Assessment Run 31 2011
CD68

The slide to be stained for CD68 comprised:

1. Appendix, 2. Liver, 3. Tonsil, 4. Brain, 5. Spleen histiocytic sarcoma
All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD68 staining as optimal included:

- A strong and distinct cytoplasmic staining of the germinal centre macrophages in the secondary follicles in the tonsil and appendix.
- A moderate to strong cytoplasmic staining of the macrophages in the interfollicular zones of the tonsil, in lamina propria of the appendix and in the Kupffer cells of the liver.
- An at least weak to moderate cytoplasmic staining of the microglial cells in the brain.
- An at least moderate cytoplasmic staining in virtually all the neoplastic cells of the histiocytic sarcoma.
- No staining in the liver cells and no or only a weak cytoplasmic staining in the epithelial cells in the appendix and tonsil.

157 laboratories participated in this assessment. 82 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for CD68, run 31**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **PG-M1**	60 1 1	Dako IDlabs NeoMarkers	28	28	5	1	90 %	95 %
mAb clone **KP1**	47 7 3 1 1 1	Dako NeoMarkers Leica/Novocastra Biocare Cell Marque Zytomed	2	43	12	3	75 %	100 %
mAb clone **514H12**	2	Leica/Novocastra	1	1	0	0	-	-
Ready-To-Use Abs								
mAb clone **PG-M1, IR613**	7	Dako	6	1	0	0	100 %	100 %
mAb clone **514H12, PA0273**	2	Leica/Novocastra	0	2	0	0	-	-
mAb clone **KP1, 790-2931**	12	Ventana	0	9	2	1	75 %	-
mAb clone **KP1, IR609**	8	Dako	0	4	4	0	50 %	-
mAb clone **KP1, N1577**	1	Dako	0	1	0	0	-	-
mAb clone **KP1, PM033**	1	Biocare	0	1	0	0	-	-
mAb clone **KP1, 168M-97**	1	Cell Marque	0	1	0	0	-	-
mAb clone **KP1, MS-397-R7**	1	NeoMarkers	0	0	1	0	-	-
Total	157		37	91	24	5	-	-
Proportion			24 %	58 %	15 %	3 %	82 %	-

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

The following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs
mAb clone **PG-M1**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution pH 9 (3-in-1) (Dako) (8/9)*, Tris-EDTA/EGTA pH 9 (6/8), Target Retrieval Solution pH 9 (Dako) (4/8), Bond Epitope Retrieval Solution 2 (Bond, Leica) (5/6), Cell Conditioning 1 (BenchMark, Ventana)(3/21) or Citrate pH 6 (2/2) as the retrieval buffer. The mAb was typically diluted in the range of 1:50– 1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 52 out of 55 (95 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **KP1**: The protocols giving an optimal result were all based HIER using either Tris-EDTA/EGTA pH 9 (1/8) or Target Retrieval Solution pH 9 (3-in-1) (Dako) (1/11) as the retrieval buffer. The mAb was typically diluted in the range of 1:500– 1:8.000 depending on the total sensitivity of the protocol employed. Using these protocol settings all of 7 (100 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **514H12**: The protocol giving an optimal result was based on Target Retrieval Solution pH 9 (3-in-1) (Dako) using Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/2) as the retrieval buffer. The mAb was diluted 1:50.

Ready-To-Use Abs
mAb clone **PG-M1** (prod. no. IR613, Dako): The protocols giving an optimal result were all based on HIER in PT-Link using either Target Retrieval Solution (TRS) pH 9 (3-in-1, Dako) or TRS pH 9 (Dako) and an incubation time of 20-30 min in the primary Ab and EnVision Flex (K8000) or Envision+ (K4007) as the detection system. Using these protocol settings all of 12 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient stains were:
- Inappropriate epitope retrieval (e.g., all of 11 protocols based on enzymatic pre-treatment for the mAb clone KP1 gave an insufficient result)
- Too low concentration of the primary Ab.

In this assessment and in concordance with the observations in the previous assessment of CD68 (run 26, 2009), the prevalent feature of an insufficient staining was a too weak or completely false negative reaction in the cells supposed to be demonstrated. Virtually all the participating laboratories were able to demonstrate CD68 in the germinal centre macrophages of the lymphatic secondary follicles, whereas the demonstration of CD68 in the interfollicular macrophages, the neoplastic cells of the histiocytic sarcoma and in particular the microglial cells of the brain was more challenging and was only seen with appropriate protocol settings, e.g., a correct titre of the mAb clones PG-M1, KP1 and 514H12 and the use of HIER.
Enzymatic pre-treatment could not be used to obtain an optimal result irrespective of the mAb clone applied. Proteolysis was seen to give not only a reduced sensitivity (as the microglial cells only showed a weak or complete negative staining) but also an impaired morphology due to digestion of the fragile cell membranes. With proteolytic pre-treatment all of 11 laboratories using KP1 and 3 out of 4 laboratories using PG-M1 obtained an insufficient result.

In this test normal brain was an efficient positive control for CD68, as all the laboratories obtaining a sufficient result could demonstrate CD68 in the microglial cells. However, in order to evaluate the specificity and a proper signal-to-noise ratio it is advisable also to use tonsil as control, in which the germinal centre B-cells must be negative. Using the mAb clone KP1 a weak to moderate cytoplasmic staining in the squamous epithelial cells should be accepted.

This was the 3rd NordiQC test for CD68. A slight increase in the proportion of sufficient stains is seen (table 2). The higher pass rate may in part be due to the specific recommendations given to the laboratories obtaining an insufficient mark in run 26. 32 of these laboratories submitted a new stain in this run. 15 followed the recommendations, of which 14 improved to good or optimal (93%). 13 laboratories did not follow the recommendations, and 4 of these (31 %) obtained a sufficient staining in the subsequent run.
3 laboratories changed their IHC system completely and 2 of these obtained a sufficient result.

Table 2. **Proportion of sufficient results for CD68 in the three NordiQC runs performed**

	Run 11 2004	Run 26 2009	Run 31 2011
Participants, n=	64	128	157
Sufficient results	73 %	70 %	82 %

Among the sufficient reactions the proportion of optimal stains in all three runs was considerably higher with clone PG-M1 than with clone KP1. This is due to a more distinct staining reaction and less cross reaction with clone PG-M1.

Conclusion

The mAb clones **PG-M1**, **KP1** and **514H12** are all recommendable antibodies for CD68. HIER (preferable in an alkaline buffer) is mandatory to obtain an optimal result. Brain and tonsil are recommended as control. In the brain, the microglial cells must show an as strong as possible cytoplasmic staining, while the background must be negative. In the tonsil the interfollicular macrophages must show a moderate to strong cytoplasmic staining, while the germinal centre B-cells should be negative.

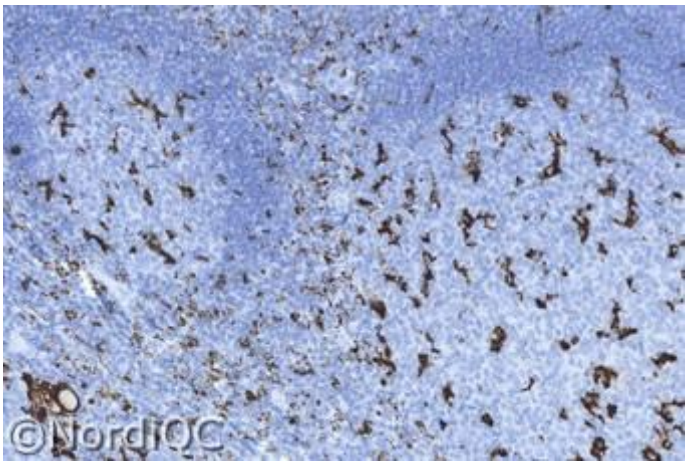

Fig. 1a
Optimal CD68 staining using the mAb clone PG-M1 optimally calibrated and with HIER. Both the germinal centre macrophages and the interfollicular macrophages show a strong and distinct cytoplasmic staining, without any background staining. Also compare with Figs. 2a & 3a – same protocol.

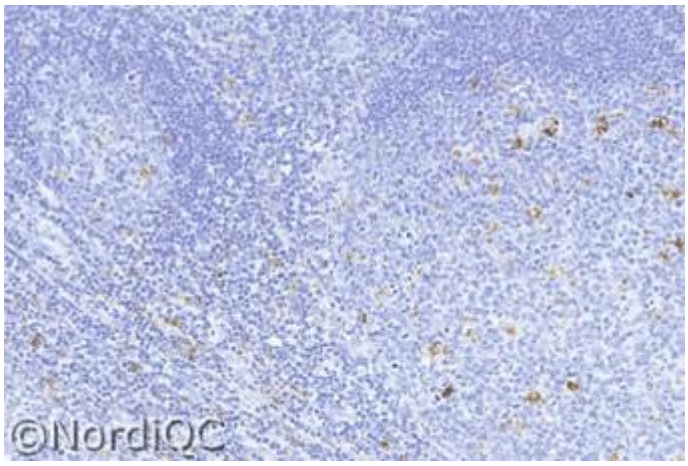

Fig. 1b
Insufficient CD68 staining using the mAb clone KP1 too diluted and no pre-treatment. Scattered macrophages are demonstrated, but both the intensity and the proportion are significantly reduced compared to the result demonstrated in Fig. 1a - same field. Also compare with Figs. 2b & 3b – same protocol.

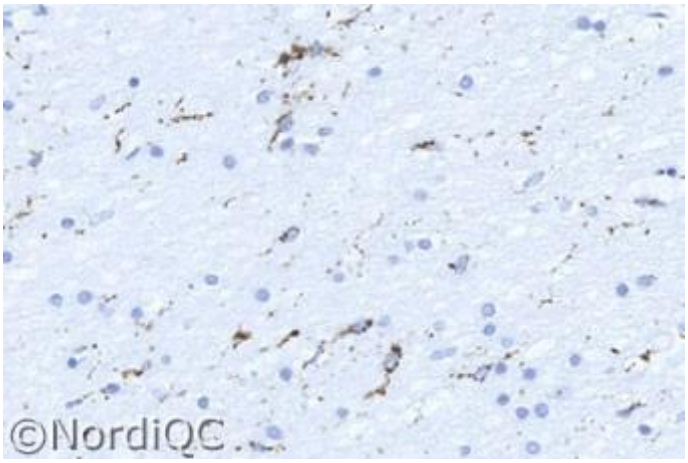

Fig. 2a
Optimal CD68 staining of the brain using same protocol as in Fig. 1a. The microglial cells show a weak to moderate distinct cytoplasmic staining reaction.

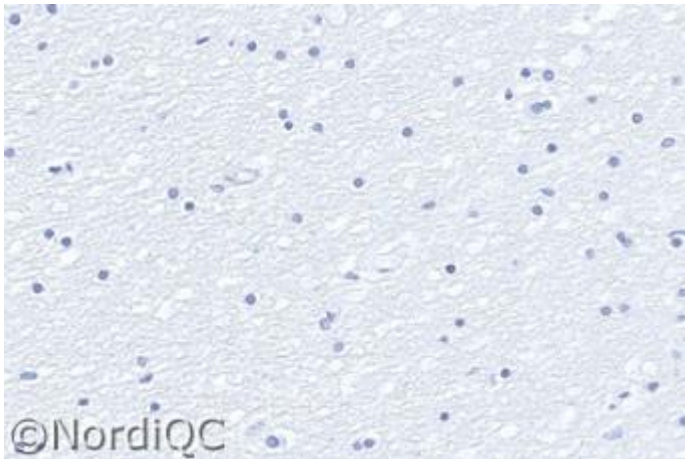

Fig. 2b
Insufficient CD68 staining of the brain using same protocol as in Fig. 1b. No staining reaction is seen in the microglial cells. Also compare with Fig. 3b – same protocol.

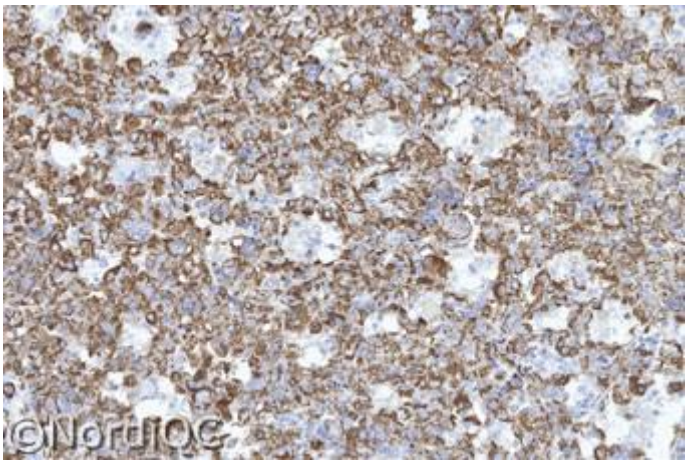

Fig. 3a
Optimal CD68 staining of the histiocytic sarcoma using same protocol as in Figs. 1a & 2a. Virtually all the neoplastic cells show a moderate to strong cytoplasmic staining reaction.

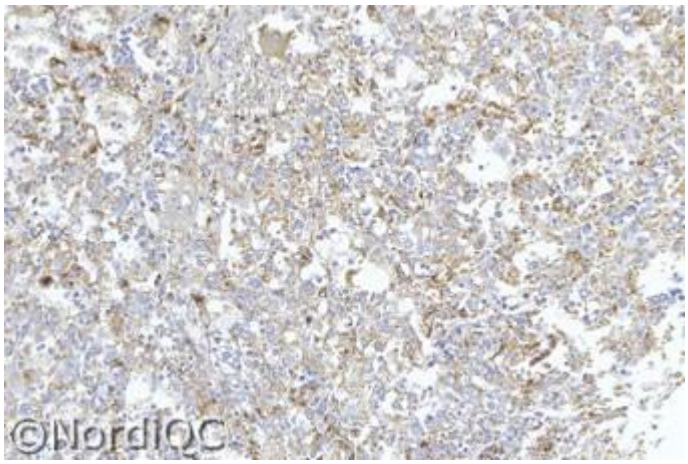

Fig. 3b
Insufficient CD68 staining of the histiocytic sarcoma using same protocol as in Figs. 1b & 2b. Only a faint and dubious staining reaction is seen in the neoplastic cells.

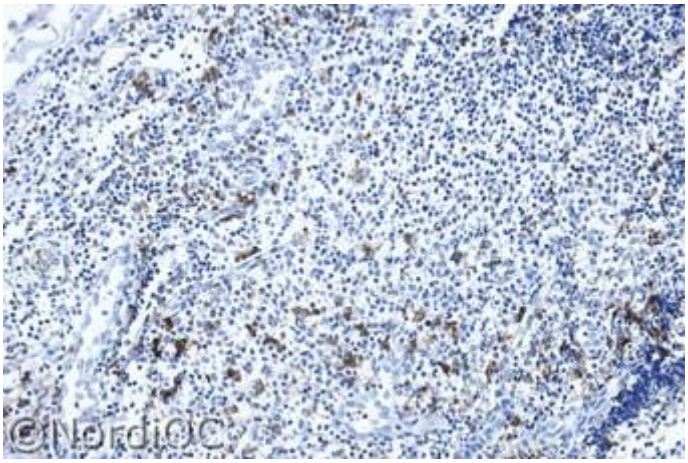

Fig. 4a
Insufficient CD68 staining of the tonsil using the mAb clone KP1 with proteolytic pre-treatment. The germinal centre macrophages show a moderate cytoplasmic staining reaction but the morphology is impaired due to excessive proteolysis and digestion of the fragile membranes of the lymphocytes. Also compare with Fig. 4b – same protocol.

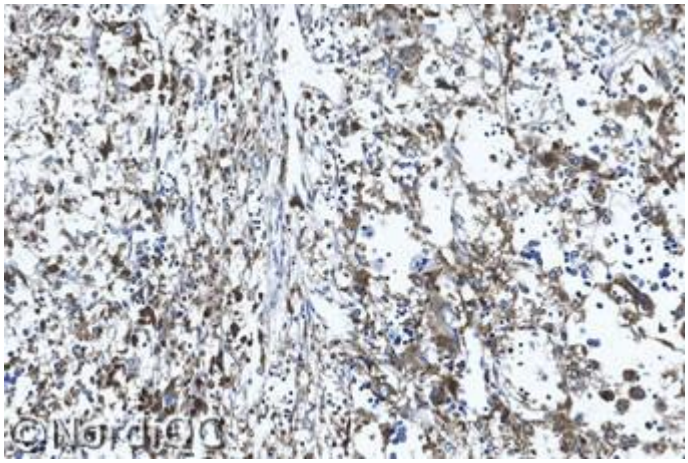

Fig. 4b
Insufficient CD68 staining of the histiocytic sarcoma using the mAb clone KP1 with proteolytic pre-treatment. The morphology is heavily impaired due to excessive proteolysis complicating the interpretation. Also compare with Fig. 4a – same protocol.

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