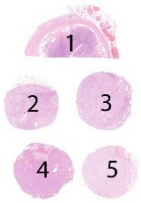
Assessment Run 76 2026

Calretinin (Calret)

Purpose
Evaluation of the technical performance and the level of analytical sensitivity and specificity of the immunohistochemical (IHC) assays used by NordiQC participants for Calret. The focus of the assessment was identification of sex cord-stromal tumours and mesotheliomas in the characterization of cancers of unknown primary origin. Relevant normal and neoplastic clinical tissues were selected to represent a broad range of Calret antigen densities (see below).

Material
The slide to be stained for Calret comprised:



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

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing Calret 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 most 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.

KEY POINTS FOR CALRET IMMUNOASSAYS
- The RTU systems from **Ventana/Roche** and **Leica Biosystems** obtained a 100% pass rate when applying vendor recommended protocol settings.
- The mAb clone **DAK-Calret 1** is less successful on Dako Omnis platform.

Participation

Number of laboratories registered for Calret, run 76	491
Number of laboratories returning slides	408 (83%)

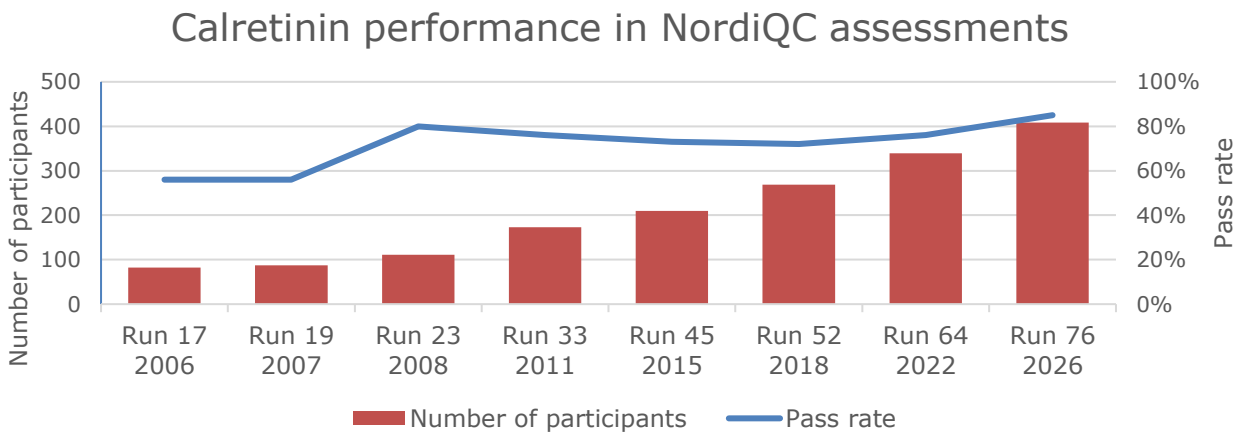
At the date of assessment, 83% of the participants had returned the circulated NordiQC slides.
In this assessment, run 76, general issues with the Danish postal service affected the distribution and return of slides to/from participants, resulting in a lower number of returned slides compared to previous assessments.
Slides received after the assessment were not included in this report. However, all returned slides were assessed, and participating laboratories with insufficient results received advice.

Results
408 laboratories participated in this assessment. 347 (85%) achieved a sufficient mark (optimal or good) – see Table 1a (page 2). Tables 1b and 1c 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).
- Use of less sensitive detection systems.

Performance history
This was the eighth NordiQC assessment of Calret and the pass rate increased to 85% in this run compared to the previous four runs (see Graph 1).

Graph 1. **Proportion of sufficient results for Calret in the eight NordiQC runs performed**



Controls
Adrenal gland and appendix are recommendable positive and negative tissue controls for Calret. 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 most 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.

Conclusion
The mAb clones **CAL6, DAK-Calret 1** and the rmAb clone **SP65** were the most widely used and could all be used to obtain an optimal result for Calret. 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 (developed for Autostainer) and concentrates, was influenced by the chosen platform and gave a low pass rate of 31% on the Omnis (Dako/Agilent) platform. The RTU systems **PA0346** (Leica Biosystems) based on the mAb clone CAL6 and **790-4467** (Ventana/Roche) based on rmAb clone SP65, using recommended protocol settings, both obtained an overall pass rate of 100% with 86% and 91% being optimal, respectively. 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.

Table 1a. **Overall results for Calret, run 76**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	60	32	14	9	5	77%	53%
Ready-To-Use antibodies	348	232	69	34	13	86%	67%
Total	408	264	83	43	18		
Proportion		65%	20%	11%	4%	85%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1b. **Concentrated antibodies and assessment marks for Calret, run 76**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **2E7**	1	Immunologic	1	-	-	-	-	-
mAb clone **CAL6**	30	Leica Biosystems	20	7	3	-	90%	67%
mAb clone **DAK-Calret 1**	19	Dako/Agilent	4	7	5	3	58%	21%
mAb clone **IHC523**	1	GenomeMe	1	-	-	-	-	-
rmAb clone **QR059**	1	Quartett	1	-	-	-	-	-
rmAb clone **SP13**	1	Diagnostic Biosystems	-	-	-	1	-	-
rmAb clone **ZR415**	1	Zeta Corporation	-	-	1	-	-	-
pAb **18-0211**	5	Invitrogen	4	-	-	1	80%	80%
pAb, **CP092C**	1	Biocare Medical	1	-	-	-	-	-
Total	60		32	14	9	5		
Proportion			54%	23%	15%	8%	77%	

1) Proportion of sufficient results (optimal or good). (≥5 assessed protocols).
2) Proportion of Optimal Results (OR).

Table 1c. **Ready-to-Use antibodies and assessment marks for Calret, run 76**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **CAL6 PA0346[3]**	21	Leica Biosystems	18	3	-	-	100%	86%
mAb clone **CAL6 PA0346[4]**	9	Leica Biosystems	5	3	1	-	89%	56%
mAb clone **DAK-Calret 1 IR627[3]**	12	Dako/Agilent	1	5	5	1	50%	8%
mAb clone **DAK-Calret 1 IR627[4]**	59	Dako/Agilent	8	17	22	12	42%	14%
mAb clone **C5G4 CCM-0222**	1	Celnovte	1	-	-	-	-	-
mAb clone **IHC523 IHC523-7**	1	GenomeMe	-	1	-	-	-	-
mAb clone **BY219 BFM-0581**	1	Bioin Biotechnology	1	-	-	-	-	-
Ab clone **DY49410 4910642**	1	Dakewe	1	-	-	-	-	-
mAb clone **MX027 MAB-0716**	1	Fuzhou Maixin	1	-	-	-	-	-
rmAb clone **SP13 232R**	2	Cell Marque	2	-	-	-	-	-
rmAb clone **SP13 AY747**	1	BioGenex	-	1	-	-	-	-
rmAb clone **BSR235 MAD-000784QD**	3	Master Diagnostica	1	1	1	-	-	-
rmAb clone **RM324 8522-C010**	3	Sakura Finetek	3	-	-	-	-	-
rmAb clone **RM324 BO6011**	1	Guangzhou Biotron	1	-	-	-	-	-
rmAb clone **SP65 790-4467[3]**	78	Ventana/Roche	71	7	-	-	100%	91%
rmAb clone **SP65 790-4467[4]**	150	Ventana/Roche	116	29	5	-	97%	77%
rmAb clone **554J1B8 PA262**	1	Abcarta	1	-	-	-	-	-
pAb **IP092**	1	Biocare Medical	-	1	-	-	-	-
pAb **HAP134**	1	PathnSitu	-	1	-	-	-	-
pAb **GT200902**	1	Gene Tech	1	-	-	-	-	-
Total	348		232	69	34	13		
Proportion			66%	20%	10%	4%	86%	

1) Proportion of sufficient results (optimal or good). (≥5 assessed 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 assessed 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 assessed protocols).

Detailed analysis of Calret, Run 76
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 (BERS2, Leica Biosystems) (2/5)* or Target Retrieval Solution (TRS) High pH (Dako/Agilent) (2/10). The mAb was diluted at 1:50 and a 3-layer detection system was applied. Using these protocol settings, 5 of 5 (100%) 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 (Dako/Agilent) (10/13), Cell Conditioning 1 (CC1, Ventana/Roche) (1/1), BERS2 (Leica Biosystems) (8/13) or Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/2). The mAb was typically diluted in the range of 1:15-1:100 and a 3-layer detection system was applied. Using these protocol settings, 23 of 25 (92%) laboratories obtained a sufficient staining result.

pAb **18-0211**: Protocols with optimal results were based on HIER using TRS High pH (Dako/Agilent) (2/2) or CC1 (Ventana/Roche) (2/3) 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, 4 of 5 (80%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for Calret for the most commonly used antibody concentrates on the four main IHC systems by optimal settings as listed above.**

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **CAL6**	-	-	9/10 (90%)	-	1/1	-	8/12 (67%)	1/2
mAb clone **DAK-Calret 1**	2/2	-	0/2	-	-	-	2/2	-
pAb **18-0211**	-	-	2/2	-	2/3	-	-	-

1) Autostainer Classical, Link 48+.
2) BenchMark GX, Ultra.
3) Bond III, MAX, Prime.

Ready-To-Use antibodies and corresponding systems
mAb clone **CAL6** product no. **PA0346**, Leica Biosystems, Leica Bond Max/III/Prime:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 15-20 min. at 100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 27 of 27 (100%) laboratories produced a sufficient staining result (optimal or good).
The product was used by 1 laboratory on a non-intended platform. Data was not included in the description above.

mAb clone **DAK-Calret 1** product no. **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 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 20 (65%) laboratories produced a sufficient staining result.
The product was used by 49 laboratories on a non-intended platform. Data was not included in the description above

rmAb clone **SP65** product no. **790-4467**, Ventana/Roche, Ventana Benchmark GT/Ultra/Ultra plus:
Protocols with optimal results were typically based on HIER in CC1 (efficient heating time for 16-64 min. at 95-100°C), 16-60 min. (36°C) incubation of the primary Ab and UltraView (760-500) with or without UltraView Amplification (760-080) or OptiView (760-700, Ventana/Roche) as detection system. Using these protocol settings, 220 of 225 (98%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly 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 Calret 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% (21/21)	86% (18/21)	100% (8/8)	63% (5/8)
Dako AS mAb DAK-Calret 1 **IR627**	50% (6/12)	8% (1/12)	90% (9/10)	50% (5/10)
Ventana BenchMark rmAb SP65 **UltraView** **790-4467**	100% (56/56)	88% (49/56)	96% (64/67)	78% (52/67)
Ventana BenchMark rmAb SP65 **OptiView** **790-4467**	100% (22/22)	100% (22/22)	98% (81/83)	77% (64/83)

* 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 Calret 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 98% (60/61) of the insufficient results. The remaining 2% (n=1) of insufficient results were characterized by a false positive staining reaction compromising interpretation.
The majority of the participating laboratories were able to demonstrate Calret in high-level antigen expressing cells, such as peripheral nerves and the neoplastic cells of the mesothelioma, whereas the demonstration of Calret 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 highly sensitive protocol settings.

The mAb clones CAL6, DAK-Calret 1 and the rmAb clone SP65 were the most widely used antibodies for demonstration of Calret and applied by 93% (378/408) of the laboratories (see Tables 1b and c).

Used as concentrated format within laboratory developed (LD) assays, the **mAb clone CAL6** provided 90% (27/30) sufficient results of which 67% (20/30) were assessed as optimal. As shown in Table 2, the mAb clone CAL6 gave superior results on the Omnis platform – 90% (9/10) of the results were assessed as optimal. The optimal protocols were based on efficient HIER in TRS pH High (3-in-1), the average dilution factor was 1:27 (range 1:15-1:50) and all laboratories applied the sensitive Envision FLEX+/++ as detection system. On the BOND platforms, 64% (9/14) of the protocols provided an optimal result. The majority was based on HIER in BERS2, and the Ab was diluted in the range of 1:50-1:100. One participant obtained an optimal result using the BERS1 HIER buffer. This buffer was less efficient in previous NordiQC assessments.

LD assays based on the **mAb clone DAK-Calret 1** provided 58% (11/19) sufficient results, 21% (4/19) were assessed as optimal. As described in the previous reports (Runs 52, 2018, and 64, 2022), the performance of the mAb clone DAK-Calret 1 depends on the chosen platform and are particularly challenging to optimize on the fully automated platforms such as Omnis and Benchmark (see Table 2). Grouped these platforms together and applying the mAb clone DAK-Calret 1, none (0/10) of the protocols were assessed as optimal, and the overall pass rate on the two platforms was only 40% (4/10). This observation was also seen in the latest NordiQC report (Run 64, 2022). Laboratories should substitute DAK-Calret 1 with alternative clones providing optimal results on these platforms as e.g., CAL6 or SP65 for Omnis and BenchMark, respectively. As outlined in previous reports , the reason for this difference in performance is unclear, but Abs with low affinity 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) due to reversing the antibody-epitope binding.

The **pAb 18-0211** provided 80% sufficient and optimal results (4/5). Although the antibody previously has proven to be challenging on the BenchMark platform, the pass rate in this assessment was 66% (2/3), both optimal. Two protocols on Omnis gave an optimal result.

In total, 85% (348/408) of the laboratories used a RTU format for demonstration of Calret. As shown in Table 3, and applying vendor recommended protocol settings (VRPS), the RTU systems PA0346 (Leica Biosystems), IR627 (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.

The most used RTU format was the rmAb clone SP65 from **Roche/Ventana, 790-4467,** applied by 56% (228/408) of all the participants. An overall high pass rate of 98% (223/228) was seen, 82% optimal

(n=187). When applying the vendor recommended protocol settings, a pass rate 100% was observed for both UltraView and OptiView protocols, 88% and 100% optimal, respectively (see Table 3). Most participants modified the protocol settings, giving slightly reduced pass rates for UltraView (96%) and OptiView (98%) detection systems. Various HIER times and incubation times for the primary Ab, were seen.

The **Dako/Agilent RTU system IR627** based on mAb clone DAK-Calret 1, intended for use on the Autostainer, was used by 22 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 by applying modifications to the basic recommendations from the vendor. Modifications providing improvements were typically based on the use of the 3-step system EnVision FLEX+ instead of the recommended 2-step system EnVision FLEX and similar observations were also seen in runs 52 and 64.
The IR627 RTU format was frequently used on non-validated platforms and in the current assessment, 46 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 28% (13/46), emphasizing that RTU formats should only be used within the intended system. Caution should 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 requires thorough technical calibration and analytical/diagnostic validation. Based on the inferior results consistently observed across three consecutive NordiQC assessments for Calret, the use of the RTU format IR627 on the Dako Omnis is not recommended. Laboratories using the Omnis platform should substitute the IR627 format with an alternative antibody, such as the concentrated mAb clone CAL6, which has demonstrated superior performance on this platform.

The **Leica Biosystems RTU system PA0346** based on the mAb clone CAL6 and for laboratories following VRPS, the pass rate was 100% (21/21) with 86% (18/21) optimal results (see Table 3). Modification of the protocol settings also provided only sufficient but slightly reduced optimal results (63%). 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.

Summary
This was the eighth NordiQC assessment of Calret (see Graph 1). The pass rate increased from 76% in run 64 to 85% in this assessment run 76 even though the number of participants has increased with 69 new laboratories.
From the previous runs, a constant increase in laboratories using the RTU products was observed going from 73% (196/269) in run 52 (2018) and 80% (272/339) in run 64 (2022) to 85% (348/408) in this run. A 100% pass rate was obtained when using RTU systems from Leica Biosystems and Roche/Ventana following the vendor recommended protocol settings. No RTU for the Dako Omnis platform is currently available, and across three consecutive NordiQC assessments for Calret, the use of the RTU format IR627 for Autostainer is not recommended on the Omnis platform, but the mAb clone CAL6 has obtained optimal results.

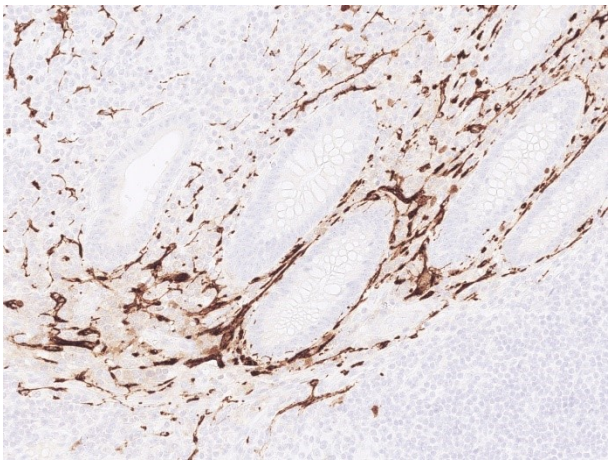


Fig. 1a
Optimal Calret staining of the appendix mucosa using the rmAb clone SP65 in an RTU format (790-4467, Ventana/Roche) using the vendor recommended protocol settings on the Ventana BenchMark Ultra. A strong, distinct cytoplasmic and nuclear staining reaction of the peripheral nerves (ganglion cells and axons) and macrophages is seen. As expected, no staining reaction is observed in the columnar epithelial cells.

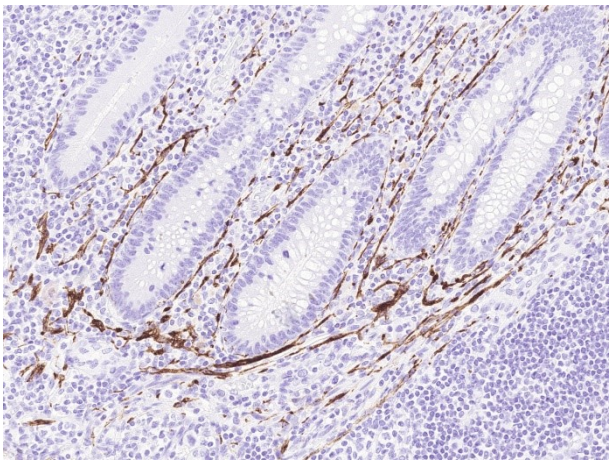


Fig. 1b
Calret staining of the appendix mucosa using the mAb clone DAK-Calret 1 in RTU format for Autostainer but applied on Dako Omnis. Although the peripheral nerves display a moderate to strong intensity, the protocol is challenged due to the use of DAK-Calret 1 on the Omnis, and thus, peripheral nerves give a false impression (too easy to stain) of the analytical sensitivity of the protocol applied.

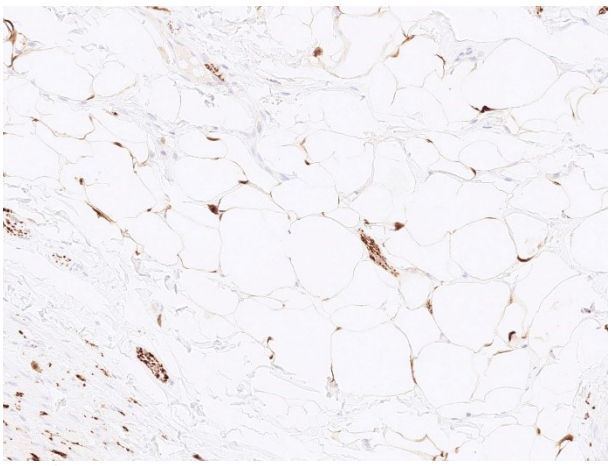


Fig. 2a
Optimal Calret staining of the appendix submucosa using the same protocol as in Fig. 1a. A weak to moderate nuclear staining reaction is seen in virtually all adipocytes.

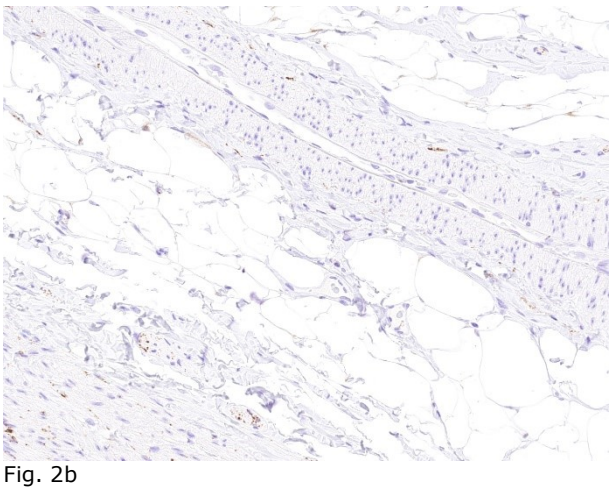


Fig. 2b
Insufficient Calret staining of the appendix submucosa using same protocol as in Fig. 1b. Virtually all adipocytes are false negative. Compare with Fig. 2a for optimal result - same field.

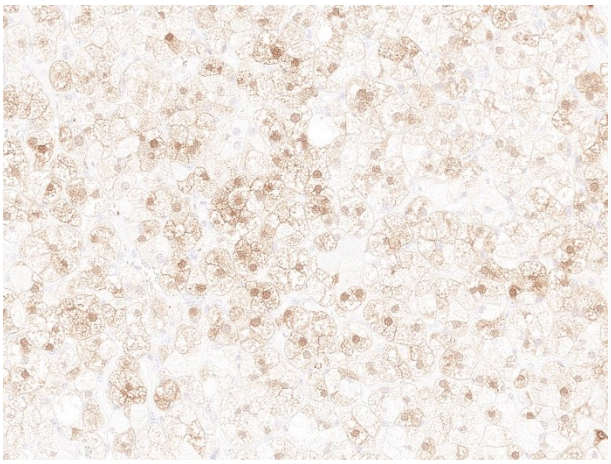


Fig. 3a
Optimal Calret staining of the adrenal gland (low-level expressor) using the same protocol as in Figs. 1a – 2a. A weak to moderate, but distinct cytoplasmic and nuclear staining reaction of most of the cortical epithelial cells is seen.

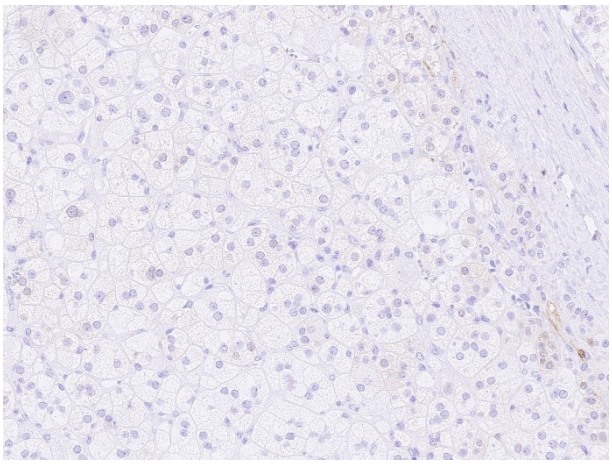


Fig. 3b
Insufficient Calret staining of the adrenal gland (low-level expressor) the same protocol as in Figs. 1b – 2b. The cortical epithelial cells are false negative or only show a barely visible cytoplasmic staining reaction. Compare with Fig. 3a for optimal result - same field.

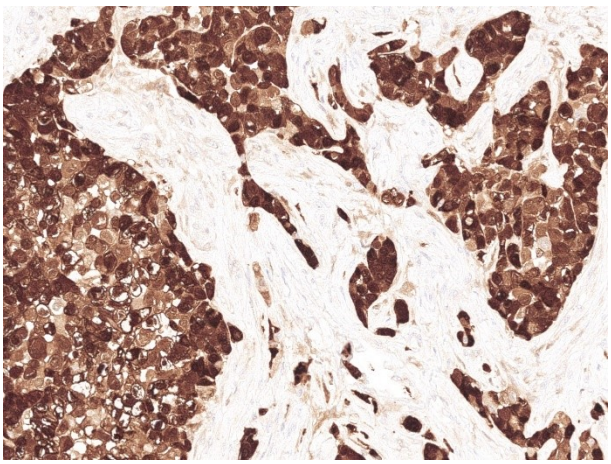


Fig. 4a
Optimal Calret staining of the mesothelioma using the same protocol as in Figs. 1a - 3a. All neoplastic cells display a strong, distinct cytoplasmic and nuclear staining reaction.

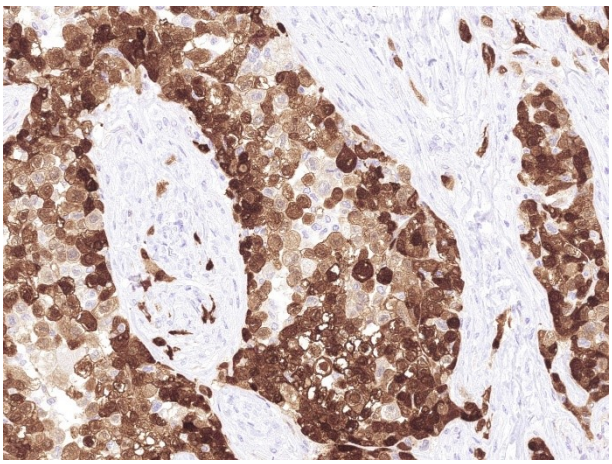


Fig. 4b
Calret staining of the mesothelioma using the same protocol as in Figs. 1b - 3b. The intensity of the staining reaction is slightly reduced. Compare with Fig. 4a – same area.

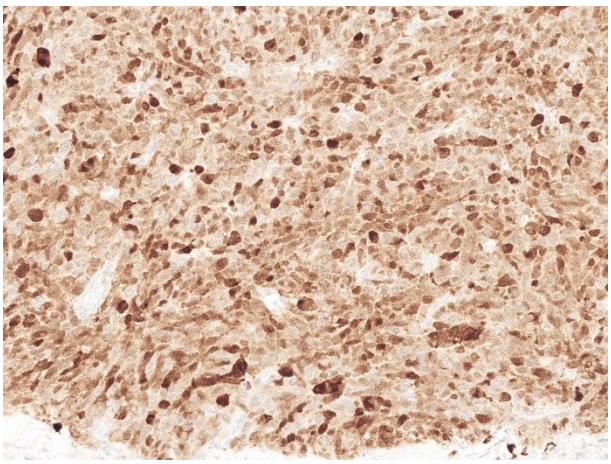


Fig. 5a
Optimal Calret staining of the granulosa cell tumour using the same protocol as in Fig. 1a - 4a. Virtually all the neoplastic cells show an at least weak to moderate, but distinct cytoplasmic and nuclear staining reaction.

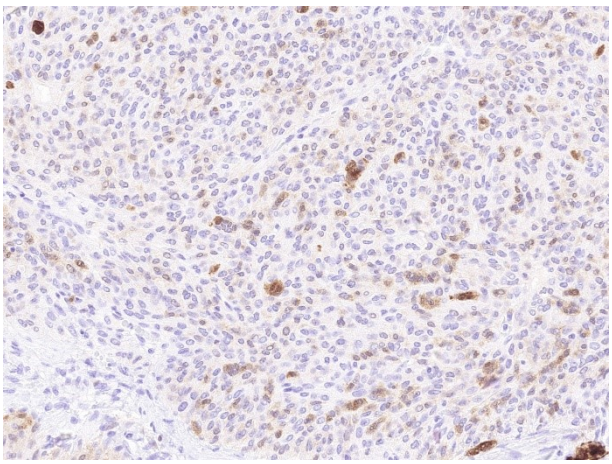


Fig. 5b
Insufficient Calret staining of the granulosa cell tumour using the same protocol as in Figs. 1b - 4b.
A too weak, predominantly cytoplasmic staining reaction in a reduced fraction of the neoplastic cells is seen. Also, a significant proportion of the neoplastic cells are false negative. Compare with Fig. 5a for optimal result - same field.

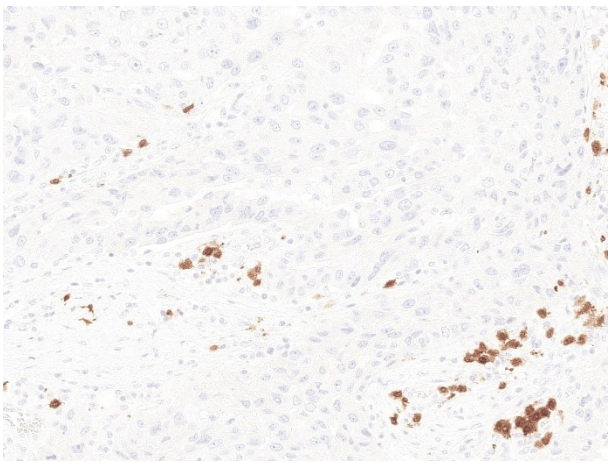


Fig. 6a
Optimal Calret staining of the lung adenocarcinoma using the same protocol as in Figs. 1a - 5a. The neoplastic cells are as expected negative, whereas dispersed macrophages display a strong, but distinct cytoplasmic and nuclear staining reaction.

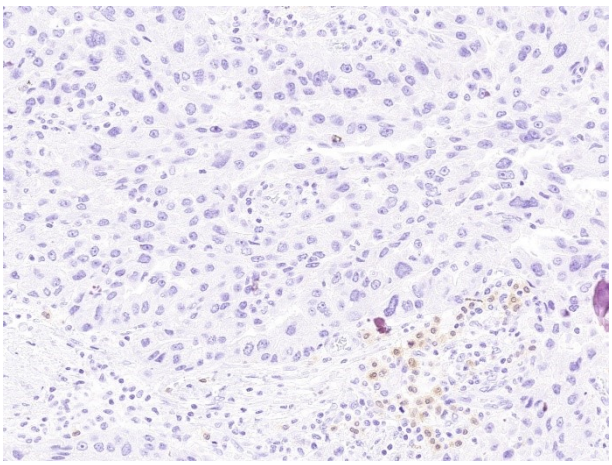


Fig. 6b
Insufficient Calret staining of the lung adenocarcinoma using the same protocol as in Figs. 1b – 5b. Although the neoplastic cells are negative as expected, the protocol provided an overall too low analytical sensitivity and here the macrophages are only faintly demonstrated.
Compare with Fig. 6a for optimal result - same field.

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