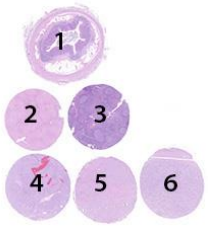
Assessment Run 55 2019 - REVISION

Alpha-smooth muscle actin (ASMA)

Purpose
Evaluation of the immunohistochemical performance with primary focus on level of technical and analytical sensitivity for the demonstration of ASMA. Relevant clinical tissue, both normal and neoplastic disorders, was selected displaying a broad spectrum of antigen expression for ASMA (see below).
This revised assessment of Run 55 2019 included modification of the scoring criteria, keeping the requirements for optimal performance as listed below but accepting a negative staining reaction of the GIST tumour as goodprovided that the assays performed as expected (good or optimal) in all other tissue cores. In the primary official assessment of run 55 a negative result in the GIST was assessed as insufficient.

Material
The slide to be stained for ASMA comprised:



1. Appendix, 2. Liver, 3. Tonsil, 4. Gastrointestinal stromal tumor (GIST),
5. Leiomyoma, 6. Leiomyosarcoma.

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 perisinusoidal cells (hepatic stellate cells) in the liver
- A strong, distinct cytoplasmic staining reaction of all neoplastic cells in the leiomyosarcoma and leiomyoma
- An at least weak, distinct cytoplasmic staining reaction of the neoplastic cells in the GIST
- A strong, distinct cytoplasmic staining reaction of smooth muscle cells throughout the specimens in the block (e.g. vessels)
- 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 55	296
Number of laboratories returning slides	291 (98%)

Results
291 laboratories participated in this assessment. 5 of these used an inappropriate Ab such as a pan-muscle marker mAb clones HHF35 and HUC (1-1). Of the remaining 286 laboratories, 167 (58%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful primary Ab (mAb clones 1A4 and asm-1)
- Poor performance of the mAb clone 1A4 on the fully automated stainer systems from Ventana (BenchMark) or Dako (Omnis)
- Too low concentration of the primary antibody

Performance history
This was the fifth NordiQC assessment of ASMA. The pass rates have constantly been relatively low 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	Run 55 2019
Participants, n=	71	106	124	234	286
Sufficient results	62%	63%	64%	60%	58%

Conclusion
The mAb clones **1A4**, **BS66** 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 but could only produce optimal results on the

Autostainer (Dako) and the BOND (Leica) platform. 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 platforms Omnis (Dako) and BenchMark (Ventana) giving no optimal staining results. In this assessment, the best performance was achieved with the mAb clone BS66 providing a 100% pass rate (7 of 7). The mAb clone BS66 was observed to be very robust and the performance was not influenced by the IHC platform used as optimal results could be obtained on both semi-and fully automated systems as Autostainer, Benchmark and Omnis. HIER in an alkaline buffer and careful calibration of the titre of the primary antibody were the main prerequisites for optimal results. Importantly, laboratories should apply an Ab that work on the in-house IHC platform, calibrate the protocols correctly and stain according to the expected antigen level of the recommended control material (see below).

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 55**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1A4**	104 8 6 5 2 1 1 1 1	Agilent/Dako Cell Marque Sigma Aldrich Thermo/NeoMarkers Zytomed Systems Biocare Genemed Diagnostic Biosystems Spring Bioscience	14	61	39	15	58%	75%
mAb clone **asm-1**	4	Leica/Novocastra	-	2	2	-	-	-
mAb clone **BS66**	7	Nordic Biosite	6	1	-	-	100%	100%
rmAb clone **EP188**	7 3	Epitomics Cell Marque	2	6	2	-	80%	100%
Ready-To-Use antibodies								
mAb clone **1A4 IR/IS611**	21	Agilent/Dako	1	17	1	2	86%	100%
mAb clone **1A4 IR/IS611**[3]	16	Agilent/Dako	-	9	4	3	56%	-
mAb clone **1A4 GA611**	15	Agilent/Dako	-	13	2	-	87%	-
mAb clone **1A4 760-2833**	59	Ventana/Roche	-	19	17	23	32%	-
mAb clone **1A4 202M-9x**	8	Cell Marque	-	2	1	5	25%	-
mAb clone **1A4 8292-C010**	1	Sakura Finetek	1	-	-	-	-	-
mAb clone **1A4 MAD-001195QD**	2	Master Diagnostica	-	1	1	-	-	-
mAb **1A4 PM001**	1	Biocare	-	1	-	-	-	-
mAb **1A4 PM001**[3]	1	Biocare	-	1	-	-	-	-
mAb clone **1A4 Kit-0006**	1	Maixin	1	-	-	-	-	-
mAb clone **asm-1 PA0943**	11	Leica/Novocastra	-	8	1	2	73%	-
Total	286		25	141	70	50		
Proportion			9%	49%	24%	18%	58%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of ASMA, Run 55
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **1A4**: Protocols with optimal results were typically based on Heat Induces Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (4/15)*, TRS ph 6.1 (3-in-1) (Dako) (1/2), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (4/20), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (2/3) or Tris-EDTA pH 9 (3/7) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:600 (Agilent/Dako, Genemed) or 1:27,000 (Sigma Aldrich). Using these protocol settings, 26 of 36 (72%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **BS66**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako) (4/4), Cell Conditioning 1 (CC1, Ventana) (1/1) and Tris-EDTA pH 9 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:150-1:1,500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 6 of 6 (100%) laboratories produced a sufficient staining result.

rmAb clone **EP188**: Protocols with optimal results were typically based on combined pre-treatment using proteolysis (Protease 2 or 3 (Ventana) for 4 min. or 12 min., respectively.) followed by HIER in CC1 (mild, Ventana). The rmAb was diluted 1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 4 of 4 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**	4/13** (31%)	1/2	0/10 (0%)	0/1	0/24 (0%)	0/2	4/9 (44%)	2/3

* 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:
One protocol with an optimal result was based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10 min. at 97°C), 20 min. incubation of the primary Ab and EnVision FLEX (K8000) as detection system. Using these protocol settings, 7 of 7 (100%) laboratories produced a sufficient staining result (optimal or good).

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 15 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 AS mAb 1A4 **IR611**	73% (8/11)	0% (0/11)	100% (10/10)	10% (1/10)
Dako Omnis mAb 1A4 **GA611**	92% (12/13)	0% (0/13)	(1/2)	(0/2)
VMS Ultra/XT/GX mAb 1A4 **760-2833**	(1/3)	(0/3)	32% (18/56)	0% (0/56)
Leica Bond III mAb Asm-1 **PA0943**	100% (6/6)	(0/6)	40% (2/5)	(0/5)

* 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 were included.

Comments
In this assessment, too weak, false negative or false positive staining reactions were the main features of insufficient results. The weak or false negative staining was seen in 42% (51 of 121) of the insufficient staining 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 and neoplastic cells of GIST could only be obtained with an optimally calibrated protocol.
In 40% of the insufficient results (48 of 121), an aberrant nuclear staining reaction of e.g. lymphocytes in the tonsil was seen and mainly observed for protocols based on mAb clone 1A4 and performed on the BenchMark stainer platforms (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.
A combination of the weak or false negative staining and the aberrant false positive nuclear staining pattern was observed in 17% of the insufficient results (20 of 121).
Two laboratories obtained an insufficient staining result because of a technical issue.

The mAb clone 1A4 was the most widely used concentrated format within a laboratory developed (LD) assay for ASMA. The mAb clone 1A4 provided a pass rate of 58% (75 of 129) but only 11% (14 of 129) were assessed as optimal (see Table 1). All protocols assessed as optimal were 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, best performance was obtained on the Dako Autostainer and Leica Bond platforms where 86% (18 of 21) and 81% (22 of 27) of the protocols produced a sufficient result respectively. None of the slides stained on either an Omnis (Dako) or a Benchmark (Ventana) platform provided an optimal result

Although the number of participants using the mAb clone BS66 within a LD-assay was low, the Ab seems to be robust as all protocols (7 of 7) obtained a sufficient staining result (see Table 1). In addition, this primary Ab provided optimal results on both the Omnis (Dako) and Benchmark (Ventana) and thus might be an alternative to the mAb clone 1A4. The Ab could be diluted in a wide spectrum of concentrations (1:150-1:1,500) as long as HIER was performed in alkaline buffer (e.g. CC1, Ventana) and primary Ab was calibrated according to the overall sensitivity of the detection systems applied.

As mentioned in the previous Run 44 (2015), the rmAb clone EP188 could produce optimal results on BenchMark Ultra (Ventana), applying combined pre-treatment using proteolysis in P2 followed by HIER in CC1. In this assessment, both protocols assessed as optimal were based on HIER in CC1 (32 min.) in combination with proteolysis in P2 (4 min.). Staining reactions of the primary Ab (diluted 1:200) were detected using OptiView with or without amplification as the detection system. The slides assessed as insufficient used similar protocol settings as protocols providing an optimal result, making it difficult to elucidate on the problems. However, the rmAb EP188 might still be a better alternative for demonstration of ASMA since it provided a significant higher pass rate (80%, 8 of 10) compared to mAb clone 1A4 (39%, 22 of 57 (all protocol settings)) on the Ventana Benchmark platform.

47% (136 of 287) of the laboratories used an RTU format for detection 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 performance characteristic (expected reaction patterns).
17 laboratories used the RTU format IS/IR611 based on the mAb clone 1A4 off-label (e.g. different protocol settings and platforms than the Autostainer (Dako)) providing a pass rate of 59% (10 of 17), with no optimal result, which was significantly lower compared to an overall pass rate of 85% (17 of 20) if the system was used on the Autostainer +/Link (Dako) (see Table 1 and Table 4).
Using the Dako RTU for Omnis (GA611), although the pass rate was 87% (13 of 15), none of the assays were assessed as optimal. This result indicates that protocols based on the mAb clone 1A4 also are challenged when applied on the fully automated instrument Omnis.

The Ventana RTU systems based on mAb clone 1A4 were the most widely used RTU systems with similar observations as for LD assays. As shown in Table 1, the Ventana RTU system (760-2833) obtained a pass rate of 32% (19 of 59) but no optimal results.

The RTU system PA0943 (Leica) based on the mAb clone asm-1 provided a pass rate of 73% (8 of 11) but, as for the Ventana and Dako Omnis RTUs, no optimal staining results were obtained.

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.

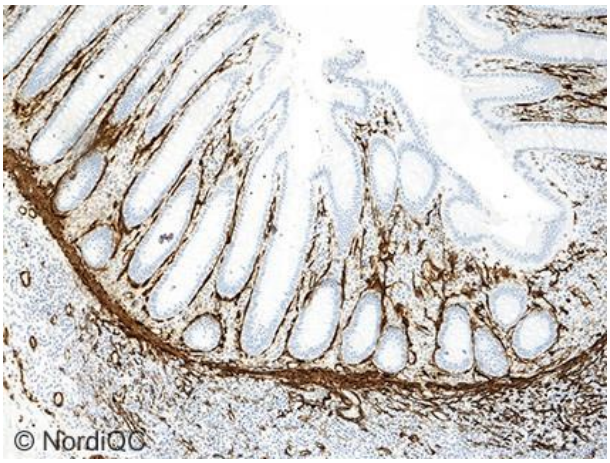


Fig. 1a (x100)
Optimal ASMA staining of the appendix using the mAb clone BS66 optimally calibrated, HIER in TRS (3-in-1) pH 9 (Dako) and a 3-step polymer based detection system (Flex+, Dako) on the Omnis (Dako). Smooth muscle cells of lamina muscularis mucosae and myofibroblasts lining the epithelial crypts show a distinct cytoplasmic staining reaction. Same protocol used in Figs. 2a-5a.

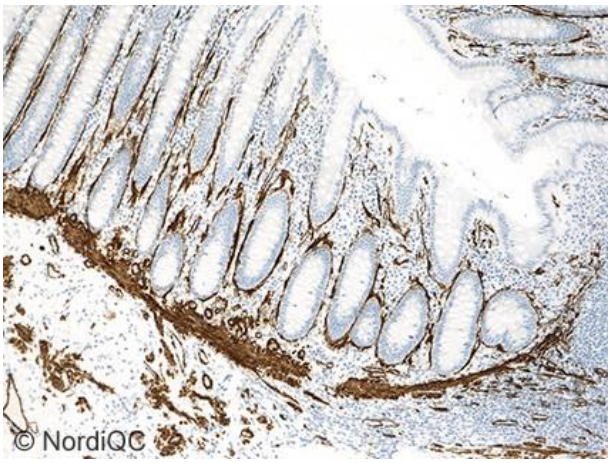


Fig. 1b (x100)
ASMA staining of the appendix using an insufficient protocol with too low technical and analytical sensitivity - based on the mAb clone 1A4 diluted 1:300, HIER in TRS (3-in-1) pH 9 (Dako) and a 3-step polymer based detection system (Flex+, Dako) on the Omnis (Dako). The protocol was almost identical to the protocol in Fig. 1a except for the choice of primary Ab. Same protocol used in Figs. 2b-5b.
Although the staining pattern is similar to the optimal protocol seen in Fig. 1a (same field), the assay provided too weak staining reaction in critical tissue specimens – compare fig. 3a-3b and 4a-4b. In this assessment, the mAb clone 1A4 seems difficult to optimize on Omnis (Dako) and Benchmark (Ventana) platforms.

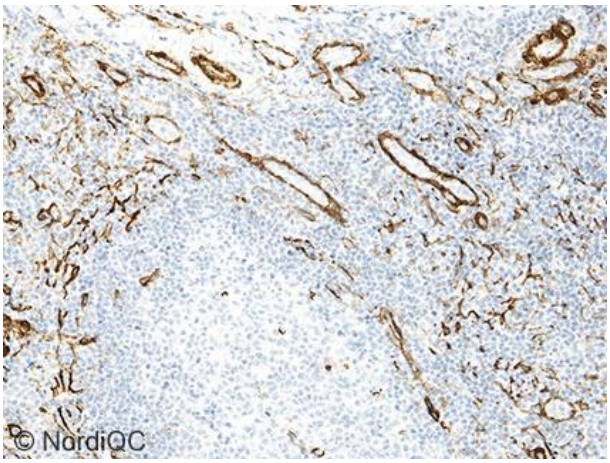


Fig. 2a (x200)
Optimal ASMA staining of the tonsil using same protocol as in Fig. 1a. The vast majority of smooth muscle cells surrounding vessels display the expected strong and distinct cytoplasmic staining reaction.

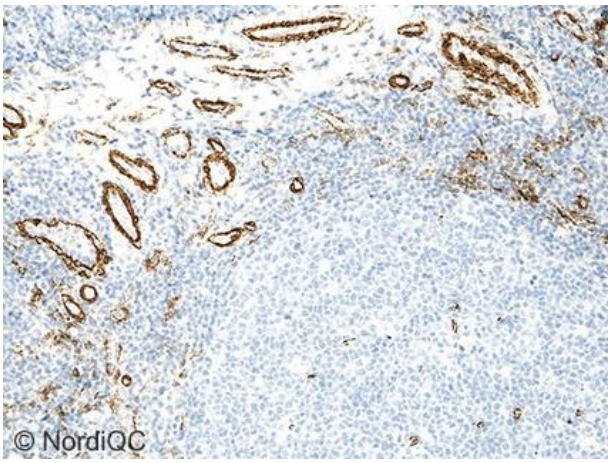


Fig. 2b (x200)
ASMA staining of the tonsil using same insufficient protocol as in Fig. 1b – same field as in Fig. 2a.
The staining pattern is similar to the optimal result seen in Fig. 2a (same field), but the assay provided a reduced analytical sensitivity with a too weak staining reaction in other tissue cores (see Figs. 3b, 4b and explanation in Fig.1b).

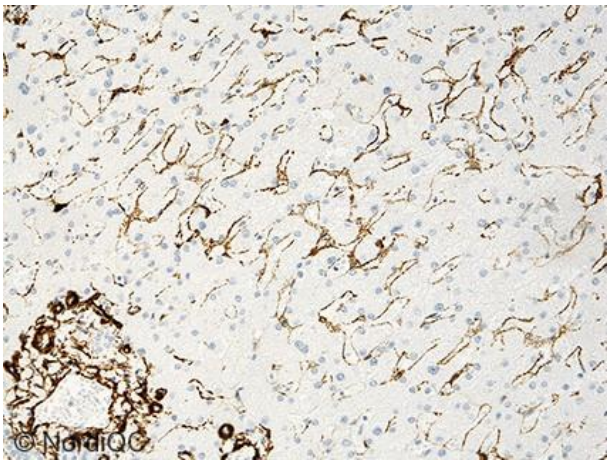


Fig. 3a (x200)
Optimal ASMA staining of the liver using same protocol as in Figs. 1a and 2a. The smooth muscle cells of the portal vessels show a moderate to strong staining reaction. Importantly, the vast majority of hepatic stellate cells (perisinusoidal smooth muscle cells) show a distinct, weak to moderate staining reaction. The hepatocytes are negative.

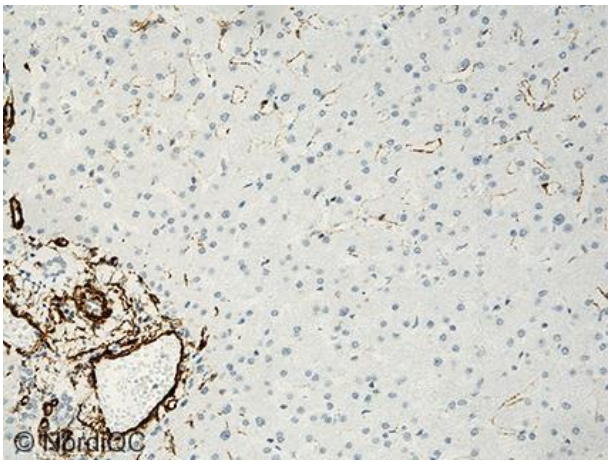


Fig. 3b (x200)
Insufficient ASMA staining of the liver using same protocol as in Figs. 1b and 2b. The proportion of positive hepatic stellate cells are significantly reduced and only display a faint staining intensity - same field as in Fig. 3a.

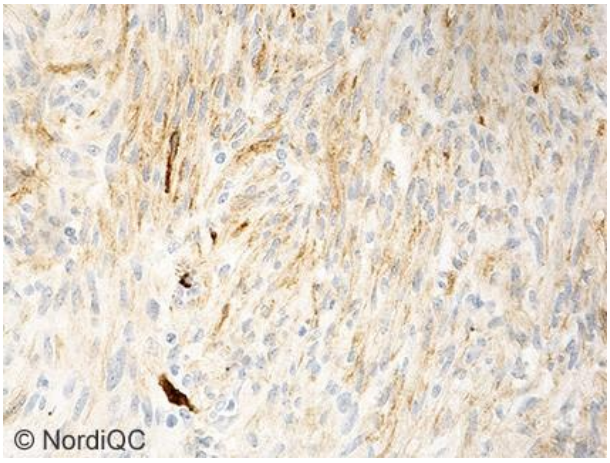


Fig. 4a (x400)
Optimal ASMA staining of the GIST using same protocol as in Figs. 1a-3a. The vast majority of the neoplastic cells show an at least weak but distinct cytoplasmic staining reaction.

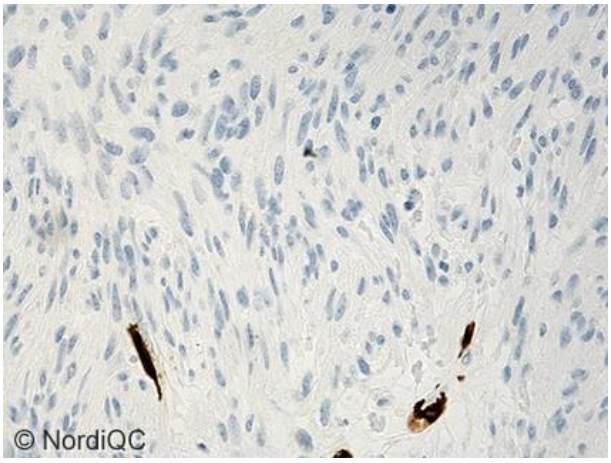


Fig. 4b (x400)
Insufficient ASMA staining of the GIST using same protocol as in Figs. 1b-3b. The neoplastic cells are false negative. Only scattered normal smooth muscle cells display strong staining intensity - same field as in Fig. 4a.

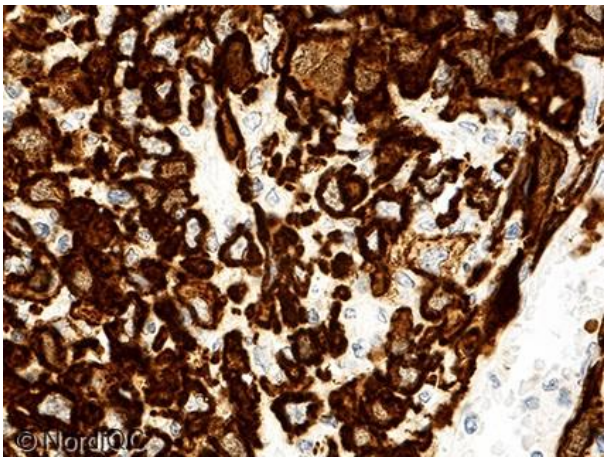


Fig. 5a (x600)
Optimal ASMA staining of the leiomyosarcoma using the same protocol as in Figs. 1a-4a. All neoplastic cells display strong cytoplasmic staining reaction.

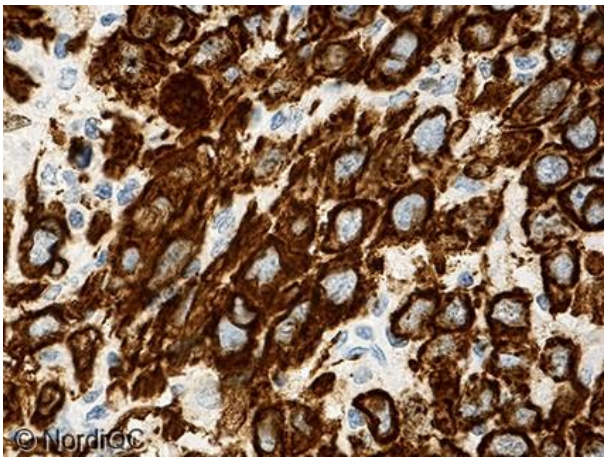


Fig. 5b (x600)
ASMA staining of the leiomyosarcoma using the same insufficient protocol as in Figs. 1b-4b. The neoplastic cells show the expected reaction pattern but the protocol, in general, provided too low technical and analytical sensitivity (see Figs. 3b, 4b and explanation in Fig. 1b).

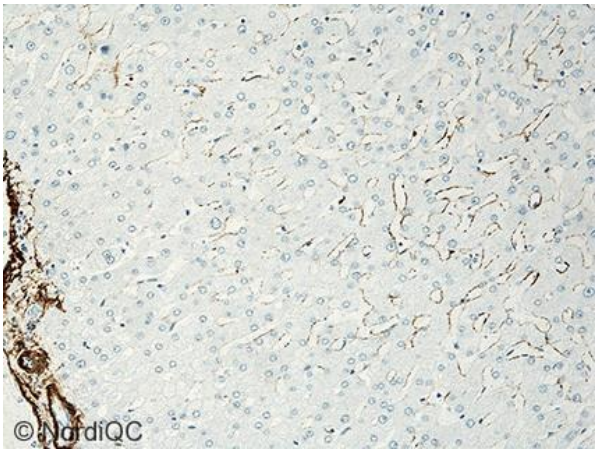


Fig. 6a (x200)
Sufficient (assessed as good) ASMA staining of the liver using the mAb clone 1A4 on the Ventana Benchmark platform, no antigen retrieval, high concentration of the primary Ab and UltraView as detection system. The majority of hepatic stellate cells display a faint to weak but distinct expression – compare with Fig. 3a and Fig 3 b. This reaction pattern was the minimum expression level accepted for ASMA to provide a sufficient result.

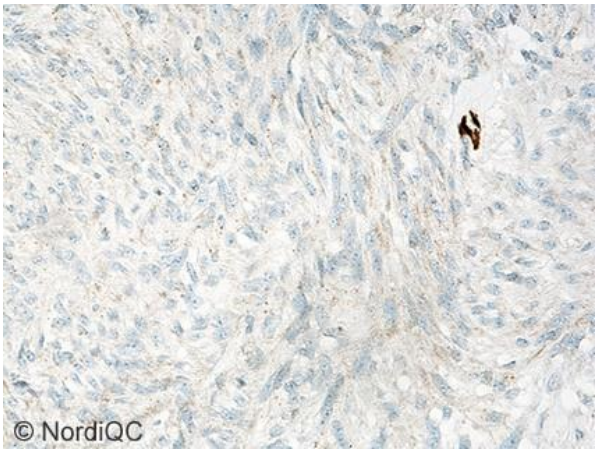


Fig. 6a (x200)
Sufficient (assessed as good) ASMA staining of the GIST using the same protocol as in Fig. 6a. The vast majority of neoplastic cells show a faint to weak cytoplasmic staining reaction. Results and staining patterns as shown in Figs. 6a and 6b were assessed as good. A sufficient level of technical and analytical sensitivity was obtained but could still be improved as shown in Figs. 3a and 4a.

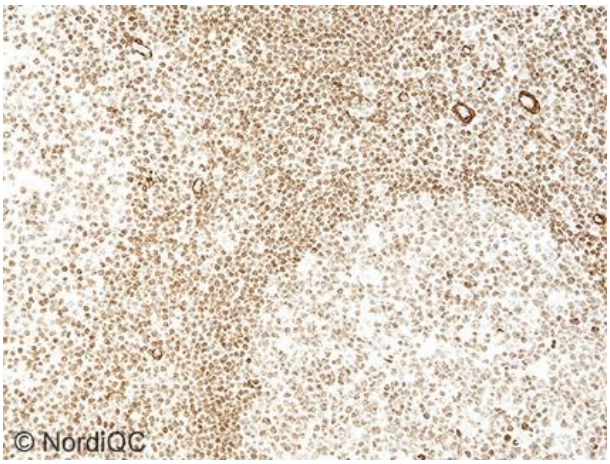


Fig. 7a (x200)
Inappropriate staining result for ASMA of the tonsil using the RTU product 760-2833 (Ventana) based on the mAb clone 1A4, HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview, Ventana).
Virtually all lymphocytes display an aberrant nuclear staining reaction compromising the interpretation – compare with Fig. 2a. This aberrant staining pattern is frequently seen on the Ventana Benchmark platform and typically related to protocol parameters increasing the technical and analytical sensitivity for mAb clone 1A4. This problem has been addressed in all previous assessment for ASMA (e.g. run 44, 2015) In this assessment the mAb clone BS66 was found be more successful on fully automated platforms as the BenchMark and Omnis.

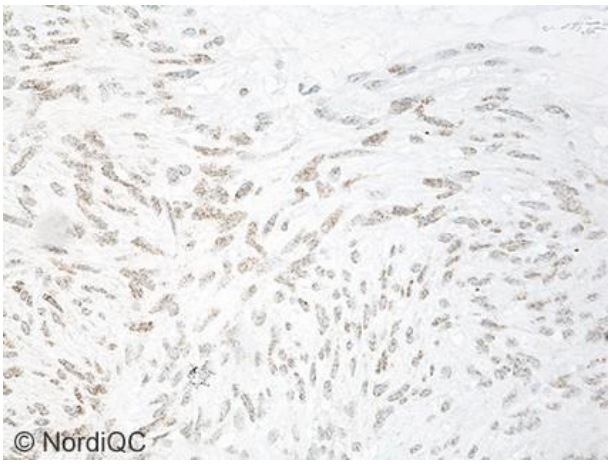


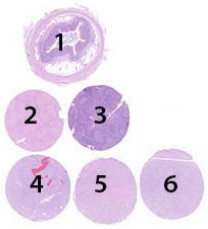
Fig. 7b (x200)
Insufficient staining result for ASMA of the GIST using same protocol as in Fig. 7b. Virtually all the neoplastic cells display an aberrant nuclear staining reaction. See explanation in Fig. 7a and compare with optimal result in Fig. 4a.

HLK/SN/MB/RR 09.12.19

Assessment Run 55 2019

Alpha-smooth muscle actin (ASMA)

Material
The slide to be stained for ASMA comprised:



1. Appendix, 2. Liver, 3. Tonsil, 4. Gastrointestinal stromal tumor (GIST),
5. Leiomyoma, 6. Leiomyosarcoma.

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 perisinusoidal cells (hepatic stellate cells) in the liver
- A strong, distinct cytoplasmic staining reaction of 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 reaction of smooth muscle cells throughout the specimens in the block (e.g. vessels)
- 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 55	296
Number of laboratories returning slides	287 (97%)

Results
287 laboratories participated in this assessment. 6 of these used an inappropriate Ab such as a pan-muscle marker mAb clones HHF35 and HUC (1-1). Of the remaining 281 laboratories, only 47 (17%) 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
- Less successful primary Ab (mAb clones 1A4 and asm-1)
- Poor performance of the mAb clone 1A4 on the fully automated stainer systems from Ventana (BenchMark) or Dako (Omnis)
- Too low concentration of the primary antibody

Performance history
This was the fifth NordiQC assessment of ASMA. The pass rates have constantly been relatively low throughout all runs but declined significantly in this run 55 (see Table 2).

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	Run 55 2019
Participants, n=	71	106	124	234	281
Sufficient results	62%	63%	64%	60%	17%

Conclusion
The mAb clones **1A4**, **BS66** 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 but could only produce optimal results on the Dako Autostainer or the Leica BOND platform. The performance of assays based on the mAb clone 1A4, both as concentrated formats and RTU systems, were challenged by automated IHC-platforms. Only 1% (2 of 158) of the slides stained on either the Ventana Benchmark or Dako Omnis platform were assessed as sufficient. In this assessment, best performance was achieved with the mAb clone BS66 providing a 100% pass rate (7 of 7). The mAb clone BS66 seems less sensitive to IHC-platform as optimal results could be obtained on both semi-and fully automated systems as the Thermo Autostainer, Ventana Benchmark or

Dako Omnis. HIER in an alkaline buffer and careful calibration of the titre of the primary antibody were the main prerequisites for 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 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 55**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1A4**	102 8 6 5 2 1 1 1 1	Agilent/Dako Cell Marque Sigma Aldrich Thermo/NeoMarkers Zytomed Systems Biocare Genemed Diagnostic Biosystems Spring Bioscience	10	14	58	45	19%	43%
mAb clone **asm-1**	4	Leica/Novocastra	0	1	2	1	-	-
mAb clone **BS66**	7	Nordic Biosite	7	0	0	0	-	-
rmAb clone **EP188**	10	Epitomics/Cell Marque	4	1	5	0	50%	60%
Ready-To-Use antibodies								
mAb clone **1A4 IR/IS611**	20	Agilent/Dako	1	5	9	5	30%	50%
mAb clone **1A4 IR/IS611**[3]	15	Agilent/Dako	0	1	9	5	-	-
mAb clone **1A4 GA611**	15	Agilent/Dako	0	1	13	1	7%	0%
mAb clone **1A4 760-2833**	59	Ventana/Roche	0	0	21	38	0%	0%
mAb clone **1A4 202M-9x**	8	Cell Marque	0	0	2	6	-	-
mAb clone **1A4 8292-C010**	1	Sakura Finetek	1	0	0	0	-	-
mAb clone **1A4 MAD-001195QD**	2	Master Diagnostica	0	0	2	0	-	-
mAb **1A4 PM001**	1	Biocare	0	0	1	0	-	-
mAb **1A4 PM001**[3]	1	Biocare	0	0	1	0	-	-
mAb clone **1A4 Kit-0006**	1	Maixin	1	0	0	0	-	-
mAb clone **asm-1 PA0943**	10	Leica/Novocastra	0	0	9	1	0%	0%
Total	281		24	23	132	102	-	
Proportion			9%	8%	47%	36%	17%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of ASMA, Run 55
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **1A4**: Protocols with optimal results were typically based on Heat Induces Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (3/27)*, Bond Epitope Retrieval Solution 2 (BERS2, Leica) (4/20), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/2) or Tris-EDTA pH 9 (2/7) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:500 (Agilent/Dako) or 1:27,000 (Sigma Aldrich). Using these protocol settings, 15 of 35 (43%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **BS66**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako) (4/4), Cell Conditioning 1 (CC1, Ventana) (1/1), CC1 (Ventana) followed by proteolysis in P3 (1/1) and Tris-EDTA pH 9 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:150-1:1500 depending on the total sensitivity of the protocol employed.

rmAb clone **EP188**: Protocols with optimal results were typically based on combined pre-treatment using proteolysis (Protease 2 or 3 (Ventana) for 4 min or 12 min, respectively.) followed by HIER in CC1 (mild, Ventana). The rmAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 5 (60%) laboratories produced a sufficient staining result (optimal or good).

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**	2/8** (25%)	0/2	0/10 (0%)	0/1	0/16 (0%)	0/2	4/7 (57%)	1/2

* 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:
One protocol with an optimal result was based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10 min. at 97°C), 20 min. incubation of the primary Ab and EnVision FLEX (K8000) as detection systems. Using these protocol settings, 3 of 6 (50%) laboratories produced a sufficient staining result (optimal or good).

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 15 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 strictly 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 AS mAb 1A4 **IR611**	25% (2/8)	0% (0/8)	40% (4/10)	10% (1/10)
Dako Omnis mAb 1A4 **GA611**	8% (1/12)	0% (0/12)	(0/1)	(0/1)
VMS Ultra/XT/GX mAb 1A4 **760-2833**	(0/6)	(0/6)	0% (0/53)	0% (0/53)
Leica Bond III mAb Asm-1 **PA0943**	(0/6)	(0/6)	(0/4)	(0/4)

* 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 were included.

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 cells expected to be demonstrated. Too weak or false negative staining reaction was seen in 72% of the insufficient results (169 of 234). 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 11%, technical issues, false positive staining reaction of e.g. epithelial cells of the appendix or poor signal-to-noise ratio was seen compromising the interpretation.

The mAb clone 1A4 was the most widely used concentrated format within a laboratory developed (LD) assay for ASMA. In general, the mAb clone 1A4 provided very poor results (19% pass rate) and only 8% (10 of 127) were assessed as optimal (see Table 1). In this run, too weak or false negative staining result was the main feature of an insufficient result and was typically caused by protocols with too low technical analytical sensitivity. Especially the neoplastic cells of the GIST but also the hepatic perisinusoidal cells was difficult to demonstrate for the vast majority of laboratories using the mAb clone 1A4 within a LD assay. All protocols assessed as optimal were 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, best performance was obtained on the Leica Bond platforms where 86% (6 of 7) of the protocols (based on HIER in BERS2) produced a sufficient result and 57% (4 of 7) were optimal. None of the slides stained on either an Omnis (Dako) or a Benchmark (Ventana) platform provided an optimal result and all (29 of 29) were assessed as insufficient (borderline or poor).
Using the mAb clone 1A4 within a LD assay, 25 laboratories omitted HIER providing a pass rate of only 12% (3 of 25), which also affected the overall large proportion of insufficient result in this run. None of these were assessed as optimal.
As observed in previous assessments for ASMA, aberrant nuclear staining reaction of e.g. lymphocytes in the tonsil is still a problem and mainly related to the BenchMark stainer platforms (Ventana). When the mAb clone 1A4 was used in relative high concentration and with HIER in CC1, the aberrant nuclear reaction was most prominent and frequently together with a too weak specific staining reaction of cells expected to be demonstrated. This staining pattern was also seen with the RTU format (760-2833, Ventana) for the Benchmark platform. The problems both with too low technical analytical sensitivity in combination with either false positive/ aberrant nuclear staining reaction seem impossible to solve for most participants.

The mAb clone asm-1 is synonymous with 1A4, giving exactly the same expression patterns/problems as described above and will not be elaborated further in this report.

Although the number of participants using the mAb clone BS66 within a LD-assay was low, the Ab seems robust and promising as all protocols (7 of 7) were assessed as optimal (see Table 1). In addition, this primary Ab provided optimal results on both the Omnis (Dako) and Benchmark (Ventana) and thus might be an alternative to the more challenging Abs (e.g. 1A4). The Ab could be diluted in a wide spectrum of concentrations (1:150-1:1,500) as long as HIER was performed in alkaline buffer (e.g. CC1, Ventana) and primary Ab was calibrated according to the over-all sensitivity of the detection systems applied.

As mentioned in the previous Run 44 (2015), the rmAb clone EP188 could produce optimal result on BenchMark Ultra (Ventana), applying combined pre-treatment using proteolysis in P2 followed by HIER in CC1. In this assessment, all (4 of 4) protocols assessed as optimal was based on HIER in CC1 (32 min) either alone or in combination with proteolysis in P2/P3 (4 and 12 min., respectively). Staining reactions of the primary Ab (diluted in the range 1:100-200) were detected using OptiView with or without amplification as the detection systems. Proteolysis was performed before HIER. The slides assessed as insufficient (typically displaying too weak staining intensity) used similar protocol settings as protocols providing an optimal result, making it difficult to elucidate on the problems. However, the rmAb EP188 is still a better alternative for demonstration of ASMA since it provided a significant higher pass rate (50%, 5 of 10) compared to mAb clone 1A4 (2%, 1 of 55 (all protocol settings)) on the Ventana Benchmark platform.

47% (133 of 281) of the laboratories used a RTU format for detection of ASMA. Ideally, a 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 performance characteristic (expected reaction patterns). Fifteen laboratories used the RTU format IS/IR611 based on the mAb clone 1A4 off-label (e.g. different protocol settings and platforms than the Autostainer (Dako)) providing a pass rate of 6% (1 of 15) which was significantly lower compared to an overall pass rate of 30% (6 of 20) if the system was used on the Autostainer +/Link (Dako) (see Table 1).

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. As shown in Table 4, the Dako RTU system (IS/IR/GA611) provided slightly higher pass rate compared to the Ventana RTU system (760-2833) but the overall performance of these systems was disappointing low. No significant difference could be seen between using official protocol recommendations given by vendors or laboratory modified protocol settings. Grouped together, the pass rate was only 7% (7 of 94) (see Table 1).

In addition, the RTU system PA043 (Leica) based on the mAb clone asm-1 provided a pass rate of 0% (all 10 protocols were assessed as insufficient). For this system, but also for the Ventana RTU system (760-2833), the official recommendations to pre-treatment is based on no antigen retrieval at all. However, and as shown in Table 4, this did not influence the final outcome as there was no difference in performance between applying vendor recommended protocol settings compared to laboratory modified protocol settings typically performing HIER in alkaline buffer.

This was the fifth assessment of ASMA in NordiQC (see Table 2). Although ASMA has been use for many years, the marker is still challenging and the pass rate in this run 55 decreased significantly compared to the latest run 44, 2015. Several elements influenced the final outcome: 1) The tissue included in the block, especially the liver (hepatic perisinusoidal cells) and the neoplastic cells of the GIST, were challenging for most participants. 2) The consistent poor performance of the mAb 1A4 on Ventana Benchmark platforms (120 laboratories) but also on the Dako Omnis (38 laboratories) accounted for the overall low pass rate obtained in this run and only 1% (2 of 158) of the users were able to produce a sufficient result. 3) A large proportion of laboratories applied protocol settings based on no antigen retrieval at all, providing a pass rate of only 6% (4 of 62) of which none were assessed as optimal 4) The mAb asm-1, primarily applied on BOND III platforms (concentrates and RTU systems), also provided a large proportion of insufficient results (93%, 13 of 14).
Importantly, laboratories should apply an Ab that work on the in-house IHC platform, calibrate the protocols correctly and stain according to the expected antigen level of the recommended control material (see below).

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.

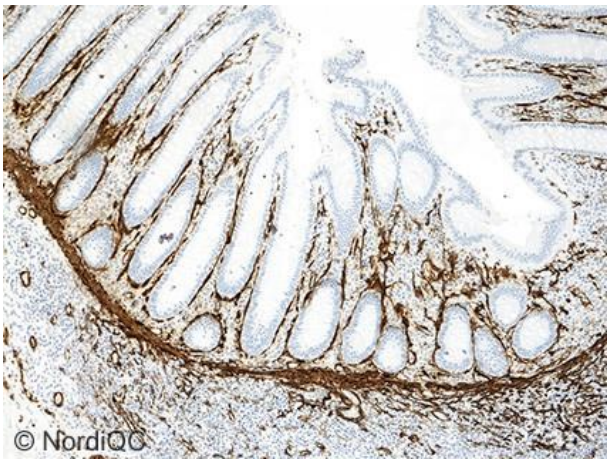


Fig. 1a (x100)
Optimal ASMA staining of the appendix using the mAb clone BS66 optimally calibrated, HIER in TRS (3-in-1) pH9 (Dako) and a 3-step polymer based detection system (Flex+, Dako) on the Omnis (Dako). Smooth muscle cells of lamina muscularis mucosae and myofibroblasts lining the epithelial crypts show a distinct cytoplasmic staining reaction. Same protocol used in Figs. 2a-5a.

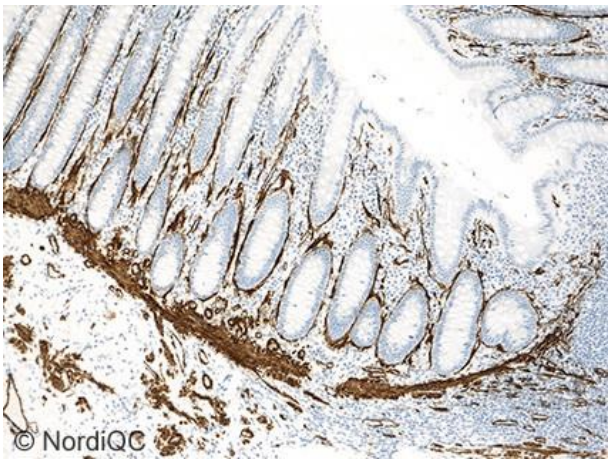


Fig. 1b (x100)
ASMA staining of the appendix using an insufficient protocol with too low sensitivity - based on the mAb clone 1A4 diluted 1:300, HIER in TRS (3-in-1) pH9 (Dako) and a 3-step polymer based detection system (Flex+, Dako) on the Omnis (Dako). The protocol was almost identical to the protocol in Fig. 1a except for the choice of primary Ab. Same protocol used in Figs. 2b-5b. Although the staining pattern is similar to the optimal protocol seen in Fig. 1a (same field), the assay provided too weak staining reaction in critical tissue specimens – compare fig. 3a-3b and 4a-4b. In this assessment, the mAb clone 1A4 seems difficult to optimize on Omnis (Dako) and Benchmark (Ventana) platforms.

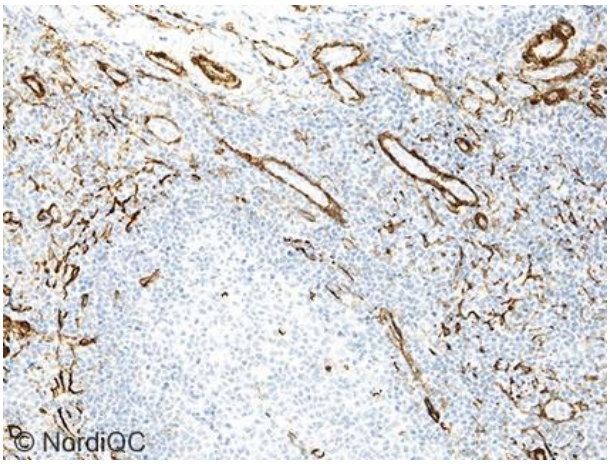


Fig. 2a (x200)
Optimal ASMA staining of the tonsil using same protocol as in Fig. 1a. The vast majority of smooth muscle cells surrounding vessels display the expected strong and distinct cytoplasmic staining reaction.

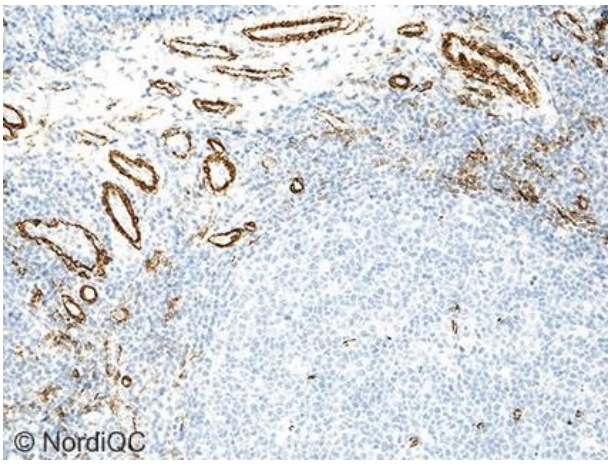


Fig. 2b (x200)
Insufficient ASMA staining of the tonsil using same protocol as in Fig. 1b – same field as in Fig. 2a. The staining pattern is similar to the optimal protocol seen in Fig. 2a (same field), but the assay provided too weak staining reaction (see explanation in Fig.1b).

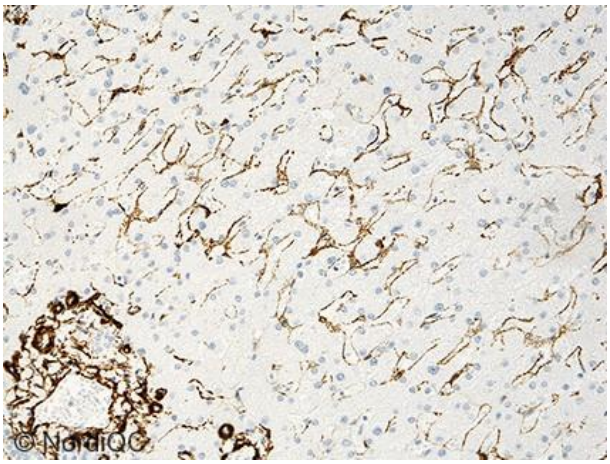


Fig. 3a (x200)
Optimal ASMA staining of the liver using same protocol as in Figs. 1a and 2a. The smooth muscle cells of the portal vessels show a moderate to strong staining reaction. Importantly, the vast majority of hepatic stellate cells (perisinusoidal smooth muscle cells) show a distinct, weak to moderate staining reaction. The hepatocytes are negative.

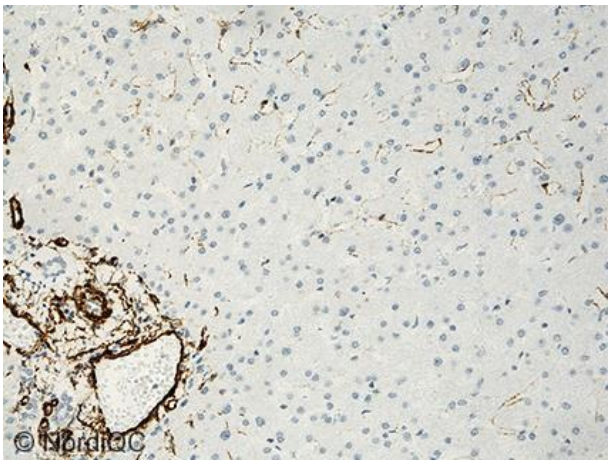


Fig. 3b (x200)
Insufficient ASMA staining of the liver using same protocol as in Figs. 1b and 2b. The proportion of positive hepatic stellate cells are significantly reduced and only display faint staining intensity - same field as in Fig. 3a.

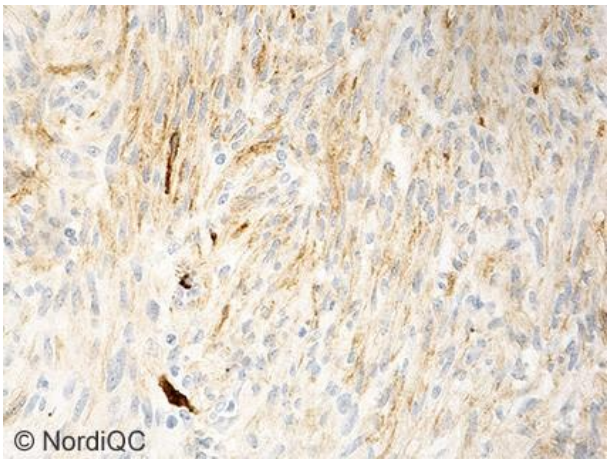


Fig. 4a (x400)
Optimal ASMA staining of the GIST using same protocol as in Figs. 1a-3a. The vast majority of the neoplastic cells show an at least weak but distinct cytoplasmic staining reaction.

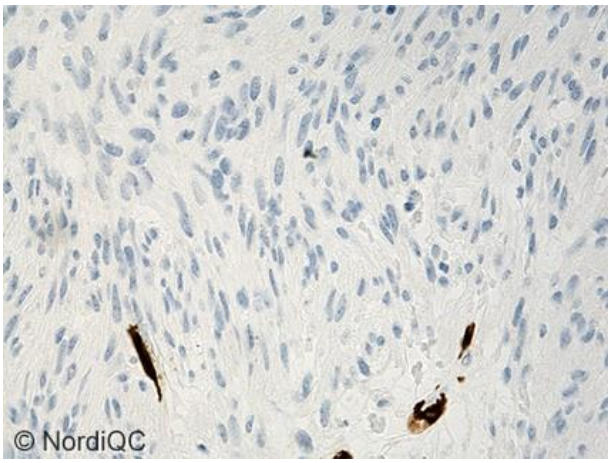


Fig. 4b (x400)
Insufficient ASMA staining of the GIST using same protocol as in Figs. 1b-3b. The neoplastic cells are false negative. Only scattered normal smooth muscle cells display strong staining intensity - same field as in Fig. 4a.

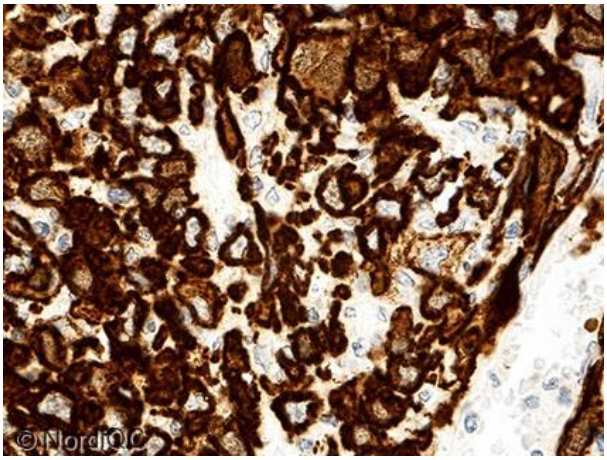


Fig. 5a (x600)
Optimal ASMA staining of the leiomyosarcoma using the same protocol as in Figs. 1a-4a. All neoplastic cells display strong cytoplasmic staining reaction.

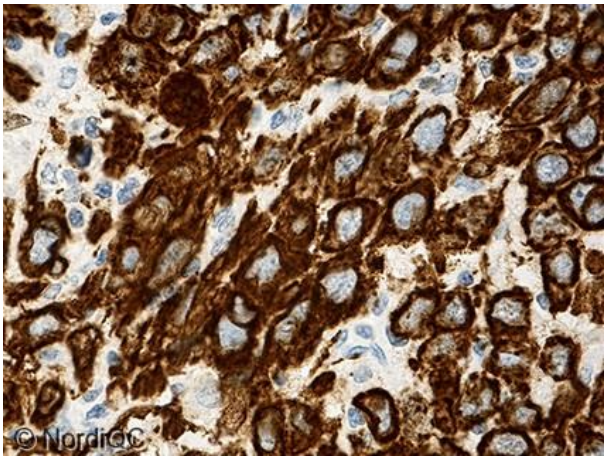


Fig. 5b (x600)
Insufficient ASMA staining of leiomyosarcoma using the same protocol as in Figs. 1b-4b. The neoplastic cells show the expected reaction pattern but the protocol, in general, provided too low sensitivity (see explanation in Fig. 1b).

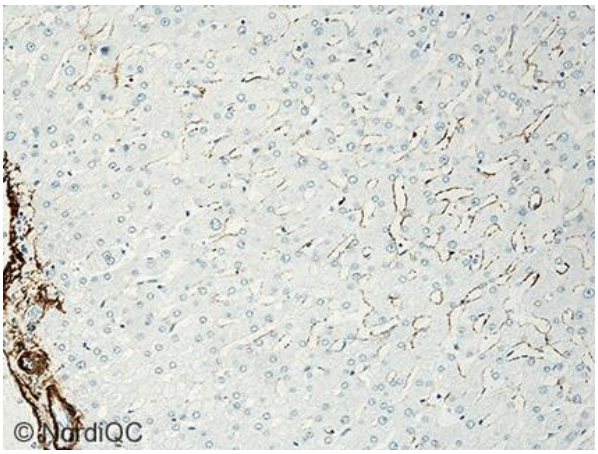


Fig. 6a (x200)
Sufficient (good) ASMA staining of the liver using the mAb clone 1A4 on the Ventana Benchmark platform, no antigen retrieval, high concentration of the primary Ab and UltraView as detection system. The majority of hepatic stellate cells display faint to weak expression – compare with Fig. 3a and Fig 3 b. This reaction pattern was the minimum of accepted expression level for ASMA to provide a sufficient result.

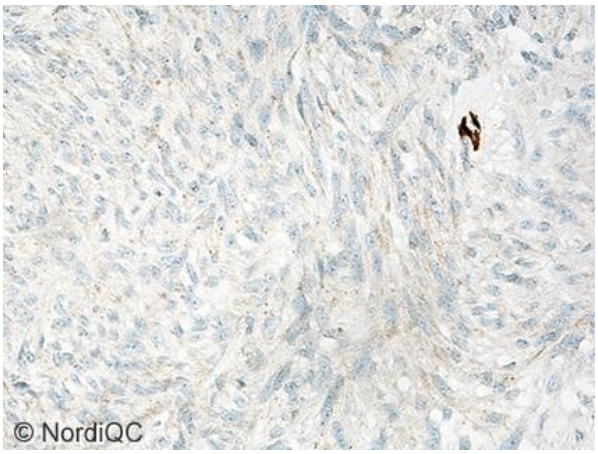


Fig. 6a (x200)
Sufficient (good) ASMA staining of the GIST using the same protocol as in Fig. 6a. The vast majority of neoplastic cells show a faint to weak cytoplasmic staining reaction. LABs able to stain the hepatic stellate cells as shown in Fig. 6a, and at the same time were able to demonstrate any specific expression level seen in GIST tumor, was assessed as good as long as the protocols detected the expected ASMA levels in the other cores (no false negative or false positive reactions). However, the protocol shown here can be optimized - compare with Fig. 4a.

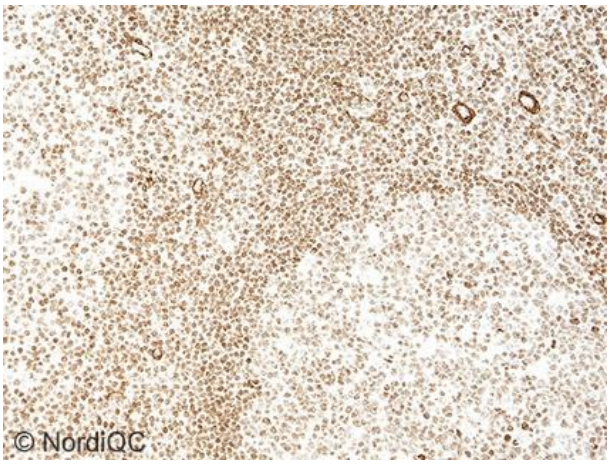


Fig. 7a (x200)
Inappropriate staining result for ASMA of the tonsil using the RTU product 760-2833 (Ventana) based on the mAb clone 1A4, HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview, Ventana).
Virtually all lymphocytes display an aberrant nuclear staining reaction compromising the interpretation – compare with Fig. 2a. This aberrant staining pattern is frequently seen on the Ventana Benchmark platform and typically related to protocol parameters increasing the analytical sensitivity. This problem has been addressed in all previous assessment for ASMA (e.g. run 44, 2015) and for LABs struggling with optimization of the protocols; it is advisable to substitute the antibody with a more robust clone e.g. BS66 or EP188.

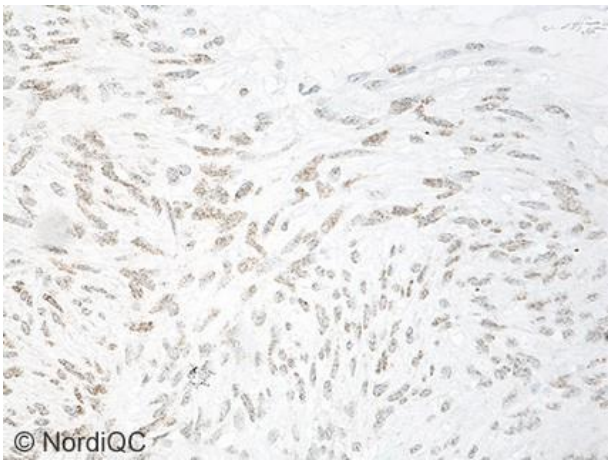


Fig. 7b (x200)
Insufficient staining result for ASMA of the GIST using same protocol as in Fig. 7b. Virtually all the neoplastic cells display an aberrant nuclear staining reaction. See explanation in Fig. 7a and compare with optimal result in Fig. 4a.

MB/LE/MV/RR 18.03.2019