## Material
The slide to be stained for S100 comprised:

1. Appendix, 2. Tonsil, 3. Schwannoma, 4-5. Malignant melanoma,
6. Colon adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a S100 staining as optimal included:

- A strong, distinct nuclear and cytoplasmic staining reaction of Schwann
  cells of peripheral nerve fibres and ganglionic satellite cells in the muscularis propria and
  submucosa in the appendix.
- A moderate to strong, distinct nuclear and cytoplasmic staining reaction of adipocytes and
  macrophages in all specimens.
- A strong, distinct nuclear and cytoplasmic staining reaction of virtually all neoplastic cells of the
  malignant melanomas (cores 4-5) and the Schwannoma.
- A weak to moderate, cytoplasmic and nuclear staining reaction of the follicular dendritic cells in the
  germinal centres of the tonsil and the Peyer's plaques in the appendix.
- No staining of other cells. The neoplastic cells in the colon adenocarcinoma, squamous epithelial
  cells in the tonsil, smooth muscle cells and columnar epithelial cells in the appendix should be
  negative.
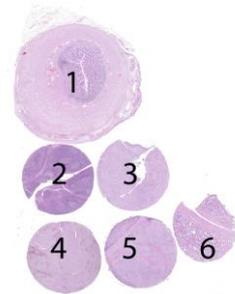
Participation

Number of laboratories registered for S100, run 50	316
Number of laboratories returning slides	299 (95%)

Results
299 laboratories participated in this assessment. 245 (82%) achieved a sufficient mark (optimal or good).
Table 1 summarizes the antibodies (Abs) used and the assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Less successful Ready-To-Use system based on the mAb clone 4C4.9 (Roche/Ventana)
- Insufficient HIER (too low temperature and/or too short efficient heating time)
- Proteolytic pre-treatment or omission of epitope retrieval
- Unexplained technical issues

Performance history
This was the fifth NordiQC assessment of S100. The overall pass rate was higher compared to all previous
runs for S100 (see Table 2).

Table 2. **Proportion of sufficient results for S100 in the five NordiQC runs performed**

	Run 7 2003	Run 20 2007	Run 34 2012	Run 45 2015	Run 50 2017
Participants, n=	63	106	200	251	299
Sufficient results	71%	75%	64%	68%	82%

Conclusion
pAbs both as concentrated and Ready-To-Use (RTU) formats were most successful for
immunohistochemical demonstration of S100. Laboratory developed assays based on pAb **Z0311** provided
the highest proportion of optimal results. Irrespective of the primary Ab applied, efficient HIER, use of
appropriate titre and incubation time tailored to the choice of IHC system were the most important
prerequisites for a sufficient staining result. Omission of HIER and/or use of proteolytic pre-treatment
provided inferior performance.
Appendix is recommended as positive and negative tissue control: The Schwann cells of peripheral nerves,
macrophages/dendritic cells and adipocytes must show a moderate to strong distinct nuclear and

cytoplasmic staining reaction. No staining reaction must be seen in other cell types including smooth muscle cells in lamina muscularis propria and columnar epithelial cells of the appendix.

In addition, tonsil should also be included as positive control. Virtually all interfollicular dendritic cells and Langerhans cells of the squamous epithelium must show a moderate to strong staining intensity whereas the follicular dendritic cells (meshwork) must at least display a weak to moderate but distinct nuclear and cytoplasmic staining reaction (latter only for pAbs).

Table 1. **Antibodies and assessment marks for S100, run 50**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4C4.9**	2 2 2 1	Immunologic Zytomed Systems Cell Marque Thermo/NeoMarkers	0	3	4	0	43%	-
mAb clone **15E2E2**	1 1	Biogenex Biocare	0	1	1	0	-	-
mAb clone **15E2E2+4C4.9**	1	Biocare	0	1	0	0	-	-
pAb **Z0311**	137	Agilent/Dako	62	60	14	1	89%	97%
pAb **NCL-L-S100p**	10	Leica/Novocastra	1	6	3	0	70%	100%
pAb **RB-9018-P**	1	Thermo/NeoMarkers	0	0	1	0	-	-
pAb **RP035**	1	Diagnostic Biosystems	0	0	1	0	-	-
Unknown	1	-	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **4C4.9 790-2914**	36	Roche/Ventana	0	20	16	0	56%	-
mAb clone **4C4.9 330M-18**	2	Cell Marque	0	2	0	0	-	-
mAb clone **4C4.9 MAD-001221QD**	3	Master Diagnostica	0	2	1	0	-	-
mAb clone **4C4.9 MON-RTU1191**	1	Monosan/Sanbio	0	1	0	0	-	-
mAb clone **4C4.9 KIT-0007**	1	Maixin	0	0	1	0	-	-
mAb clone **15E2E2+4C4.9 PM089**	1	Biocare	0	1	0	0	-	-
rmAb clone **EP32 AN713**	1	Biogenex	0	1	0	0	-	-
rmAb clone **EP32 8442-C010**	1	Sakura	0	1	0	0	-	-
pAb **IS/IR504**	26	Agilent/Dako	0	22	4	0	85%	-
pAb **IS/IR504**[3]	5	Agilent/Dako	0	5	0	0	100%	-
pAb **GA504**	21	Agilent/Dako	1	19	1	0	95%	100%
pAb **GA504**[4]	6	Agilent/Dako	3	2	0	1	83%	-
pAb **760-2523**	28	Roche/Ventana	0	23	2	3	82%	-
pAb **PA0900**	6	Leica/Novocastra	0	6	0	0	100%	-
pAb **E031**	1	Linaris	0	1	0	0	-	-
Total	299		67	178	49	5	-	
Proportion			23%	59%	16%	2%	82%	

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 Agilent/Dako semi-automatic system (Autostainer) but used by laboratories on different platforms (e.g. Leica BOND III).
4) RTU system developed for the Agilent/Dako full-automated systems (Omnis) but used by laboratories on different platforms (e.g. Ventana Benchmark) or manually.

Detailed analysis of S100, Run 50
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
pAb **Z0311**: Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (CC1, Ventana) (39/68)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) or TRS pH 9 (11/27), Tris-EDTA/EGTA pH 9 (3/4), Tris-HCL pH 9 (1/1), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/9), Citrate pH 6 (1/2), TRS pH 6 (Dako) (1/2) or TRS pH 6.1 (3-in-1) (Dako) (2/3) as retrieval buffer. The pAb was typically diluted in the range of 1:1,000-1:4,000 depending on the total sensitivity of the protocol employed. Using these protocol settings, 76 of 78 (97%) laboratories produced a sufficient staining result (optimal or good). One protocol with an optimal result was based on no pre-treatment at all.
* (number of optimal results/number of laboratories using this HIER buffer)

pAb **NCL-L-S100p**: One protocol with an optimal result was based on HIER for 32 min. using CC1 (1/2) as retrieval buffer. The pAb was diluted 1:1,000, OptiView was used as detection system and IHC performed on BenchMark Ultra (Ventana).

Table 3. **Proportion of optimal results for S100 for the most commonly used antibody as concentrate on the 3 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
pAb **Z311**	6/11** (55%)	0/1	3/4	-	30/46 (65%)	-	3/6 (50%)	0/3

* 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
pAb, product no. **GA504**, Agilent/Dako, Omnis:
One protocol with an optimal result was based on HIER using TRS High pH 9 (GV804) (efficient heating time 30 min. at 97°C) and 17.5 min. incubation of the primary Ab and EnVision FLEX (GV800) as detection system. Using these protocol settings, 14 of 14 (100%) laboratories produced a sufficient staining result.

Table 4 summarises 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 basic protocol settings. Only protocols performed on the specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for S100 for the most commonly used RTU IHC systems**

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS pAb **IS/IR504**	80% (8/10)	0% (0/10)	88% (14/16)	0% (0/16)
Dako Omnis pAb **GA504**	100% (15/15)	7% (1/15)	83% (5/6)	0% (0/6)
Leica BOND MAX/III pAb **PA0900**	0% (0/0)	0% (0/0)	100% (6/6)	0% (0/6)
VMS Ultra/XT pAb **760-2523**	100% (6/6)	0% (0/6)	77% (17/22)	0% (0/22)
VMS Ultra/XT mAb 4C4.9 **790-2914**	33% (1/3)	0% (0/3)	58% (19/33)	0% (0/33)

* 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 S100 assessments, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 57% (31 of 54) of the insufficient results. The

majority of participating laboratories were able to demonstrate S100 in Schwann cells of peripheral nerves, whereas the demonstration of S100 in the follicular dendritic cells of the germinal centres in the tonsil and the Peyer's plaques in the appendix was more challenging and only seen with appropriate protocol settings. This follicular dendritic cell meshwork must at least display a weak to moderate staining intensity and is in concordance with the guidelines and requirements for an optimal S100 staining reaction given by the International Ad Hoc Expert Committee (Appl Immunohistochem Mol Morphol. 2015 Jan;23(1):1-18.). It was observed, that lack of reaction of this meshwork resulted in weaker or too weak staining intensity of the Schwannoma and both malignant melanomas – especially in the melanoma tissue core no. 4.

For the remaining insufficient results, the staining patterns were characterized by a poor signal-to-noise ratio and/or false positive staining reaction compromising interpretation.

54% (160 of 299) of the laboratories used concentrated Ab format within laboratory developed (LD) assays for S100. The pAb Z0311 was the most widely used Ab and could be used to obtain optimal staining results as shown in Tables 1 and 3. Used within a LD assay, the pAb Z0311 gave an overall pass rate of 89% (122 of 137) and 45% (62 of 137) were optimal.

HIER, preferable in an alkaline buffer, in combination with a careful calibration of the primary Ab seem to be the most critical parameters for sufficient and optimal results. Omission of HIER or performing proteolytic pre-treatment was less successful. Using HIER and a titer of the primary Ab Z0311 in the range of 1:1,000-1:4,000, 97% (74 of 76) of the protocols provided a sufficient result of which 61% (46 of 76) was optimal. In comparison, using the same primary Ab and titer range but omitting HIER or using proteolytic pre-treatment, 75 % (9 of 12) of protocols gave an sufficient result of which only 8% (1 of 12) were assessed as optimal.

In this run, there was no significant difference in the overall performance related to the choice of the detection system. Using optimal protocol settings for the pAb Z0311 as concentrate, the pass rates for 2- and 3-step multimer/polymer detection systems were 96% (47 of 49) and 100% (27 of 27) of which 59% (29 of 49) and 63% (17 of 27) were optimal.

Precise calibration of the primary Ab was central for optimal performance. If HIER was used in combination with a titer below 1:1,000, a poor signal-to-noise ratio was frequently seen hampering the interpretation. If a titer in the range of 1:5,000-10,000 was used, a general too weak staining reaction was seen.

Ten laboratories used the pAb NCL-L-S100p within a LD assay and 70% (7 of 10) produced a sufficient result of which 10% (1 of 10) were assessed as optimal. The single protocol with an optimal result was based on HIER (CC1, Ventana), diluted 1:1,000 (32 min. incubation) and using OptiView (Ventana) as detection system. The pAb was mainly applied on the Leica BOND platforms, BOND-III/MAX (7 of 10), giving an overall pass rate of 72% (5 of 7) of which none were assessed as optimal. The two remaining protocols used proteolytic digestion with the "Bond Enzyme Pre-treatment KIT" and both were assessed as insufficient (borderline).

46% (139 of 299) of the laboratories used Ready-To-Use (RTU) systems for detection of S100. Although the pass rate was relative high for the RTU systems based on pAb IS/IR/GA504 (Dako), pAb 760-2523 (Ventana) and pAb PA0900 (Leica), the proportion of optimal results was very low, if used as "true plug and play systems" from the respective vendors/manufacturers (see Table 1). Grouped together, only one laboratory (1%, 1 of 81) obtained an optimal score. In general, too weak or completely absent staining reaction of the follicular dendritic cell meshwork was seen. In addition the neoplastic cells of the Schwannoma and malignant melanomas showed a reduced intensity. As shown in Table 4, the laboratory modified protocol settings for pAb IS/IR/GA504 (Dako) and pAb 760-2523 (Ventana) provided a relatively high proportion of sufficient results but no optimal results. Despite using protocols with enhanced analytical sensitivity no significant improvement was seen. Re-calibration of the RTU formats of the primary Abs from the respective manufacturers, focusing on low-level expressing cellular structures should be considered.

For the RTU system pAb 760-2523 (Ventana), the parameters causing insufficient results were typically too short HIER time in CC1 or omission of HIER. For the RTU systems pAb IS/IR/GA504 (Dako), the parameters causing insufficient results were typically omission of HIER or unexplained technical issues.

None of the participants using the RTU system based on the pAb 0900 (Leica) used the vendor recommended protocol settings based on enzymatic pre-treatment. All laboratory modified protocols, typically adjusting pre-treatment (e.g. using HIER instead of enzymatic digestion) and/or prolonged incubation time in primary ab, provided a sufficient staining result (all were assessed as good).

The RTU system 790-2914 (Ventana) based on the mAb 4C4.9, gave a low proportion of sufficient results of which none were optimal (see Table 1). The majority of laboratories used modified protocol settings (see table 4). The typical pattern of insufficient results was either a too weak staining intensity of cellular

structures expected to be demonstrated or a false positive staining reaction of e.g. smooth muscle cells of lamina muscularis propria in the appendix. If the RTU format was used with HIER in CC1 for 8 min., any incubation time with the primary Ab and UltraView or OptiView as the detection system a pass rate of 87% (13 of 15 protocols) was seen. Using the same conditions but with omission of HIER, 56% (5 of 9 protocols) provided an insufficient result caused by a too weak staining reaction. Finally, and again applying the same conditions as listed above but with an average HIER time of 27 min. (range 16-36 min.), 83% (10 of 12 protocols) provided an insufficient result due to poor-signal-to noise ration and/or false positive staining of cells expected to be negative. The problem is illustrated is figs. 6-7. On the Benchmark XT/Ultra, the protocol recommendations for this RTU product is based on HIER for 16-36 min. in CC1 depending on the total sensitivity of the detection system in use (UltraView or OptiView) and a short incubation time of the primary Ab for 4 min. Using these protocol settings, 33% (1 of 3) obtained a sufficient mark (good).

This was the fifth assessment of S100 in NordiQC (Table 2). A pass rate of 82% was obtained, which is a significant improvement compared to 68% in run 45, 2015. However, caution must be taken as only 23% of the protocols submitted provided an optimal result and especially the performance of the RTU systems as "true plug-and–play assays" needs to be optimized. The frequent use of non-HIER methods (omission of retrieval or proteolytic pre-treatment) by participants also influenced the overall pass rate and proportion of optimal staining results. Altogether, 14% (41 of 298) of the protocols assessed were based on non-HIER methods and 68% (28 of 41) produced a sufficient mark of which only 2% (1 of 41) were optimal. In comparison, 86% (258 of 299) of the protocols assessed were based on HIER methods and 84% (217 of 258) obtained a sufficient mark of which 26% (66 of 258) were optimal. In this run, the overall pass rate of the RTU systems based on pAbs from the three major vendors was comparable to LD assays based on pAb Z0311 as concentrate. However, the proportion of optimal results was significant higher within the LD assays (pAb Z0311) as 45% (62 of 137) were assessed as optimal compared to 1% for the RTU systems.

Controls
Appendix and tonsil is recommended as positive and negative tissue controls for S100. In the appendix virtually all adipocytes, Schwann cells and dendritic cells must be stained as strongly as possible without any staining reaction of the smooth muscle or epithelial cells. In the tonsil, interfollicular dendritic cells and Langerhans cells of the squamous epithelium, must display a moderate to strong staining intensity whereas the follicular dendritic cell meshwork of the germinal centers must show an at least weak to moderate nuclear and cytoplasmic staining reaction.
As a supplement, the Ad Hoc Committee (see ref. above) also recommends the use of pancreas as positive tissue control in which endocrine cells of islets of Langerhans must show weak to strong cytoplasmic and nuclear staining reaction (all Abs for S100).
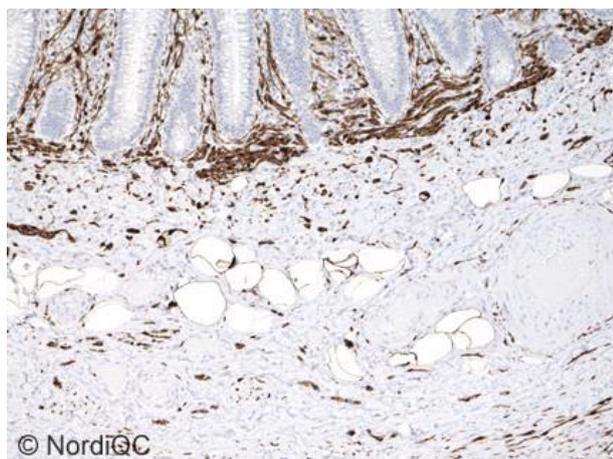

Fig. 1a (x100)
Optimal staining reaction for S100 of the appendix using the pAb Z0311 as concentrate, HIER in an alkaline buffer (TRS pH 9) and a polymer based detection system (FLEX, Agilent/Dako) - same protocol used in Figs. 2a - 4a. The lipocytes and Schwann cells of the peripheral nerves show a moderate to strong staining reaction without any background staining - compare with Fig.1b.
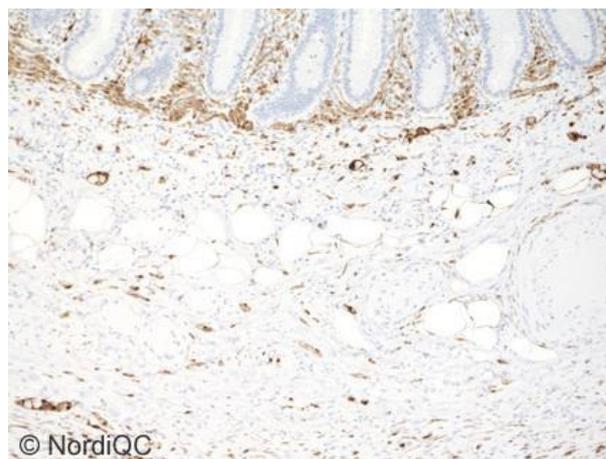

Fig. 1b (x100)
Insufficient staining reaction for S100 of the appendix using the pAb Z0311 as concentrate (too diluted), HIER in an alkaline buffer (too short time) and a polymer based detection system (FLEX, Agilent/Dako) - same protocol used in Figs. 2b - 4b. The intensity of the staining reaction is significantly reduced and the majority of lipocytes are negative or only show a weak staining reaction - compare with Fig. 1a (same field).
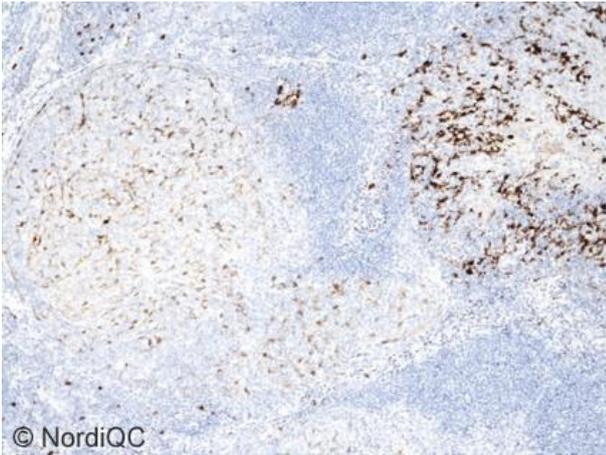
Fig. 2a (x100)
Optimal staining reaction for S100 of the tonsil using the same protocol as in Fig. 1a. The majority of the interfollicular dendritic and Langerhans cells in the squamous epithelium (crypts) display strong staining reaction, while the follicular dendritic cells in germinal centres show a weak to moderate but distinct nuclear and cytoplasmic staining reaction - compare with Fig. 2b.
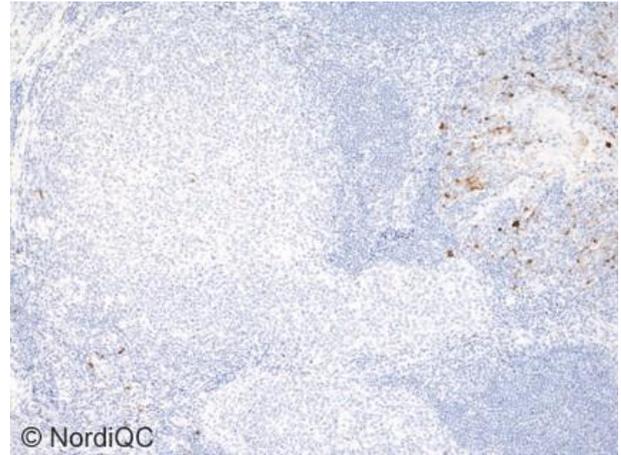

Fig. 2b (x100)
Insufficient staining reaction for S100 of the tonsil using same protocol as in Fig. 1b - same field as in Fig. 2a. The proportion of positive cells and the intensity of the staining reaction are significantly reduced.
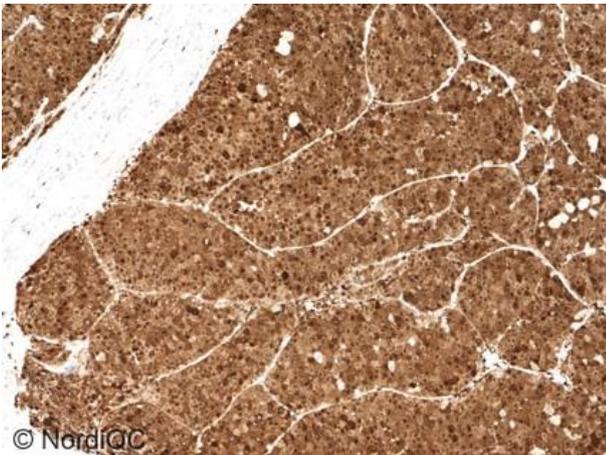
Fig. 3a (x100)
Optimal staining reaction for S100 of the malignant melanoma, tissue core no. 4, using same protocol as in Figs. 1a and 2a. All neoplastic cells show a strong nuclear and cytoplasmic staining reaction – compare with Fig. 3b.
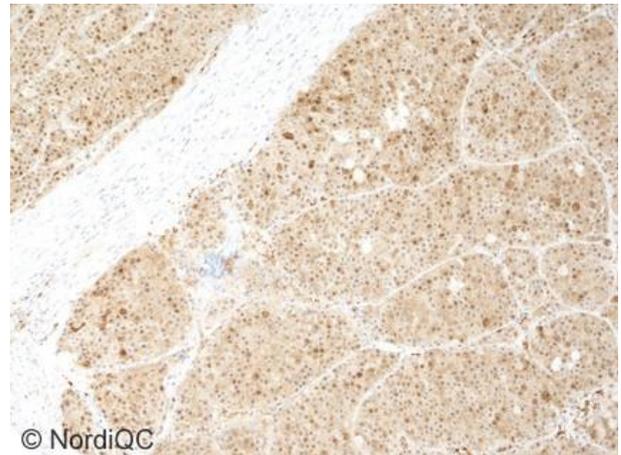

Fig. 3b (x100)
Insufficient staining reaction for S100 of the malignant melanoma, tissue core no. 4, using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The majority of the neoplastic cells display a reduced staining intensity. The tumour cells of this challenging melanoma only display a weak nuclear and cytoplasmic staining reaction.
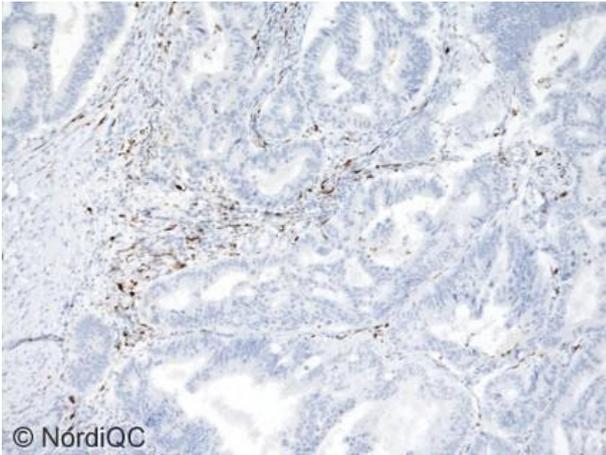
Fig. 4a (x100)
Optimal staining reaction for S100 of the colon adenocarcinoma using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal macrophages show a strong, distinct nuclear and cytoplasmic staining reaction – compare with Fig. 4b.
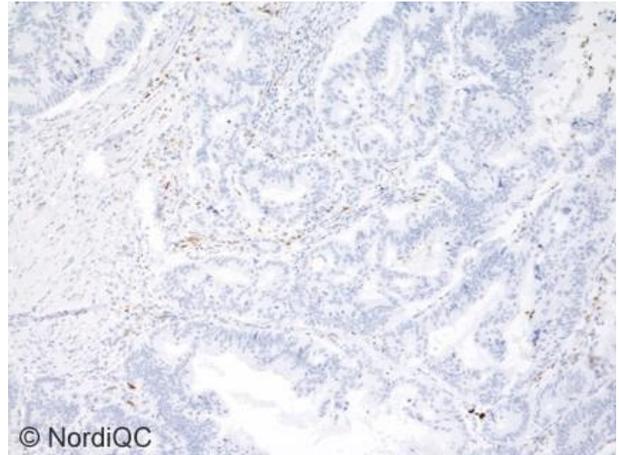

Fig. 4b (x100)
Insufficient staining reaction for S100 of the colon adenocarcinoma, using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
Only few scattered stromal macrophages are identified and display a faint staining reaction.
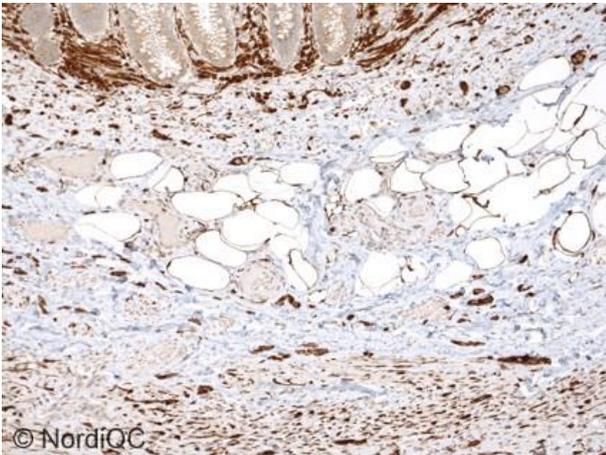
Fig. 5a (x100)
Insufficient staining reaction for S100 of the appendix using the pAb Z0311 as concentrate (too high concentration), HIER in an alkaline buffer (TRS pH9) and a polymer based detection system (FLEX, Agilent/Dako). Both the lipocytes and Schwann cells of the peripheral nerves are strongly stained but in addition smooth muscle cells in the muscularis propria and the epithelium of the appendix show a weak to moderate non-specific staining reaction - compare with Fig. 1a.
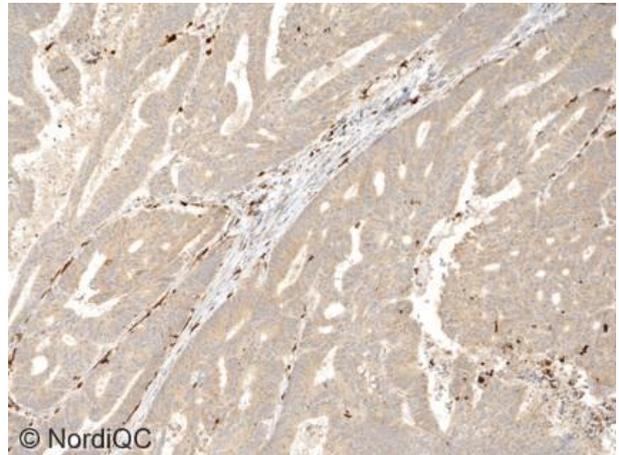
Fig. 5b (x100)
Insufficient staining reaction for S100 of the colon adenocarcinoma using the same protocol as in Fig. 5a. The stromal macrophages show a strong, distinct nuclear and cytoplasmic staining reaction but the neoplastic cells of the colon adenocarcinoma display an aberrant and false positive cytoplasmic staining reaction - compare with Fig. 4a.
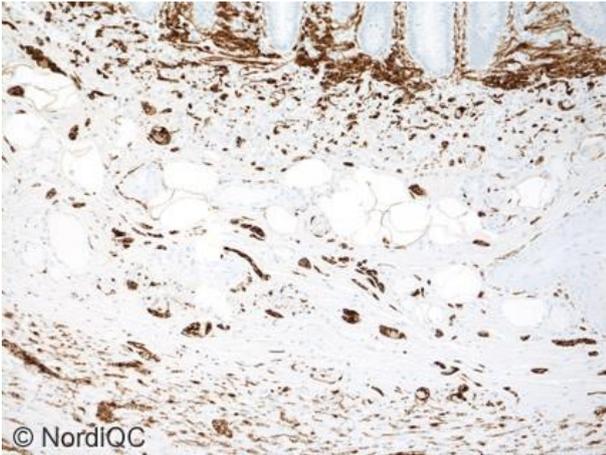
Fig. 6a (x100)
Insufficient staining reaction for S100 of the appendix using the mAb clone 4C4.9 as RTU format (790-2914) with no pre-treatment and UltraView (Roche/Ventana) as the detection system and performed on the Ventana Benchmark Ultra. The lipocytes only show a faint staining reaction and also the melanoma, tissue core no. 4, was inadequately demonstrated – see Fig. 6b.
This pattern with too low analytical sensitivity was typically seen using the protocol settings listed. If the RTU format was applied with HIER for more than 8 min. a poor signal-to-noise ratio was seen - see Figs. 6b, 7a and 7.b and read the description of the problem in the report –also compare with the optimal result in Fig. 1a.

Fig. 6b (x100)
Insufficient staining reaction for S100 of the malignant melanoma, tissue core no. 4, using the same protocol as in Fig. 6a. The neoplastic cells only display a faint to weak predominantly nuclear staining reaction – compare with the optimal result shown in Fig. 3a.
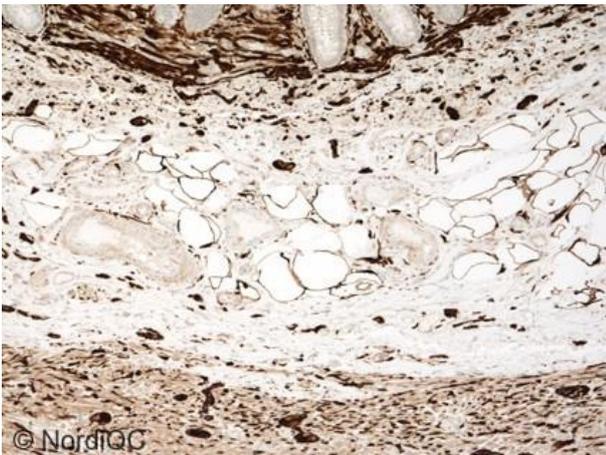

Fig. 7a (x400)
Insufficient staining reaction for S100 of the appendix using the mAb clone 4C4.9 as RTU format (790-2914) with HIER in CC1 (36 min.) and UltraView (Roche/Ventana) as detection system and performed on the Ventana Benchmark Ultra. An aberrant cytoplasmic staining reaction in e.g. smooth muscle cells of the vascular structures and the epithelium of the appendix complicates the interpretation. For this RTU system, prolonged HIER time (>8 min.) typically provided a false positive staining result. 83% of the protocols assessed using these conditions were giving an insufficient mark - compare with the optimal result in Fig. 1a.
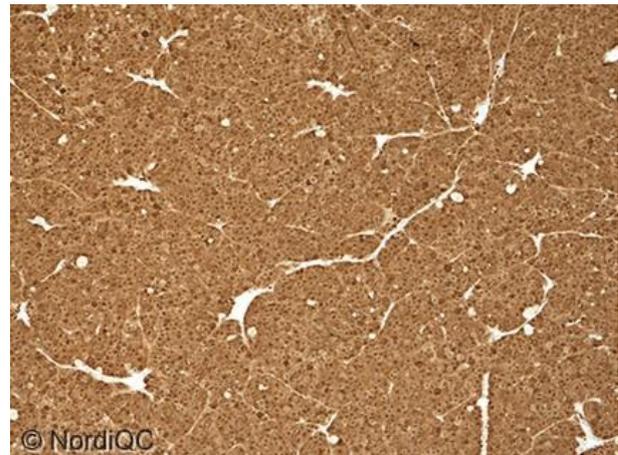

Fig. 7b (x400)
Insufficient staining reaction for S100 of the malignant melanoma, tissue core no. 4, using the same protocol as in Fig. 7a. Although, the neoplastic cells displays a strong staining intensity as expected, the assay is inadequate due to a false positive staining result in cellular structures expected to be negative (see Fig. 7a). Therefore, it is strongly advisable to include adequate control material (both positive and negative tissue controls) to elucidate if the assay has the appropriate analytical sensitivity and specificity.

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