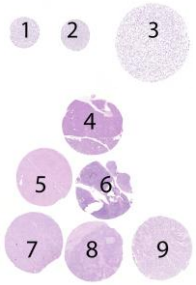
Assessment Run B22 2016
Ki67

Material
The slide to be stained for Ki67 comprised the following 9 materials:

No.	Material	Ki67 proliferation index *
1.	Cell line 1. Horizon Discovery**	80-90%
2.	Cell line 2. Horizon Discovery**	70-80%
3.	Cell line 3. Horizon Discovery**	80-90%
4.	Pancreas	< 1% of the epithelial cells of the exocrine glands and ducts
5.	Liver	< 1% of the hepatocytes
6.	Tonsil	80-90% of the germinal centre B-cells
7.	Breast ductal carcinoma	20-30%
8.	Breast ductal carcinoma	70-80%
9.	Breast ductal carcinoma	5-10%



* Ki67 prolifereation index as characterized by NordiQC reference laboratories using the mAb clone MIB1
** The cell lines were not included in the assessment. Data will be analyzed subsequently by digital image analysis.

All materials were fixed in 10% neutral buffered formalin.

Criteria for assessing 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 in dispersed epithelial cells of the exocrine pancreatic glands and large ducts.
- A moderate to strong, distinct nuclear staining reaction of the appropriate proportion of the neoplastic cells in the breast carcinomas no. 7-9.
- A nuclear staining reaction only in scattered hepatocytes (<1%).
- No or only a weak background and cytoplasmic staining reaction.

Participation

Number of laboratories registered for Ki67, run B22	428
Number of laboratories returning slides	409 (96%)

Results
409 laboratories participated in this assessment. 382 (93%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Insufficient HIER
- Too low concentration of the primary Ab

Performance history
This was the fifth NordiQC assessment of Ki67. A significant increase in the pass rate was seen compared to run B13 in 2012 (see table 2).

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

	Run 5 2001	Run 19 2007	Run B7 2009	Run B13 2012	Run B22 2016
Participants, n=	42	100	124	229	409
Sufficient results	71%	73%	77%	73%	93%

Conclusion
The mAb clones **BS4**, **GM001**, **K2**, **MIB-1**, **UMAB107** 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 widely used RTU systems based on clones MIB-1, Dako, and 30-9, Ventana, showed slightly superior pass rates compared to laboratory developed assays for Ki67.
Normal tonsil is at present the best recommendable positive tissue control tissue for Ki67. 80-90% of the germinal centre B-cells must show a moderate to strong, distinct nuclear reaction. Especially low-level Ki67 expressing B-cells in the light zone must be demonstrated.

Table 1. **Antibodies and assessment marks for Ki67, run B22**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BS4**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **GM001**	1	Genemed	1	0	0	0	-	-
mAb clone **K2**	2 1	Zytomed Leica/Novocastra	2	1	0	0	-	-
mAb clone **MIB-1**	122 1	Agilent/Dako VWR/Immunologic	72	36	13	2	88%	90%
mAb clone **UMAB107**	7	ZSBio	2	4	1	0	86%	80%
rmAb clone **SP6**	7 5 3 3 3 1 1	Thermo/Neomarkers Cell Marque Biocare Spring Bioscience Zytomed Master Diagnostica Diagnostic Biosystems	17	5	1	0	96%	95%
pAb **RB-1510**	1	Thermo/Neomarkers	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **GM001 60-0040-7**	1	Genemed	1	0	0	0	-	-
mAb clone **K2 PA0230**	4	Leica/Novocastra	2	2	0	0	-	-
mAb clone **Ki88 AM370**	1	Biogenex	0	1	0	0	-	-
mAb **MIB-1 IR626/IS626**	65	Agilent/Dako	34	25	5	1	91%	94%
mAb **MIB-1 GA626**	31	Agilent/Dako	25	5	1	0	97%	100%
mAb clone **MIB-1 AM297**	1	Biogenex	1	0	0	0	-	-
mAb clone **MM1 PA0118**	9	Leica/Novocastra	0	8	1	0	-	-
mAb clone **MX006 MAB-0672**	1	Maixin	0	1	0	0	-	-
rmAb clone **SP6 275R**	4	Cell Marque	2	1	1	0	-	-
rmAb clone **SP6 PRM 325**	1	Biocare	0	1	0	0	-	-
rmAb clone **SP6 MAD-000310QD**	1	Master Diagnostica	0	1	0	0		
rmAb clone **30.9 790-4286**	131	Roche/Ventana	121	9	1	0	99%	100%
Total	409		282	100	24	3	-	
Proportion			69%	24%	6%	1%	93%	

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

Detailed analysis of Ki67, Run B22
The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies
mAb clone **BS4**: One protocol with an optimal result was based on HIER using Tris-EDTA pH 9 as retrieval buffer, a titre of 1:200 and visualized by a 2-step polymer based detection kit (KDB-10007, Nordic Biosite).

mAb clone **GM001**: One protocol with an optimal result was based on HIER using Tris-EDTA pH 9 as retrieval buffer, a titre of 1:120 and visualized by a 2-step polymer based detection kit (GK6005, Gene Tech).

mAb clone **K2**: Protocols with optimal results were based on HIER using Citrate pH 6 as retrieval buffer. The mAb was diluted 1:200-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **MIB-1**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (16/20)*, TRS low (Dako) (2/2), Cell Conditioning 1 (CC1, Ventana) (40/62), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (5/18), PT Module 1 buffer (Thermo) (1/1), Borg Decloaker (Biocare) (1/1), Tris-EDTA pH 9 (5/7), Citrate (1/4) and Unknown (1/1) 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, 103 of 114 (90%) laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **UMAB107**: Protocols with optimal results were based on HIER using Tris-EDTA pH 9 (2/4) or Citrate pH 6 (1/1) as retrieval buffer.
The mAb was diluted 1:100-1:200 depending on the total sensitivity of the protocol employed.
Using these protocol settings, 4 of 5 (80%) laboratories produced a sufficient staining result.

rmAb clone **SP6**: Protocols with optimal results were based on HIER using CC1 (Ventana) (9/10), TRS pH 9 (3-in-1) (Dako) (3/3), BERS2 (Leica) (2/3), Montage EDTA Antigen Retrieval Solution pH 8 (1/1), Citrate pH 6 (1/3) or Unknown (1/1) as retrieval buffer.
The rmAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed.
Using these protocol settings, 19 of 20 (95%) laboratories produced a sufficient staining result.

pAb **RB-1510**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer, a titre of 1:12,000 and visualized by a 2-step multimer based detection kit (UltraView, Ventana).

Table 3. **Proportion of optimal results for Ki67 for the most commonly used antibody as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer / Omnis**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	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 **MIB-1**	16/20** (80%)	2/2	39/61 (64%)	-	5/16 (31%)	0/3

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

Ready-To-Use antibodies and corresponding systems
mAb clone **K2** product no. **PA0230**, Leica/Novocastra, BOND III/MAX:
Protocols with optimal results were based on HIER using BERS2 (Bond Leica) (efficient heating time 20-30 min. at 99-100°C) and 15 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 2 of 2 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **MIB-1**, product no. **IR626/IS626**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS low pH 6.1 (efficient heating time 10-20 min. at 95-99°C), 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 49 of 52 (94%) laboratories produced a sufficient staining result.

mAb clone **MIB-1**, product no. **GA626**, Dako, Omnis:
Protocols with optimal results were typically based on HIER using TRS low pH 6.1 (efficient heating time 30 min. at 97°C), 20 min. incubation of the primary Ab and Envision FLEX (GV800) Envision FLEX+ (GV800+GV821) as detection system. Using these protocol settings, 27 of 27 (100%) laboratories produced a sufficient staining result.

rmAb clone **30.9**, product no. **790-4286**, Roche/ Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 24-64 min.) and 12-36 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings, 126 of 126 (100%) laboratories produced a sufficient staining result.

Comments
In this assessment and in concordance with the previous NordiQC runs for Ki67, the prevalent feature of an insufficient staining was a too weak or completely false negative staining reaction of cells expected to be demonstrated and thus characterized by a reduced Ki67 proliferation index (PI) compared to the levels expected. Virtually all laboratories were able to demonstrate Ki67 in high-level expressing cells as germinal centre B-cells in the dark zones, whereas low-level Ki67 expressing cells in the light zones could only be demonstrated with an optimally calibrated protocol. Too weak staining or false negative staining reaction of the light zone B-cells in particular had a significant impact on the Ki67 PI in the breast carcinoma core no. 7. This core was expected to show a Ki67 PI of 20-30%, which according to guidelines suggested by the St Gallen consensus meetings[1] would indicate a "high" Ki67 PI. In the results assessed as too weak, typically a Ki67 PI of <20% was seen in this tumour and thus classified as "low" Ki67.
Too weak or false negative staining pattern was seen in 78% of the insufficient results (21 of 27 laboratories). Too weak staining in combination with excessive background staining, impaired morphology or excessive counterstaining characterized the remaining insufficient results.

37% (150 of 409) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for Ki67. The well-characterized mAb clone MIB-1 and rmAb clone SP6 were the two most widely used Abs and could both be used to obtain an optimal staining result. However, also the newly launched mAbs clones BS4, GM001, K2 and UMAB107 all provided optimal results. Irrespective of the Ab applied, efficient HIER and careful calibration of the primary Ab were the two most central parameters for optimal results. Choice of detection system was of less importance. Both 2-step and 3-step multimer/polymer based detection systems could be used to obtain optimal staining results, provided that the titre of the primary Ab was adjusted correctly.
For the most commonly used Ab, mAb clone MIB-1, optimal results could be obtained on the main IHC systems from Dako, Leica and Ventana, as listed in table 3. For unexplained reasons, MIB-1 showed an inferior performance on the Leica, Bond IHC system compared to the other IHC systems and in particular compared to the Dako IHC systems. Despite a sensitive 3-step polymer based detection system is used as default setting on the Bond IHC system, only 31% of the laboratories, using the identified "optimal" protocol settings regarding HIER conditions and Ab titre, obtained an optimal mark. 19% produced a staining evaluated as insufficient. In comparison, 80% of the protocols applied on the Dako IHC system were evaluated as optimal and none insufficient. No explanations for this difference could be identified from the submitted protocols.

Ready-To-Use (RTU) antibodies were used by 63% (259 of 409) of the laboratories. Optimal results could be obtained with the RTU systems from the three main IHC system providers, Dako, Leica and Ventana. The Ventana RTU system based on the rmAb clone 30-9 (790-4286) was in this assessment the most widely used assay and gave an overall impressive pass rate of 99%. Optimal results could be obtained both by the vendor recommended protocol settings (16 min. incubation of the primary Ab, HIER in CC1 for 64 min. and UltraView as detection kit) and by laboratory modified protocols adjusting incubation time of the primary Ab, HIER time and choice of detection system. The combination of reduced incubation time of the primary Ab to e.g. 8-12 min. and reduced HIER time for 8-32 min. was slightly less successful, but still provided a sufficient result typically assessed as "Good".

The Dako RTU system IR626/IS626 for Autostainer based on the mAb MIB-1 provided a pass rate of 91%. Both vendor recommended (20 min. incubation of the primary Ab, HIER in TRS Low pH 6.1 for 20 min. and EnVision FLEX as detection kit) and laboratory modified protocol settings (adjusting HIER time and/or using TRS High pH as HIER buffer) could produce optimal results. No overall reasons for insufficient results could be identified.
The Dako RTU system GA626 for Omnis based on mAb clone MIB-1 provided a slightly higher overall pass rate of 97%. Both vendor recommended protocol settings (20 min. incubation of the primary Ab, HIER in

TRS Low pH 6.1 for 30 min. and EnVision FLEX as detection kit) and laboratory modified protocol settings (using TRS High pH for HIER and/or using EnVision FLEX+ as detection kit) could provide optimal results.

The Leica RTU system PA0230 based on the mAb clone K2 for Bond gave a pass rate of 100%. Optimal results were obtained by the vendor recommended protocol settings (15 min. incubation of the primary Ab, HIER in ER2 for 20 min. and Refine as detection kit) and by slightly laboratory modified protocol settings prolonging HIER to 30 min.

This was the fifth NordiQC assessment of Ki67. The pass rate improved from 73% in latest assessment run B13, 2013, to 93% in the current run.
The access to and extended use of optimally calibrated RTU systems for Ki67 on all the three main IHC systems from Dako, Leica and Ventana has improved the overall pass rate.
In run B13, 39% of the participants (90 of 229) used one of the above-mentioned RTU systems. Compared to this run, 57% (231 of 409) of the participants used the same RTU systems with a total pass rate of 97%. In comparison, the pass rate for LD assays based on same clones was 88% (111 of 126 laboratories) in this run and 87% (97 of 112) in run B13.

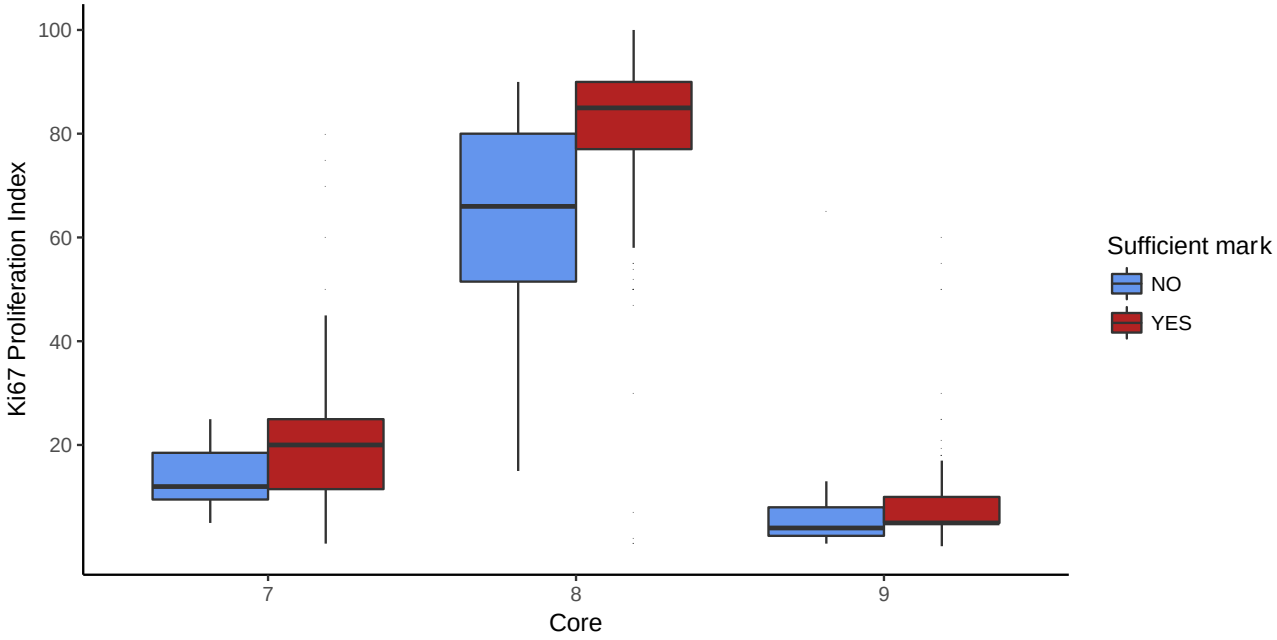
Scoring
The participants were asked to submit scoring of the three breast carcinomas (core 7-9). 306 of the 409 (75%) laboratories submitted scores. The median submitted scores can be seen in table 4.

Table 4. **Median Ki67 proliferation index of the NordiQC participants in 3 breast carcinomas**

Ki67 Proliferation Index (%)	Core 7	Core 8	Core 9
Median participant score	20	82	5
Score range	1-80	1-100	0.5-65
NordiQC reference score	20-30	70-80	5-10

Overall, the median values were similar to the expected reference values from the NordiQC assessor group. The median scores of the breast cancer cores no. 7 and 9 were in the lower end of the range estimated by the NordiQC reference labs (20-30 and 5-10), while the median scores for core no. 8 were slightly above the estimated range (70-80). However, the submitted scores varied significantly, see ranges in table 4. When stratifying for the assessment marks, analysis revealed that scores among participant that had received an insufficient mark ("Borderline" or "Poor") reported lower scores than laboratories that had received a sufficient mark. However, there was a significant overlap between the two groups as seen in fig 1.

Figure 1. Comparison of participant Ki67 Proliferation Indices



Controls

In this assessment and as observed in the previous runs for Ki67, tonsil is recommendable as positive and negative tissue control. Virtually all B-cells in the dark zones of the germinal centres must show a moderate to strong nuclear staining reaction, while an at least weak to moderate staining reaction must be seen in most B-cells in the light zones.

No staining reaction must be seen in the vast majority of mantle zone B-cells.

Liver or pancreas can be used primarily as supplementary negative tissue controls in which <1% of hepatocytes and epithelial cells of the exocrine pancreatic glands should be positive (inflammatory and reactive conditions can induce an elevated Ki67 score. Leukocytes can for unexplained reasons show a weak nuclear staining reaction.

The recommendations of the above mentioned tissue controls for IHC are concordant to the guidelines published by the International Ad Hoc Expert Committee[2].

3 different cell lines (Horizon Discovery, Cambridge UK) were included in this assessment, primarily to evaluate if this material in combination with digital image analysis can be used to evaluate staining quality for Ki67 and potentially be used as standard reference material for validation of the precision of Ki67 IHC assays. Subsequent analysis will be performed by NordiQC and published at a later stage.

[1]Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thurlimann B, Senn HJ. Tailoring therapies-improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann Oncol. 2015;26:1533–46. doi: 10.1093/annonc/mdv221.

[2]Torlakovic EE, Nielsen S, Francis G, Garratt J, Gilks B, Goldsmith JD, Hornick JL, Hyjek E, Ibrahim M, Miller K, Petcu E, Swanson PE, Zhou X, Taylor CR, Vyberg M. Standardization of positive controls in diagnostic immunohistochemistry: recommendations from the International Ad Hoc Expert Committee. Appl Immunohistochem Mol Morphol. 2015 Jan;23(1):1-18. doi: 10.1097/PAI.0000000000000163. Review. PubMed PMID: 25474126.

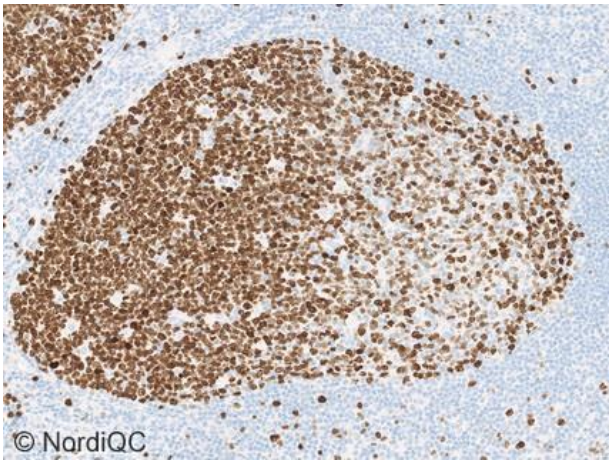


Fig. 1a
Optimal staining for Ki67 of the tonsil using the mAb clone MIB1 properly calibrated and with HIER in an alkaline buffer.
A moderate to strong, distinct nuclear staining reaction is seen in 80-90 % of the germinal centre B-cells in both the dark and the light zone.
Also compare with Figs. 2a - 5a – same protocol.

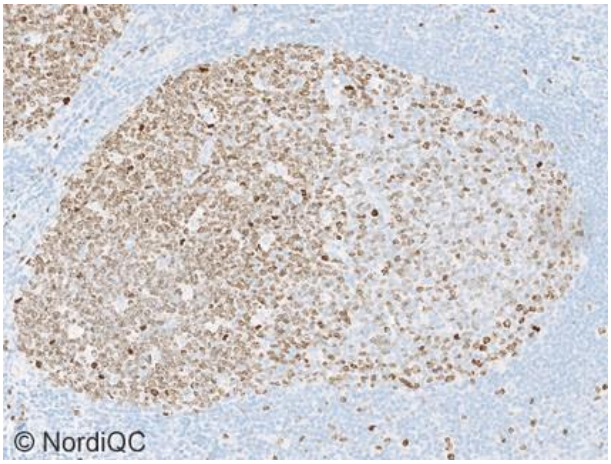


Fig. 1b
Insufficient staining for Ki67 of the tonsil using the mAb clone MIB1 with a protocol providing a too low sensitivity, most likely due to a too low concentration of the primary Ab.
The majority of the germinal centre B-cells are demonstrated, but especially the B-cells in the light zone only show a weak and equivocal nuclear staining reaction – same field as in Fig. 1a.
Also compare with Figs. 2b - 5b - same protocol.

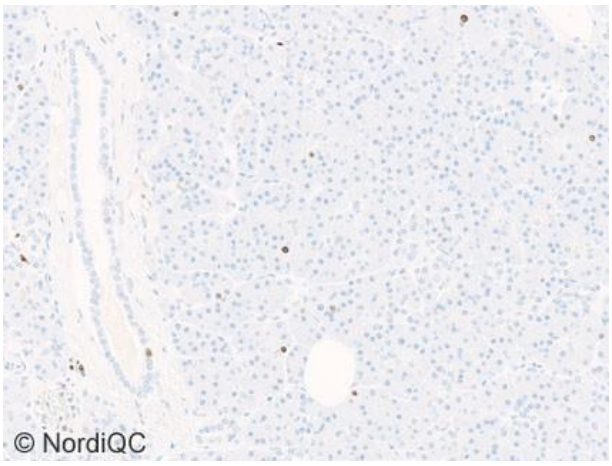


Fig. 2a
Optimal staining for Ki67 of the pancreas using same protocol as in Fig. 1a.
Dispersed epithelial cells of the exocrine glands and large ducts show a distinct nuclear staining reaction.
The nuclear staining reaction for Ki67 is easily identified even at a low magnification (x100).

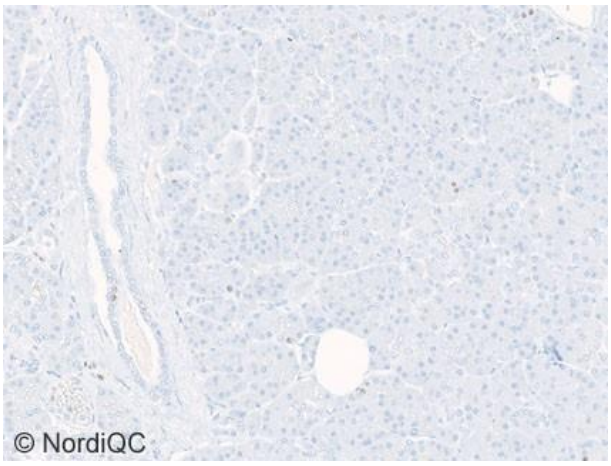


Fig. 2b
Insufficient staining for Ki67 of pancreas using same protocol as in Fig. 1b. - same field as in Fig. 2a.
The intensity and proportion of positive cells is significantly reduced compared to the result in Fig. 2a.
Also compare with Fig. 3b – same protocol.

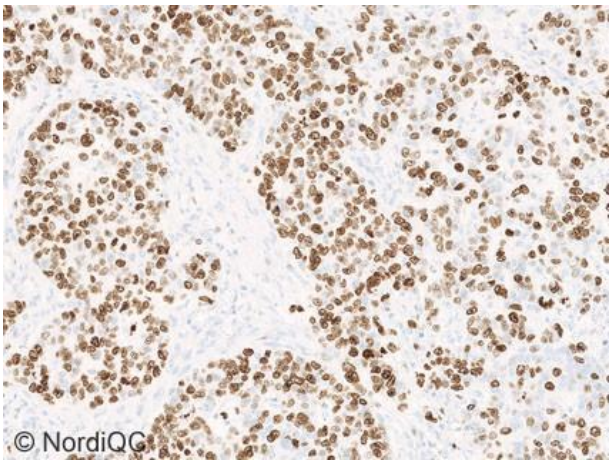


Fig. 3a
Optimal staining for Ki67 of the breast carcinoma, tissue core no. 8 using same protocol as in Figs. 1a and 2a.
>80% of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

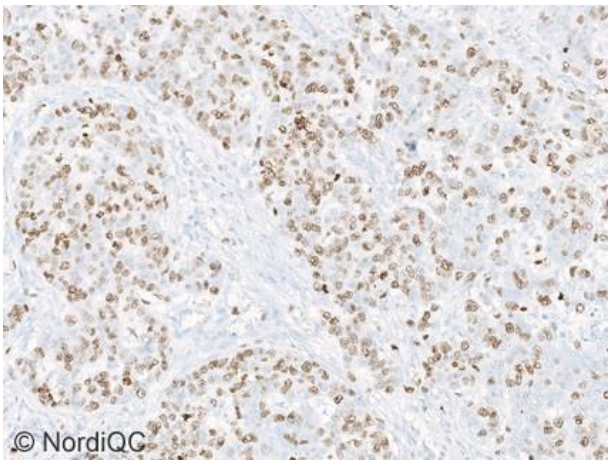


Fig. 3b
Insufficient staining for Ki67 of the breast carcinoma, tissue core no. 8 using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
The intensity and proportion of positive cells is significantly reduced compared to the result in Fig. 3a.

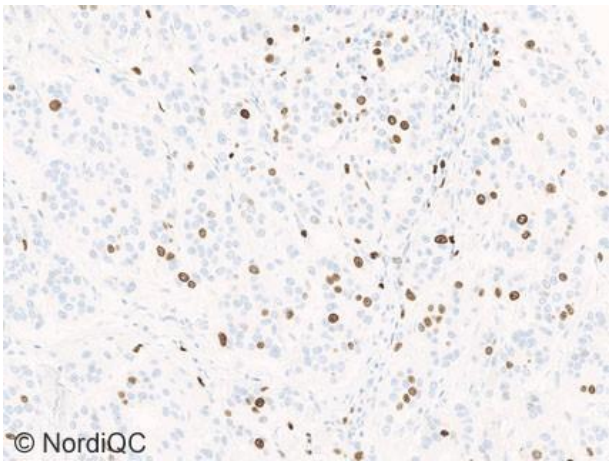


Fig. 4a
Optimal staining for Ki67 of the breast carcinoma, tissue core no. 7 using same protocol as in Figs. 1a - 3a.
20-30% of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

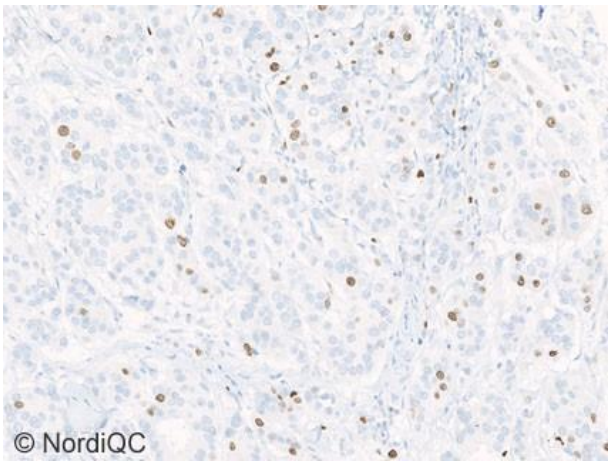


Fig. 4b
Insufficient staining for Ki67 of the breast carcinoma, tissue core no. 7 using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
The intensity and proportion of positive cells is significantly reduced compared to the result in Fig. 3a.

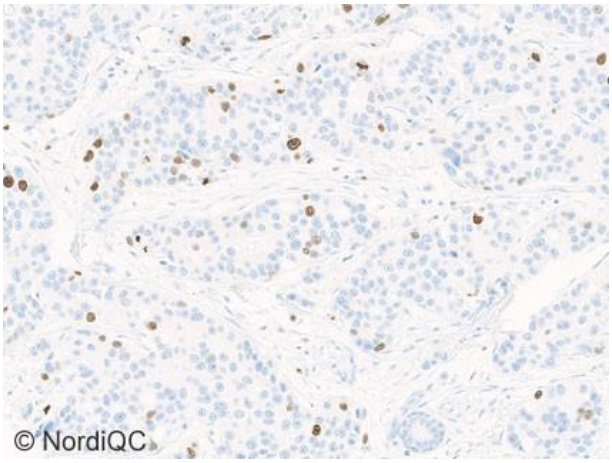


Fig. 5a
Optimal staining for Ki67 of the breast carcinoma, tissue core no. 9 using same protocol as in Figs. 1a - 4a.
5-10% of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

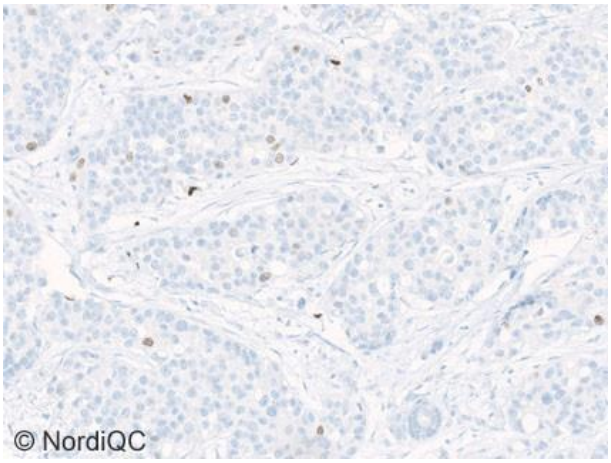


Fig. 5b
Insufficient staining for Ki67 of the breast carcinoma, tissue core no. 9 using same protocol as in Figs. 1b - 4b - same field as in Fig. 5a.
Only scattered cells show a distinct nuclear staining reaction.

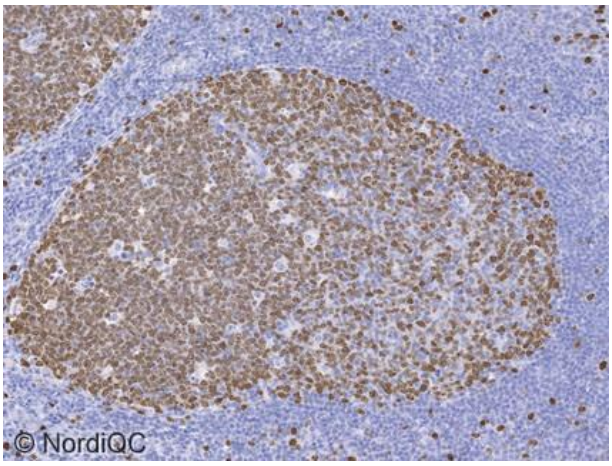


Fig. 6a
Insufficient staining for Ki67 of the tonsil using the mAb clone MIB1 with a protocol complicating the interpretation.
An excessive counterstaining is applied, which in particular affects the ability to identify the nuclear staining reaction for Ki67 in low-level expressing structures as B-cell in the light zone.
Also compare with Fig. 6b, same protocol.

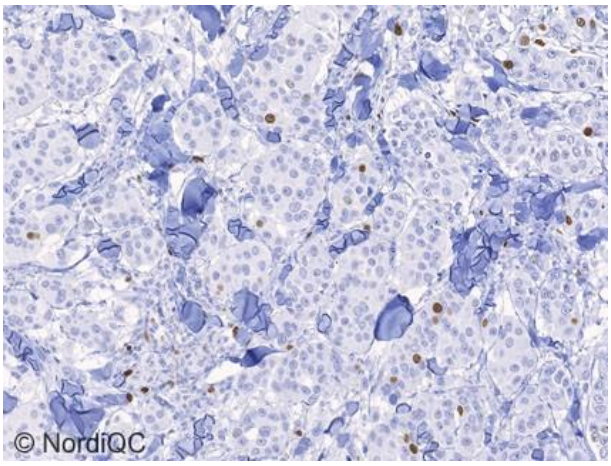


Fig. 6b
Insufficient staining for Ki67 of the breast carcinoma, tissue core no. 7 using same protocol as in Fig. 6a.
The excessive counterstaining compromises the identification of Ki67 and a reduced proportion of Ki67 positive cells is observed.
At the same time also the stromal tissue is stained by the counterstaining, altogether obscuring the interpretation.

SN/RR/LE 07.12.2016