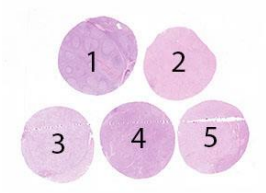
Assessment Run 72 2024
Ki67

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for Ki67, focused on grading neuroendocrine tumors and distinction between low and high grade neuroendocrine neoplasias.
Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for Ki67 (see below).

Material
The slide to be stained for Ki67 comprised:



1. Tonsil, 2. Liver, 3. Seminoma, 4. High grade, colon neuroendocrine carcinoma. 5. Low grade pancreatic neuroendocrine tumor.

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 in the tonsil.
- A nuclear staining reaction only in scattered hepatocytes (<1%) in the liver.
- An at least weak to moderate, distinct nuclear staining reaction in the majority of neoplastic cells in the seminoma.
- An at least weak to moderate, distinct nuclear staining reaction of 30-70% of the neoplastic cells in the high grade colon neuroendocrine carcinoma.
- A nuclear staining reaction in scattered neoplastic cells (≤2%%) in the low grade pancreatic neuroendocrine tumor.

KEY POINTS FOR Ki67 IMMUNOASSAYS
- The widely used mAb clone **MIB-1** and rmAb clone **SP6** gave a high pass rate both as concentrated and RTU formats.
- The Leica Biosystem RTU system **PA0230** based on mAb clone K2 and the Ventana/Roche RTU system **790-4286** based on rmAb clone 30-9 were most successful giving pass rates of 100% and 98%, respectively.
- The mAb clone **MM1**, Leica Biosystems cannot be recommended neither as concentrated nor RTU format.

Participation

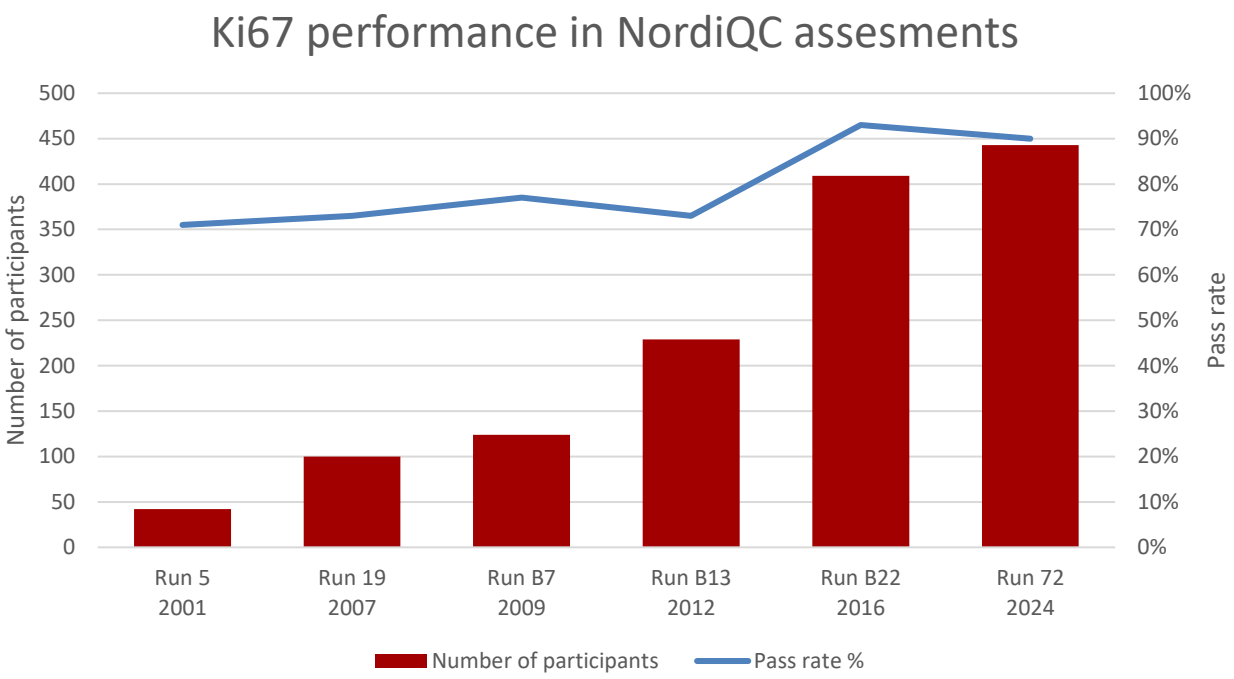
Number of laboratories registered for Ki67, run 72	467
Number of laboratories returning slides	443 (95%)

Results
443 laboratories participated in this assessment. 399 (90%) achieved a sufficient mark (optimal or good), see Table 1a (see page 3). Tables 1b and 1c summarizes antibodies (Abs) used and assessment marks (see page 3 and 4).

The most frequent causes of insufficient staining reactions were:
- Less successful primary Abs
- Inefficient HIER

Performance history
This was the sixth NordiQC assessment of Ki67. A similar pass rate was seen compared to run B22, breast modulein 2016 (see Graph 1, page 2), despite different neoplasias and associated cut-off levels being applied in the two runs.

Graph 1. **Proportion of sufficient results for Ki67 in the six NordiQC run performed.**



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 – most cells should display a moderate intensity, see Fig. 1a. No staining reaction must be seen in the vast majority of mantle zone B-cells.
Liver can be used primarily as supplementary negative tissue controls in which <1% of hepatocytes should be positive (inflammatory and reactive conditions can induce an elevated Ki67 score). Leukocytes can for unexplained reasons show a weak nuclear staining reaction and in particular observed by protocols giving a high analytical sensitivity.
The recommendations of the above mentioned tissue controls for IHC are concordant to the guidelines published by the International Ad Hoc Expert Committee[1].

Conclusion
The mAb clones **MIB-1** and **K2** 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.
RTU Abs showed a superior pass rates compared to concentrated Abs for Ki67 (92% and 83%, respectively). RTU systems from all main vendors, Ventana/Roche (790-4286), Dako/Agilent (IR/IS626, GA626) and Leica Biosystems (PA0230) all obtained high proportion of sufficient and optimal results.

[1]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.

Table 1a. **Overall results for Ki67, run 72**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	106	63	25	16	2	83%	59%
Ready-To-Use antibodies	337	241	70	22	4	92%	71%
Total	443	304	95	38	6		
Proportion		69%	21%	9%	1%	90%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1b. **Concentrated antibodies and assessment marks for Ki67, run 72**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **BS4**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **K2**	1	Zytomed	0	0	1	0	-	-
mAb clone **MIB-1**	75	Dako/Agilent	41	20	12	2	81%	55%
	2	ImmunoLogic	2	0	0	0	-	-
	1	PathnSitu	1	0	0	0	-	-
	1	Zeta Corporation	1	0	0	0	-	-
mAb clone **MM1**	1	Leica Biosystems	0	0	1	0	-	-
mAb clone **ZM67**	1	Zeta Corporation	1	0	0	0	-	-
rmAb clone **EP5**	1	Epitomics	0	1	0	0	-	-
rmAb clone **SP6**	1	Biocare Medical	0	1	0	0	-	-
	10	Cell Marque	9	0	1	0	90%	90%
	2	Diagnostic Biosystems	1	1	0	0	-	-
	2	Epredia	2	0	0	0	-	-
	2	Master Diagnostica	2	0	0	0	-	-
	1	Zytomed Systems	0	0	1	0	-	-
rmAb clone **IHC167**	2	GenomeMe	1	1	0	0	-	-
rmAb clone **QR015**	1	Quartett	1	0	0	0	-	-
Ab clone **1A1-D3**	1	Wondfo	0	1	0	0	-	-
Total	106		63	25	16	2		
Proportion			59%	24%	15%	2%	83%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1c. **Ready-To-Use antibodies and assessment marks for Ki67, run 72**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **GM010 8473-C010**	2	Sakura Finetek	2	0	0	0	-	-
mAb clone **GM027 8276-C010**	1	Sakura Finetek	1	0	0	0	-	-
mAb clone **GM027 GT209402**	1	Gene Tech	1	0	0	0	-	-
mAb **MIB-1 IR626/IS626 (VRPS)[3]**	4	Dako/Agilent	4	0	0	0	-	-
mAb **MIB-1 IR626/IS626 (LMPS)[4]**	22	Dako/Agilent	15	3	4	0	82%	68%
mAb **MIB-1 GA626 (VRPS)[3]**	63	Dako/Agilent	29	28	6	0	90%	46%
mAb **MIB-1 GA626 (LMPS)[4]**	26	Dako/Agilent	20	3	3	0	88%	77%
mAb clone **MIB-1 Z2305MP**	3	Zeta Corporation	0	3	0	0	-	-
mAb clone **MIB-1 API3156 AA**	2	Biocare Medical	0	1	1	0	-	-
mAb clone **MM1 PA0118/PA0410 (VRPS)[3]**	9	Leica Biosystems	0	2	5	2	22%	0%
mAb clone **MM1 PA0118/PA0410 (LMPS)[4]**	5	Leica Biosystems	0	4	1	0	80%	0%
mAb clone **K2 PA0230 (VRPS)[3]**	4	Leica Biosystems	3	1	0	0	-	-
mAb clone **K2 PA0230 (LMPS)[4]**	5	Leica Biosystems	4	1	0	0	100%	80%
rmAb clone **MX002 RMA-0731**	1	Fuzhou Maixin	1	0	0	0	-	-
rmAb clone **SP6 275R-10/17/18**	3	Cell Marque	3	0	0	0	-	-
rmAb clone **SP6 PRM 325**	1	Biocare Medical	0	1	0	0	-	-
rmAb clone **SP6 MAD-000310QD**	2	Master Diagnostica	0	2	0	0	-	-
rmAb clone **SP6 BFM-0003**	1	Bioin Biotechnology	0	1	0	0	-	-
rmAb clone **SP6 BRB040**	1	Zytomed Systems	1	0	0	0	-	-
rmAb clone **30.9 790-4286 (VRPS)[3]**	57	Ventana/Roche	51	5	0	1	98%	89%
rmAb clone **30.9 790-4286 (LMPS)[4]**	122	Ventana Roche	104	15	2	1	98%	85%
rmAb clone **BP6045 KFO**	1	Biolynx Biotechnology	1	0	0	0	-	-
Ab clone **176B3C4 PA028**	1	Abcarta	1	0	0	0	-	-
Total	337		241	70	22	4		
Proportion			71%	21%	7%	1%	92%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.
3) Vendor recommended protocol settings – RTU product used in compliance to protocol settings, platform and package insert.
4) Laboratory modified protocol settings for a RTU product applied either on the vendor recommended platform(s) or other platforms.

Detailed analysis of Ki67, Run 72
The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies
mAb clone **MIB-1**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) (6/6)*, Cell Conditioning 1 (CC1, Ventana/Roche) (33/51) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (6/19) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 60 of 72 (83%) laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **SP6**: Protocols with optimal results were based on HIER using CC1 (Ventana/Roche) (4/4), TRS pH 9 (Dako/Agilent) (2/2), BERS2 (Leica Biosystems) (5/9), Tris-EDTA buffer (2/2) or Unknown (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:50-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings, 16 of 17 (94%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for Ki67 for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **MIB-1**	2/2**	-	4/4	-	33/47 (70%)	-	6/19 (32%)	-
rmAb clone **SP6**	1/1	-	1/1	-	4/4	-	5/8 (63%)	-

* 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)
1) Autostainer Classical, Link 48.
2) BenchMark GX, XT, Ultra, Ultra plus
3) Bond III, Prime

Ready-To-Use antibodies and corresponding systems
mAb clone **K2** product no. **PA0230**, Leica Biosystems, BOND III/MAX/PRIME:
Protocols with optimal results were typically based on HIER using BERS2, efficient heating time 20-30 min. at 99-100°C, and 15-20 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 8 of 8 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **MIB-1**, product no. **IR626/IS626**, Dako/Agilent, Autostainer Classical/Autostainer Link 48:
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 (3-in-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, 14 of 14 (100%) laboratories produced a sufficient staining result.

mAb clone **MIB-1**, product no. **GA626**, Dako/Agilent, Omnis:
Protocols with optimal results were typically based on HIER using TRS Low pH or TRS High pH, efficient heating time 20-30 min. at 97°C, 20-25 min. incubation of the primary Ab and Envision FLEX (GV800) or Envision FLEX+ (GV800+GV821) as detection system. Using these protocol settings, 80 of 87 (92%) laboratories produced a sufficient staining result.

rmAb clone **30.9**, product no. **790-4286**, Ventana/Roche, BenchMark GX/XT/Ultra/Ultra plus:
Protocols with optimal results were typically based on HIER using CC1, 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, 174 of 178 (98%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 asessed protocols). The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **Proportion of sufficient and optimal results for Ki67 in the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako Autostainer mAb MIB-1 **IR/IS626**	(4/4)	(4/4)	91% (10/11)	64% (7/11)
Dako Omnis mAb MIB-1 **GA626**	90% (57/63)	46% (29/63)	88% (23/26)	77% (20/26)
Leica Bond mAb MM1 **PA0118/PA0410**	22% (2/9)	0% (0/9)	80% (4/5)	0% (0/5)
Leica Bond mAb K2 **PA0230**	(4/4)	(3/4)	(4/4)	(3/4)
Ventana BenchMark rmAb 30.9 **790-4286 UltraView**	100% (49/49)	94% (46/49)	99% (71/72)	85% (61/72)
Ventana BenchMark rmAb 30.9 **790-4286 OptiView**	88% (7/8)	63% (5/8)	96% (47/49)	88% (43/49)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer were included.

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 level expected. This staining pattern was seen in 93% of the insufficient results (41 of 44). A poor signal-to-noise ratio or an excessive background staining characterized the remaining three insufficient results.

24% (106 of 443) 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. Irrespective of the clone applied, efficient HIER and careful calibration of the primary Ab were the two most central parameters for optimal results.

For the most commonly used Ab, mAb clone **MIB-1**, an overall pass rate of 86% (57% optimal) was seen. Optimal results could be obtained on the main IHC systems from Dako, Leica and Ventana, as listed in Table 2. For unexplained reasons, MIB-1 showed an inferior performance on the Leica Bond IHC system compared to the IHC systems from the other main vendors. Despite a sensitive 3-step polymer-based detection system is used as default setting on the Bond IHC system, only 30% of the laboratories, using the identified "optimal" protocol settings regarding HIER conditions and Ab titre, obtained an optimal mark. 58% produced a staining evaluated as sufficient (good or optimal). The insufficient results for clone MIB1 on Bond were typically related to a poor-signal-to noise ratio of Ki67 compromising the scoring of PI and/or a reduced number of Ki67 positive cells. In comparison, 100% (6 of 6) of the protocols applied on the Dako/Agilent IHC systems (Omnis or Autostainer) were evaluated as optimal. No explanations for this difference could be identified from the submitted protocols. This was in concordance to the results found in the previous NordiQC Ki67 run B22.

The rmAb clone **SP6** as a concentrate was used by 18 laboratories, giving an overall pass rate of 89% (78% optimal), and optimal results on the main IHC systems (see Table 2). rmAb clone SP6 gave a better performance on the Bond platforms compared to mAb clone MIB-1, with an overall pass rate of 88% (7 of 8) if using the "optimal" protocol settings.

Ready-To-Use (RTU) antibodies were used by 76% (337 of 443) of the laboratories. Optimal results could be obtained with the RTU systems from the three main IHC system providers, Dako/Agilent, Leica Biosystems and Ventana Roche.

The Ventana/Roche 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 98%. 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 or OptiView as detection kit) and by laboratory modified protocols adjusting incubation time of the primary Ab and/or HIER time. No significant changes in pass rates and proportion of optimal results were observed between the use of UltraView and OptiView, as typically seen for type I IHC assays in NordiQC assessments. 66% of the participants applied UltraView as detection

system (n=119) giving a pass rate of 99%, 89% optimal compared to OptiView used by 32% (n=57) with a pass rate of 95%, 84% optimal.
The Dako/Agilent RTU system **GA626** for Omnis based on mAb clone MIB-1 provided a pass rate of 90% when using the recommended protocol settings (see Table 3). 29% modified the protocol settings, typically changing to HIER in TRS High pH and/or minor adjustments in incubation times. Five laboratories used EnVision Flex+ as detection system, all with an optimal result.

Four participants used the Dako/Agilent RTU system **IR626/IS626** for Autostainer based on the mAb MIB-1 as recommended by vendor, all four optimal. As for the Omnis RTU, the modified protocols were typically using HIER in TRS High pH and/or minor adjustments in incubation times.
11 participants used the Autostainer RTU on other platforms. Similar to the observations for the concentrated format, the MIB1 RTU format IR626/IS626 showed an inferior performance on Bond IHC platforms as 3 of 5 protocols gave an insufficient result. In contrast mitigation of the RTU format to Ventana Benchmark was more successful as an optimal result was obtained in all five protocols based on this combination. However, in this context, it must be emphasized that modifications of vendor recommended protocol settings for RTU systems including migration of the RTU Abs to another platform than the intended, require a meticulous validation process for the end-users. As seen in this and previous assessments, modifications can be very successful but may also generate sub-optimal or aberrant results and therefore must be carefully monitored.

The RTU system **PA0230** from Leica Biosystems based on the mAb clone K2 for Bond gave a pass rate of 100% (8 of 8), despite the protocol settings. Four participants modified the protocol by changing HIER to BERS2 for 30 min. or prolonged the incubation time of the primary Ab.

In concordance with the results seen in run B22, the Leica Biosystems RTU **PA0118/PA0410** based on mAb clone MM1 showed an inferior performance compared to the RTU based on clone K2. In this run, nine laboratories followed the vendor recommend protocol settings, with a disappointing pass rate of 22%, no optimal. Five participants prolonged HIER or incubation time of primary Ab giving a pass rate of 80%, however, no optimal. Overall, the insufficient results were caused by a generally too weak staining reaction for Ki67 and reduced PI in the neoplasias included in the TMA circulated.

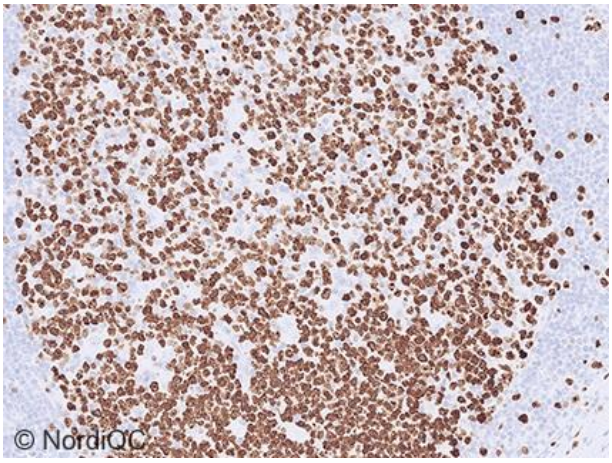


Fig. 1a
Optimal staining reaction for Ki67 of the tonsil using the rmAb clone 30.9 RTU 790-4286 (Ventana/Roche), using the vendor recommended protocol settings with UltraView as detection system.
A moderate to strong, distinct nuclear staining reaction is seen in 80-90 % of the germinal centre B-cells in both the dark and the light zone.
Also compare with Figs. 2a - 5a – same protocol.

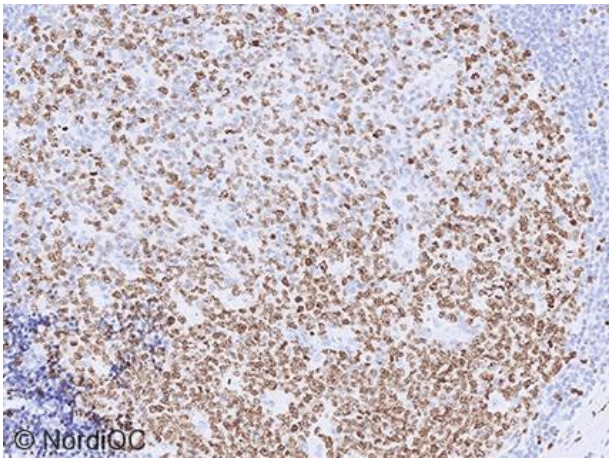


Fig. 1b
Insufficient staining reaction for Ki67 of the tonsil using the mAb clone MIB-1 with a protocol providing a too low analytical sensitivity, most likely due to a too low concentration of the primary Ab. The staining was performed on a BenchMark platform, using HIER in an alkaline buffer and UltraView as detection system.
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.

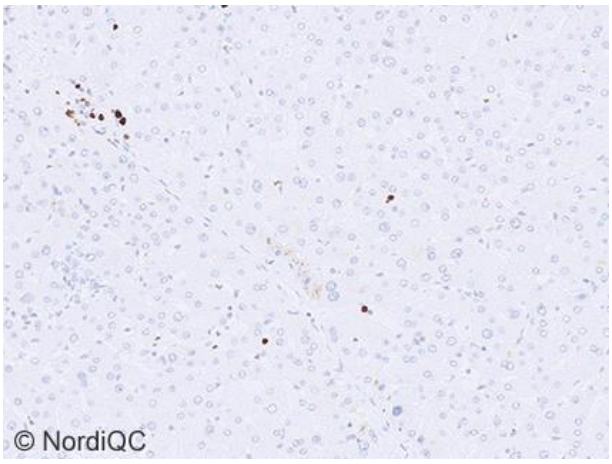


Fig. 2a
Optimal staining reaction for Ki67 of the liver using same protocol as in Fig. 1a.
Scattered hepatocytes show a distinct nuclear staining reaction.

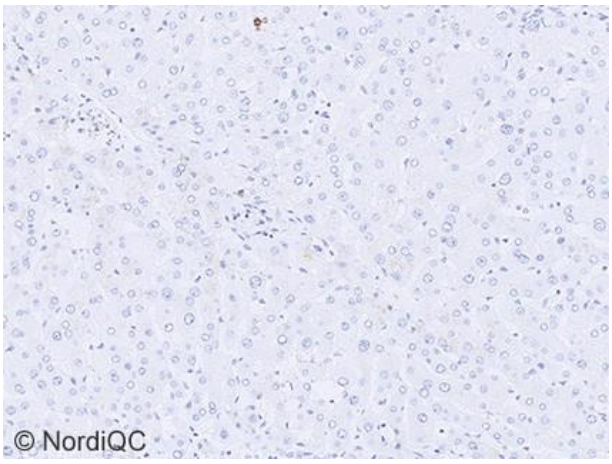


Fig. 2b
Staining reaction for Ki67 of liver using same insufficient protocol as in Fig. 1b. - same field as in Fig. 2a.
No staining reaction is seen.

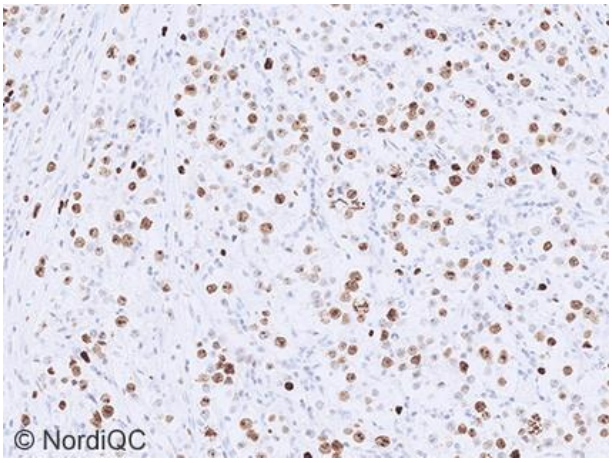


Fig. 3a
Optimal staining reaction for Ki67 of the seminoma using same protocol as in Figs. 1a and 2a.
The vast majority of the neoplastic cells show a distinct nuclear staining reaction, and no background staining is seen.

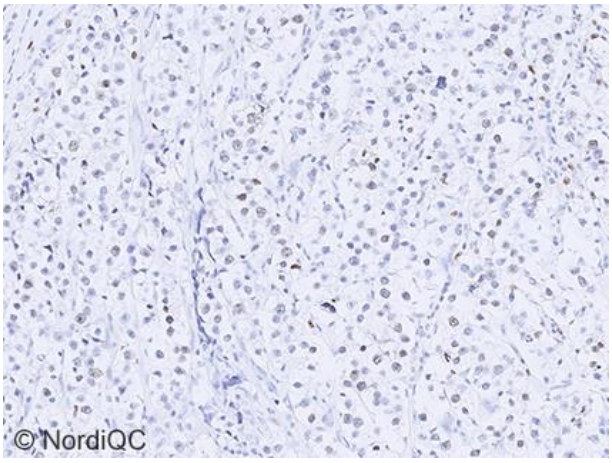


Fig. 3b
Insufficient staining reaction for Ki67 of the seminoma using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
The intensity and proportion of positive cells is significantly reduced compared to the result in Fig. 3a.

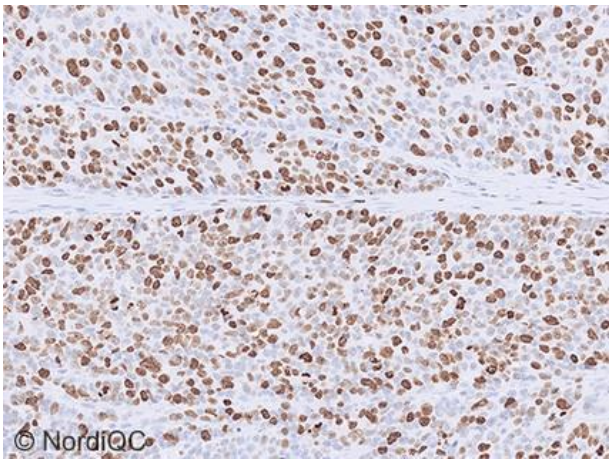


Fig. 4a
Optimal staining reaction for Ki67 of the high grade colon neuroendocrine carcinoma, using same protocol as in Figs. 1a - 3a.
>50% of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

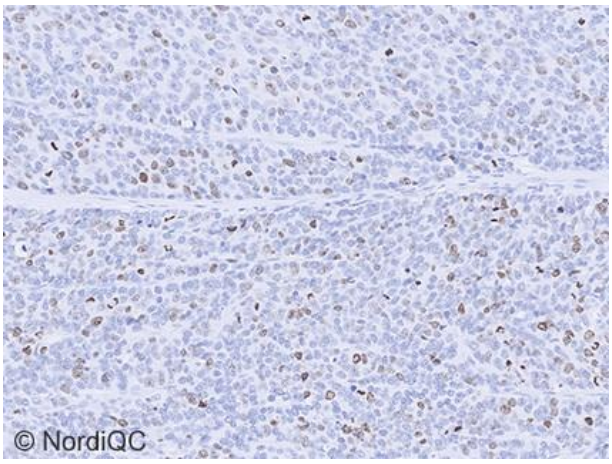


Fig. 4b
Insufficient staining reaction for Ki67 of the high grade colon neuroendocrine carcinoma, using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
The intensity and proportion of positive cells is significantly reduced compared to the result in Fig. 4a.

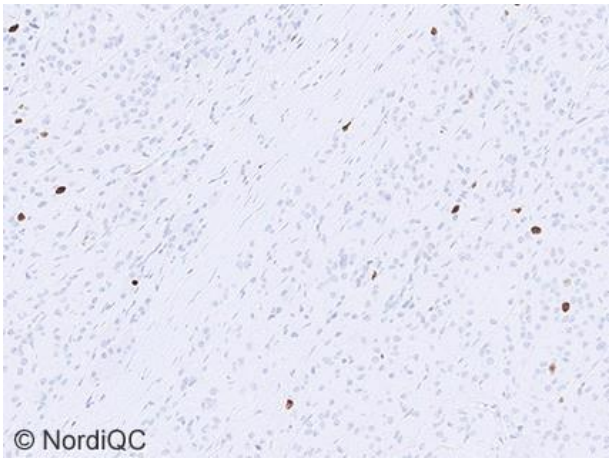


Fig. 5a
Optimal staining reaction for Ki67 of the low grade pancreatic neuroendocrine tumor, using same protocol as in Figs. 1a - 4a.
<2% of the neoplastic cells show a distinct nuclear staining reaction and no background staining is seen.

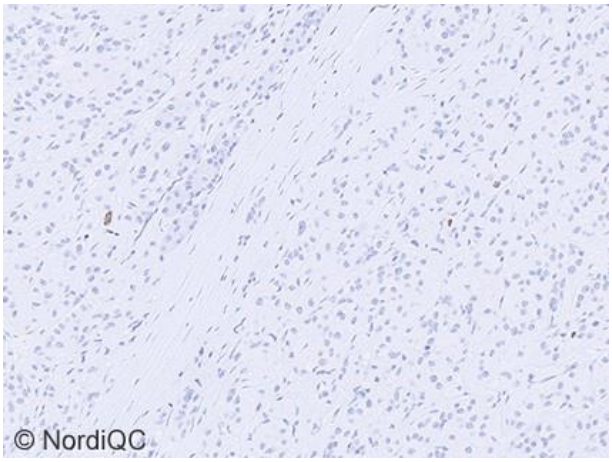


Fig. 5b
Staining reaction for Ki67 of the low grade pancreatic neuroendocrine tumor, using same protocol as in Figs. 1b - 4b - same field as in Fig. 5a.
Only scattered cells show a faint nuclear staining reaction.

HLK/LE/SN 28.11.2024