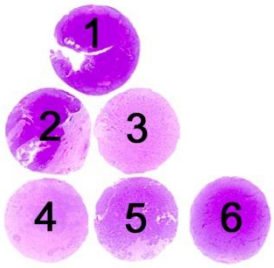
Assessment Run B7 2009
Ki67

The slide to be stained for Ki-67 comprised:

No.	Tissue	Ki-67 score*
1.	Tonsil (24 h in NBF)	80-90 % (germinal centre cells)
2.	Tonsil (48 h in NBF)	80-90 % (germinal centre cells)
3.	Breast ductal carcinoma	2 (10-24 %)
4.	Breast ductal carcinoma	1 (1-9 %)
5.	Breast ductal carcinoma	2 (10-24 %)
6.	Breast ductal carcinoma	4 (≥ 50 %)



*Ki-67-score and staining pattern as characterized by NordiQC reference laboratories using the mAb clone MIB1 and the rmAb clone 30-9.

All tissues were fixed in 10% neutral buffered formalin for 24 – 48 hours.

Criteria for assessing a Ki-67 staining as optimal included:

- A strong nuclear staining in 80-90% of the germinal centre lymphocytes in both the light and the dark zone and in the basal and suprabasal squamous epithelial cells of the tonsils.
- A strong, distinct nuclear staining of the appropriate proportion of the neoplastic cells in the breast ductal carcinomas no. 3-6.
- No or only a weak background staining reaction.

124 laboratories participated in this assessment. 77 % achieved a sufficient mark. The antibodies (Abs) and marks are summarized in table 1.

Table 1. **Abs and scores for Ki-67, run B7**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **MIB1**	83	Dako	29	32	17	5	73 %	76 %
mAb clone **MM1**	6	Novocastra	1	4	1	0	83 %	100 %
mAb clone **Ki-S5**	1	Dako	0	0	0	1	-	-
rmAb clone **SP6**	7 1 1 1	NeoMarkers BioCare Master Diagnostica Zytomed	5	1	3	1	60 %	67 %
Ready-To-Use Abs								
rmAb clone **30-9, 790-4286**	12	Ventana	10	2	0	0	100 %	100 %
mAb clone **MIB1, IR626**	7	Dako	6	1	0	0	100 %	100 %
mAb clone **K-2, 800-2910**	3	Ventana	1	1	1	0	-	-
mAb clone **7B11, ZM-0165**	1	Zymed	1	0	0	0	-	-
mAb clone **DVB-2, IP080**	1	BioCare	0	1	0	0	-	-
Total	124		53	42	22	7	-	-
Proportion			43 %	34 %	18 %	5 %	77 %	81 %

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

Following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs

mAb clone **MIB1**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using Tris-EDTA/EGTA pH 9 (9/22)*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako, (10/18), Cell Conditioning 1 (CC1, BenchMark, Ventana) (6/18), EDTA/EGTA pH 8 (1/2) or Citrate pH 6 (3/14) as retrieval buffer. The mAb was typically diluted in the range of 1:40– 1:600 depending on the total sensitivity of the

protocol employed. Using these protocol settings 54 out of 71 (76 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **MM1**: The protocol giving an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 (1/3) as retrieval buffer. The mAb was diluted 1.50. Using these protocol settings 3 out of 3 (100 %) laboratories produced a sufficient staining.

rmAb clone **SP6**: The protocols giving an optimal result were all based on HIER using Tris-EDTA/EGTA pH 9 (2/2), CC1 (BenchMark, Ventana) (1/4)*, Bond Epitope Retrieval Solution 1 (Bond, Leica) (1/1) or Citrate pH 6 (1/3) as retrieval buffer. The rmAb was typically diluted in the range of 1:100– 1:600 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 out of 9 (67 %) laboratories produced a sufficient staining (optimal or good).

Ready-To-Use Abs
rmAb clone **30-9**, prod. no 790-4286, Ventana: The protocols giving an optimal result were all based on HIER using CC1, mild or standard, an incubation time of 12-32 min in the primary Ab and iView or ultra View as the detection system. Using these protocol settings all of 12 (100 %) laboratories produced a sufficient staining.

mAb clone **MIB1**, prod. no IR626, Dako: The protocols giving an optimal result were all based on HIER using Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) or Target Retrieval Solution pH 6.1 (FLEX TRS low pH) for 20 min in the PT-Link, an incubation time of 20 min in the primary Ab and EnVision Flex (K8000/K8002) as the detection system. Using these protocol settings all of 6 (100 %) laboratories produced a sufficient staining.

mAb clone **K-2**, prod. no 800-2910, Ventana: The protocol giving an optimal result was based on HIER using CC1 mild, an incubation time of 32 min in the primary Ab and ultra View as the detection system.

mAb clone **7B11**, prod. no ZM-0165, Zymed: The protocol giving an optimal result was based on HIER using Citrate pH 6 in a pressure cooker and an incubation time of 60 min in the primary Ab and PV8000 (Zhongshan) as the detection system.

The most frequent causes of an insufficient staining in this run were:
- Too low concentration of the primary antibody
- Insufficient HIER (use of citrate pH 6.0 and/or too short heating time)
- Excessive HIER.

The prevalent features of the insufficient results were either a generally too weak or false negative reaction, which was observed in 69 % of the insufficient results. In 24 % the insufficient results was caused by excessive HIER and finally 7 % were insufficient due to an excessive background reaction. The weak and false negative reaction was characterized by a too low proportion of positive cells in all 4 breast carcinomas, but especially in the two carcinomas no. 3 & 5 with a moderate Ki-67 positivity of 10-24 %. As noticed in the last assessment of Ki-67 in run 19, 2007 tonsil is a recommendable positive control for Ki-67. In the optimal results, 80-90 % of the germinal centre cells showed a strong and distinct nuclear reaction, while a weak reaction and a reduced proportion of positive cells in the germinal centres typically was an indicator of an insufficient staining.
In some protocols based on the mAb clone MIB1 an aberrant cytoplasmic reaction was seen in both scattered neoplastic cells and smooth muscle cells. No plausible reason for this reaction could be identified, but the reaction pattern was frequently observed when the clone was applied relatively concentrated and with efficient HIER.
HIER is mandatory for an optimal Ki-67 result irrespective of the clone applied. In the literature and in previous assessment of Ki-67and from the literature, it has been shown that efficient HIER, e.g., using a pressure cooker and/or an alkaline buffer is superior to less efficient HIER methods, e.g., citrate pH 6.0 in a domestic microwave oven. However the HIER method has to be adjusted to give a high sensitivity along with an acceptable morphology. In the current run some laboratories obtained an insufficient result due to excessive HIER (typically as a consequence of too long heating time and/or too high temperature). The impaired morphology was observed both in the tonsils and in the breast carcinomas, typically characterized by severe wrinkling of the nuclei and a generally poor cell morphology.

This was the second NordiQC Ki-67 assessment (and the first assessment in the breast module). An almost identical proportion of sufficient results have been obtained in these two runs as shown in table 2.

Table 2. **Sufficient results with Ki-67 in the two NordiQC runs**

	Run 19 2007	Run B7 2009
Participants, n=	100	124
Sufficient results	73 %	77 %

Conclusion

The mAb clones **7B11**, **MIB1** and **MM1** and the rmAb clones **30-9** and **SP6** are all recommendable Abs for Ki-67. Efficient HIER is mandatory to obtain an optimal result and must be carried out to provide an optimal balance between sensitivity and morphology. The primary Ab concentration should be carefully calibrated. Normal tonsil is an appropriate control tissue: 80-90% of the germinal centre cells must show a distinct strong nuclear reaction with no or only a faint cytoplasmic reaction.

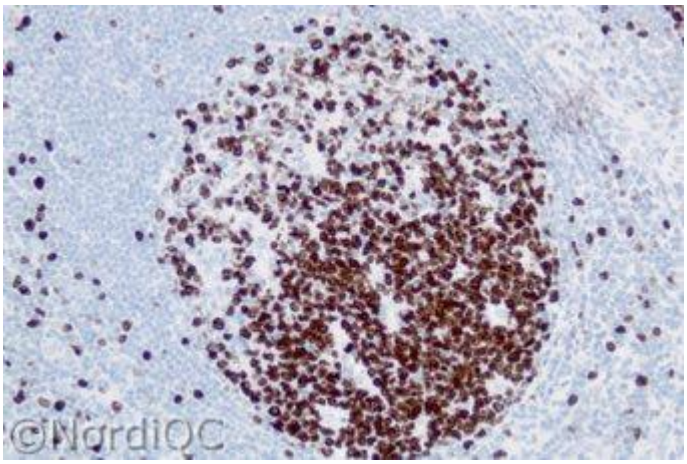


Fig. 1a
Optimal staining for Ki-67 of the tonsil using the mAb clone MIB1 as Ready-To-Use and with HIER in TRS low pH 6.1, (Dako).
A strong and distinct nuclear staining is seen in 80-90 % of the germinal centre B-cells.
Also compare with Figs. 2a & 3a – same protocol.

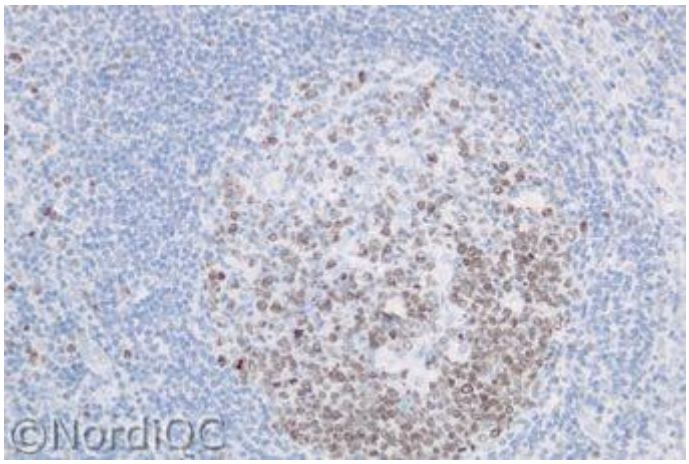


Fig. 1b
Insufficient staining for Ki-67 of the tonsil using the mAb clone MIB1 too diluted and with HIER in citrate pH 6.0.
The majority of the germinal centre B-cells is demonstrated, but shows a weak and diffuse nuclear staining – same field as in Fig. 1a. Also compare with Figs. 2b & 3b - same protocol.

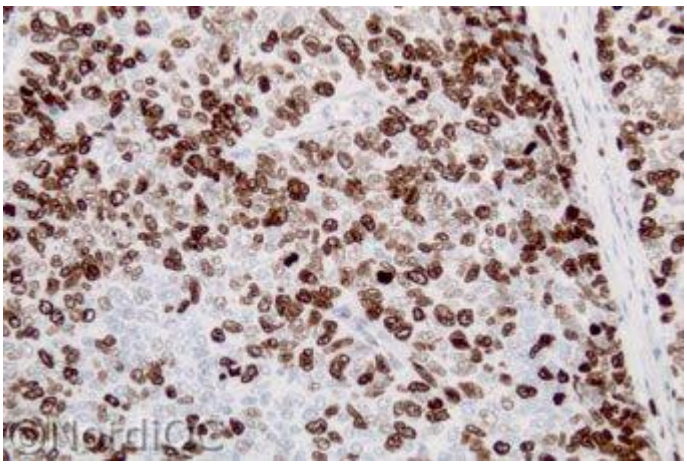


Fig. 2a
Optimal staining for Ki-67 of the breast carcinoma no. 6 using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong and distinct nuclear staining.

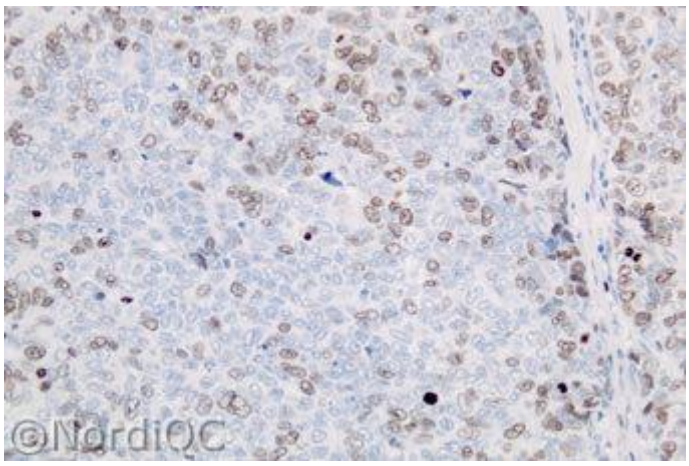


Fig. 2b
Insufficient staining for Ki-67 of the breast carcinoma no. 6 using same protocol as in Fig. 1b.- same field as in Fig. 2a.
The intensity and proportion of the positive cells is significantly reduced compared to the result in Fig. 2a.

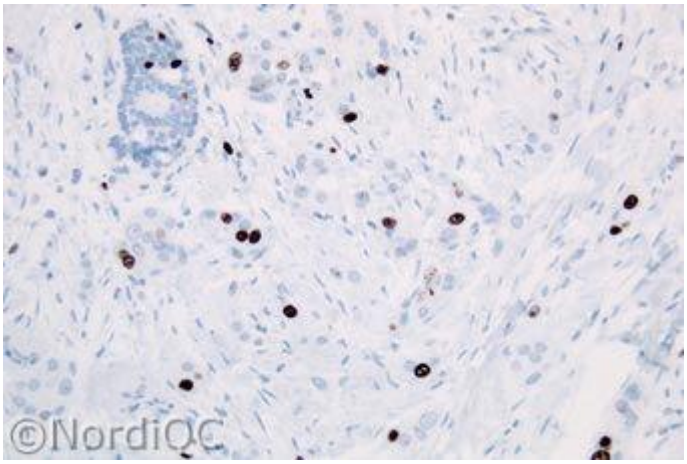


Fig. 3a
Optimal staining for Ki-67 of the breast carcinoma no. 3 using same protocol as in Figs. 1a & 2a.
The neoplastic cells show a distinct nuclear staining and no background reaction is seen.

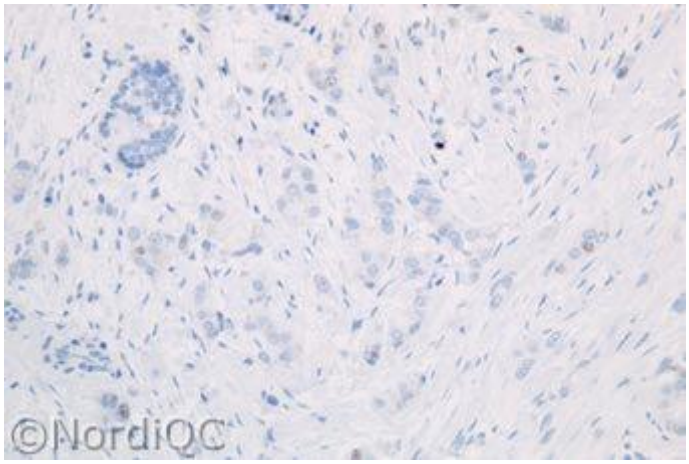


Fig. 3b
Insufficient staining for Ki-67 of the breast carcinoma no. 3 using same protocol as in Figs. 1b & 2b - same field as in Fig. 3a.
Only a significant reduced proportion of the neoplastic cells show a weak and dubious staining.

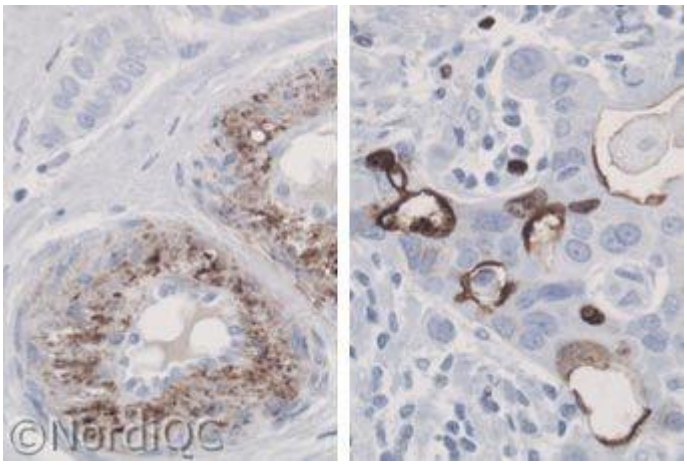


Fig. 4a
Aberrant staining pattern of the mAb clone MIB1. This was typically seen when a sensitive protocol was used based on HIER in and alkaline buffer and a high conc. of the primary Ab.
Left: Vascular smooth muscle cells show a moderate cytoplasmic staining.
Rght: Scattered neoplastic cells of the breast carcinoma no. 3 show a cytoplasmic and membranous staining.

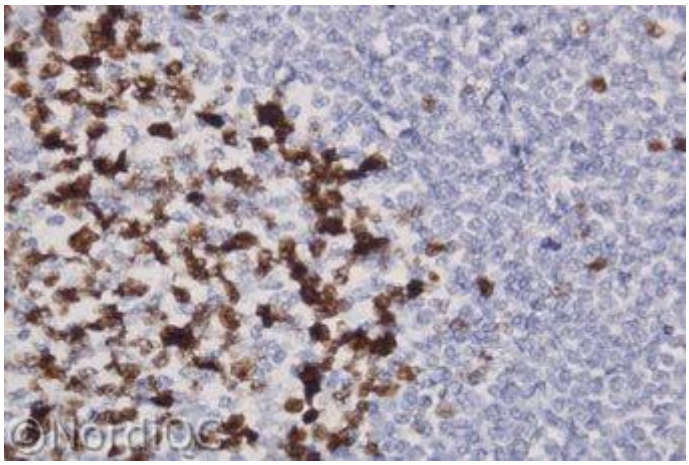


Fig. 4b
Insufficient staining (borderline) of the tonsil using the mAb clone MIB1 with excessive HIER.
The germinal centre B-cells are demonstrated, but all nuclei including the mantle zone B-cells show a severely impaired morphology.

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