was 75 % (73 out of 98 laboratories) for the laboratories participating in both runs.

Conclusion
The mAb clones **DAK-Calret1**, **5A5**, and **CAL6**, the rmAb clone **SP65** and the pAb **18-0211** are all recommendable Abs for CR. Efficient HIER in an alkaline buffer in combination with a sensitive and specific IHC system is mandatory for optimal performance. In this assessment the RTU systems based on the rmAb clone **SP65** for CR from Ventana and the mAb clone **CAL6** from Leica gave the highest proportion of sufficient results.
Normal adrenal gland is an appropriate control: The majority of the cortical epithelial cells must show an at least weak to moderate nuclear and cytoplasmic staining reaction.
Biotin based detection systems can not be recommended due to the risk of a false positive staining reaction due to endogenous biotin.

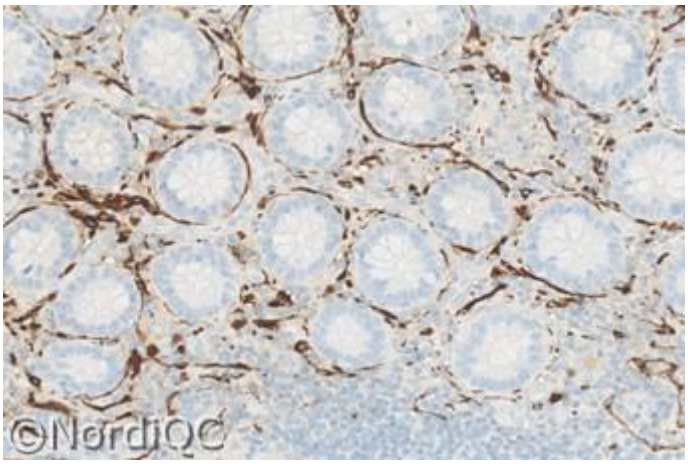


Fig. 1a
Optimal staining for CR of the appendix using the rmAb clone SP65 as RTU, HIER in an alkaline buffer and a 2-step multimer based detection system. Virtually all the peripheral nerves show a distinct, moderate to strong nuclear and cytoplasmic staining reaction, while the columnar epithelial cells are negative.

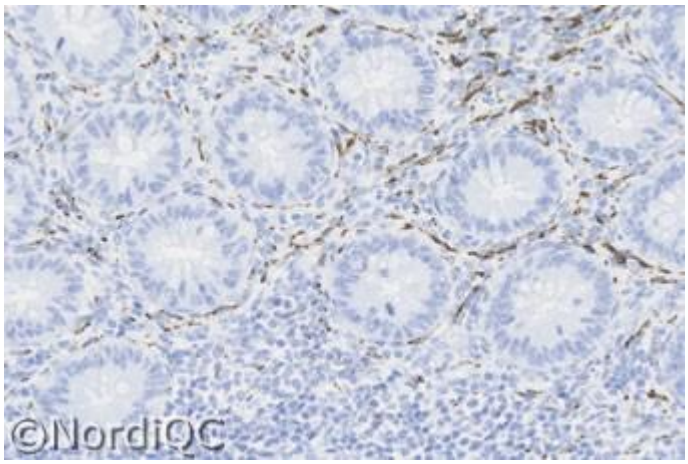


Fig. 1b
Insufficient staining for CR of the appendix using the mAb clone DAK-Calret 1 by a protocol with a too low sensitivity (too low. conc. of the primary Ab) - same field as in Fig. 1a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result obtained in Fig. 1a.
Also compare with Figs. 2b. - 4b., same protocol.

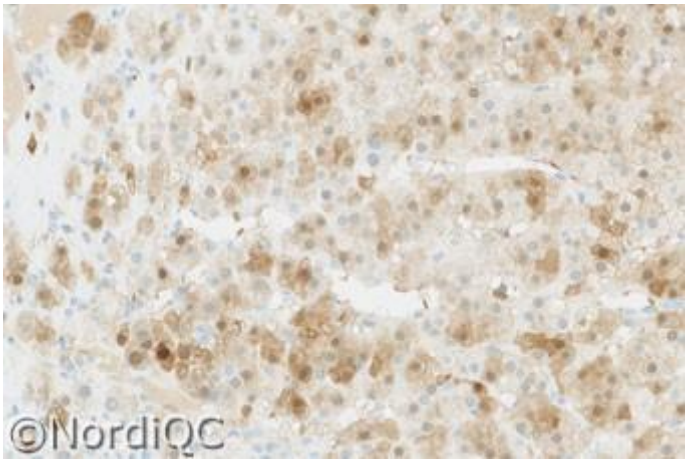


Fig. 2a
Optimal staining for CR of the adrenal gland using same protocol as in Fig. 1a.
The majority of the cortical epithelial cells show a weak to moderate nuclear and cytoplasmic staining reaction. No background staining is seen.

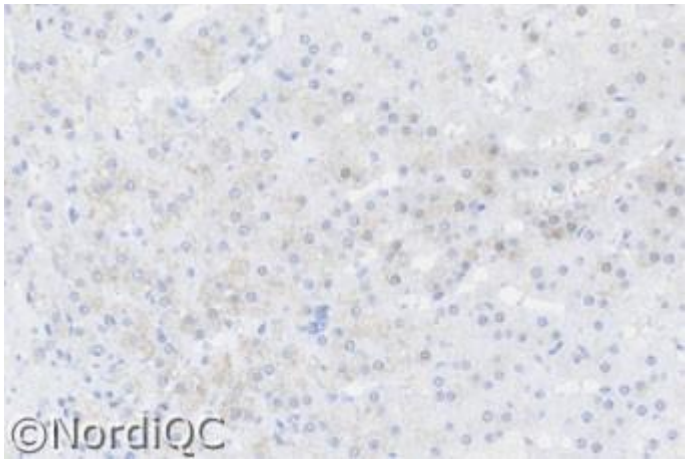


Fig. 2b
Insufficient staining for CR of the adrenal gland using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only scattered cortical epithelial cells show a weak and equivocal staining reaction.
Also compare with Fig. 3b., same protocol.

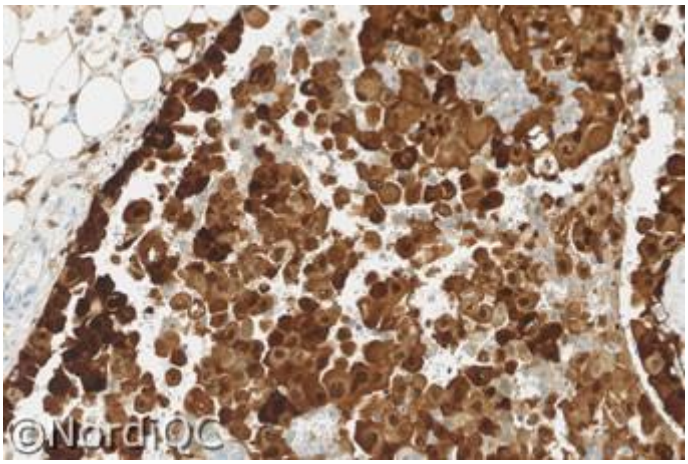


Fig. 3a
Optimal staining for CR of the mesothelioma using same protocol as in Figs. 1a. & 2a.
Virtually all the neoplastic cells show a distrinct, moderate to strong nuclear and cytoplasmic staining reaction.
Also the fat cells (left corner, top) show a weak to moderate specific staining.

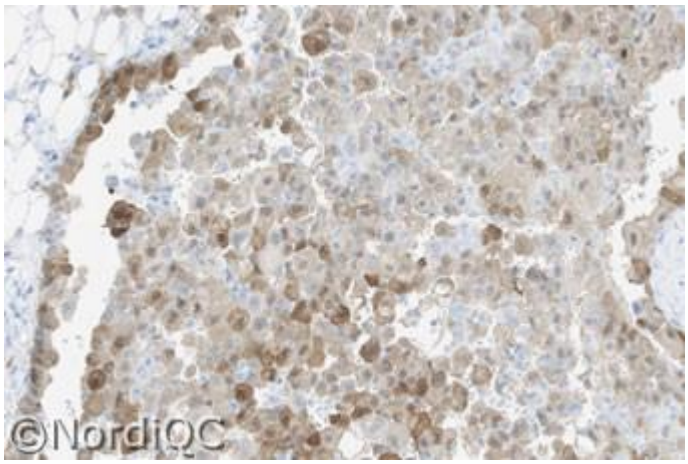


Fig. 3b
Insufficient staining for CR of the mesothelioma using same protocol as in Figs. 1b. & 2b. – same field as in Fig. 3a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result obtained in Fig. 3a.

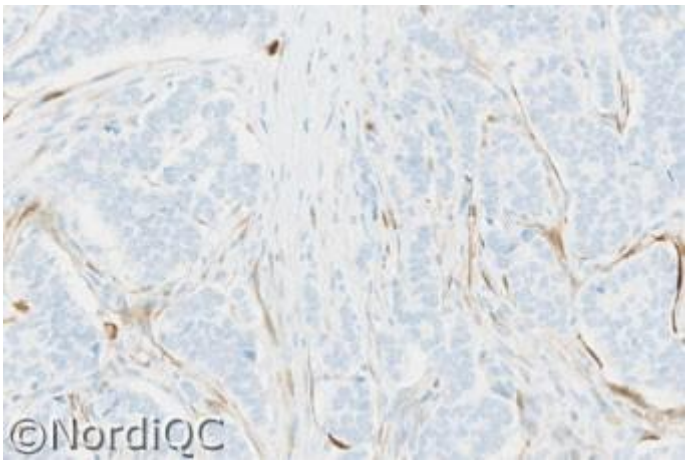


Fig. 4a
Optimal staining for CR of the breast ductal carcinoma using same protocol as in Figs. 1a. - 3a.
The neoplastic cells are negative, while scattered stromal cells show a moderate nuclear and cytoplasmic staining reaction.

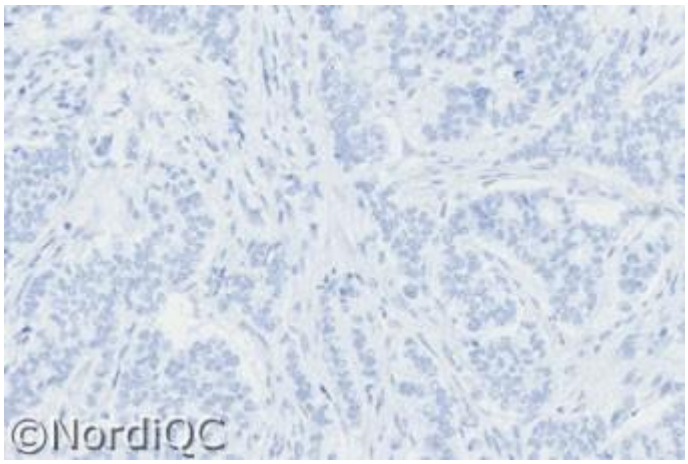


Fig. 4b
Staining for CR of the breast ductal carcinoma using same insufficient protocol as in Figs. 1b. - 3b. – same field as in Fig. 4a.
No staining is seen in any cells.

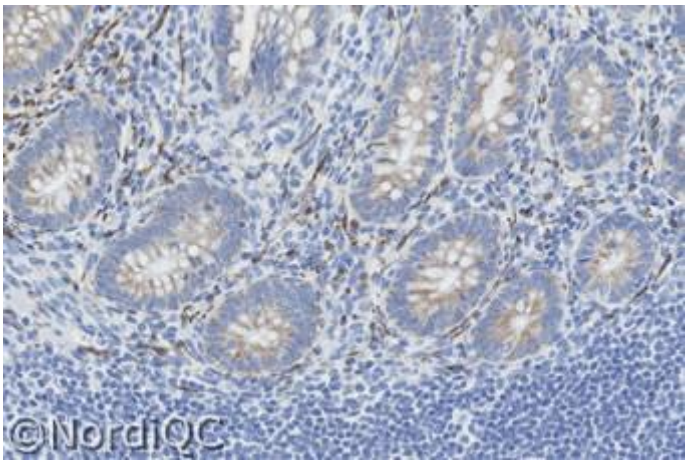


Fig. 5a
Insufficient staining for CR of the appendix using a too low concentration of the primary Ab combined with HIER in an alkaline buffer and a biotin based detection system.
The specific staining reaction in the peripheral nerves is significantly reduced and at the same time a false positive cytoplasmic staining reaction of endogenous biotin in the epithelial cells is seen – also compare with Fig. 5b – same protocol

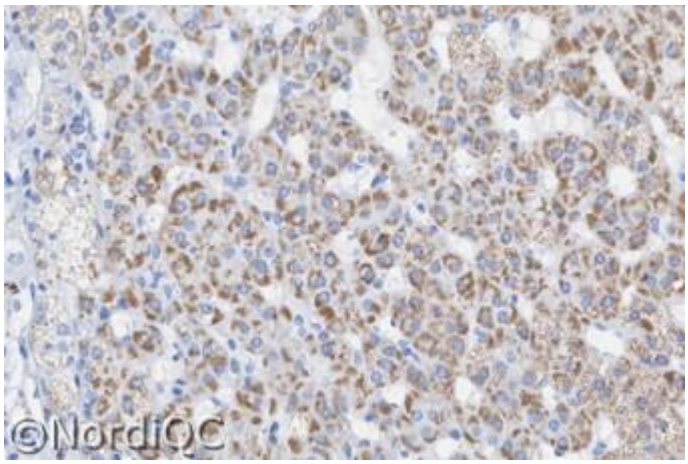


Fig. 5b
Insufficient staining for CR of the adrenal gland using same protocol as in Fig. 5a.
No nuclear staining reaction in the cortical epithelial cells is seen and only a moderate granular cytoplasmic staining reaction of endogenous biotin is seen.

SN/MV/LE 5-12-2011