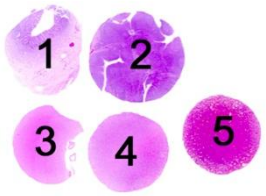
Assessment Run 26 2009
CD68

The slide to be stained for CD68 comprised:
1. Appendix, 2. Tonsil, 3. Brain, 4. Juvenile xantogranuloma, 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 appendix and tonsil.
- An at least moderate cytoplasmic staining of the macrophages in the interfollicular zones in the tonsil and in lamina propria in the appendix.
- An at least weak to moderate cytoplasmic staining of the microglial cells in the brain.
- An at least moderate cytoplasmic staining of the majority of the neoplastic cells of the Juvenile xantogranuloma and the histiocytic sarcoma.
- No staining of the germinal centre B-cells in the tonsil.

A weak cytoplasmic reaction in the epithelial cells in the appendix and tonsil was accepted, when the mAb clone KP1 was used.

128 laboratories participated in this assessment. 70 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and scores for CD68, run 26**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **PG-M1**	57 1	Dako ID Labs	25	20	10	3	78 %	84 %
mAb clone **KP1**	42 5 3 1 1	Dako NeoMarkers Novocastra Cell Marque Master Diagnostica	11	22	14	5	63 %	79 %
mAb clone **514H12**	1	Novocastra	1	0	0	0	-	-
Ready-To-Use Abs								
mAb clone **PG-M1, IR613**	3	Dako	2	0	1	0	-	-
mAb clone **KP1, 790-2931**	8	Ventana	0	6	2	0	75 %	-
mAb clone **KP1, IR609**	4	Dako	0	3	1	0	-	-
mAb clone **KP1, N1577**	1	Dako	0	1	1	0	-	-
Total	128		39	51	30	8	-	-
Proportion			30 %	40 %	24 %	6 %	70 %	83 %

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 Tris-EDTA/EGTA pH 9 (10/19)*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako, (8/9), Bond Epitope Retrieval Solution 2 (Bond, Leica) (4/6), Cell Conditioning 1 (BenchMark, Ventana) (2/12) or Citrate pH 6 (1/7) as retrieval buffer. The mAb was typically diluted in the range of 1:50– 1:400 depending on the total

sensitivity of the protocol employed. Using these protocol settings 42 out of 50 (84 %) 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 on HIER using Tris-EDTA/EGTA pH 9 (4/14), Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako, (3/6), Target Retrieval Solution pH 6.1 (Dako) (1/1), Cell Conditioning 1 (BenchMark, Ventana) (1/13), Cell Conditioning 2 (BenchMark, Ventana) (1/3) or Citrate pH 6 (1/7)as retrieval buffer. The mAb was typically diluted in the range of 1:500 – 1:.5.000 depending on the total sensitivity of the protocol employed. Using these protocol settings 26 out of 33 (79 %) laboratories produced a sufficient staining.

mAb clone **514H12**: the protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/1) as retrieval buffer. The mAb was diluted 1:40.

Ready-To-Use Abs
mAb clone **PG-M1**, prod. no IR610, Dako: The protocols giving an optimal result were all based on HIER in PT-Link using Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH), an incubation time of 20 or 30 min in the primary Ab and EnVision Flex (K8000/SM802) as the detection system. Using these protocol settings 2 out of 3 (67 %) laboratories produced a sufficient staining.

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

In this assessment 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 secondary follicles, whereas the demonstration of CD68 in the microglial cells and the neoplastic cells of the juvenile xantogranuloma was only seen with appropriate protocol settings, e.g., a correct titre of the mAb clones PG-M1 or KP1 and the use of HIER. Enzymatic pre-treatment could not be used to obtain an optimal result irrespective of the mAb clone applied. 7 out of 7 laboratories using the mAb clone KP1 combined with proteolysis all obtained an insufficient result. The vendors' data sheets for mAb clone KP1 give misleading information concerning the epitope retrieval: NeoMarkers / Thermo Scientific recommends proteolysis as pre-treatment for clone KP1, while Dako recommends HIER for clone KP1 when sold as a concentrate and as Ready-To-Use prod. no. IR609, but recommends proteolysis for the Ready-To-Use prod. no. N1577.
Only few laboratories obtained an insufficient result due to an excessive background reaction (when the mAb clone KP1 was used too concentrated).
Normal brain was in this test useful to evaluate the sensitivity of the protocols 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.

This was the second NordiQC assessment of CD68. In spite of a marked increase in the number of participants, a constant proportion of sufficient results have been seen as shown in table 2:

Table 2. **Sufficient results with CD31 in the two NordiQC runs**

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

Conclusion
The mAb clones PG-M1, KP1 and 514H12 are all recommendable CD68 Abs. HIER (preferable in an alkaline buffer) is mandatory to obtain an optimal result. Brain (or liver or appendix as demonstrated in run 11) is recommended as positive control. In the brain, the microglial cells must show an as strong as possible positive staining, while the background must be negative.

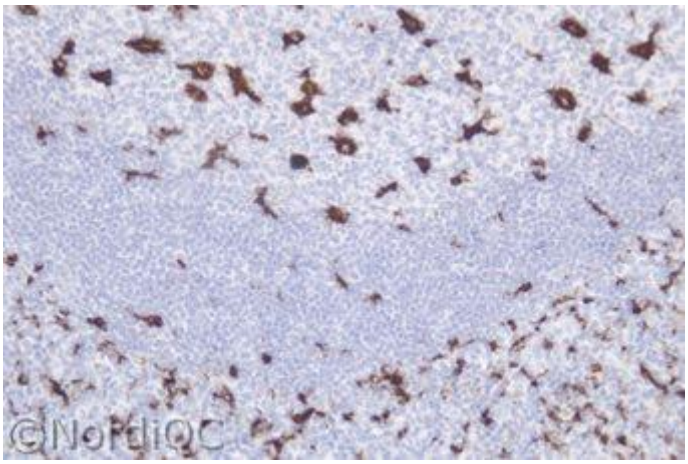


Fig. 1a
Optimal staining for CD68 using the mAb clone PG-M1 optimally calibrated and with HIER.
Both the germinal centre macrophages and the interfollicular macrophages show a strong and distinct cytoplasmic staining, without any background staining.
Also compare with Figs. 2a & 3a – same protocol.

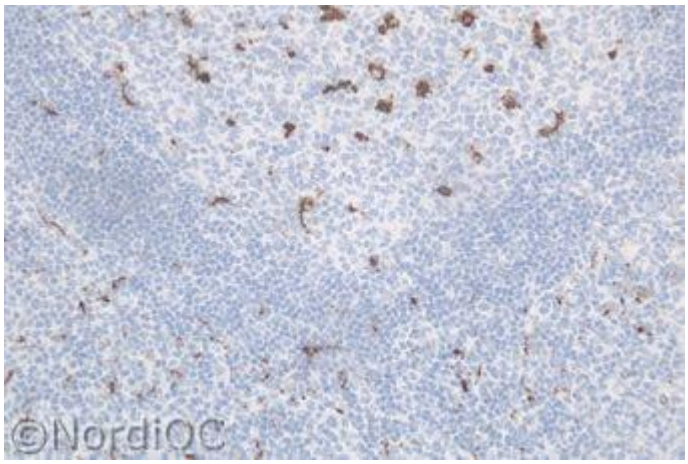


Fig. 1b
Insufficient staining for CD68 using the mAb clone PG-M1 too diluted.
Scattered macrophages are demonstrated, but both the intensity and the proportion is reduced compared to the result demonstrated in Fig. 1a - same field. Also compare with Figs. 2b & 3b – same protocol.

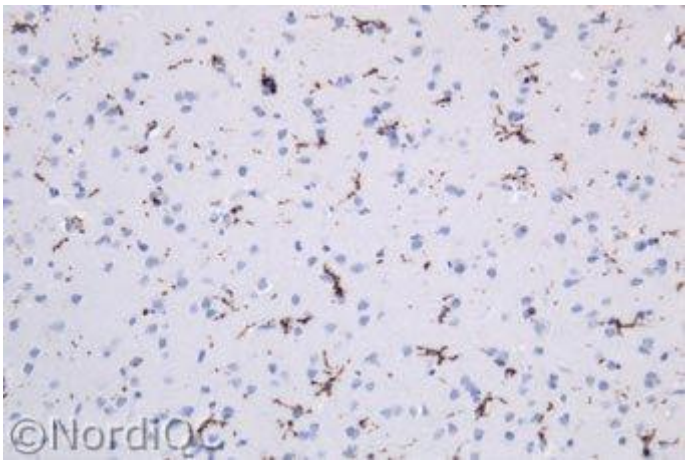


Fig. 2a
Optimal staining for CD68 of the brain using same protocol as in Fig. 1a. The microglial cells show a moderate to strong and distinct cytoplasmic reaction.

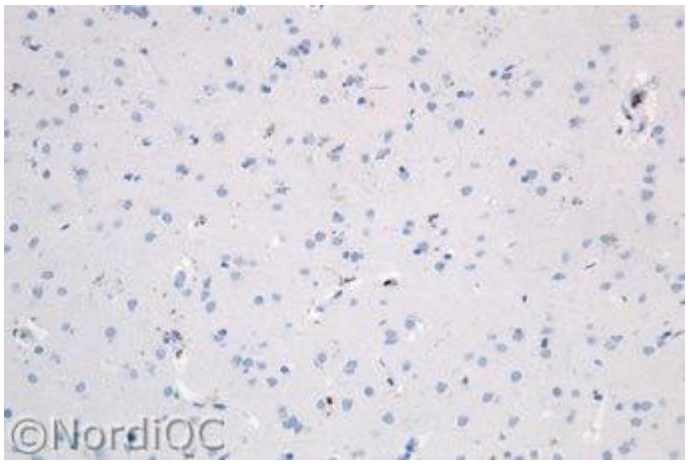


Fig. 2b
Insufficient staining for CD68 of the brain using same protocol as in Fig. 1b. Only scattered microglial cells show a faint and dubious reaction. Also compare with Fig. 3b – same protocol.

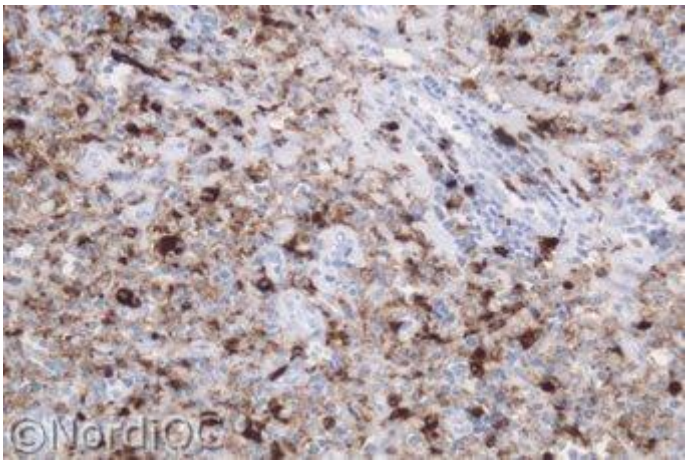


Fig. 3a
Optimal staining for CD68 of the juvenile xantogranuloma using same protocol as in Figs. 1a & 2a. The majority of the neoplastic cells show a weak to moderate cytoplasmic reaction, while entrapped normal macrophages show a strong staining reaction.

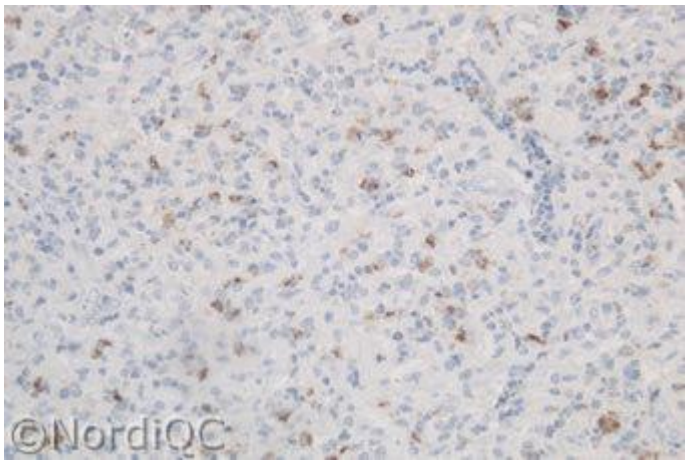


Fig. 3b
Insufficient staining for CD68 of the juvenile xantogranuloma using same protocol as in Figs. 1b & 2b. None or only a faint reaction is seen in the neoplastic cells, while only scattered normal macrophages show a distinct reaction.

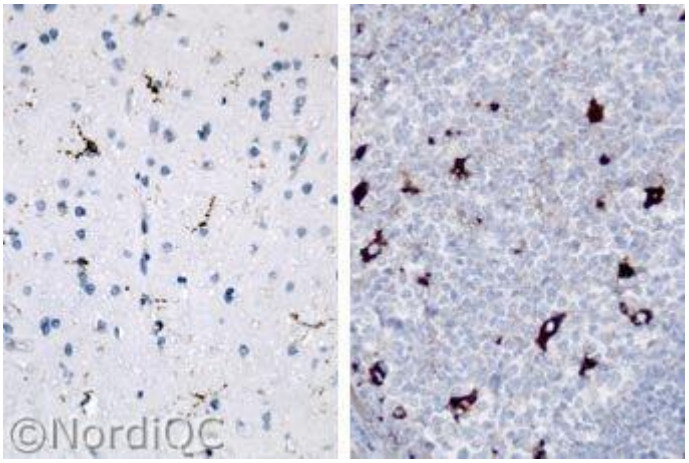


Fig. 4a
Optimal staining for CD68 using the mAb clone KP1 optimally calibrated and with HIER.
Left: Brain: The microglial cells show a moderate to strong and distinct cytoplasmic reaction.
Right: Tonsil: The germinal centre macrophages show a strong and distinct cytoplasmic staining.
Also compare with Figs. 4b, left & right – same mAb clone, but with proteolytic pre-treatment.

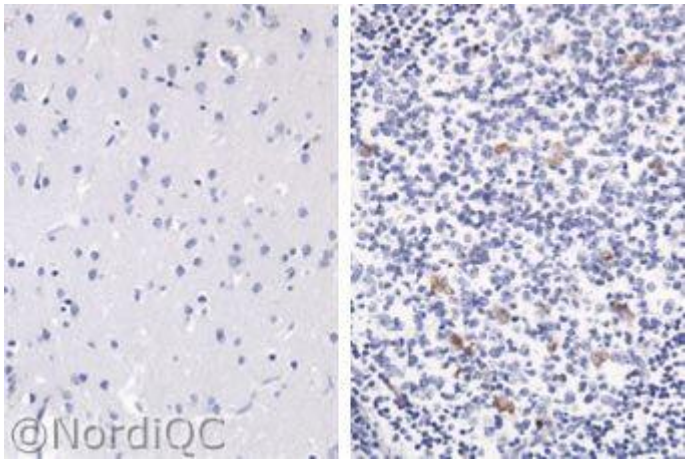


Fig. 4b
Insufficient staining for CD68 using the mAb clone KP1 with proteolytic pretreatment.
Left: Brain: No staining is seen in the microglial cells.
Right: Tonsil: The germinal centre macrophages only show a weak reaction and the morphology is impaired due to excessive proteolysis and digestion of the fragile membranes of the lymphocytes. Also compare with Figs. 4a, left & right – same clone, but with HIER.

SN/HN/MV/LE 14-7-2009