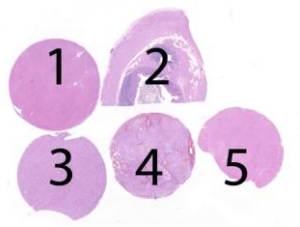
Assessment Run 44 2015

Alpha-smooth muscle actin (ASMA)

Material
The slide to be stained for ASMA comprised:

1. Liver, 2. Appendix, 3. Leiomyosarcoma, 4. GIST, 5. Leiomyoma



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing 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 the perisinusoidal cells (hepatic stellate cells) in the liver
- A moderate to strong, distinct cytoplasmic staining reaction of virtually all neoplastic cells in the leiomyosarcoma and leiomyoma
- An at least weak, distinct cytoplasmic staining reaction of the majority of neoplastic cells in the GIST
- A strong, distinct cytoplasmic staining in smooth muscle cells in virtually all vessels throughout the specimens in the block

Participation

Number of laboratories registered for ASMA, run 44	248
Number of laboratories returning slides	242 (98%)

Results
242 laboratories participated in this assessment. 8 of these used an inappropriate Ab such as a pan-muscle marker (mAb clones HHF35 and SC28). Of the remaining 234 laboratories, 60% achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Omission of HIER
- Too low concentration of the primary antibody
- Less successful performance of the mAb clone 1A4 on Ventana BenchMark stainer platform

Performance history
This was the fourth NordiQC assessment of ASMA and as shown in table 2 the pass rates have been constant at a relatively low level throughout all runs.

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

	Run 10 2004	Run 21 2007	Run 27 2009	Run 44 2015
Participants, n=	71	106	124	234
Sufficient results	62%	63%	64%	60%

Conclusion
The mAb clones **1A4**, **asm-1** and rmAb clone **EP188** could all be used to obtain an optimal staining result. Using the two most widely used ASMA antibodies (clone 1A4 and asm-1) HIER and careful calibration of the titre of the primary antibody were the main prerequisites for optimal results.
The performance of clone 1A4 seems to be influenced by the stainer platform as a significantly reduced proportion of sufficient results was observed when used on the Ventana BenchMark platform compared to Dako Autostainer and Leica BOND platforms.
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 majority of the 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 44**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1A4**	98 6 5 1 1 1 1 1 1 1	Dako Thermo/NeoMarkers Sigma Aldrich AbD Serotec Biocare BioGenex Genemed Immunologic Spring Bioscience Zytomed	34	49	23	10	72%	85%
mAb clone **asm-1**	10	Leica/Novocastra	2	4	4	0	60%	100%
mAb clone **BS66**	1	Nordic Biosite	0	1	0	0	-	-
rmAb clone **E184**	1	Biocare	0	0	0	1	-	-
rmAb clone **EP188**	1	Epitomics	1	0	0	0	-	-
rmAb clone **SP171**	1	Spring Bioscience	0	0	1	0	-	-
Unknown	2	Unknown	1	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **1A4 IR/IS611**	44	Dako	23	13	7	1	82%	91%
mAb clone **1A4 760-2833**	44	Ventana/Cell Marque	0	6	29	9	14%	-
mAb clone **1A4 202M-9x**	3	Cell Marque	0	0	2	1	-	-
mAb clone **1A4 MAD-001195QD**	3	Master Diagnostica	0	0	3	0	-	-
mAb **1A4 PM001**	1	Biocare	0	0	1	0	-	-
mAb clone **1A4 AM128-5M**	1	BioGenex	0	1	0	0	-	-
mAb clone **1A4 Kit-0006**	1	Maixin	0	0	1	0	-	-
mAb clone **asm-1 PA0943**	5	Leica/Novocastra	1	3	1	0	80%	100%
Total	234		62	77	73	22	-	
Proportion			27%	33%	31%	9%	60%	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of ASMA, Run 44
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **1A4**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (8/12)*, TRS pH 9 (Dako) (7/14), Cell Conditioning 1 (CC1, Ventana) (1/32), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (5/10), BERS1 (Leica) (1/3), DIVA Decloaker (Biocare) (2/3) or Tris-EDTA pH9 (9/16) as retrieval buffer. The mAb was typically diluted in the range of 1:7,500-1:27,000 (Sigma Aldrich) and 1:50-1,000 (other vendors e.g. Dako). Using these protocol settings 73 of 86 (85%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **asm-1**: Two protocols with optimal results were both based on HIER using BERS2 (Leica) (2/4) as retrieval buffer. The mAb was diluted in the range of 1:50-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 4 of 4 (100%) laboratories produced a sufficient staining result.

rmAb clone **EP188**: One protocol with an optimal result was based on a combined pre-treatment using proteolysis (Protease 2, Ventana for 4 min.) followed by HIER (CC1 mild (Ventana). The mAb was diluted 1:200 using OptiView + amplification (760-700+760-099, Ventana) as detection system.

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

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	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**	14/25** (56%)	0/2	1/29 (3%)	0/2	5/7 (71%)	1/3

* 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
mAb clone **1A4**, product no. **IS611/IR611**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 95-98°C), 20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings 32 of 35 (91%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **asm-1**, product no. **PA0943**, Leica, Bond Max:
One protocol with an optimal result was based on HIER using BERS2 pH 9 (Bond, Leica) (efficient heating time 20 min. at 100°C), 15 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.

Comments
In this assessment and in concordance with the observations in previous NordiQC assessments of ASMA, the prevalent feature of an insufficient staining reaction was a too weak or false negative staining reaction of the majority of the cells expected to be demonstrated. Too weak or false negative staining reaction was seen in 71% of the insufficient results (67 of 95.) 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 and neoplastic cells of GIST could only be obtained with an optimally calibrated protocol. In 17% of the insufficient results both a too weak specific staining reaction and an aberrant nuclear staining reaction was seen. In the remaining 12% a poor signal-to-noise ratio was seen compromising the interpretation.

The mAb clone 1A4 was the most widely used Ab as concentrated format within a laboratory developed (LD) assay for ASMA. HIER preferable in an alkaline buffer in combination with a careful calibration of the primary Ab seem to be the most critical parameters. Both 2- and 3-step polymer based detection systems could be used to obtain an optimal result. Omission of HIER was one of the main reasons for an insufficient result. 69% (11/16) of protocols omitting HIER gave an insufficient staining result and none were assessed as optimal. The titre of mAb clone 1A4 for an optimal result was highly depending on the analytical sensitivity of the IHC protocol but also on the source of the concentrated format. The Dako format of mAb clone 1A4 thus gave an optimal result if applied in the range of 1:50-1,000, whereas the range was 1:7,000 – 27,000 for the Sigma Aldrich format of mAb 1A4.
As also observed in previous assessments for ASMA, aberrant false positive nuclear staining reaction in liver cells, lymphocytes and epithelial cells was seen. This pattern was mainly observed, when the mAb clone 1A4 was used with HIER in CC1 on the BenchMark stainer platforms, Ventana. The aberrant nuclear reaction was most prominent, when the clone was used relatively concentrated and frequently both a too weak specific staining reaction and an aberrant nuclear staining reaction was observed at the same time. The overall pass rate and proportion of optimal results for mAb clone 1A4 within a LD assay was influenced by the stainer platform as shown in table 3. If the Ab was applied on either the Dako Autostainer system or the Leica Bond system and used as a concentrate in the ranges as listed above with HIER in an alkaline buffer, 59% of the laboratories obtained an optimal staining result with an overall pass rate of 97%. In comparison, only 3% of the laboratories using same clone and similar protocol settings on the Ventana BenchMark platform obtained an optimal result with an overall pass rate of 67%.

Despite limited data available, rmAb clone EP188 might be an alternative to mAb clone 1A4 when using Ventana BenchMark platforms. In this assessment one protocol based on a combined pre-treatment using proteolysis in P2 followed by HIER in CC1 thus provided an optimal staining result on BenchMark ULTRA.

Dako and Ventana Ready-To-Use (RTU) systems based on mAb clone 1A4 were the most widely used RTU systems with similar observations as for LD assays. The Dako RTU system was most successful providing an overall pass rate of 82%. Optimal results could both be obtained by using the official protocol recommendations given by Dako but also by laboratory modified protocol settings (typically adjusting incubation time of the primary Ab and/or choice of detection system).
The Ventana RTU system showed an inferior performance giving a pass rate of 14%, none optimal. Neither official protocol recommendations given by Ventana nor laboratory modified protocol settings gave an optimal 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 majority of the 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.

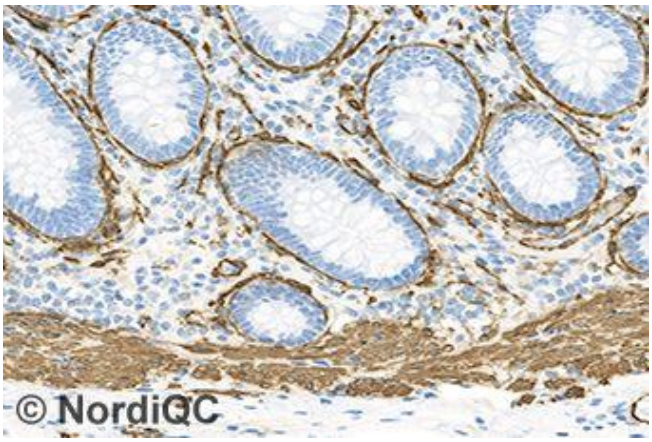


Fig. 1a (x200)
Optimal staining result for ASMA of the appendix using the mAb clone 1A4 as Ready-To-Use format (Dako) with HIER in TRS high pH and performed on the Autostainer Link48 platform (Dako). Both the smooth muscle cells of lamina muscularis mucosae and myofibroblasts lining the epithelial crypts show a distinct staining reaction.

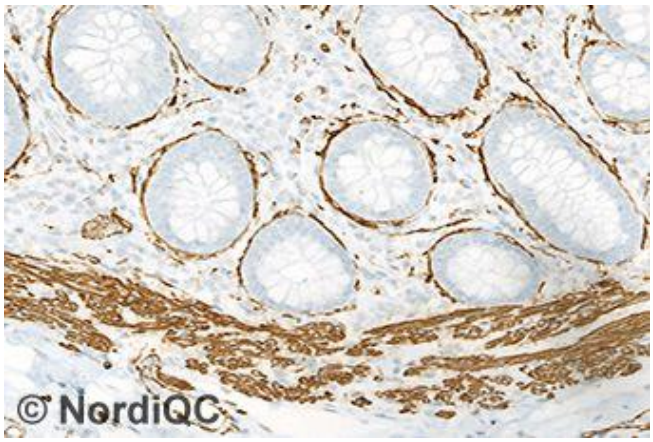


Fig. 1b (x200)
Staining result for ASMA of the appendix using the mAb clone 1A4 as a concentrate. The protocol was based on HIER in CC1 mild and performed on the BenchMark XT (Ventana) using a 2- step multimer based detection system, same field as in Fig. 1a. Both the smooth muscle cells of lamina muscularis mucosae and myofibroblasts lining the epithelial cryps show a distinct staining reaction. However also compare with Figs. 2b – 4b, same protocol. The protocol overall provided a too low sensitivity.

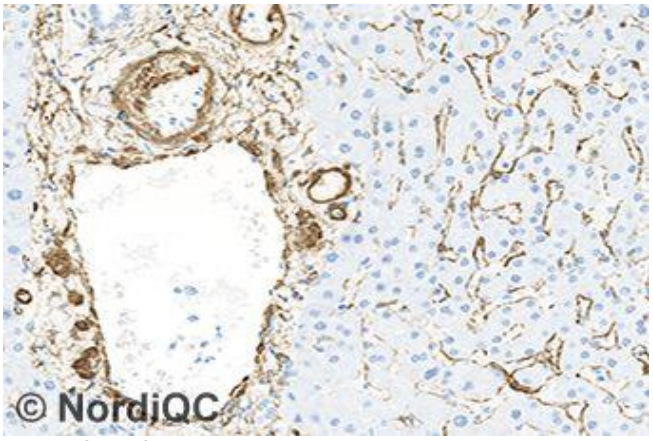


Fig. 2a (x200)
Optimal staining result for ASMA of the liver using same protocol as in Fig. 1a. The smooth muscle cells of the portal vessels show a moderate to strong staining reaction. However, most importantly the vast majority of perisinusoidal smooth muscle cells show a distinct, weak to moderate staining reaction. No background staining is seen.

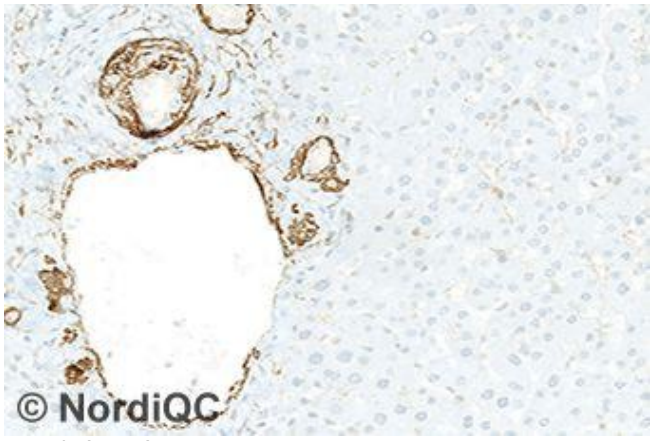


Fig. 2b (x200)
Insufficient staining result for ASMA of the liver using same protocol as in Fig. 1b – same field as in Fig. 2a. Only the smooth muscle cells of the portal vessels are demonstrated, while the perisinusoidal smooth muscle cells are virtually negative. Also compare with Fig. 4b, same protocol.

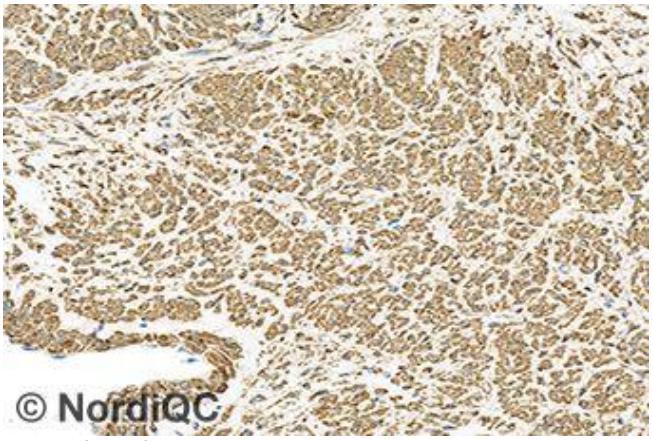


Fig. 3a (x100)
Optimal staining result for ASMA of the leiomyoma using same protocol as in Figs. 1a and 2A. Virtually all the neoplastic cells show a distinct, moderate to strong staining reaction with no background staining.

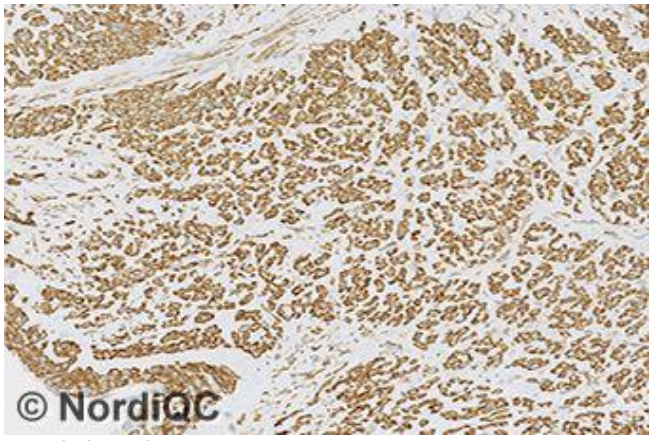


Fig. 3b (x100)
Staining result for ASMA of the leiomyoma using same insufficient protocol as in Figs. 1b and 2b. Virtually all the neoplastic cells show a distinct, moderate to strong staining reaction with no background staining – same field as in Fig. 3a. However, also compare with Fig. 4b – same protocol.

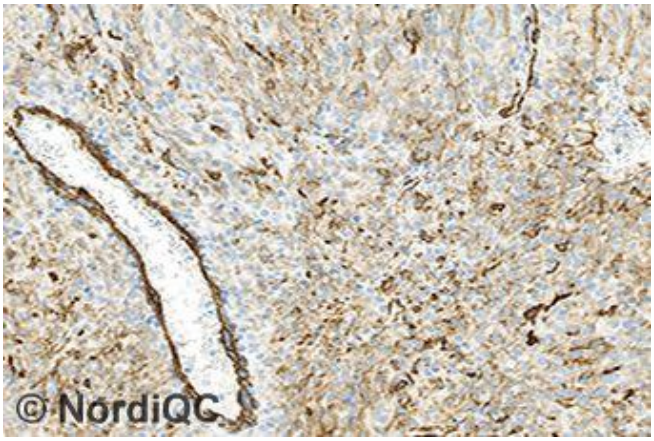


Fig. 4a (x100)
Optimal staining result for ASMA of the GIST using same protocol as in Figs. 1a – 3a. The majority of neoplastic cells show a moderate and distinct staining reaction with no background reaction.

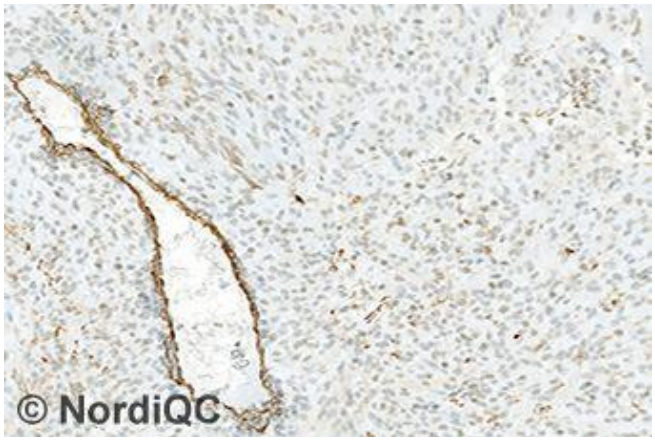


Fig. 4b (x100)
Insufficient staining result of the GIST using same protocol as in Figs. 1b - 3b. Only smooth muscle cells in vessels are demonstrated, while the neoplastic cells are unstained except for a weak diffuse nuclear staining reaction – same field as in Fig. 4a.

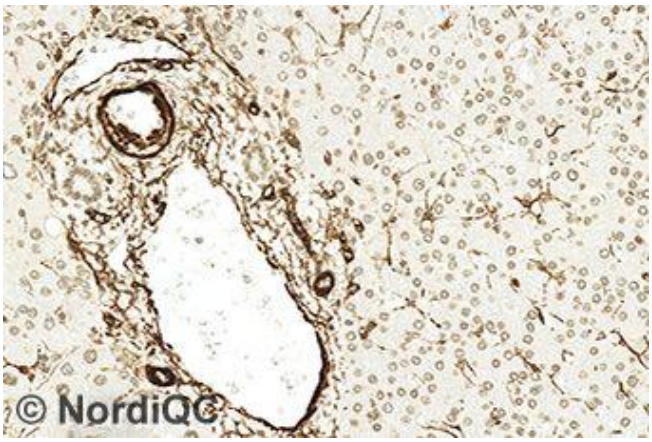


Fig. 5a (x200)
Inappropriate staining result for ASMA of the liver using the mAb clone 1A4 as concentrate on the BenchMark XT (Ventana). The protocol was based on HIER in CC1 standard and a 3-step multimer based detection system (OptiView) increasing the technical sensitivity compared to the protocol used in Figs. 1b – 4b. The perisinusoidal smooth muscle cells are demonstrated, but liver cells and epithelial cells of bile ducts show an aberrant nuclear staining reaction compromising the interpretation. Also compare with Fig. 5b, same protocol.

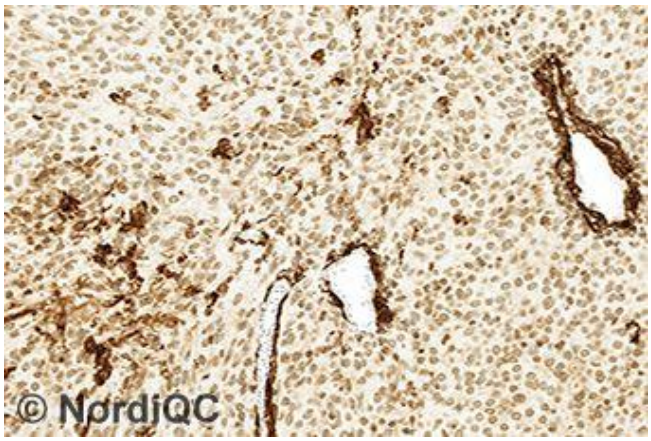


Fig. 5b (x200)
Insufficient staining result for ASMA of the GIST using same protocol as in Fig. 5b. Smooth muscle cells in vessels are demonstrated, whereas the neoplastic cells only show an aberrant nuclear staining reaction and no specific cytoplasmic staining reaction as expected can be identified.

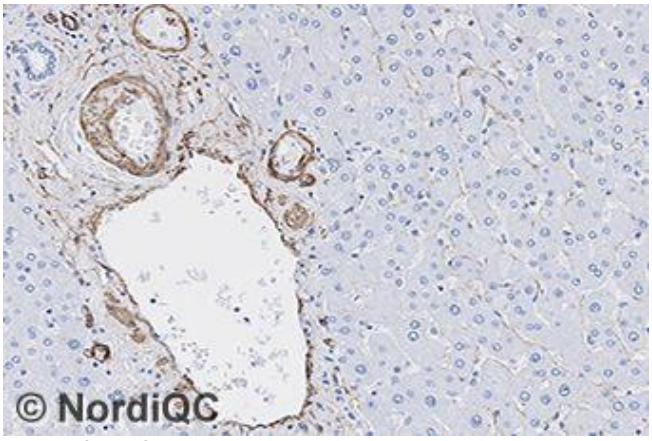


Fig. 6a (x200)
Insufficient ASMA staining of the liver using the mAb clone 1A4 in a protocol omitting HIER – same field as in Fig. 2a. Scattered perisinusoidal smooth muscle cells are demonstrated, but the intensity and proportion of cells is reduced compared to the level expected. Also compare with Fig. 6b, same protocol.

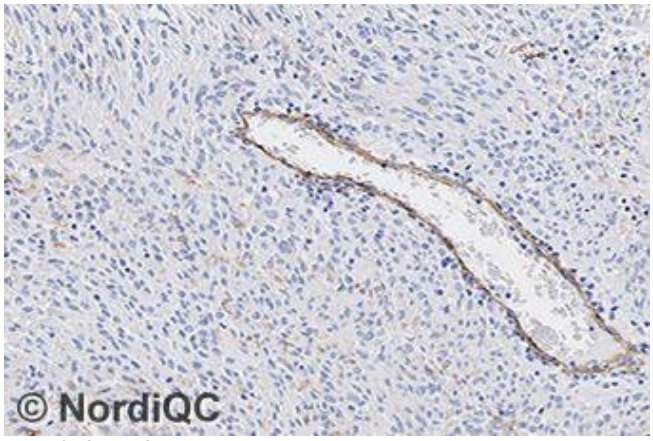


Fig. 6b (x200)
Insufficient staining result for ASMA of the GIST using same protocol as in Fig. 6a. The staining reaction in both smooth muscle cells in vessels and neoplastic cells is reduced compared to the level expected and obtained by optimal protocol settings - compare with Fig. 4a.

SN/RR/LE/MV 24-6-2015