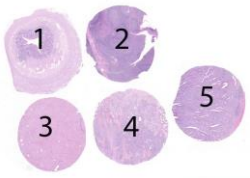
Assessment Run 46 2016
CD31



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 CD31 staining as optimal included:

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

Participation

Number of laboratories registered for CD31, run 46	277
Number of laboratories returning slides	263 (95%)

Results
263 laboratories participated in this assessment. 200 (76%) 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:
- Too low concentration of the primary Ab
- Less sensitive detection systems
- Insufficient HIER
- Less successful primary Abs (in particular 1A10)

Performance history
This was the fifth NordiQC assessment of CD31. The pass rate increased compared to previous runs as shown in table 2.

Table 2. **Proportion of sufficient results for CD31 in the five NordiQC runs performed**

	Run 11 2004	Run 26 2009	Run 32 2011	Run 38 2013	Run 46 2016
Participants, n=	59	116	167	213	263
Sufficient results	66%	52%	60%	62%	76%

Conclusion
The mAb clone **JC70A** was the only Ab providing an optimal demonstration of CD31. As a concentrated format in a laboratory developed (LD) assay, optimal staining results could be obtained on all main platforms from Dako, Leica and Ventana. Efficient HIER in an alkaline buffer or TRS low pH (Dako) in combination with appropriate calibration of the concentration of the primary Ab and use of a sensitive 3-step detection system gave the highest proportion of optimal results in the LD assays.
The Dako Ready-To-Use systems based on the mAb clone JC70A were the most successful assays with an overall pass rate of 89-100%.
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 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1A10**	1 1	Leica/Novocastra Thermo/Pierce	0	0	0	2	-	-
mAb clone **BS50**	1	Nordic Biosite	0	0	1	0	-	-
mAb clone **JC70A**	111 10 3 1 1 1	Dako/Agilent Thermo/Neomarkers Immunologic Cell Marque Leica/Novocastra Zytomed	62	40	20	5	80%	84%
mAb clone **UC-CD31**	1	Immunologic	0	0	1	0	-	-
rmAb clone **EP78**	1 1	Cell Marque Epitomics	0	0	2	0	-	-
Ready-To-Use antibodies								
mAb clone **1A10 PA0250**	5	Leica/Novocastra	0	0	0	5	0%	-
mAb clone **BC2 PM347**	1	Biocare	0	1	0	0	-	-
mAb clone **JC70A 760-4378**	54	Ventana/Roche	23	10	18	3	61%	67%
mAb **JC70A IR/IS610**	47	Dako/Agilent	27	15	3	2	89%	95%
mAb **JC70A GA610**	19	Dako/Agilent	15	4	0	0	100%	100%
mAb clone **JC70A MAD-02048QD**	2	Master Diagnostica	0	1	1	0	-	-
mAb clone **JC70A PA0414**	1	Leica/Novocastra	1	0	0	0	-	-
mAb clone **JC70A MAB-0031**	1	Maixin	1	0	0	0	-	-
Total	263		129	71	46	17	-	
Proportion			49%	27%	18%	6%	76%	

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

Detailed analysis of CD31, Run 46

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) (2/7)*, TRS pH 9 (Dako) (1/5), TRS pH 6.1 (Dako) (8/12), Cell Conditioning 1 (CC1, Ventana) (36/70), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (11/13), BORG Decloaker pH 9.5 (Biocare) (1/1), DIVA Decloaker pH 6 (Biocare) (1/1) or Tris-EDTA pH 9 (2/12) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:400. Using these protocol settings, 94 of 112 (84%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

Table 3. **Proportion of optimal results for CD31 for the most commonly used antibody as concentrate on the 3 main IHC systems***

Concentrated antibody	**Dako Autostainer / Omnis**		**Ventana BenchMark XT / GX /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 **JC70A**	3/11** (27%)	8/10 (80%)	35/67 (52%)	-	8/10 (80%)	0/3

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use antibodies and corresponding systems
mAb clone **JC70A**, product no. **760-4378**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 24-64 min.) and 16-48 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings, 30 of 45 (67%) laboratories produced a sufficient staining result.

mAb clone **JC70A**, product no. **IS610/IR610**, 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 95-99°C), 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 39 of 41 (95%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **JC70A**, product no. **GA610**, Dako, Dako Omnis:
Protocols with optimal results were based on HIER using TRS pH 9 (efficient heating time 24-30 min. at 97°C), 20-30 min. incubation of the primary Ab and EnvisionFlex/FLEX+ (GV800/GV800+GV821) as detection system. Using these protocol settings, 17 of 17 (100%) laboratories produced a sufficient staining result.

mAb clone **JC70A** product no. **PA0414**, Leica/Novocastra, Leica Bond:
One protocol with an optimal result was based on HIER using BERS2 (Bond, Leica) (efficient heating time 20 min. at 99-100°C), 20 min. incubation of the primary Ab and Bond Polymer Refine Detection (Leica DS9800) as detection system.

mAb clone **JC70A**, product no. **MAB-0031**, Maixin, manual staining:
One protocol with an optimal result was based on HIER using Tris-EDTA pH 9 (Water bath, efficient heating time 20 min. at 100°C), 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

Comments
In this assessment and in concordance with the previous NordiQC assessments of CD31, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of the cells and structures expected to be demonstrated. This pattern was observed in 98% of the insufficient results (63 of 64). Virtually all laboratories were able to demonstrate CD31 in high level antigen expressing structures such as endothelial cells of the large vessels in the appendix and portal tracts in the liver, whereas demonstration of CD31 in low expressing structures as hepatic sinusoidal endothelial cells and activated B-cells in the mantle zones of the tonsil were more challenging.

Only the mAb clone JC70A could be used to provide an optimal staining result, as seen in table 1. Applied as a concentrated format within a laboratory developed (LD) assay, optimal results could be obtained on the most widely used IHC platforms, as shown in table 2.
A careful calibration of the titre of the primary Ab, efficient HIER either in an alkaline buffer or special formulated citrate based buffers TRS low pH (Dako) or DIVA (Biocare) in combination with a 3-step polymer/multimer based detection system provided the highest proportion of optimal results. E.g. on the Ventana BenchMark platform a pass rate of 100% (17 of 17) was seen of which 82% were scored as optimal, when the mAb was applied in the range of 1:20-100, HIER was performed in CC1 for 32-64 min. and OptiView (760-700) selected as detection system. If one or more of these basic protocol settings were changed, a less successful result was observed. E.g using the settings listed for Ab titre and HIER but with the 2-step multimer based UltraView (760-500) as detection system a pass rate of 81% (18 of 22) was seen, of which only 32% were scored as optimal.

Dako and Ventana Ready-To-Use (RTU) systems based on mAb clone JC70A were the most widely used RTU systems with similar observations as the LD assays.
The Dako RTU systems IR/IS610 and GA610 for Autostainer and Omnis, respectively were most successful providing an overall pass rate of 89 and 100%. Optimal results primarily were obtained by using the official protocol recommendations given by Dako using HIER in TRS High pH 9 and FLEX+, mouse as detection system. For all five insufficient results by the RTU system IS/IR610, a significant laboratory modified protocol was applied, such as dilution of the RTU format, omission of mouse linker or performing the assay on a non-Dako IHC platform.
The Ventana RTU system based on product id. 760-4378 provided a pass rate of 61%. An optimal result typically was based on a laboratory modified protocol using a 3-step detection system as OptiView or UltraView + amplification. In contrast, the vendor recommended protocol, which includes the 2-step visualization system Ultraview, could not provide an optimal staining reaction.

This was the fifth NordiQC assessment of CD31 and ever since the low pass rate of 52% observed in run 26, 2009 the pass rate has consistently increased (see table 2). In this run a pass rate of 76% was seen, which is very satisfactory, especially as many new laboratories participated for the first time.
The primary reason for the improvement seems to be an increased use of mAb clone JC70A at the expense of the less successful mAb clone 1A10. In run 26 mAb clone JC70A was used by 88% (102 of 116) and mAb clone 1A10 by 12% (14 of 116) of the laboratories. In the current run 95% (251 of 263) used mAb clone JC70A and only 3% (7 of 263) used mAb clone 1A10. At the same time harmonization of recommendable protocol settings has been observed. E.g., in run 26, 7% of the laboratories used proteolytic pre-treatment as epitope retrieval compared to <1% in this run (in all NordiQC assessments for CD31 proteolytic pre-treatment method has been inferior to HIER).

Controls
Liver and tonsil are recommendable positive tissue controls for CD31. In liver an at least weak to moderate, distinct staining reaction in virtually all hepatic sinusoidal endothelial cells must be seen. Endothelial cells of the portal tract vessels must show a moderate to strong staining reaction. In tonsil the majority of activated mantle zone B-cells must display an at least weak to moderate, distinct membranous staining reaction, whereas plasma cells and endothelial cells must show a strong staining reaction. Appendix can be used as negative tissue control, as no staining reaction of the epithelial cells should be seen (only intraepithelial lymphocytes should be demonstrated).

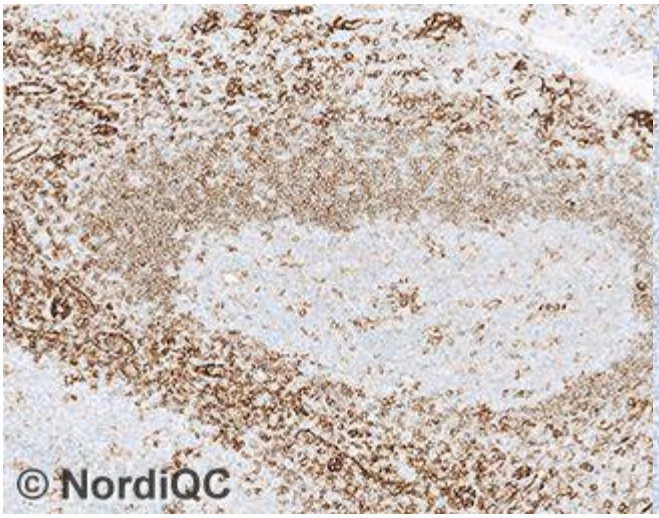


Fig. 1a
Optimal CD31 staining of the tonsil using the mAb clone JC70A as Ready-To-Use format (GA610, Dako), with HIER in TRS High pH 9 for 30 min., a 3-step polymer based detection kit and performed on Omnis, Dako.
Even at low magnification (4x) a distinct positive staining reaction of mantle zone B-cells, plasma cells and endothelial cells can be identified
Also compare with Figs. 2a – 4a, same protocol.

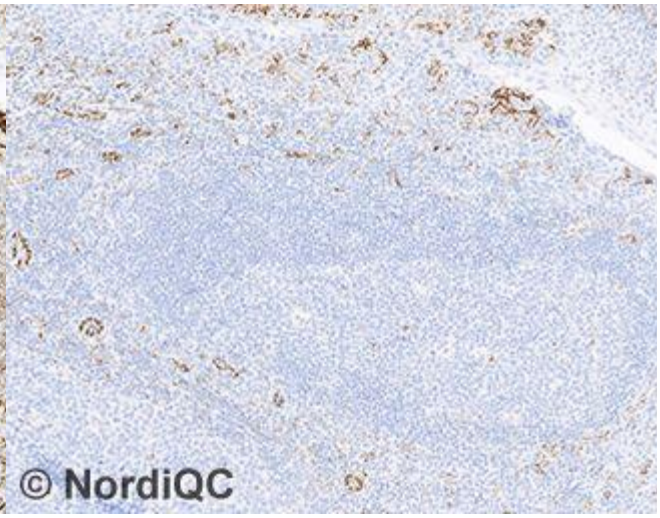


Fig. 1b
Insuffcient CD31 staining of the tonsil using the mAb clone JC70A as Ready-To-Use format (IR610) same field as in Fig. 1a.
The primary Ab was applied by a laboratory modified protocol reducing the HIER time and a 2-step polymer system.
At 4x magnification, only endothelial cells can be identified.
Also compare with Figs. 2b - 4b – same protocol.

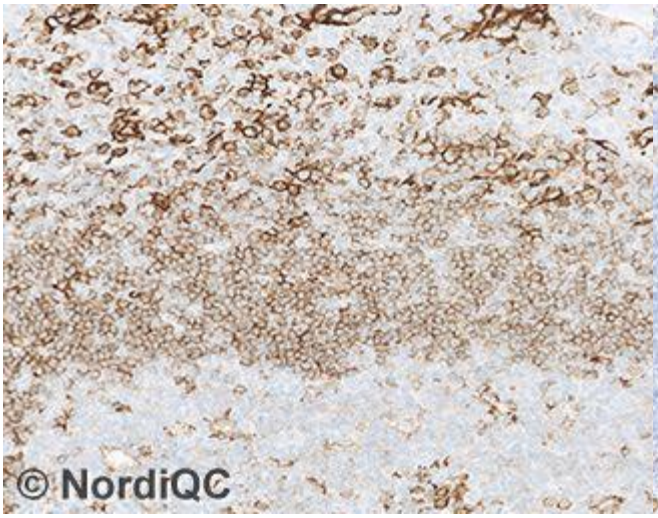


Fig. 2a
Optimal CD31 staining of the tonsil using same protocol as in Fig. 1a.
At 200x a moderate and distinct membranous staining reaction is seen in the majority of mantle zone B-cells, while plasma cells and endothelial cells show an intense staining reaction.
No background reaction is seen.

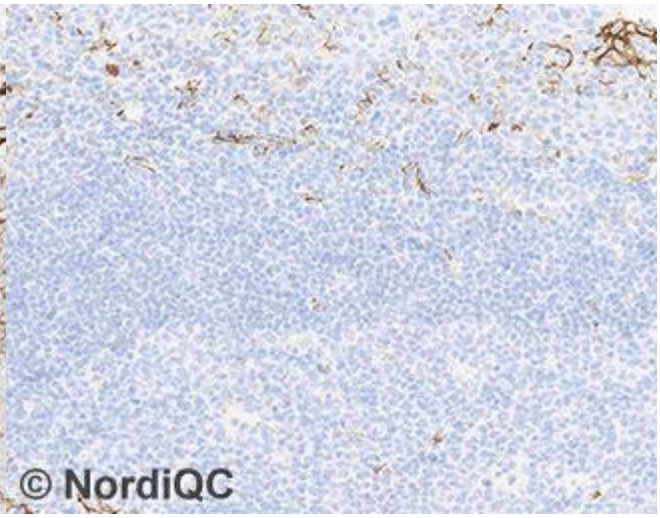


Fig. 2b
Insufficient CD31 staining of the tonsil using same protocol as in Fig. 1b - same field as in Fig 2a.
Only endothelial cells and plasma cells are demonstrated.
Mantle zone B-cells are negative
Also compare with Fig. 4b – same protocol.

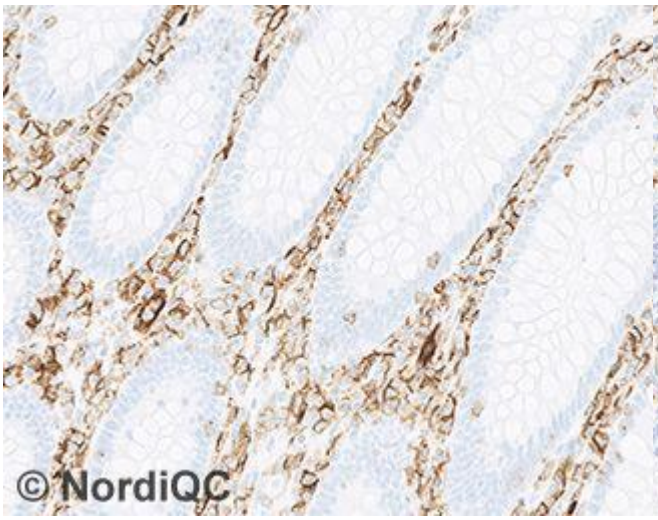


Fig. 3a
Optimal CD31 staining of the appendix using same protocol as in Figs. 1a and 2a.
Both endothelial cells, plasma cells and macrophages in lamina propria and intraepithelial T-cells are distinctively demonstrated.
No staining reaction of epithelial cells is seen.

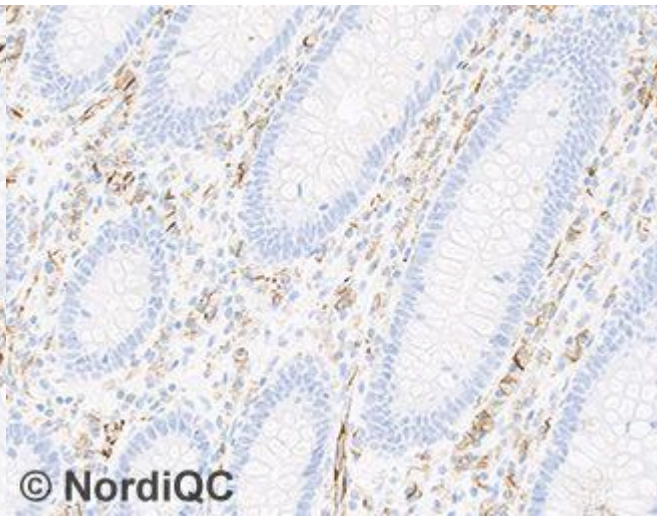


Fig. 3b
Insufficient CD31 staining of the appendix using the same protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
The intensity and proportion of plasma cells and macrophages is significantly reduced compared to the level expected and obtained by optimal protocol settings and only endothelial cells are distinctively demonstrated.
Intraepithelial T-cells are negative.

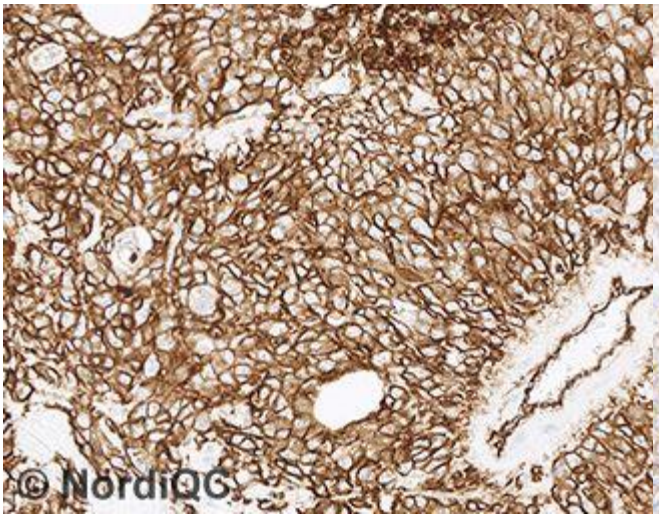


Fig. 4a
Optimal CD31 staining of the angiosarcoma using same protocol as in Figs. 1a - 3a.
Virtually all neoplastic cells show a distinct predominantly membranous staining reaction.
No background reaction is seen.

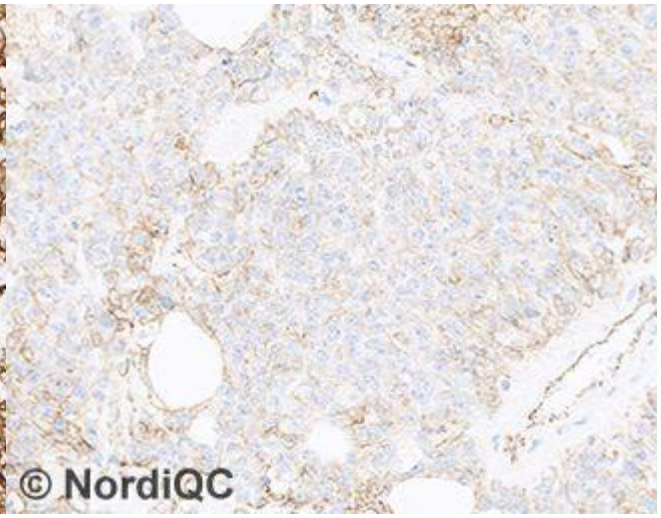


Fig. 4b
CD31 staining of the angiosarcoma using the same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
The intensity and proportion of neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained by optimal protocol settings.
Normal endothelial cells, bottom right, are demonstrated stressing these cells cannot reliably be used as internal positive tissue control.
Tonsil should be used as external positive tissue control to monitor a correct analytical sensitivity (see Fig. 1a).

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