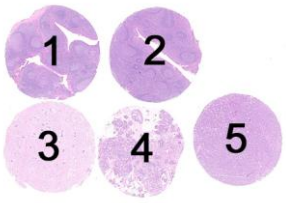
Assessment Run B13 2012
Ki67

Material
The slide to be stained for Ki67 comprised the following five tissues:

No.	Tissue	Ki67 score*
1.	Tonsil, fixed 24 hours	80-90 % of the germinal centre B-cells
2.	Tonsil, fixed 48 hours	80-90 % of the germinal centre B-cells
3.	Breast carcinoma	1 – 9 %
4.	Breast carcinoma	10 – 24 %
5.	Breast carcinoma	≥ 50 %



* Ki67 score 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 Ki67 staining as optimal included:

- A moderate to strong, distinct nuclear staining reaction in 80-90% of the germinal centre B-cells in both the light and the dark zone and in the vast majority of the suprabasal squamous epithelial cells.
- A moderate to strong, distinct nuclear staining reaction of the appropriate proportion of the neoplastic cells in the breast carcinomas no. 3-5.
- No or only a weak background and cytoplasmic staining reaction.

229 laboratories participated in this assessment. 89 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for Ki67, run B13**

Concentrated Abs:	N	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **7B11**	2	Invitrogen	1	0	1	0	-	-
mAb clone **BGX-297**	1	Biogenex	0	1	0	0	-	-
mAb clone **K2**	1	Immunologic	1	0	0	0	-	-
mAb clone **MIB-1**	101 1	Dako DBS	69	20	10	3	87 %	87 %
mAb clone **MM1**	9	Leica/Novocastra	5	2	1	1	78 %	100 %
rmAb clone **EP5**	1	Epitomics	0	0	1	0	-	-
rmAb **SP6**	8 7 1 1 1	Thermo/NeoMarkers Cell Marque Biocare Master Diagnostica Spring	10	5	2	1	83 %	92 %
pAb **A0047**	1	Dako*	1	0	0	0	-	-
pAb **RB-1510**	1	Thermo/NeoMarkers	1	0	0	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Ready-To-Use Abs:								
mAb clone **MIB-1 IR/IS626**	38	Dako	32	3	2	1	92 %	97 %
mAb clone **MM1 PA0118**	2	Leica/Novocastra	1	1	0	0	-	-
mAb clone **MM1 RTU-Ki67-MM1**	2	Leica/Novocastra	1	1	0	0	-	-
mAb clone **MM1 PM375**	1	Biocare	0	1	0	0	-	-
rmAb clone **30-9**	48	Ventana	43	4	1	0	98 %	98 %

790-4286								
rmAb clone **EP5 ZA-0502**	1	Zhongshan	0	1	0	0	-	-
Total	229		166	39	18	6	-	
Proportion			72 %	17 %	8 %	3 %	89 %	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below
* Product has been discontinued by the vendor

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

Concentrated Abs
mAb clone **7B11**: The protocol giving an optimal result was based on heat induced epitope retrieval (HIER) using EDTA/EGTA pH 8* as the retrieval buffer. The mAb was diluted 1:100. Using these protocol settings 1 out of 2 (50 %) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this reagent)

mAb clone **K2**: The protocol giving an optimal result was based on HIER using Citrate pH 6 as the retrieval buffer. The mAb was diluted 1:100.

mAb clone **MIB-1:** The protocols giving an optimal result were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (12/16), TRS pH 9 (Dako) (7/9), TRS pH 6.1 (Dako) (2/3), Cell Conditioning 1 (CC1; BenchMark, Ventana) (29/43), Bond Epitope Retrieval Solution 2 (BERS 2; Bond, Leica) (2/5) BERS 1 (Bond, Leica) (1/2), Diva Decloaker pH 6.2 (Biocare) (2/3), Tris-EDTA/EGTA pH 9 (10/14), EDTA/EGTA pH 8 (1/1) or Citrate pH 6 (3/6) as the retrieval buffer.
The mAb was typically diluted in the range of 1:50-1:600 depending on the total sensitivity of the protocol employed. Using these protocol settings 87 out of 100 (87 %) laboratories produced a sufficient staining result.

mAb clone **MM1:** The protocols giving an optimal result were all based on HIER using BERS 2 (Bond, Leica) (5/6) as the retrieval buffer.
The mAb was diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 out of 6 (100 %) laboratories produced a sufficient staining result.

rmAb clone **SP6**: the protocols giving an optimal result were all based on HIER using either TRS pH 9 (3-in-1) (Dako) (2/2), TRS pH 6.1 (Dako) (1/1), CC1 (BenchMark, Ventana) (5/8), BERS 2 (Bond, Leica) (1/2) and BERS 1 (Bond, Leica) (1/1) as the retrieval buffer.
The rmAb was typically diluted in the range of 1:30-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings 12 out of 13 (92 %) laboratories produced a sufficient staining result.

pAb **RB-1510**: the protocol giving an optimal result was based on HIER using CC1 (BenchMark, Ventana) as the retrieval buffer. The pAb was diluted 1:200.

Ready-To-Use Abs
mAb clone **MIB-1** (prod. no. IR/IS626, Dako): The protocols giving an optimal result were typically based on HIER in PT-Link (heating time for 10-30 min at 95°C- 99°C) using TRS pH 6.1, TRS pH 9 (3-in-1) (Dako) or TRS pH 9 (Dako) as HIER buffer, an incubation time of 20-30 min in the primary Ab and EnVision Flex or EnVision Flex+ (K8000/K8002) as the detection system. Using these protocol settings 35 out of 36 (97 %) laboratories produced a sufficient staining result.

mAb clone **MM1** (product. no. PA0118, Leica/Novocastra): The protocol giving an optimal result was based on HIER using BERS 2 (Bond, Leica), an incubation time of 15 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system. Using these protocol settings 2 out of 2 (100 %) laboratories produced a sufficient staining result.

mAb clone **MM1** (product. no. RTU-KI67-MM1, Leica/Novocastra): The protocol giving an optimal result was based on HIER using BERS 2 (Bond, Leica), an incubation time of 15 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system. Using these protocol settings 1 out of 1 (100 %) laboratories produced a sufficient staining result.

rmAb clone **30-9** (prod. no 790-4286, Ventana): The protocols giving an optimal result were typically based on HIER using mild or standard CC1, an incubation time of 8-32 min in the primary Ab and iView (760-91), UltraView (760-500) or OptiView (760-700) as the detection system. Using these protocol settings 47 out of 48 (98 %) laboratories produced a sufficient staining result.

The most frequent causes of an insufficient staining in this run were:

- Too low concentration of the primary antibody
- Insufficient HIER (too short heating time)
- Excessive HIER
- Inadequate deparaffination

In this NordiQC assessment for Ki67 the features of the insufficient results irrespective of the primary Ab applied were characterized by either a generally too weak staining reaction, a poor signal-to-noise ratio or a significantly impaired morphology.
A too weak staining reaction was seen in 50 % of the insufficient staining results (n=12) and characterized by a too low proportion and staining intensity of the positive cells in all the 3 breast carcinomas and of the germinal centre B-cells of the two tonsils. This staining pattern was typically caused by insufficient HIER e.g., a too short efficient heating time and/or a too low concentration of the primary Ab including attempts to dilute Ready-To-Use (RTU) Abs.
A significantly impaired morphology compromising the interpretation was also a frequent feature observed and most likely was caused by excessive HIER e.g. typically as a consequence of a too long heating time and/or too high temperature, but can also be seen if the sections are dried after the IHC reaction has been completed and before the slides have been mounted. This was typically characterized by a severe wrinkling of the nuclei and/or "empty" nuclei virtually without nuclear content and a generally very poor cellular morphology.
The impaired morphology was also seen as severe nuclear bubbling and presence of paraffin remnants in the tissues. This pattern most likely was caused by inadequate deparaffination by the use of "3-in-1" deparaffination-dehydration-HIER methods. In the majority of the protocols based on the use of "3-in-1" methods an optimal staining result were seen and the insufficient results might be related to individual modifications of the procedures.
Finally a few insufficient staining results were characterized by a false positive nuclear staining reaction of virtually all cells including mantle zone B-cells, endothelial cells etc. and was probably caused by a technical error as drying out of the sections during the IHC staining.

As also noticed in the previous assessment of Ki67 (run B7, 2009) tonsil is a recommendable control for Ki-67. In the optimal results, 80-90 % of the germinal centre B-cells showed a moderate too strong and distinct nuclear staining reaction, while a weak staining reaction and a reduced proportion of positive cells in the germinal centres typically was an indicator of an insufficient staining. In the interfollicular areas dispersed lymphocytes also showed a moderate to strong nuclear staining reaction, while the vast majority of the mantle zone B-cells were negative.

Many primary Abs could be used to obtain an optimal staining result and both the concentrated formats and RTU formats/systems of the respective Abs gave a high proportion of sufficient staining results. In this assessment the RTU format/system, Dako of the most widely used mAb clone MIB1 gave a superior pass rate of 97 % compared to a pass rate of 87 % when the same mAb clone was used by an in-house validated assay. A similar high pass rate was also observed for the RTU format/system of the rmAb clone 30-9, Ventana.

This was the 4th assessment of Ki67 in NordiQC (Table 2). In this run a significantly higher pass rate was achieved compared to the previous runs performed. As the same tissues were used for the material circulated in the runs B7 and B13, this indicates generated real improvement of laboratory performance. The extended use of properly calibrated RTU systems for Ki67 being used by 45 % of the laboratories also has a high impact on the overall pass rate.

Table 2. **Proportion of sufficient results for Ki67 in the four NordiQC runs performed**

	Run 5 2001	**Run 19 2007**	**Run B7 2009**	**Run B13 2012**
Participants, n=	42	100	124	229
Sufficient results	71 %	73 %	77 %	89 %

Conclusion
The widely used mAb clones **MIB1** and **MM1** and the rmAb clones **30-9** and **SP6** are all recommendable Abs for Ki67. Efficient HIER is mandatory to obtain an optimal result and must be carried out to provide an optimal balance between the sensitivity and preserved morphology.
The RTU systems showed superior pass rates compared to the in-house validated assays for Ki67.
Normal tonsil is an appropriate control tissue or Ki67. 80-90% of the germinal centre
B-cells must show a moderate to strong, distinct nuclear reaction and only dispersed lymphocytes in the interfollicular areas should be demonstrated.

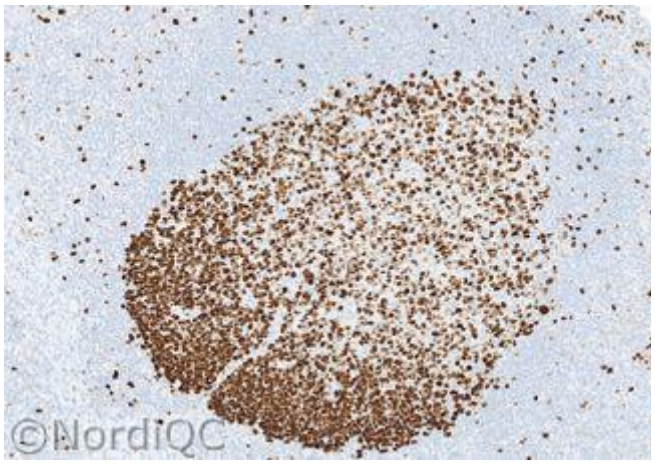


Fig. 1a
Optimal staining for Ki67 of the tonsil fixed for 24 hours in NBF using the mAb clone MIB1 properly calibrated and with HIER in an alkaline buffer.
A moderate to strong, distinct nuclear staining reaction is seen in 80-90 % of the germinal centre B-cells in both the dark and the light zone.
Also compare with Figs. 2a & 3a – same protocol.

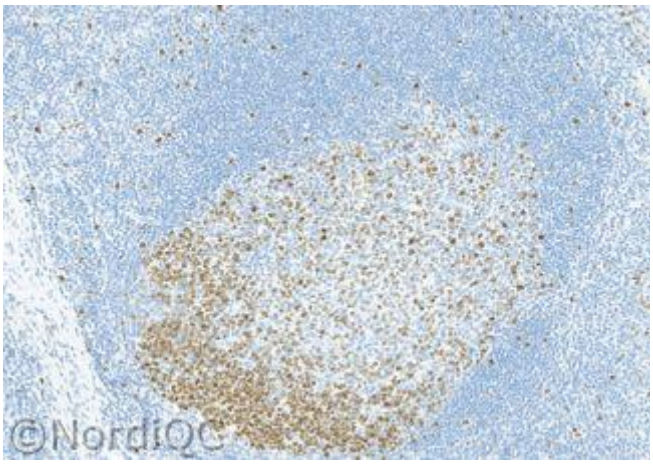


Fig. 1b
Insufficient staining for Ki67 of the tonsil fixed for 24 hours in NBF using the mAb clone MIB1 with a protocol providing a too low sensitivity, most likely due to a too low concentration of the primary Ab.
The majority of the germinal centre B-cells are demonstrated, but especially the B-cells in the light zone only show a weak and diffuse nuclear staining reaction – same field as in Fig. 1a.
Also compare with Figs. 2b & 3b - same protocol.

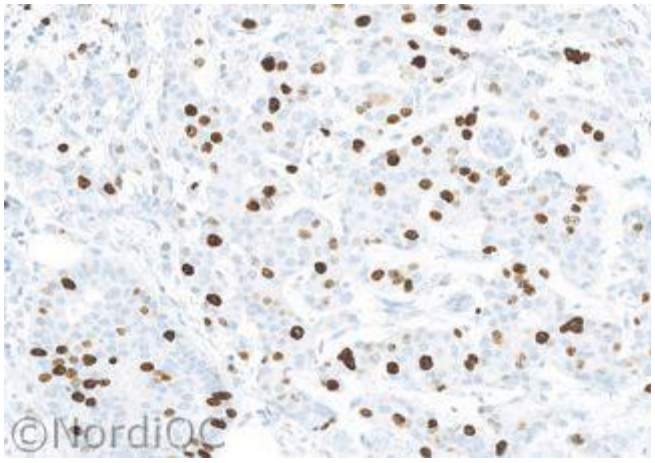


Fig. 2a
Optimal staining for Ki67 of the breast carcinoma no. 4 using same protocol as in Fig. 1a.
≥ 10 and < 24 % of the neoplastic cells show a moderate to strong and distinct nuclear staining reaction. The nuclear staining reaction for Ki67 is easily interpreted.

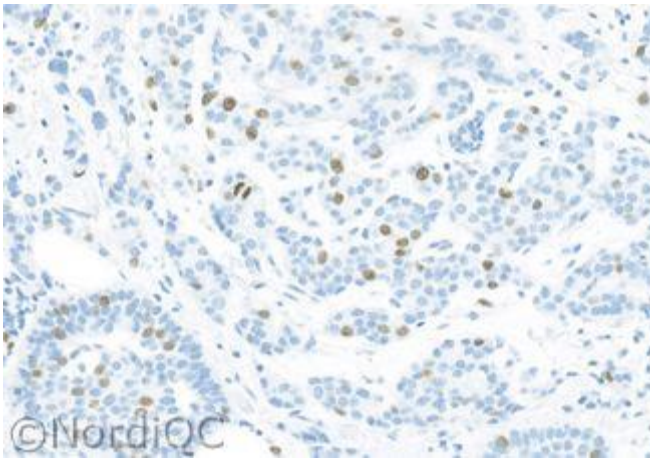


Fig. 2b
Insufficient staining for Ki67 of the breast carcinoma no. 4 using same protocol as in Fig. 1b. - same field as in Fig. 2a.
The intensity and proportion of the positive cells is significantly reduced compared to the result in Fig. 2a.
Also compare with Fig. 3b – same protocol.

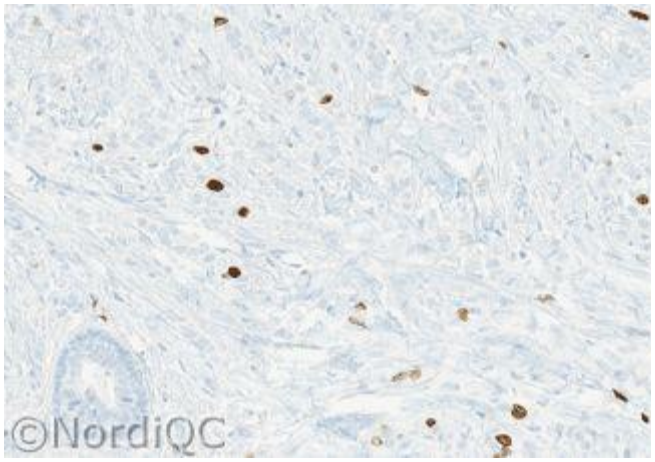


Fig. 3a
Optimal staining for Ki67 of the breast carcinoma no. 3 using same protocol as in Figs. 1a & 2a.
≥ 1 and < 9 % of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

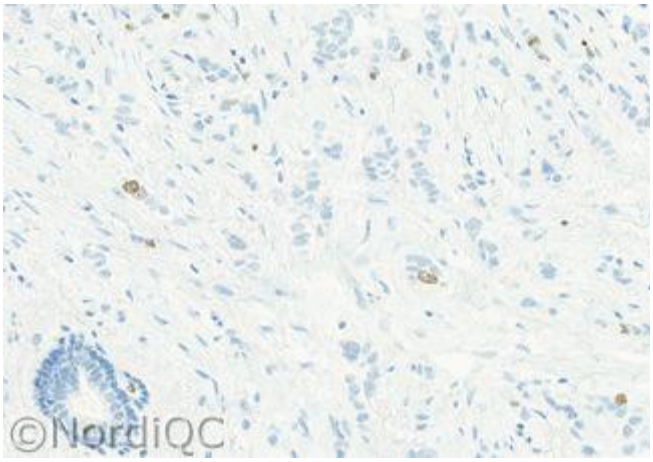


Fig. 3b
Insufficient staining for Ki67 of the breast carcinoma no. 3 using same protocol as in Figs. 1b & 2b - same field as in Fig. 3a.
The intensity and proportion of the positive cells is significantly reduced compared to the result in Fig. 3a.

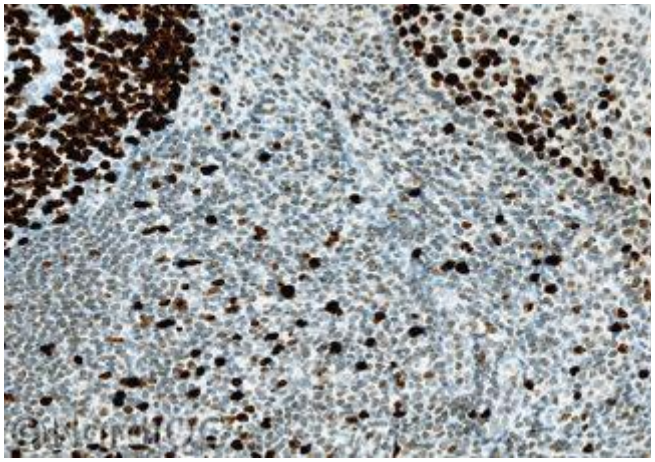


Fig. 4a
Insufficient staining for Ki67 of the tonsil as a false positive staining reaction is seen. Virtually all cells show a positive nuclear staining reaction. The germinal centre B-cells, top left, show an intense nuclear staining reaction, but also the vast majority of the mantle zone B-cells and interfollicular lymphocytes show a positive nuclear staining reaction. A too high concentration of the primary Ab and/or inadequate wash in buffers may be the cause for this aberrant staining pattern.

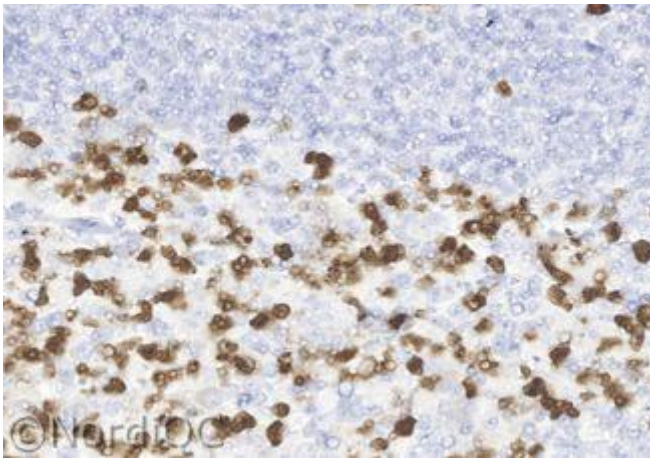


Fig. 4b
lnsufficient staining for Ki67 of the tonsil as an impaired morphology is observed
The germinal centre B-cells are demonstrated, but the interpretation is compromised by an extensive nuclear bubbling in all cells. This artefact most likey was caused by inadequate deparaffination occasionally seen by the use of 3-in-1 (deparaffination-dehydration-HIER) protocols.

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