Alpha-smooth muscle actin (ASMA)

Purpose

Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC assays for ASMA performed by the NordiQC participants, identifying smooth muscular origin of cancers of unknown origin and differentiation between leiomyoma and schwannoma. Relevant clinical tissues, both normal and neoplastic disorders, were selected to display a broad spectrum of antigen expression for ASMA (see below).

Material

The slide to be stained for ASMA comprised:

1. Appendix, 2. Tonsil, 3. Liver, 4. Leiomyoma, 5. Leiomyosarcoma, 6. Schwannoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing an ASMA staining as optimal included:

- A strong, distinct cytoplasmic staining reaction of all smooth muscle cells in the muscularis propria, lamina muscularis mucosae and myofibroblasts lining crypts and surface epithelium of the appendix.
- An at least weak to moderate, distinct cytoplasmic staining reaction of the majority of perisinusoidal cells (hepatic stellate cells) in the liver.
- A strong, distinct cytoplasmic staining reaction of all neoplastic cells in the leiomyosarcoma and leiomyoma.
- A strong, distinct cytoplasmic staining reaction of smooth muscle cells throughout the specimens in the block (e.g. vessels).
- No more than a weak, focal staining reaction (<10%) in the neoplastic cells in the schwannoma.
- No staining reaction of other cells, including lymphocytes (all specimens), squamous epithelial cells of the tonsils, columnar epithelial cells of the appendix and hepatocytes in the liver.

Participation

Number of laboratories registered for ASMA, run 59	323
Number of laboratories returning slides	262* (81%)

*Two laboratories used an inappropriate antibody. Data is not included below.

The number of laboratories returning slides has decreased in this run 59 compared to previous assessments, due to the COVID-19 pandemic. All slides returned after the assessment will be assessed, and receive advice if the result is insufficient but will not be included in this report.

Results

262 laboratories returned slides for this assessment. Two of these used an inappropriate Ab as a pan-muscle marker mAb clone HHF35 or mAb clone SMMS-1 for heavy chain myosin. Of the remaining 260 laboratories, 180 (69%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 3).

The most frequent causes of insufficient staining reactions were:
- Poor performance of the mAb clone 1A4 on the fully automated stainer system from Ventana (BenchMark)
- Too high concentration of the primary Ab
- Too low concentration of the primary Ab
- Omission of HIER

Performance history
This was the sixth NordiQC assessment of ASMA. The pass rates have constantly been relatively low throughout all runs. A pass rate of 69% was seen, which is the highest level obtained in all NordiQC assessments of ASMA (see Graph 1).

Graph 2. **Proportion of sufficient results for ASMA in the six NordiQC runs performed**

ASMA performance in NordiQC assessments
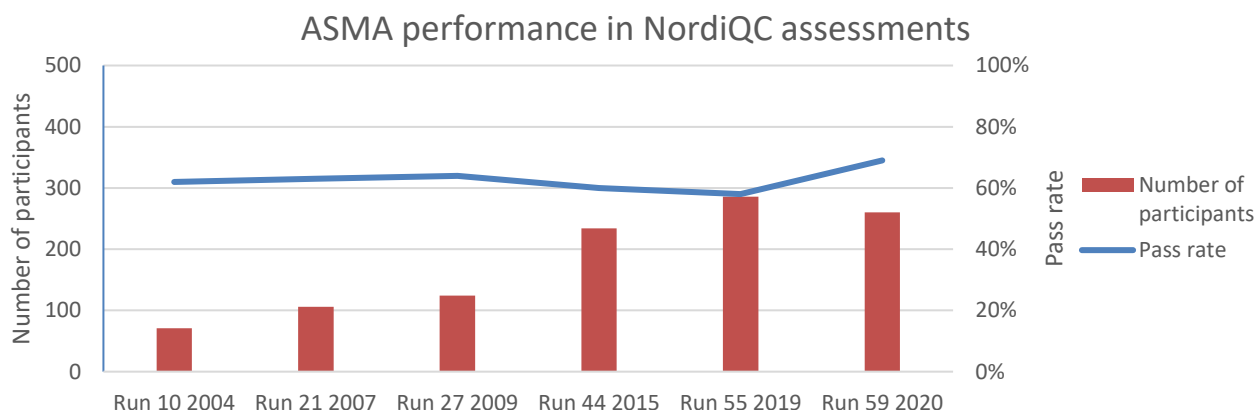
Conclusion
The mAb clones **1A4**, **BS66, asm-1** and rmAb clone **EP188** could all be used to obtain an optimal staining result. The mAb clone 1A4 was used by the majority of laboratories.
The performance of assays based on the mAb clone 1A4, both as concentrated format and RTU systems, were challenged when applied on the fully automated IHC platform BenchMark (Ventana) giving only three optimal staining results. For the mAb clone 1A4, the majority of insufficient staining results were characterized by an aberrant false positive nuclear staining reaction, which was mostly seen when the clone was applied on the BenchMark (Ventana) platform with HIER as pre-treatment. The recently launched and very promising mAb clone BS66 was observed to provide unreproducible results, as different staining results was obtained when applying exactly same protocol settings. The product was during the assessment terminated by the vendor due to lot-to-lot inconsistency.
Irrespective of the Ab, HIER in an alkaline buffer and careful calibration of the titre of the primary antibody were the main prerequisites for optimal results. The Ready-To-Use (RTU) systems from Dako and Leica based on mAb clone 1A4 and asm-1, respectively, were in this assessment most successful and provided high proportions of sufficient and optimal results.

Appendix and liver are recommendable positive and negative tissue controls for ASMA. Virtually all smooth muscle cells in vessels, appendiceal muscularis mucosae and lamina propria must show a moderate to strong cytoplasmic staining reaction, while the vast majority of perisinusoidal cells (hepatic stellate cells) in the liver must show an at least weak to moderate staining reaction. No staining reaction should be seen in appendiceal columnar epithelial cells, lymphocytes or liver cells.

Table 1. **Antibodies and assessment marks for ASMA, run 59**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **1A4**	62 6 4 1 1 2 1 1 1 1 1	Agilent/Dako Cell Marque Sigma Aldrich Thermo Fisher Scientific Zytomed Systems Biocare Genemed Diagnostic Biosystems Spring Bioscience Abcam Zeta Corporation	28	34	14	5	77%	35%
mAb clone **asm-1**	4	Leica/Novocastra	1	3	-	-	-	-
mAb clone **BS66**	50	Nordic Biosite	19	16	15	-	70%	38%
rmAb clone **EP188**	11 2	Epitomics Cell Marque	1	6	6	-	54%	8%
Ready-To-Use antibodies								
mAb clone **1A4 IR/IS611 (VRPS)**[3]	7	Agilent/Dako	4	2	-	1	86%	57%
mAb clone **1A4 IR/IS611 (LMPS)**[4]	17	Agilent/Dako	3	10	4	-	77%	18%
mAb clone **1A4 GA611 (VRPS)**[3]	12	Agilent/Dako	7	5	-	-	100%	58%
mAb clone **1A4 GA611 (LMPS)**[4]	12	Agilent/Dako	4	6	1	1	83%	33%
mAb clone **1A4 760-2833 (VRPS)**[3]	4	Ventana/Roche	-	-	3	1	-	-
mAb clone **1A4 760-2833 (LMPS)**[4]	40	Ventana/Roche	-	18	13	9	45%	0%
mAb clone **1A4 202M-9x**	6	Cell Marque	-	-	4	2	0%	0%
mAb clone **1A4 8292-C010**	1	Sakura Finetek	1	-	-	-	-	-
mAb clone **1A4 MAD-001195QD**	2	Master Diagnostica	-	2	-	-	-	-
mAb **1A4 28-10M**	1	Biogenex	-	1	-	-	-	-
mAb clone **asm-1 PA0943 (VRPS)**[3]	4	Leica Biosystems	4	-	-	-	-	-
mAb clone **asm-1 PA0943 (LMPS)**[4]	6	Leica Biosystems	2	3	-	1	83%	33%
Total	260		74	106	60	20		
Proportion			28%	41%	23%	8%	69%	

1) Proportion of sufficient stains (optimal or good) (≥5 asessed protocols).
2) Proportion of Optimal Results (≥5 asessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 asessed protocols).

Detailed analysis of ASMA, Run 59
The following protocol parameters were central to obtain optimal staining:

mAb clone **1A4**: 28 of 81 (35%) protocols were assessed as optimal, of which 59 laboratories used Heat Induced Epitope Retrieval (HIER) as pretreatment, while 22 did not perform any pretreatment. Protocols with optimal results based on HIER used Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (9/12)*, TRS, High pH (Dako) (2/6), Cell Conditioning 1 (CC1, Ventana) (3/18), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (6/14), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (2/3) or Tris-EDTA pH 9 (3/6) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:500 (Agilent/Dako, Genemed) or 1:27,000 (Sigma Aldrich). Using these protocol settings, 44 of 59 (83%) laboratories produced a sufficient staining result, 25 (42%) optimal.
* (number of optimal results/number of laboratories using this HIER buffer)

Protocols without HIER, but same dilution range as above, provided a sufficient staining result in 75% (15 of 20), but only 15% (3 of 20) optimal.

mAb clone **BS66**: 19 of 50 (38%) protocols were assessed as optimal, of which 13 laboratories used HIER as single pretreatment, while 6 used HIER pretreatment followed by proteolysis.
Protocols with optimal results based on HIER as single pretreatment used TRS pH 9 (Dako) (8/13) and CC1 (Ventana) (5/25) as retrieval buffer. The mAb was typically diluted in the range of 1:500-1:3,200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 23 of 30 (77%) laboratories produced a sufficient staining result.
Protocols with optimal results based on a combined pretreatment used HIER in CC1 and Protease 3 (Ventana) (6/10). The mAb was diluted in the range of 1:200-1:400. Using these protocol settings, 7 of 8 (88%) laboratories produced a sufficient staining result.

rmAb clone **EP188**: One protocol with an optimal result was based on combined pre-treatment using proteolysis (Protease 2 (Ventana) for 4 min.) followed by HIER in CC1 (Ventana) for 32 min. The rmAb was diluted 1:200. Using these protocol settings, 2 of 3 laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for ASMA for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibodies	Dako/Agilent Autostainer		Dako/Agilent Omnis		Ventana/Roche BenchMark 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 **1A4**	9/12 (75%)	-	2/5 (40%)	-	3/15 (20%)	-	6/13 (46%)	2/2
mAb clone **BS66**	-	-	8/12 (67%)	-	11/32 (34%)	-	-	-

* 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 **1A4**, product no. **IS611/IR611**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 20-30 min. at 97°C), 10-30 min. incubation of the primary Ab and EnVision FLEX (K8000) as detection system. Using these protocol settings, 9 of 11 (82%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **1A4**, product no. **GA611,** Dako, Omnis:
Protocols with optimal results were based on HIER using TRS pH 9 (efficient heating time 30 min. at 97°C), 20-30 min. incubation of the primary Ab and EnVision Flex+ (GV800/GV021) as detection system. Using these protocol settings, 15 of 15 (100%) laboratories produced a sufficient staining result.

mAb clone **asm-1**, product no. **PA0943**, Leica, Bond Max, Bond III:
Protocols with optimal results were typically based on HIER using BERS1 or BERS2 (efficient heating time 10-20 min. at 100°C) or no pretreatment, 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.
Using these protocol settings, 9 of 9 (100%) laboratories produced a sufficient staining result.

mAb clone **1A4**, product no. **8292-C010**, Sakura Finetek, Tissue-Tek Genie Advanced Stainer:
One protocol with an optimal result was based on HIER using Tissue-Tek Genie High pH Antigen Retrieval Solution (efficient heating time 60 min. at 98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro Detection Kit, DAB (8826-K250) as detection system.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 asessed protocols). The performance was evaluated both as "true" plug-and-play systems performed accordingly 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 4. **Proportion of sufficient and optimal results for ASMA for the most commonly used RTU IHC systems**

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako Autostainer Link 48+ mAb 1A4 **IR611**	6/7 (86%)	4/7 (57%)	3/4	2/4
Dako Omnis mAb 1A4 **GA611**	12/12 (100%)	7/12 (58%)	9/11 (82%)	3/11 (27%)
Ventana BenchMark Ultra/XT/GX mAb 1A4 **760-2833**	0/4	0/4	18/40 (45%)	0/40 (0%)

* 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 were included.

Comments

In this assessment, false positive, too weak or false negative staining reactions were the main features of insufficient results.

In 48% of the insufficient results (38 of 80), a false positive staining reaction was observed either as a cytoplasmic staining reaction in the schwannoma or an aberrant nuclear staining reaction of e.g. lymphocytes in the tonsil. 76% (29 of 38) of the false positive staining reaction was characterized by an aberrant nuclear staining reaction in one or more of the tissues included. This observation was in concordance with previous NordiQC assessments for ASMA. All protocols applied the mAb clone 1A4, typically on a Benchmark platform (Ventana). The aberrant staining reaction was in particular prominent when protocols with high level of technical and analytical sensitivity was applied e.g. high titer of the primary Ab and/or efficient HIER. An aberrant nuclear reaction could also be observed on the Bond platform (Leica) and Autostainer Link 48 (Dako/Agilent) but to a lesser degree. The remaining 24% of the false positive staining reactions were characterized by a distinct and excessive cytoplasmic staining reaction in the majority of neoplastic cells in the schwannoma and epithelial cells of the appendix. This pattern was only observed for the mAb clone BS66 and rmAb clone EP188 and typically within a poorly calibrated protocol.

A weak or false negative staining reaction was seen in 31% (25 of 80) of the insufficient results and was typically caused by protocols with too low technical and analytical sensitivity. The majority of the laboratories were able to demonstrate ASMA in cells with high-level antigen expression as smooth muscle cells in appendiceal muscularis mucosae, smooth muscle cells in large vessels and neoplastic cells of the leiomyosarcoma, whereas demonstration of ASMA in cells with low-level antigen expression as hepatic perisinusoidal cells could only be obtained with an optimally calibrated protocol.
A combination of a weak or false negative staining reaction and the aberrant false positive nuclear staining reaction was observed in 14% of the insufficient results (11 of 80).
Six laboratories (7%) obtained an insufficient staining result because of other technical issues.

57% (148 of 260) of the laboratories used a concentrated format within a laboratory developed (LD) assay for detection of ASMA.

The mAb clone 1A4 was the most widely used concentrated format within a LD assay. The mAb clone 1A4 provided a pass rate of 77% (62 of 81) but only 35% (28 of 81) were assessed as optimal (see Table 1). All protocols assessed as optimal were typically based on HIER (preferable in an alkaline buffer) in combination with a careful calibration of the primary Ab. Both 2- and 3-step polymer-based detection systems could be used to obtain an optimal result. As shown in Table 3, the best performance was obtained on the Dako Autostainer where 75% (9 of 12) of the protocols produced a sufficient result.

The number of participants using the recently launched mAb clone BS66 within a LD-assay increased in this run 59, compared to the last run 55, from 7 to 50. The mAb clone BS66 provided a pass rate of 70% (35 of 50), 38% (19 of 50) optimal. 87% (13 of 15) of the insufficient results were characterized by an excessive false positive cytoplasmic staining reaction in especially the schwannoma but also observed in the epithelial cells of the appendix and finally also displayed as a general reduced signal-to-noise ratio (see Figs. 6a-6b). The false positive staining reaction was in particular prominent when protocols with high level of technical and analytical sensitivity was applied e.g. high titer of the primary Ab and/or efficient HIER. mAb clone BS66 has in the recent assessment run 55 been very successful and in runs 55 and 59 been used as the reference standard method in NordiQC to validate the test performance characteristics and to define the expected reaction patterns for the assessment tissue materials circulated. The Ab has been very promising and in run 55 recommended to participants with insufficient results, as mAb clone BS66 in contrast to mAb clone 1A4 could be used to provide optimal results on all main fully automated IHC

platforms. The inferior performance of mAb clone BS66 and of particular importance the aberrant positive staining reaction of the schwannoma was unexpected and not reproducible. It was observed that e.g. the schwannoma could be either negative or positive applying virtually exactly same protocol settings and titres. During the data analysis no evident reason for the discrepancy could be identified including comparison of e.g. lot-to-lot differences, but only, as mentioned above, too high titer of the Ab might be a contributing factor. During the assessment the vendor of the mAb clone BS66 has informed that the sale has been terminated *"due to decreased specificity and inconsistency between batches"*. This communication from the vendor underlines the observations generated in the assessment, but also introduce a new situation where it is necessary to identify a new reference standard method for ASMA. Finally, it is not possible for NordiQC to suggest protocol recommendations for inferior performance of the mAb clone 1A4 on e.g. BenchMark IHC platforms (Ventana) at the moment.

The rmAb clone EP188 could produce sufficient results on BenchMark Ultra (Ventana), applying a combined pre-treatment using proteolysis in P2 or P3 followed by HIER in CC1. The slides assessed as insufficient used similar protocol settings as protocols providing an optimal result, making it difficult to elucidate on the problems. The insufficient results were characterized by e.g. poor morphology, poor signal-to-noise ratio and in two protocols an aberrant reaction in T-cells were observed.

43% (112 of 260) of the laboratories used an RTU format for the demonstration of ASMA. Ideally, an RTU format of a primary Ab should be used within a system that has been thoroughly validated, providing precise information on vendor recommended protocol settings, equipment, reagents and test performance characteristics (expected reaction patterns).

24 laboratories used the RTU format IS/IR611 based on the mAb clone 1A4. Only seven laboratories used it on the Autostainer Link 48+ (Dako) with recommended protocol settings, with a pass rate of 86% (6 of 7) – see Table 1. 13 laboratories used the IS/IR611 on a different stainer platform. Ten used the Dako Omnis, with a pass rate of 90% (9 of 10).

Using the Dako RTU for Omnis (GA611) with recommended protocol settings, the pass rate was 100% (12 of 12), 58% optimal. When modifying the protocol, a pass rate of 82% was observed, 27% optimal (see Table 4). Five laboratories omitted the use of mouse linker – none obtained optimal results.

The Ventana RTU system based on mAb clone 1A4 was the most widely used RTU system with similar observations as for LD assays. Four laboratories used the RTU as recommended by Ventana, which resulted in no sufficient results (see Table 4). As shown in Table 4, the Ventana RTU system (760-2833) with modified protocol settings obtained a pass rate of 45% (18 of 40) but no optimal results. When calibrating the protocol to increase the technical and analytical sensitivity, an aberrant nuclear staining result was seen. If the protocol was calibrated to avoid the aberrant nuclear staining a reduced analytical sensitivity was seen. Few laboratories added a blocking step after incubation of primary Ab, and it seems to increase the technical and analytical specificity and eliminate/reduce the aberrant nuclear staining reaction and still maintain a sufficient level of analytical sensitivity. However, the protocol settings were only used by few participants, and further validation is needed. For further information contact Ventana/Roche.

The RTU system PA0943 (Leica) based on the mAb clone asm-1 applied as recommended by Leica was very successful and only provided optimal results (see Table 1). When modifying the protocol settings, a pass rate of 100% (5 of 5) was seen, 40% optimal. One laboratory used the RTU on a different platform, with an insufficient result.

Controls
Appendix and liver are recommendable positive and negative tissue controls for ASMA. Virtually all smooth muscle cells in vessels, appendiceal muscularis mucosae and lamina propria must show a moderate to strong cytoplasmic staining reaction, while the vast majority of perisinusoidal cells (hepatic stellate cells) in the liver must show an at least weak to moderate, distinct staining reaction. No staining reaction should be seen in appendiceal columnar epithelial cells, lymphocytes or liver cells.
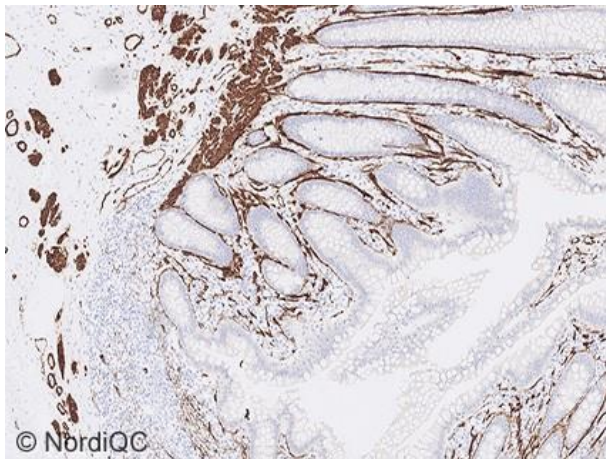
Fig. 1a
Optimal ASMA staining of the appendix using the mAb clone BS66 optimally calibrated, HIER in CC1 (Ventana) combined with proteolysis and a 3-step polymer-based detection system (OptiView) on the BenchMark Ultra (Ventana). Smooth muscle cells of lamina muscularis mucosae and myofibroblasts lining the epithelial crypts show a distinct cytoplasmic staining reaction, while epithelial cells are negative.
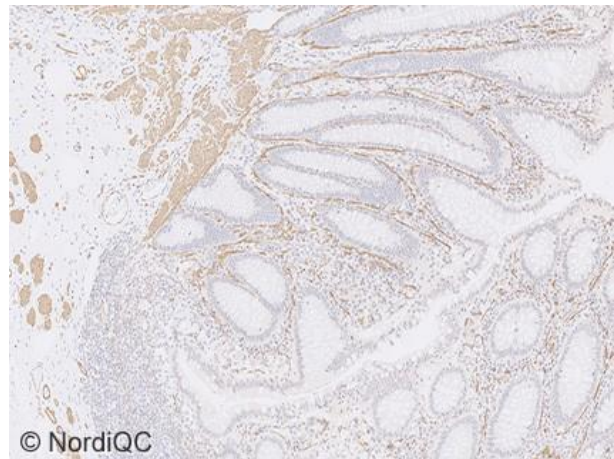Same protocol used in Figs. 2a-5a.

Fig. 1b
ASMA staining of the appendix using the RTU product 760-2833 (Ventana) based on the mAb clone 1A4, protocol settings applied as recommended by Ventana. Same protocol used in Figs. 2b-5b.
Although the staining pattern is similar to the optimal result seen in Fig. 1a (same field), the assay provided a much weaker staining reaction.
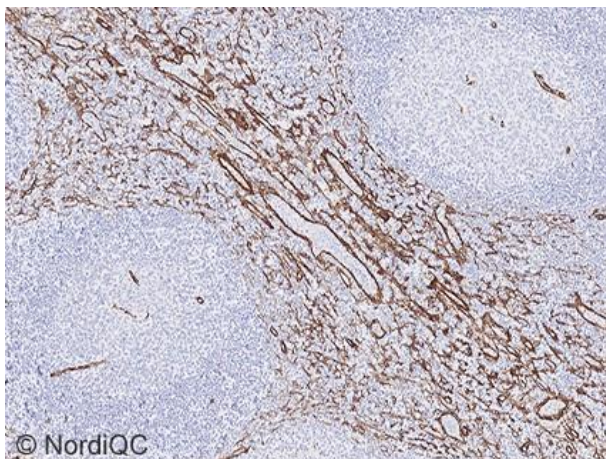
Fig. 2a
Optimal ASMA staining of the tonsil using same protocol as in Fig. 1a. The vast majority of smooth muscle cells in both large and smaller vessels display the expected strong and distinct cytoplasmic staining reaction.
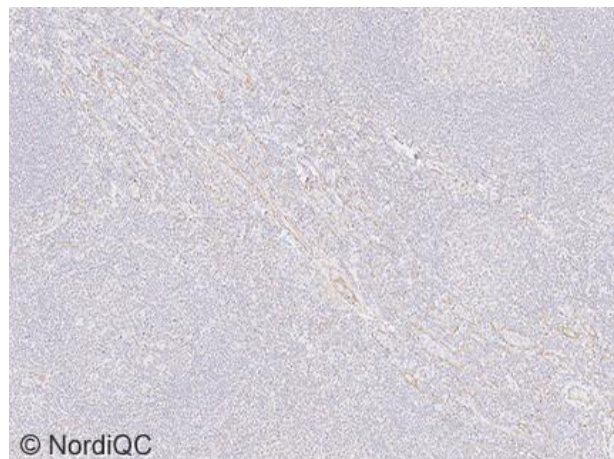
Fig. 2b
Insufficient ASMA staining of the tonsil using same protocol as in Fig. 1b – same field as in Fig. 2a. Virtually all smooth muscle cells are negative or only faintly positive.
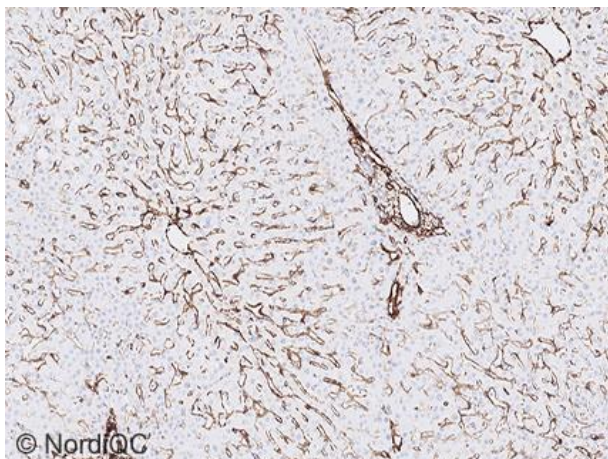
Fig. 3a
Optimal ASMA staining of the liver using same protocol as in Figs. 1a and 2a. The smooth muscle cells of the portal vessels show a moderate to strong staining reaction. Importantly, the vast majority of hepatic stellate cells (perisinusoidal smooth muscle cells) show a distinct, weak to moderate staining reaction. The hepatocytes are negative.
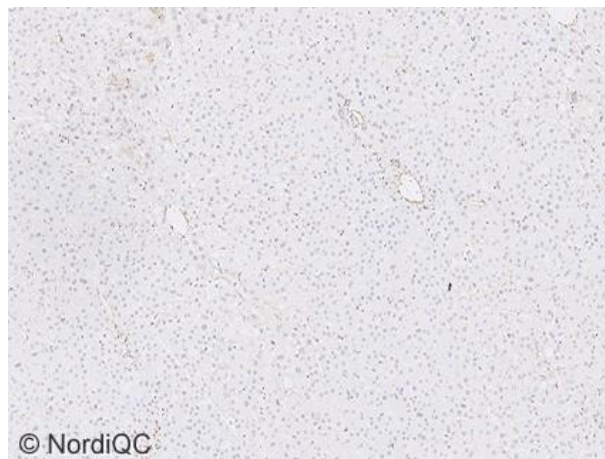

Fig. 3b
Insufficient ASMA staining of the liver using same protocol as in Figs. 1b and 2b. The proportion of positive hepatic stellate cells are significantly reduced and only display a faint staining intensity - same field as in Fig. 3a.
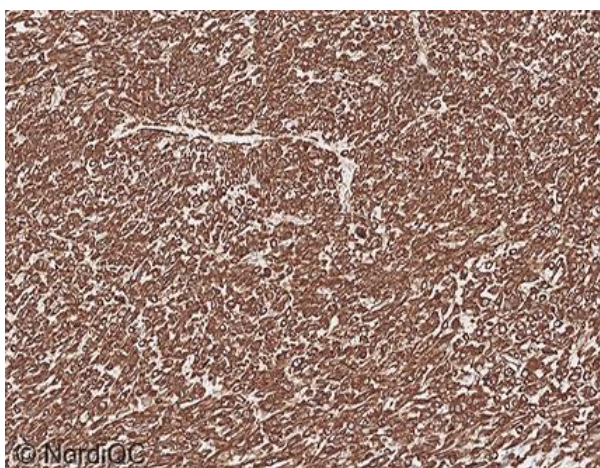
Fig. 4a
Optimal ASMA staining of the leiomyosarcoma using same protocol as in Figs. 1a-3a. Virtually all neoplastic cells show a strong distinct cytoplasmic staining reaction.
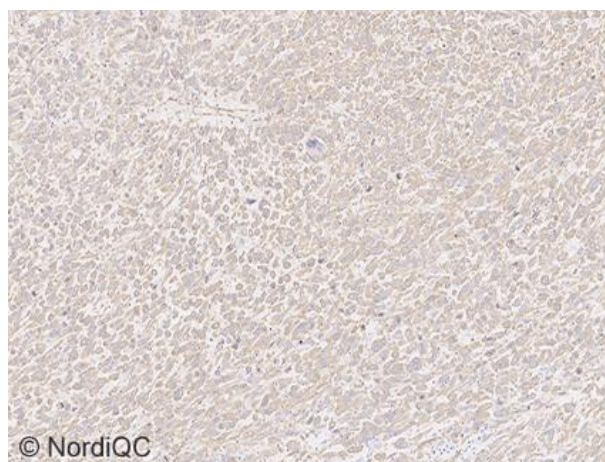

Fig. 4b
Insufficient ASMA staining of the leiomyosarcoma using same protocol as in Figs. 1b-3b. The neoplastic cells only display a weak staining reaction - same field as in Fig. 4a.
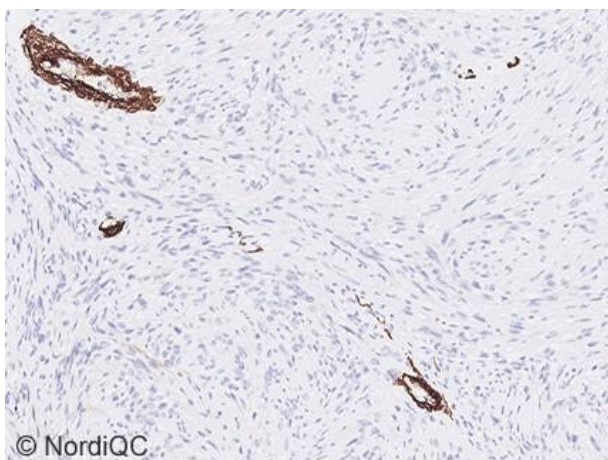

Fig. 5a
Optimal ASMA staining of the schwannoma using the same protocol as in Figs. 1a-4a. All neoplastic cells are negative. Only scattered normal vascular smooth muscle cells display a strong staining intensity.
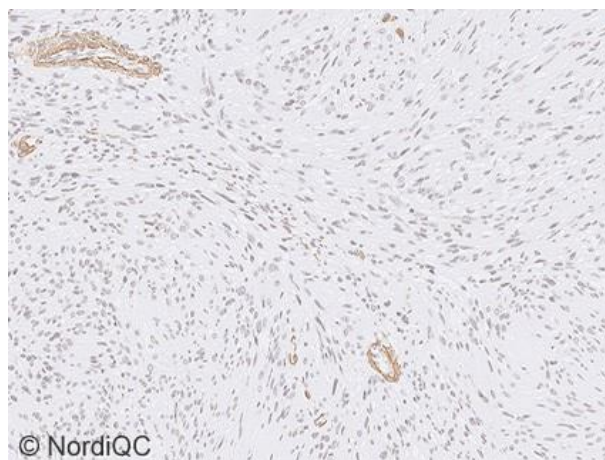

Fig. 5b
Insufficient ASMA staining of the schwannoma using same protocol as in Figs. 1b-4b. An aberrant nuclear staining reaction is seen in the majority of cells - same field as in Fig. 5a.
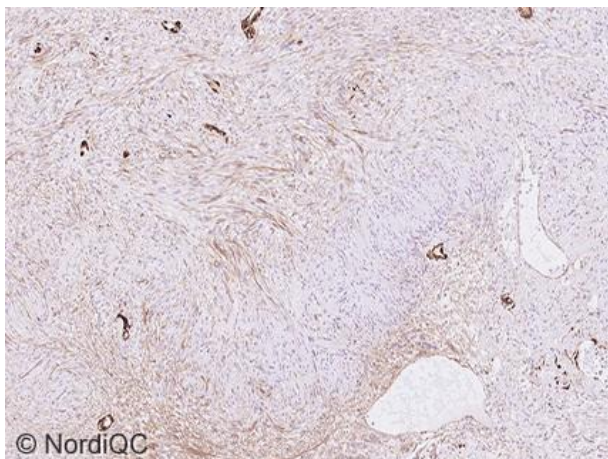
Fig. 6a
Insufficient ASMA staining of the schwannoma using the mAb clone BS66 on the Ventana Benchmark platform, applying a "too sensitive" protocol, primarily caused by a too high concentration of the mAb BS66. The majority of neoplastic cells show a weak cytoplasmic staining reaction – same field as in Fig. 5a.
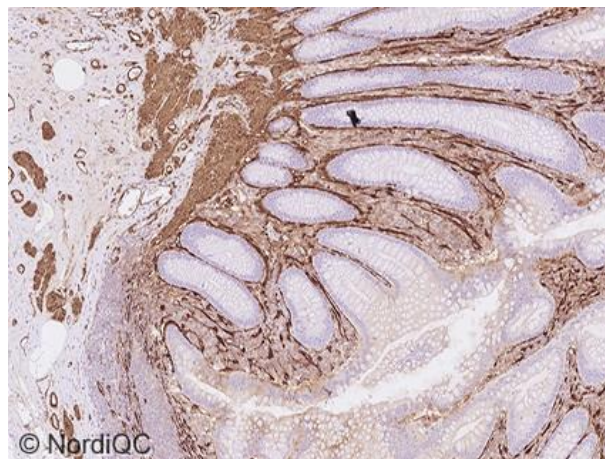

Fig. 6b
Insufficient ASMA staining of the appendix using the same protocol as in Fig. 6a. A false positive staining reaction is seen in some epithelial cells, and a moderate diffuse background reaction is seen in lamina propria – same field as in Fig. 1a.

HLK/RR/LE/SN 26.06.2020