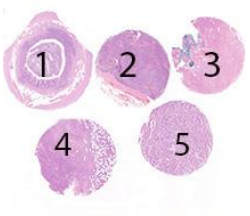
Assessment Run 62 2021
CD31

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CD31, typically identifying tumour cell invasion in vascular structures and angiosarcomas in the characterization of cancers of unknown origin. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for CD31 (see below).

Material
The slide to be stained for CD31 comprised:



1. Appendix, 2. Tonsil, 3. Liver, 4. Angiosarcoma, 5. Colon adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD31 staining as optimal included:

- A strong and distinct, predominantly membranous staining reaction of virtually all normal endothelial cells and plasma cells in all tissue cores.
- An at least weak to moderate, distinct membranous staining reaction of activated B- and T-cells, in particular the vast majority of mantle zone B-cells in the tonsil and intraepithelial T-cells in the appendix.
- An at least weak to moderate, predominantly membranous staining reaction of virtually all hepatic sinusoidal endothelial cells.
- A strong, predominantly membranous staining reaction of all neoplastic cells in the angiosarcoma.
- No staining reaction of the epithelial cells in the appendix, tonsil, liver or neoplastic cells of the colon adenocarcinoma.

Participation

Number of laboratories registered for CD31, run 62	364
Number of laboratories returning slides	342 (94%)

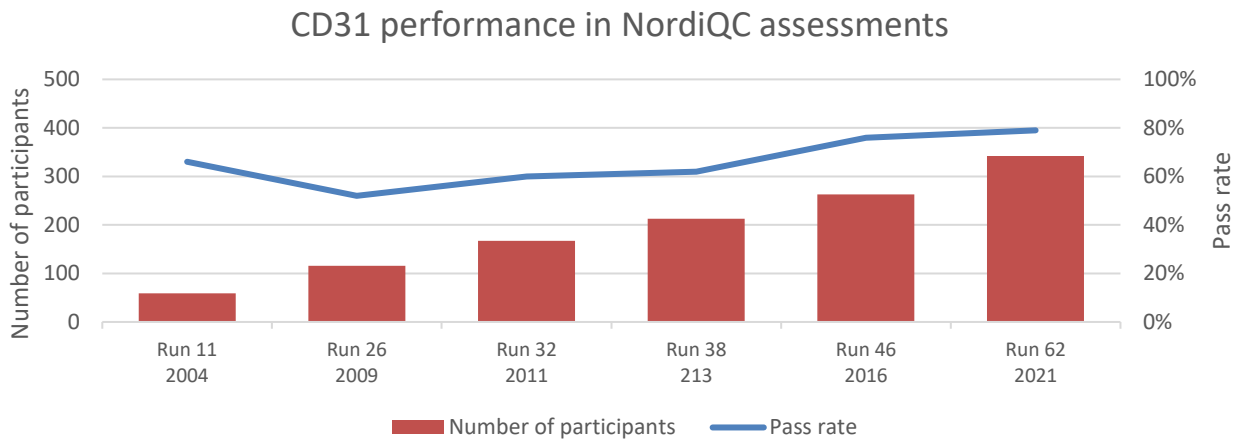
Results
At the date of assessment, 94% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

342 laboratories participated in this assessment and 79% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Omission of Heat Induced Epitope Retrieval (HIER).
- Inefficient HIER (use of "standard" citric based buffer e.g., BERS1).
- Use of less sensitive detection systems.
- Less successful RTU formats/systems.
- Less successful primary antibodies.

Performance history
This was the sixth NordiQC assessment of CD31. The pass rate increased slightly compared to the previous run and a consistent improvement has been observed in the four latest assessments (see Graph 1).

Graph 1. **Proportion of sufficient results for CD31 in the six NordiQC runs performed**



Conclusion

The mAb clones **JC70A**, **BS50**, **MX032**, **IHC031**, **1A10** and the rmAb clone **RM247** could all produce an optimal result for CD31. The vast majority of assays were based on the mAb clone JC70A and, used as a concentrated format within a laboratory developed (LD) assay, optimal staining results could be obtained on all main platforms from Dako/Agilent, Leica Biosystems and Ventana/Roche. The RTU systems GA610 (Omnis, Dako/Agilent) and PA0414 (Bond III/MAX, Leica Biosystems) provided superior performance for demonstration of CD31. For all assays based on the mAb clone JC70A, both concentrates and RTU formats, efficient HIER in an alkaline buffer or TRS low pH (Dako/Agilent) in combination with use of a sensitive 3-step detection system, gave the highest proportion of sufficient and optimal results.

Liver and tonsil are recommendable positive and negative tissue controls for CD31:

Virtually all hepatic sinusoidal cells and activated tonsillar mantle zone B-cells must show an at least weak to moderate, and distinct membranous, staining reaction, while other endothelial cells and plasma cells must show a strong staining reaction. No staining of hepatocytes, epithelial cells and muscle cells should be seen.

Table 1. **Antibodies and assessment marks for CD31, Run 62**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **JC70A**	94 10 3 2 2 1 1 1	Dako/Agilent Cell Marque Leica Biosystems Thermo Scientific Immunologic Zytomed Systems Diagnostic Biosystem Zeta Corporation	58	37	17	2	83%	51%
mAb clone **1A10**	1	Thermo Scientific	1	0	0	0	-	-
mAb clone **BS50***	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **9G11**	1	BioGenex	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **JC70A, IR610[3]**	14	Dako/Agilent	11	1	2	0	86%	79%
mAb clone **JC70A, IR610[4]**	20	Dako/Agilent	11	6	3	0	85%	55%
mAb clone **JC70A, GA610[3]**	33	Dako/Agilent	32	1	0	0	100%	97%
mAb clone **JC70A, GA610[4]**	23	Dako/Agilent	14	5	4	0	83%	61%
mAb clone **JC70A, PA0414[3]**	5	Leica Biosystems	5	0	0	0	100%	100%
mAb clone **JC70A, PA0414[4]**	7	Leica Biosystems	7	0	0	0	100%	100%
rmAb clone **JC70A, 760-4378[3]**	13	Ventana/Roche	0	6	7	0	46%	0%
rmAb clone **JC70A, 760-4378[4]**	75	Ventana/Roche	39	15	20	1	72%	52%
mAb clone **JC70A, 131M-97/98**	16	Cell Marque	6	4	6	0	63%	38%
mAb clone **JC70A, MAD-002048QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **JC70A, PM131**	1	Biocare Medical	0	0	1	0	-	-
mAb clone **MX032, MAB-0720**	2	Fuzhou Maixin Biotech	1	1	0	0	-	-
mAb clone **IHC031, IHC031**	1	GenomeMe	1	0	0	0	-	-
mAb clone **GM100, GT232102**	1	Gene Tech	0	1	0	0	-	-
mAb clone **BC2, PM347**	1	Biocare Medical	0	1	0	0	-	-
mAb clone **1A10, PA0250[3]**	2	Leica Biosystems	0	0	2	0	-	-
mAb clone **1A10, PA0250[4]**	3	Leica Biosystems	1	0	1	1	-	-
mAb clone **BS50, MAD-000790QD***	2	Vitro SA	0	1	1	0	-	-
mAb clone **C12A6, CCM-0534**	1	Celnovte Biotechnology	0	0	1	0	-	-
rmAb clone **RM247, 8282-C010**	4	Sakura Finetek	2	0	1	1	-	-
Total	342		190	80	66	6	-	
Proportion			56%	23%	19%	2%	79%	

1) Proportion of sufficient results (optimal or good) (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).
* Discontinued May, 2021

Detailed analysis of CD31, Run 62
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **JC70A**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (7/12)*, Cell Conditioning 1 (CC1, Ventana/Roche) (37/64), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (8/14) or TRS pH 6 (3-in-1) (Dako/Agilent) (5/7) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:100. Using these protocol settings, 68 of 76 (89%) laboratories produced a sufficient staining result (optimal or good). One protocol obtaining an optimal result was based on proteolytic pre-treatment.
* (number of optimal results/number of laboratories using this HIER buffer)

Table 2. **Proportion of optimal results for CD31 for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibody	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark GX / XT / Ultra**		**Leica Biosystems 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 **JC70A**	1/3	3/4	5/6 (83%)	1/2	28/51 (55%)	-	6/10 (60%)	0/4

* 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 **JC70A**, product no. **IR610**, Dako/Agilent, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 20 min. at 97-99°C), 20 min. incubation of the primary Ab and EnVision FLEX+ (K8002) as detection systems. Using these protocol settings, 12 of 13 (92%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **JC70A**, product no. **GA610**, Dako/Agilent, Omnis:
Protocols with optimal results were typically 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/GV821) as detection system. Using these protocol settings, 46 of 46 (100%) laboratories produced a sufficient staining result.

mAb clone **JC70A**, product no. **760-4378**, Ventana/Roche, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min. at 98-100°C) and 24-48 min. incubation of the primary Ab and UltraView (760-500) with amplification (760-080) or OptiView (760-700) as detection system. Using these protocol settings, 41 of 42 (98%) laboratories produced a sufficient staining result.

mAb clone **JC70A** product no. **PA0414**, Leica Biosystems, Bond III/MAX:
Protocols with optimal results were typically based on HIER using BERS2 (efficient heating time 20 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 8 of 8 (100%) laboratories produced an optimal staining result.

mAb clone **MX032**, product no. **MAB-0720**, Fuzhou Maixin Biotech, Titan S:
One protocol with an optimal result was based on HIER using High pH buffer (DNS-0811) (efficient heating time 20 min. at 99°C), 30 min. incubation of the primary Ab and Titan Super Detection Kit (TT-0805) as detection system.

rmAb clone **RM247**, product no. **8282-C010**, Sakura FineTek, Tissue-Tek Genie Advanced:
Protocols with optimal results were based on HIER using Tissue-Tek Genie High pH Antigen Retrieval Solution (efficient heating time 30-45 min. at 97-98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro Detection Kit (8826-K250) as detection system.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly according to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **Proportion of sufficient and optimal results for CK7 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb JC70A **IR610**	86% (12/14)	79% (11/14)	90% (9/10)	40% (4/10)
Dako Omnis mAb JC70A **GA610**	100% (33/33)	97% (32/33)	84% (16/19)	68% (13/19)
Leica Bond III/MAX mAb JC70A **PA0414**	100% (5/5)	100% (5/5)	100% (7/7)	100% (7/7)
VMS Ultra/XT/GX mAb JC70A **760-4378**	46% (6/13)	0% (0/13)	72% (54/75)	52% (39/75)

* 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 integrated.

Comments

In this assessment and in concordance with the previous NordiQC runs for CD31, the prevalent feature of an insufficient result was a too weak or completely false negative staining reaction of cells expected to be demonstrated. This staining pattern was seen in 92% (66/72) of the insufficient results. Virtually all laboratories were able to demonstrate CD31 in large vascular structures (all specimens) and the neoplastic cells of the angiosarcoma, whereas demonstration of CD31 in mantle zone B-cells in the tonsil, intraepithelial T-cells in the appendix and hepatic sinusoidal endothelial cells was more challenging, requiring an optimally calibrated protocol.

The mAb clone JC70A was the most widely used antibody for the demonstration of CD31 and applied by 94% (322/342) of the laboratories (see Table 1). Used as concentrated format within LD assays, the mAb clone JC70A provided 83% (95/114) sufficient results of which 51% (58/114) were assessed as optimal. As shown in Table 2, the mAb clone JC70A could provide optimal results on the four main automated platforms. The most prevalent causes of insufficient staining results were use of HIER in "standard" citric based buffer or use of a less sensitive detection system. Using HIER in an alkaline buffer (all protocol settings) and applying a 3-step polymer/multimer detection system (e.g., EnVision FLEX+, Bond Refine or OptiView), 94% (61/65) produced a sufficient staining result and 74% (48/65) were assessed as optimal. In comparison, using similar protocol settings and a 2-step polymer/multimer detection system (e.g., UltraView or Envision Flex), 31% (9/29) of the protocols provided a sufficient result and only 14% (4/29) were given an optimal mark

As mentioned above, use of "standard" citric based HIER buffers (pH 6) also seems to impact the overall performance as none (0/7) of the results based on protocols with these buffers were assessed as optimal and only 42% (3/7) provided a sufficient result. However, for protocols based on the modified citric based buffer TRS pH 6 (3-in-1) from Dako/Agilent, 85% (6/7) provided sufficient results and 71% (5/7) were optimal.

The titer of the primary antibody was of less significance provided that HIER was performed in alkaline buffer and a 3-step multimer/polymer detection system was applied. Almost identical proportion of sufficient and optimal results was obtained within the typical dilution range of 1:20-1:100 compared to a dilution factor of > 1:100, giving 75% (36/48) versus 77% (17/22) optimal results, respectively.

In total, 66% (225/342) of the laboratories used a RTU format and almost all (92%, 208/225) were based on the mAb clone JC70A. As shown in Table 3 applying vendor recommended protocol settings (VRPS), the most successful RTU systems for demonstration of CD31 were Dako/Agilent GA610 and Leica Biosystems PA0414. Grouped together, these RTU systems provided 100% (38/38) sufficient results of which 97% (37/38) were assessed as optimal. The basic protocol settings for these two RTU systems are based on HIER in an alkaline buffer, TRS pH 9 (Dako/Agilent) or BERS2 (Leica Biosystems), and use of 3-step polymer detection systems, Envision Flex+ (Dako/Agilent) or Bond Refine (Leica Biosystems).

In comparison, only 46% (6/13) of the participants applying the official recommendations to the RTU system 760-4378 (Ventana/Roche), also based on the mAb clone JC70A, obtained a sufficient result and none were optimal. Using laboratory modified protocol settings (LMPS), the proportion of sufficient and optimal results increased to 72% (54/75) and 52% (39/75), respectively, and could in particular be related to the use of a 3-step multimer detection system (UltraView with amplification or OptiView). Using the optimal protocol settings for this RTU system as described above, the proportion of sufficient results were 98 % (41/42) of which 86% (36/42) were optimal. Using exactly the same overall protocol settings but substituting the 3-step multimer detection systems (UltraView with amplification or OptiView) with the 2-step multimer detection system UltraView as recommended in the package insert for the RTU system, the proportion of sufficient result declined to 31% (8/26) and none were optimal. This observation

emphasizes, that the less sensitive and vendor recommended detection system UltraView cannot provide the required level of analytical sensitivity for the RTU system 760-4378, and thus, should be substituted. This product has been developed together with Cell Marque for the Ventana Benchmark platforms, and Cell Marque have their own product line (131M-97/98) based on mAb clone JC70A of which all protocols (16/16) were applied on the Ventana Benchmark platforms in this assessment, displaying completely the same reaction patterns as described above.

The Leica RTU system PA0250 based on the mAb clone 1A10 being used by five participants has consistently over the previous assessments (run 26, 32, 38 and 46) provided inferior results, and including this run, only 4% (1/24) of all protocols have produced a sufficient result. Thus, participants are encouraged to substitute the RTU system PA0250 with the RTU system PA0414, providing the required analytical sensitivity and specificity for the demonstration of CD31 that in this assessment showed superior performance.

This was the sixth NordiQC assessment of CD31 and a minor increase in the pass rate was seen compared to the previous run 46 2016 (see Graph 1). The vast majority of assays were based on the mAb clone JC70A (94%; 322/342) and the most prevalent feature of an optimal result, was use of HIER in an alkaline buffer in combination with use of a sensitive 3-step detection system. Using these fundamental parameters, 95% (202/213) produced a sufficient result of which 79% (169/213) were optimal. Importantly, protocols must stain according to the expected antigen levels, and both tonsil and liver are essential immunohistochemical critical assay performance controls (ICAPCs) assisting to monitor the required level of the analytical sensitivity and specificity of the assay (see below).

Controls

Tonsil and liver are recommended as positive and negative tissue controls for CD31. In tonsil, the vast majority of all mantle zone B-cells cells must show an at least weak to moderate, but distinct membranous staining reaction, while endothelial and plasma cells must show a strong, predominantly membranous staining reaction. The squamous epithelial cells must be negative. In liver, the hepatic sinusoidal endothelial cells must display weak to moderate, distinct membranous staining reaction, while hepatocytes are negative.

The recommendations of the mentioned tissue controls for IHC are concordant with the guidelines published by the International Ad Hoc Expert Committee[1].

[1]Torlakovic EE, Nielsen S, Francis G, Garratt J, Gilks B, Goldsmith JD, Hornick JL, Hyjek E, Ibrahim M, Miller K, Petcu E, Swanson PE, Zhou X, Taylor CR, Vyberg M. Standardization of positive controls in diagnostic immunohistochemistry: recommendations from the International Ad Hoc Expert Committee. Appl Immunohistochem Mol Morphol. 2015 Jan;23(1):1-18. doi: 10.1097/PAI.0000000000000163. Review. PubMed PMID: 25474126.

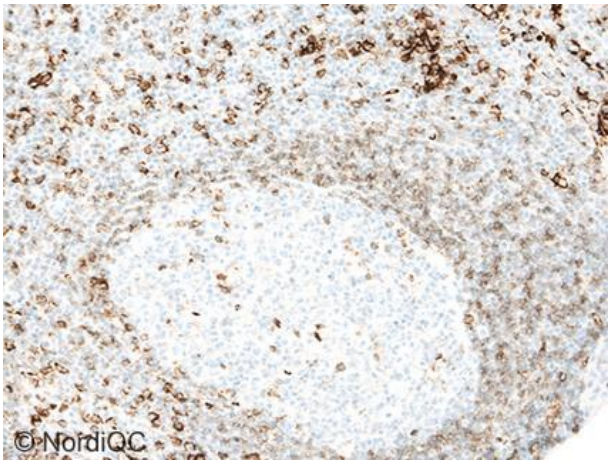


Fig. 1a (x200)
Optimal staining for CD31 of the tonsil, performed on the BenchMark Ultra (Ventana/Roche) using the mAb clone JC70A as concentrate diluted 1:100, efficient HIER in CC1 and OptiView as the detection system - same protocol used in Figs. 2a - 5a. The vast majority of mantle zone B-cells show a weak to moderate staining reaction. Virtually all endothelial and plasma cells show a strong, predominantly membranous staining reaction.

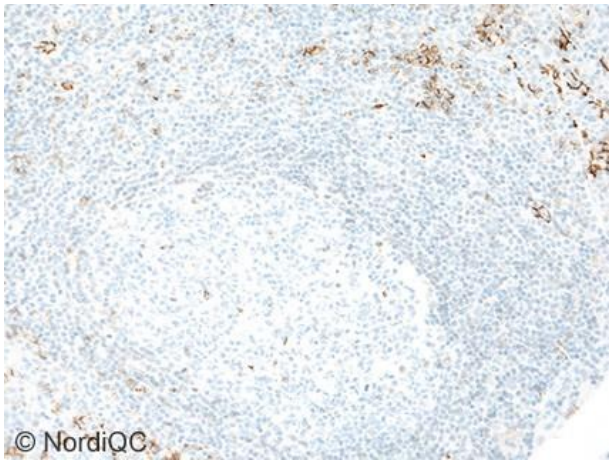


Fig. 1b (x200)
Insufficient staining for CD31 of the tonsil using the mAb clone JC70A within a LD-assay on BenchMark Ultra, applying efficient HIER in CC1, optimal dilution range of the primary antibody (1:25) but with the less sensitive UltraView as the detection system - same protocol used in Figs. 2b – 5b. The mantle zone B-cells are false negative and endothelial cells and plasma cells only display weak to moderate intensity. This pattern was typically seen using 2-step polymer/multimer detection systems e.g., UltraView and/or if HIER was performed in "standard" citric based buffers – same field as in Fig. 1a.

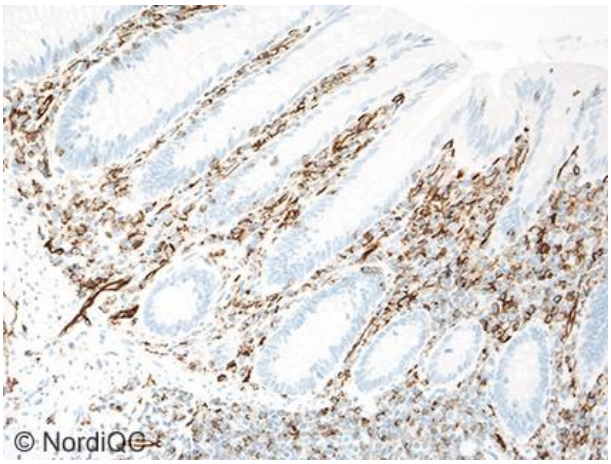


Fig. 2a (x200)
Optimal CD31 staining of the appendix using same protocol as in Fig. 1a. Virtually all endothelial and plasma cells show a strong, predominantly membranous staining reaction. Importantly, a weak but distinct staining reaction is seen in intraepithelial T-cells, while the epithelium is negative.

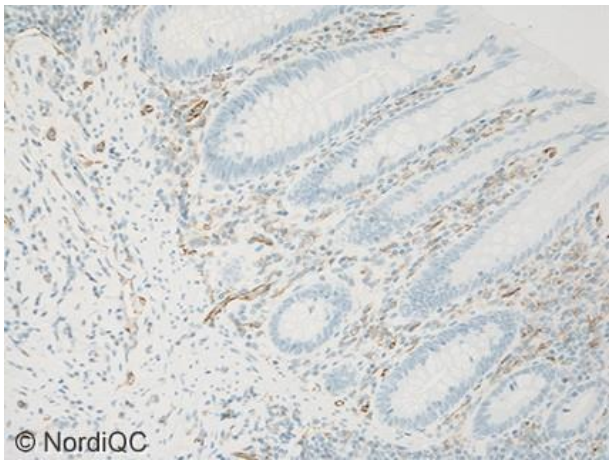


Fig. 2b (x200)
Insufficient CD31 staining of the appendix using same protocol as in Fig. 1b. The proportion and staining intensity of plasma cells is significantly reduced, endothelial cells are only weakly demonstrate and intraepithelial T-cells false negative - same field as in Fig. 2a.

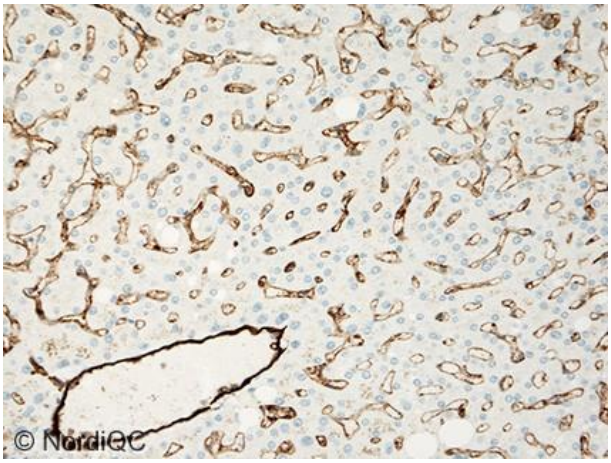


Fig. 3a (x200)
Optimal CD31 staining of the liver using same protocol as in Figs. 1a and 2a. Virtually all hepatic sinusoidal endothelial cells display a moderate and distinct membranous staining reaction, whereas normal vessels are strongly labelled. The hepatocytes are as expected negative.

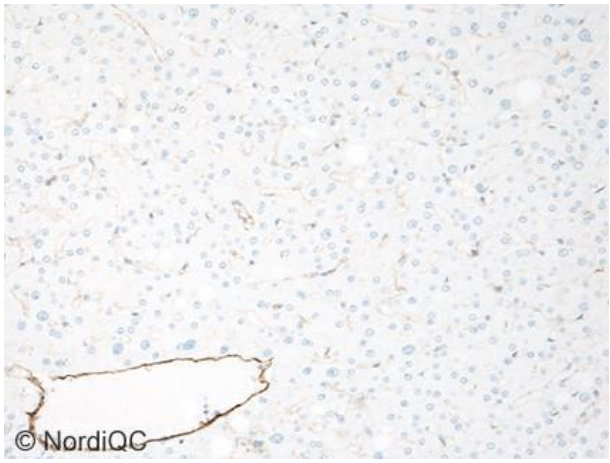


Fig. 3b (x200)
Insufficient CD31 staining of the liver using the same protocol as in Figs. 1b and 2b. Virtually all hepatic sinusoidal endothelial cells are false negative or only faintly demonstrated. Only endothelial cells of large vessels are distinctively demonstrated but display reduced staining intensity - same field as in Fig. 3a.

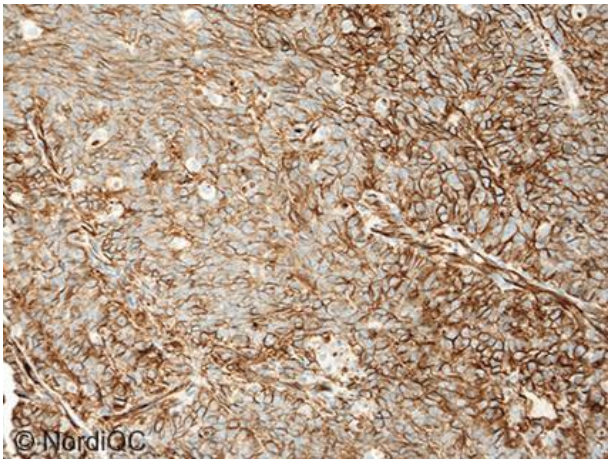


Fig. 4a (x200)
Optimal CD31 staining of the angiosarcoma using same protocol as in Figs. 1a - 3a. All neoplastic cells display a strong and distinct membranous staining reaction.

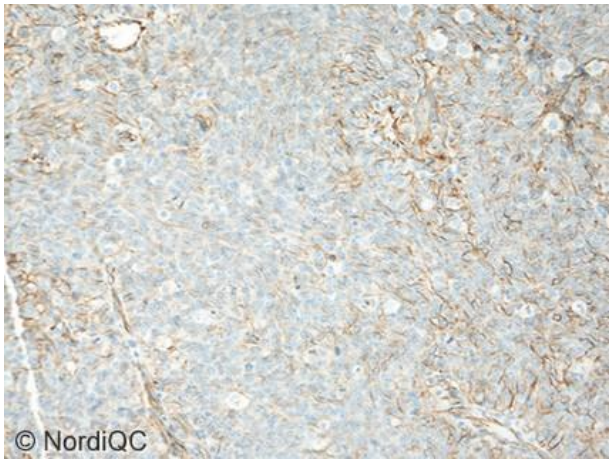


Fig. 4b (x200)
Insufficient CD31 staining of the angiosarcoma using the same protocol as in Figs. 1b - 3b. The vast majority of the neoplastic cells are false negative or only faintly demonstrated - same field as in Fig. 4a.

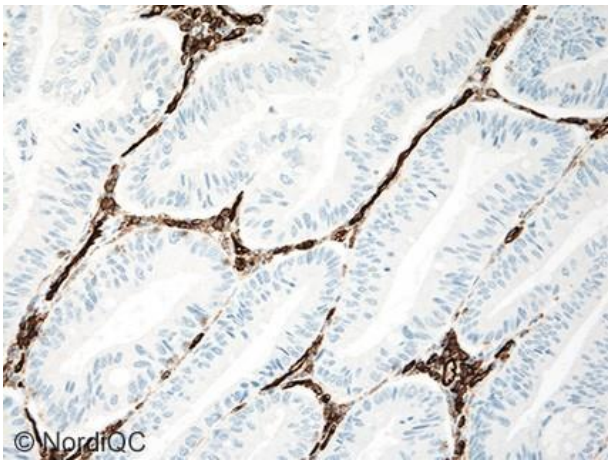


Fig. 5a (x200)
Optimal CD31 staining of the colon adenocarcinoma, using same protocol as in Figs. 1a - 4a. The neoplastic cells are negative as expected. In the stromal compartment of the tumour, both endothelial and plasma cells display a strong, distinct membranous staining reaction.

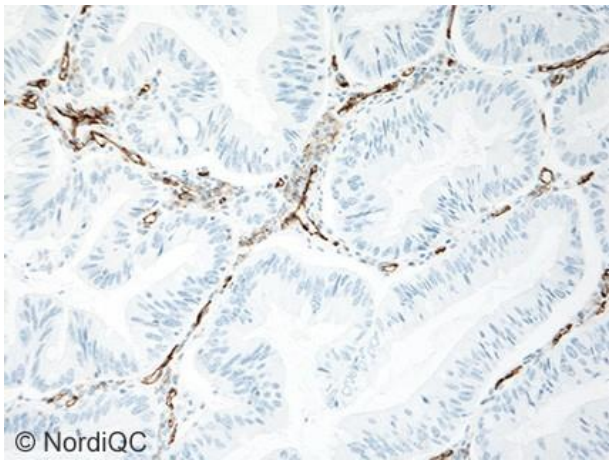


Fig. 5b (x200)
Insufficient CD31 staining of the colon adenocarcinoma, using same protocol as in Figs. 1b - 4b. The neoplastic cells are negative as expected, but endothelial cells in the stromal compartment of the tumour show a reduced staining intensity and a significant proportion of plasma cells are false negative– same field as in Fig. 5a.

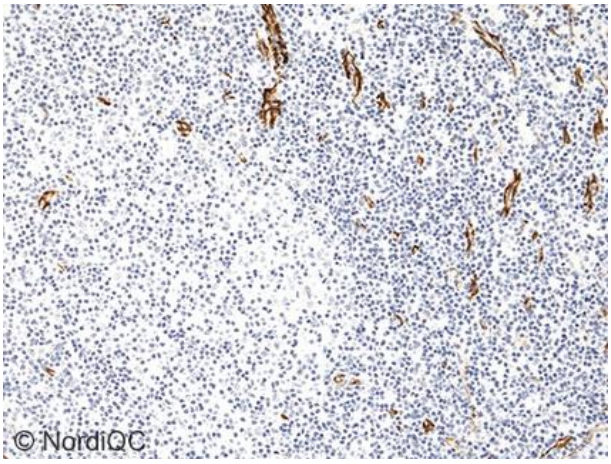


Fig. 6a (x200)
Insufficient CD31 staining of the tonsil using the mAb clone 9G11 within a LD-assay based on proteolytic pre-treatment – same protocol used in Fig. 6 b. The expression of CD31 in lymphocytes e.g., mantle zone B-cells and plasma cells is undetectable due to over-digestion of the fragile membranes, and thus providing an impaired morphology and extraction of the target analyte – compare with Fig 1a.
It is not recommended to use enzymatic pre-treatment for demonstration of CD31, risking false negative results and thereby misdiagnose tumours of unknown origin – see Fig. 6b.

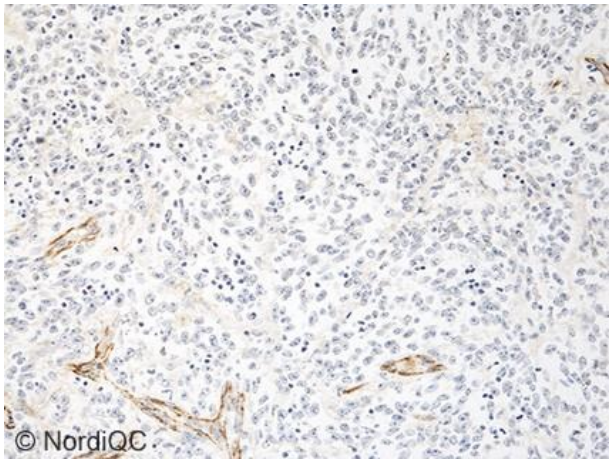


Fig. 6b (x200)
Insufficient CD31 staining of the angiosarcoma using same protocol as in Fig. 6a. All the neoplastic cells are false negative – compare with Fig. 4a. The normal endothelial cells are stained but being unreliable as internal positive tissue control due to the high antigen expression level compared to the level seen in the neoplastic cells with risk to misclassification of the tumour in the diagnostic work-up of cancers of unknown origin. Importantly, laboratories should use appropriate tissue controls and obtain results / staining patterns accordingly to the recommendations given by the International Ad Hoc Expert Committee[1] (see above).

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