Assessment Run 38 2013
CD31

Material
The slide to be stained for CD31 comprised:

1. Appendix, 2. Tonsil, 3. Liver, 4. Angiosarcoma

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 normal endothelial cells and plasma cells in all the cores.
- An at least weak to moderate, distinct membranous staining reaction of the activated B- and T-cells – in particular the mantle zone B-cells in the tonsil and the 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 neoplastic cells in the angiosarcoma.
- No staining reaction of the epithelial cells of the appendix and tonsil.

213 laboratories participated in this assessment. Of these, 131 (62%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks.

Table 1. **Antibodies and assessment marks for CD31, run 38**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **JC70A**	113 11 2 1 1 1 1	Dako NeoMarkers Cell Marque Immunologic Biocare ZytoMed Maixin Biotechnology	50	38	33	9	68 %	92 %
mAb clone **1A10**	7	Leica/Novocastra	-	1	3	3	14%	-
rAb clone **EP3095**	1	Millipore	-	-	1	-	-	-
mAb clone **UC-CD31**	1	Immunologic	-	-	1	-	-	-
rAb clone **EP78**	1	Epitomics	-	-	1	-	-	-
mAb clone **BS50**	1	Nordic Biosite	-	-	-	1	-	-
Ready-To-Use antibodies								
mAb clone **JC70A, IR610/IS610**	34	Dako	15	16	3	0	91 %	97 %
mAb clone **JC70A, 760-4378**	28	Ventana / Cell Marque	2	7	15	4	32 %	67 %
mAb clone **JC70A, PM131-97**	1	Cell Marque	0	0	1	0	-	-
mAb clone **JC70A, MAD-002048QD**	1	Master Diagnostica	1	0	0	0		
mAb clone **JC70A, GM0823-02**	1	Gene Tech	0	0	0	1	-	-
mAb clone **BC2, PM347**	1	Biocare	0	1	0	0	-	-
mAb clone **1A10 PA0250**	5	Leica/Novocastra	0	0	0	5	0%	0%
mAb clone **1A10**	1	Monosan	0	0	0	1	-	-

MON-RTU1040							
Total	213	68	63	58	24	131	
Proportion		32 %	30%	27 %	11 %	62 %	

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 38
The following protocol parameters were central to obtain an optimal staining

Concentrated Antibodies
mAb clone **JC70A**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Tris-EDTA/EGTA pH 9 (4/17)*, Target Retrieval Solution pH 9 (3-in-1) (Dako) (2/9), Target Retrieval Solution pH 9 (Dako) (3/7), Target Retrieval Solution pH 6.1 (Dako) (9/12), Bond Epitope Retrieval Solution 1 (Bond, Leica) (2/6), Bond Epitope Retrieval Solution 2 (Bond, Leica) (9/12), HIER buffer High (Thermo S./ Labvision) (1/1) or Cell Conditioning 1 (BenchMark, Ventana) (20/57). The mAb was typically diluted in the range of 1:10–1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 87 of 95 (92 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

Ready-To-Use (RTU) Antibodies
mAb clone **JC70A** (prod. no. IR610, Dako): Protocols with an optimal result were all based on HIER in PT-Link using Target Retrieval Solution pH 9 (3-in-1) or Target Retrieval Solution pH 9 (heating time 10-20 min. at 95-100°C), 20-30 min. incubation with the primary Ab and EnVision Flex/Flex+ (K8000/K8002) as the detection systems. Using these protocol settings 33 of 34 (97 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **JC70A** (prod. no. 760-4378, Ventana): Protocols with optimal results were based on HIER using mild or standard Cell Conditioning 1, 32 min. incubation of the primary Ab and UltraView (760-500) with amplification (760-080) or OptiView (760-700) as detection systems. Using these protocol settings 4 of 6 (67%) laboratories produced a sufficient staining (optimal or good).

mAb clone **JC70A** (prod. no. MAD-002048QD, Master Diagnostica): The protocol with an optimal result was based on HIER using EDTA/EGTA pH 8 (heating time 20 min. at 95°C, 10 min. incubation of the primary Ab and Quanto (MAD-021881QK) as detection system.

Table 2 summarizes the overall proportion of optimal staining results, using the most frequently used concentrated Ab on the three most commonly used IHC stainer platforms.

Table 2. **Optimal results for CD31 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
Buffer	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**	33 % 5/15**	73 % 8/11	36 % 20/55	- -	75 % 9/12	33 % 2/6

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

The most frequent causes of insufficient stains were:

- Less successful primary antibodies
- Too low concentration of the primary antibody
- Inappropriate epitope retrieval (use of proteolysis)
- Use of non-alkaline HIER buffer or insufficient HIER (too short efficient HIER time)
- Use of low sensitive detection systems

In concordance with the previous NordiQC assessment of CD31 (run 32, 2011), the prevalent feature of an insufficient result was a too weak or a false negative staining of the cells expected to be demonstrated. The majority of the laboratories were able to demonstrate CD31 in high level antigen expressing structures such as endothelial cells of the large vessels in the appendix and in the portal rooms in the liver, whereas the demonstration of CD31 in the hepatic sinusoidal endothelial cells and the activated B-cells in the mantle zones of the tonsil were more challenging and required an optimally calibrated protocol. If these critical staining quality indicators displayed a very faint staining reaction or were absent, the neoplastic

cells of the angiosarcoma were negative or showed an equivocal staining reaction.

In this assessment, the overall pass rate was highly influenced by the choice of primary Ab, epitope retrieval procedure and detection system used.

Optimal staining result could only be obtained with the mAb clone JC70A. Used as a concentrate with HIER based on either BERS2 (Leica) or TRS low pH 6.1 (Dako), mAb JC70A provided the highest proportion of sufficient (100% and 91%, respectively) and optimal results (75% and 73%, respectively) (Table 2). If the concentrated format of the mAb JC70A was used in the "optimal" dilution range 1:10-1:100, alkaline HIER buffers (pH 8-9) provided a slightly higher pass rate of 75% (n=66/88) out of which 41% (n=36/88) were assessed as optimal, compared to protocols based on standard acidic (pH 6) HIER buffers (overall pass rate of 60% (n=6/10), 10% (n=1/10) was optimal).
No protocol based on enzymatic digestion (n=6) was assessed as optimal and only one produced a sufficient result.

The proportion of sufficient results was influenced by the choice of the detection system used. Protocols based on the same dilution range as mentioned above, HIER performed in an alkaline buffer and applying a 2-step polymer/multimer based detection system, provided a sufficient staining result in 55% (n=21/38) and 18% (n=7/38) gave an optimal result. If the same protocol settings were applied with 3-step polymer/multimer based detection system (EnVision FLEX+ (Dako), Optiview (Ventana) or Bond Refine (Leica)), 92% (n=31/34) produced a sufficient staining result and 68% (n=23/34) assessed as optimal.

All protocols except for one (n=11/12) based on the mAb 1A10, either as concentrate or as a Ready-To-Use (RTU) format, were assessed as insufficient. The main feature was lack of staining in challenging cellular structures such as hepatic sinusoidal endothelial cells and activated B-cells in the mantle zones of the tonsils giving false negative results. Even though the protocol settings applied for the mAb clone 1A10 have been similar to the laboratories producing optimal staining results with the mAb clone JC70A (Figs. 4a and 4b), no participants using the mAb 1A10 have ever obtained an optimal score in this or in previous NordiQC runs for CD31.

In this run, the Dako RTU system for CD31 based on the mAb clone JC70A (IR610/IS610) provided a higher pass rate and proportion of optimal results, compared to both in-house validated protocols and the Ventana RTU system for CD31 based on the same clone. The Dako RTU system gave a pass rate of 97 % out of which 44 % were optimal. In comparison the RTU system from Ventana (760-4378) based on the same mAb gave a pass rate of 32 % out of which 7 % were assessed as optimal.

Controls
Liver was found to be a recommendable control for CD31. In liver tissue an at least weak to moderate, distinct staining reaction must be seen in the majority of the hepatic sinusoidal endothelial cells. Alternatively, tonsil can be used as positive control, provided that the activated B- and T-cells, particular the mantle zone B-cells display an at least weak to moderate and distinct membranous staining. It is crucial that these structures (critical staining quality indicators for CD31) are demonstrated as this indicates a highly sensitive protocol has been applied.
No staining reaction must be seen in hepatocytes or in epithelial cells of tonsil and appendix.

Performance history
This was the 4th NordiQC assessment of CD31 (Table 3). A similar pass rate was achieved in this run compared to run 32, 2011.

Table 3. **Proportion of sufficient results for CD31 in the four NordiQC runs performed**

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

Several parameters may contribute to the consistent low pass rate and lack of improvement. The persistent use of the less successful mAb clone 1A10, inappropriate epitope retrieval for the mAb clone JC70A (use of a standard acidic (pH 6) HIER buffer or proteolysis), poorly calibrated Abs or use of low sensitive detection systems all have a negative impact on the pass rate.

Conclusion
The mAb clone **JC70A** is the most robust marker for CD31. Efficient HIER with an alkaline buffer or TRS low pH 6.1 (Dako) in combination with a sensitive and specific IHC system gave the highest proportion and optimal results. The mAb clone JC70A provided an optimal result on the 3 main IHC platforms (Ventana,

Dako and Leica). The Ready-To-Use system based of the mAb clone JC70A from Dako gave the highest proportion of sufficient and optimal results.
Liver is recommended as control tissue. Hepatic sinusoidal endothelial cells must show an as strong as possible staining, while the liver cells must be negative.

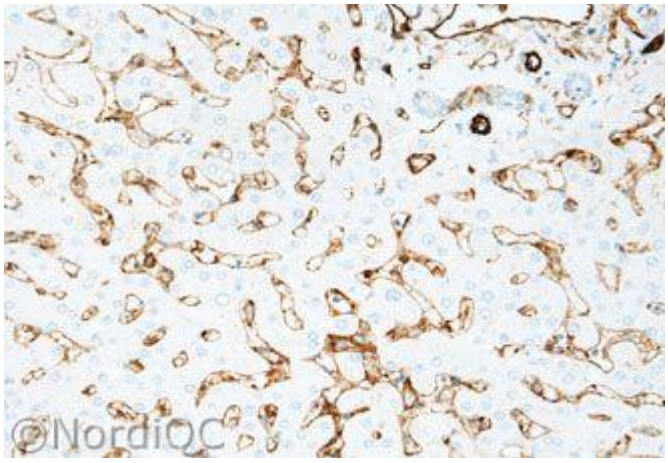

Fig. 1a
Optimal staining for CD31 of the liver using the mAb clone JC70A as as concentrate, HIER in an alkaline buffer and a 3-step multimer based detection system.
Virtually all the hepatic sinusoidal endothelial cells show a moderate predominantly membranous staining reaction.

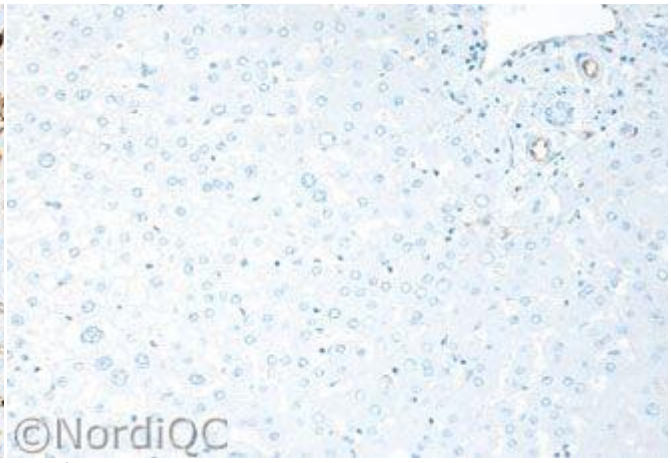

Fig. 1b
Insufficient staining for CD31 of the liver using the mAb clone JC70A by a protocol with a too low sensitivity (too low. conc. of the primary Ab and a 2-step multimer based detection system) - same field as in Fig. 1a.
All hepatic sinusoidal endothelial cells are negative compared to the result obtained in Fig. 1a.
Also compare with Figs. 2b - same protocol.

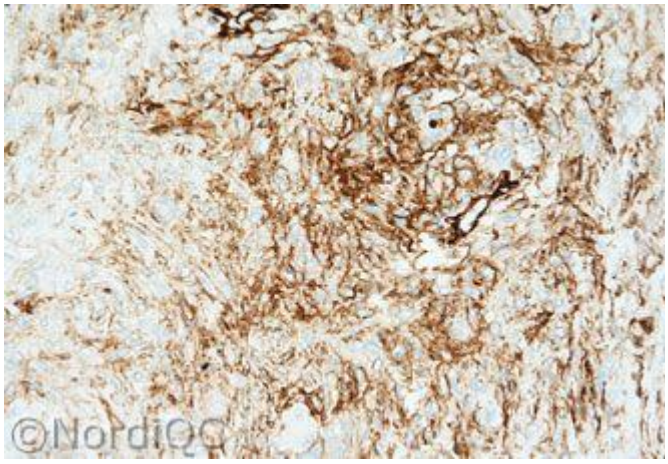

Fig. 2a
Optimal staining for CD31 of the angiosarcoma using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong predominantly membranous staining reaction.

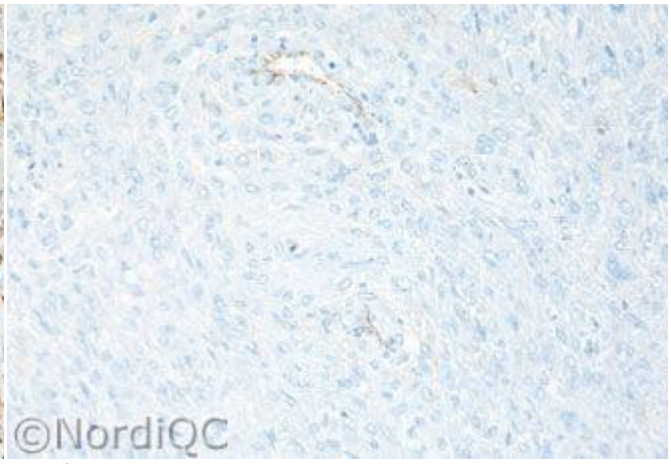

Fig. 2b
Insufficient staining for CD31 of the angiosarcoma using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only the large vessels display a too weak and equivocal staining reaction and all the neoplastic cells of the angiosarcoma is completely negative.

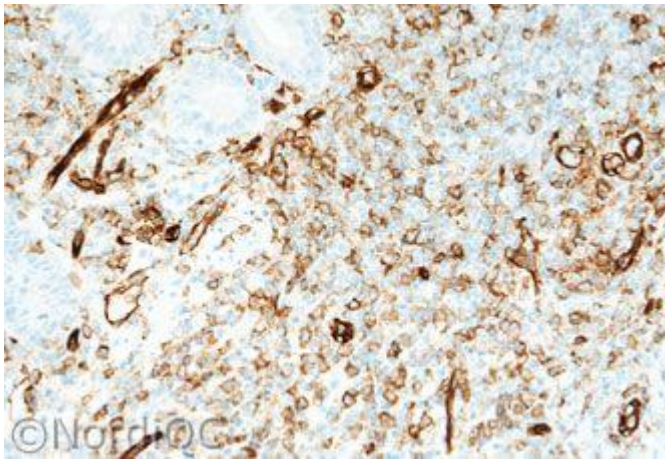

Fig. 3a
Optimal staining for CD31 of the appendix using same protocol as in Fig. 1a & 2a.
All the vessels show a distinct, moderate to strong staining reaction, whereas activated B- and T-cells displays a weak to moderate, distinct membranous staining.

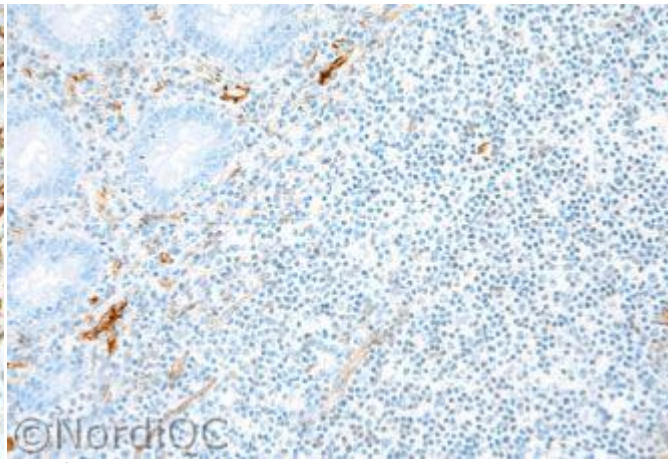

Fig. 3b
Insufficient staining for CD31 of the appendix using same protocol settings as in Fig. 3a. except for using enzymatic pre-treatment.
Only the endothelial cells of the vessels show a weak to moderate staining, while activated B- and T-cells are all false negative - same field as in Fig. 3a.

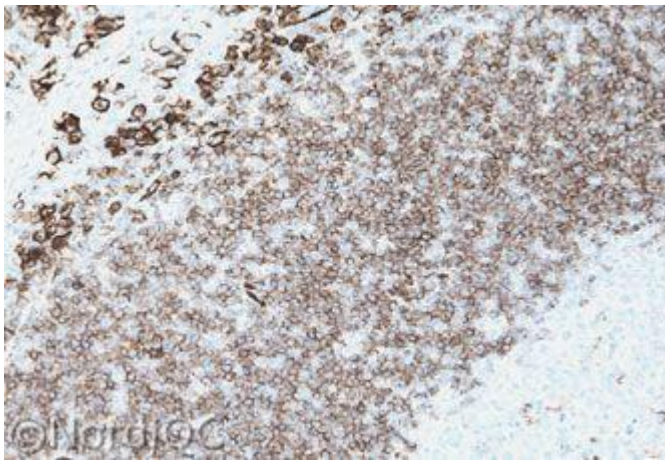

Fig. 4a
Optimal staining for CD31 of the tonsil using the mAb clone JC70A (dil. 1:50), HIER in TRS low pH6.1 (Dako) and Envision Flex+ as the detection system.
The B- and T-cells – in particular the mantle zone B-cells show an at least weak to moderate, distinct membranous staining.

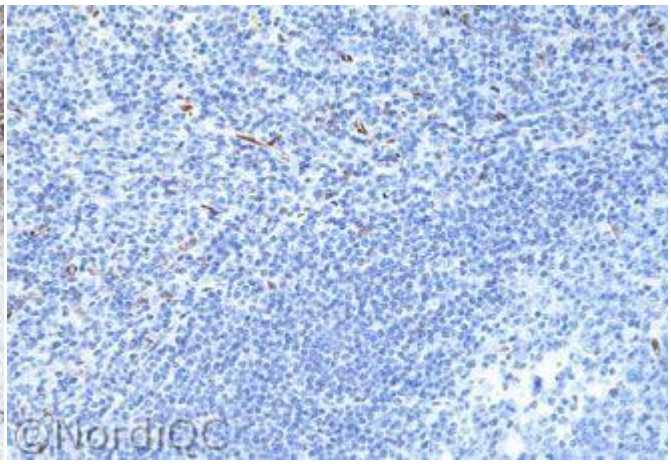

Fig. 4b
Insufficient staining for CD31 of the tonsil using exactly the same protocol settings as in Fig. 4a. except for using the mAb 1A10 (dil. 1:50). Virtually all the B- and T-cells are false negative and only faint reaction is seen in the vessels.

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