BAP1 (BRCA1-Associated Protein 1)

Purpose
Evaluation of the technical performance, the level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for BAP1, identifying malignant mesothelioma. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for BAP1 (see below).

Material
The slide to be stained for BAP1 comprised:

1. Appendix, 2. Tonsil, 3. Lung adenocarcinoma, 4-5. Malignant mesothelioma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a BAP1 staining as optimal included:

- An at least weak to moderate, distinct nuclear staining reaction in virtually all of the mantle zone and interfollicular lymphocytes in tonsil.
- A moderate to strong, distinct nuclear staining reaction in virtually all germinal center B-cells and squamous epithelial cells in tonsil.
- An at least weak to moderate nuclear staining reaction of the majority of smooth muscle cells and at least a moderate nuclear staining reaction in virtually all epithelial cells in appendix.
- An at least weak to moderate nuclear staining reaction in the neoplastic cells in the lung adenocarcinoma.
- An at least moderate nuclear staining reaction of most stromal cells in both malignant mesotheliomas.
- No nuclear staining of neoplastic cells in both malignant mesotheliomas. A weak cytoplasmic staining reaction was accepted providing read-out not being compromised.

KEY POINTS FOR BAP1 IMMUNOASSAYS
- The mAb clones **C-4, BSB-109** and rmAb clone **EPR22826-65** are recommendable Abs.
- All polyclonal Abs produced inferior results, mostly exhibiting a false positive staining reaction in the malignant mesotheliomas.
- Highest proportion of sufficient results was achieved on Ventana Benchmark stainer platforms, especially when **OptiView with OptiView Amplification Kit** was applied, resulting in a pass rate of 81% (65/80) for these protocols.
- IHC for BAP1 showed a relatively low inter- and intra-laboratory reproducibility as identical protocol settings provided different results.

Participation

Number of laboratories registered for BAP1, run 71	241
Number of laboratories returning slides	224 (93%)

Results
At the date of assessment, 93% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

224 laboratories participated in this assessment. 63% achieved a sufficient mark (optimal or good), see Table 1a (see page 3). Tables 1b and 1c summarize the antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:

- Inappropriate concentration of the primary antibody.
- Inefficient or inappropriate Heat Induced Epitope Retrieval (HIER).
- Use of a less sensitive detection system.
- Unexplained technical issues

Performance history
This was the second NordiQC assessment of BAP1. The pass rate has slightly decreased compared to the previous assessment in run 65, 2022 (see Graph 1), whereas the number of participants increased by 37% from 163 in run 65 to 224 in this run.

Graph 1. **Proportion of sufficient results for BAP1 in the eight NordiQC runs performed**
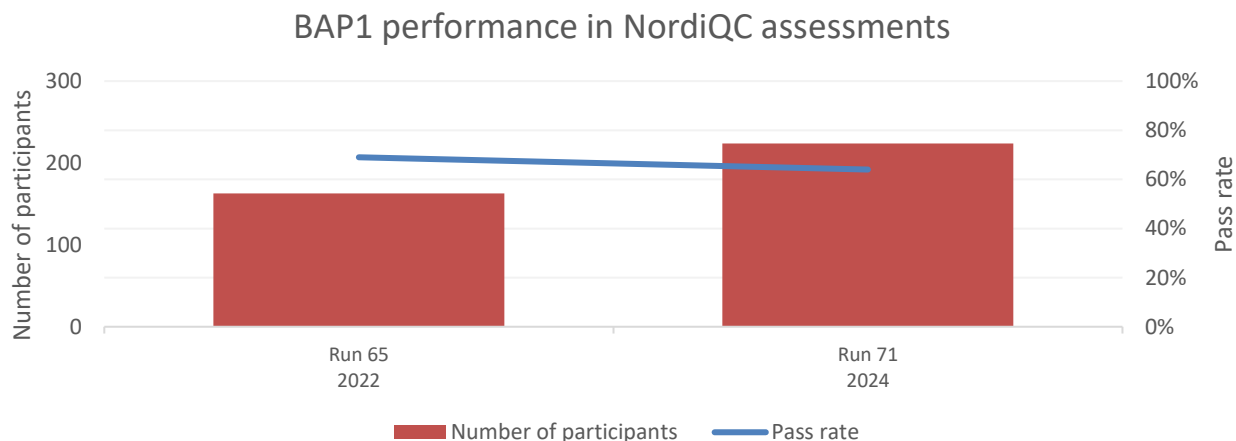
Controls
At present and according to publications and data generated in the NordiQC assessments, appendix and/or tonsil are recommended as external positive tissue controls for BAP1. In appendix, virtually all epithelial cells should show an at least moderate nuclear staining reaction, and an at least weak nuclear staining reaction must be seen in virtually all lymphocytes, smooth muscle and stromal cells. In tonsil, an at least weak nuclear staining reaction in most mantle zone lymphocytes must be seen, whereas an at least weak to moderate nuclear staining reaction should be seen in virtually all germinal center lymphocytes. In addition to external controls, it is of highest importance that stromal cells within the tumor tissues tested exhibit a distinct nuclear staining reaction serving as internal positive tissue control (see Fig. 4a). A reliable interpretation of the results in the tumor directly depends on clear demonstration of the internal positive control and if both entities are negative the IHC test is not conclusive.

Conclusion
The mAb clones **C-4**, **BSB-109** and the rmAb clone **EPR22826-65** could all provide an optimal result for the demonstration of BAP1. All protocols based on pAbs produced inferior results, with the majority of insufficient outcomes characterized by false positive BAP1 staining reactions in malignant mesotheliomas. Optimal results could be obtained on all the main fully automated staining platforms from Dako/Agilent, Ventana/Roche and Leica Biosystems, however most were achieved on the Ventana Benchmark together with the use of OptiView and OptiView Amplification Kit as a detection system, accounting for 73% (40/55) of all optimal results obtained in this assessment. Similar to the previous assessment run 65 (2022), the vast majority of all protocols (68%, 152/224) were based on the mAb clone C-4.
HIER in an alkaline buffer, precise calibration of the primary Ab and in particular use of a sensitive 3-step polymer or multimer based detection system were the main prerequisites for a successful result. BAP1 IHC showed a relatively low reproducibility as identical protocol settings including choice of reagents and stainer platforms could give different results ranging from optimal to insufficient.
It is important to optimize and calibrate the protocol settings thoroughly to provide the expected staining reaction in the on-slide external control tissues - (see above), however when interpreting BAP1 results on clinical samples, it is of utmost importance that the stromal cells intermingling between the tumor tissue evaluated exhibit- a distinct nuclear staining reaction serving as final internal control for the adequacy of the IHC assay.

Table 1a. **Overall results for BAP1, run 71**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR.[2]
Concentrated antibodies	183	48	69	56	10	64%	26%
Ready-To-Use antibodies	41	7	18	11	5	61%	17%
Total	224	55	87	67	15		
Proportion		25%	39%	30%	7%	63%	

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

Table 1b. **Concentrated antibodies and assessment marks for BAP1, Run 71**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb **C-4**	124	Santa Cruz	33	48	36	7	65%	27%
	10	Immunologic	2	7	1	0	90%	20%
	4	Histopathology	0	1	3	0	-	-
	3	Zeta Corporation	1	0	1	1	-	-
	2	Nordic Biosite	0	0	2	0	-	-
	1	Monosan	1	0	0	0	-	-
mAb **BSB-109**	24	BioSB*	4	11	9	0	63%	17%
	3	LSBio	3	0	0	0	-	-
mAb **IHC761**	1	GenomeMe	1	0	0	0	-	-
rmAb **EPR22826-65**	7	Abcam	3	1	3	0	57%	43%
rmAb **QR119**	1	Quartett	0	1	0	0	-	-
rmAb **ZR454**	1	Histopathology	0	0	1	0	-	-
pAb **AB199396**	1	Abcam	0	0	0	1	-	-
pAb **HPA026803**	1	Sigma-Aldrich	0	0	0	1	-	-
Conc total	183		48	69	56	10		
Proportion			26%	38%	31%	5%	64%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
* including distributed by Gennova (n=6)

Table 1c. **Ready-To-Use antibodies and assessment marks for BAP1, Run 71**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR.[2]
mAb clone **BSB-109 BSB 3300/1/2**	28	Bio SB	7	12	7	2	68%	25%
mAb clone **C-4 PDM595**	5	Diagnostic BioSystems	0	4	1	0	80%	0%
mAb clone **C-4 Z2318MP**	2	Zeta Corporation	0	1	1	0	-	-
mAb clone **C-4 MAB-1143**	1	Fuzhou Maixin Biotech	0	0	0	1	-	-
rmAb clone **BAP1/8959R AND45**	1	BioGenex	0	0	1	0	-	-
rmAb clone **EPR22826-65 8353-C010**	1	Sakura Finetek	0	1	0	0	-	-
pAb **PA525**	1	Abcarta	0	0	1	0	-	-
pAb **API 3247 AA**	2	Biocare Medical	0	0	0	2	-	-
RTU total	41		7	18	11	5		
Proportion			17%	44%	27%	12%	61%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).

Detailed analysis of BAP1, Run 71
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb **C-4**: Protocols with optimal results were based on HIER in an alkaline buffer using either Target Retrieval Solution (TRS) High pH (Dako/Agilent) (3/25), Cell Conditioning 1 (CC1, Ventana/Roche) (33/81) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (1/26) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 84 of 121 (69%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer).

mAb clone **BSB-109**: Protocols with optimal results were based on HIER using CC1 (Ventana/Roche) (7/13) 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, 11 of 13 (85%) laboratories produced a sufficient staining, 54% (7/13) optimal.

rmAb clone **EPR22826-65**: Protocols with optimal results were all based on HIER in an alkaline buffer using TRS High pH (Dako/Agilent) (1/3) or CC1 (Ventana/Roche) (2/2) as retrieval buffer. The rmAb was diluted in the range of 1:25-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 3 of 5 (60%) laboratories produced an optimal staining.

Table 2. **Proportion of optimal results for BAP1 for the most commonly used antibody concentrates on the four main IHC systems***

Concentrated antibody	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 **C-4**	0/8** (0%)	-	3/24 (13%)	0/2	32/72 (44%)	-	1/25 (4%)	-
mAb clone **BSB-109**	0/2	-	0/8 (0%)	-	7/13 (54%)	-	0/2	1/1
rmAb clone **EPR22826-65**	-	-	1/3	-	2/2	-	0/2	-

* 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 XT, Ultra, Ultra plus
3) Bond III

Ready-To-Use antibodies
mAb clone **BSB-109**, product no. **BSB 3300/1/2**, BioSB:
Protocols with optimal results were typically based on HIER using CC1 (Ventana/Roche) as retrieval buffer (efficient heating time 64 min.), 32-52 min. incubation of the primary Ab and OptiView with OptiView Amplification Kit (Ventana/Roche, 760-700 + 760-099/860-099) as detection system. Using these protocol settings, 6 of 6 (100%) laboratories produced a sufficient staining result, 5 of 6 (83%) optimal.

Comments
In this assessment a slight decrease in pass rate to 63% (142/224) was observed compared to 69% (113/163) obtained in the previous BAP1 assessment in run 65, 2022. The majority of insufficient results were characterized by three main reaction patterns: excessive background (24/82, 29%), poor signal-to-noise ratio (23/82, 28%) and a too weak or completely false negative staining result (20/82, 24%). In the remaining insufficient results (15/82, 18%) a granular staining interfering interpretation, cytoplasmic staining or false positive reaction with or without false negative staining were seen. This clearly demonstrates the present challenge to optimize reproducible IHC assays for BAP1 with adequate technical quality and diagnostic accuracy. In this assessment most participants were able to detect stromal cells at least weakly in the two mesotheliomas, whereas labeling of nuclei of normal cells in the tonsil and appendix and neoplastic cells in the lung adenocarcinoma was more challenging with suboptimal protocols. For slides exhibiting either an excessive background staining or poor signal-to-noise ratio, the interpretation of presence or absence of BAP1 expression in the malignant cells of the mesotheliomas was hindered (see Fig. 4b).

Similar to the previous run, 83% (183/224) of the laboratories used Abs as concentrated format within laboratory developed (LD) tests for BAP1. The mAb **clone C-4** was the most widely used clone, being applied by 79% (144/183) of the participants and achieved a pass rate of 65% (93/144), 26% (37/144) optimal (see Table 1b). Sufficient results could be obtained on the three main fully automated stainer platforms, however the proportions of optimal results differed between platforms (see Table 2) with the most successful results seen on the Ventana Benchmark, whereas an inferior performance being observed on the Leica Bond. The main prerequisites for a sufficient staining with mAb clone C-4 were efficient HIER in an alkaline buffer and careful calibration of the titre of the primary Ab. Nevertheless, data analysis revealed a high proportion of conflicting results when similar protocol settings were applied by different participants (see Figs. 1-4a and b), which might indicate fluctuations in the quality of reagents used within the assays and/or reproducibility issues of the IHC systems applied. 99% (142/144) of participants used an at least a 3-step polymer/multimer based detection system as BAP1 has shown to require sensitive protocol settings for adequate staining reaction. The highest proportion of sufficient and optimal results were obtained when OptiView together with OptiView Amplification Kit (Ventana/Roche) was applied on the Ventana Benchmark platforms. This was done by 56% (80/144) of the participants and a pass rate of 81% (65/80), 48% (38/80) optimal, was achieved. However, it is well-known from previous NordiQC assessments and also observed in this BAP1 assessment, that assays based on OptiView with OptiView Amplification Kit (tyramide based) can be challenging to calibrate and reproduce and might induce a risk of

aberrant granular precipitation of the chromogen in structures expected to be negative compromising the read-out.

Within a LD assay based on using a primary antibody concentrate, the mAb clone **BSB-109** provided almost identical pass and optimal rates of 67% (18/27) and 26% (7/27), respectively, compared to the performance of mAb clone C-4. Based on the data from this assessment, an optimal result could only be achieved on the Ventana Benchmark Ultra staining platform, whereas the proportion of assessment marks "Good" on Dako Omnis was 56% (5/9) and 100% (2/2) on Leica Bond III. The overall pass rate on Ventana Ultra was 92% (11/12), 58% (7/12) optimal. All optimal results were based on the use of OptiView with OptiView Amplification Kit as the detection system.

The rmAb clone **EPR22826-65** was used by 7 participants as a LD assay based on a primary Ab concentrate and achieved a pass rate of 57% (4/7), 43% (3/7) being optimal. Optimal staining results could be achieved on the Ventana Benchmark Ultra and Dako Omnis platforms (see Table 2). Both protocols on Ventana Benchmark Ultra were based on HIER in CC1 for 40-64 min., antibody incubation for 32 min with a dilution factor of 1:25 or 1:100 and OptiView as detection system. One optimal protocol on Dako Omnis used HIER in TRS High pH for 24 min., diluted the antibody with Renoir Red (Biocare Medical, PD904) to 1:500 and used FLEX with dual linker as the detection system.

Currently there are no RTU systems on the market specifically validated for the main staining platforms from Dako/Agilent, Ventana/Roche and Leica Biosystems. Nevertheless, 18% (41/224) of participants used BAP1 in an RTU format, achieving a pass rate of 61% (25/41), 17% (7/41) optimal. The primary antibody product **BSB 3300/1/2** based on mAb clone **BSB-109** from BioSB was the most used RTU product, attaining a pass rate of 68% (19/28), 25% (7/28) being optimal. Similar to the results seen with the corresponding concentrate, optimal stainings were all achieved on the Ventana Benchmark platforms mainly when OptiView with OptiView Amplification Kit was used as a detection system. 2/2 protocols performed on Leica Bond gave a result assessed as Good. Both were based on HIER in BERS2 for 30 min., 15 min. incubation with the primary RTU Ab and Bond Refine as detection system. The pass rate for slides stained on Dako Omnis was 20% (1/5, assessed as Good) with all 5 being based on HIER for 30 min. in TRS High pH, FLEX+ as a detection system and ab incubation ranging between 10-40 min. (27 min. for slide assessed as Good).

This was the second assessment of BAP1 in NordiQC (see Graph 1). The pass rate was 63%, which is slightly reduced compared to the level of 69% achieved in the previous run 65 in 2022, however the proportion of optimal results decreased significantly from 42% to 25%. The results of laboratories who took part in both NordiQC BAP1 assessments were slightly superior to those participating for the first time in run 71 with respective pass rates being 68% (97/143) and 56% (45/81). It was encouraging to see that for the group of laboratories (n=41) failing in the first BAP1 assessment, run 65, and participating again in this second run, a pass rate of 54% was seen indicating tailored recommendations being beneficial to optimize IHC assays. When concentrating only on participants who took part in both assessments with exactly the same staining protocol, a difference in performance is seen, with proportions of sufficient results in run 65 being 84% (49/58), 50% (29/58) optimal compared to 76% (44/58) and 28% (16/58), respectively, in run 71. This indicates that the decrease in both the overall pass rate and especially the proportion of optimal results, at least in part, was impacted by external technical parameters, such as the accuracy of staining platforms, quality of reagents used and/or tissues circulated compromising the reproducibility of the implemented and applied protocols. As indicated in this report, the IHC testing reproducibility for BAP1 seems to be relatively challenging and is most likely caused by a combination of several parameters. The lack of reproducibility has been observed both in the two assessment runs for BAP1 but also internally at NordiQC in the development, optimization and validation process of the reference IHC assay for BAP1. In this process different antibodies, IHC stainer platforms and protocol settings have been evaluated. The selected method was based on the rmAb clone **EPR22826-65** and performed on the Ventana BenchMark Ultra using OptiView with Amplification as detection system. Despite being meticulously calibrated, the same protocol could give optimal results with a perfect signal-to-noise ratio and also inappropriate results with either an excessive aberrant granular cytoplasmic staining reaction or a too low level of analytical sensitivity confirming reproducibility challenges for "the total analytical part" of the BAP1 IHC assay. At present the limitation to mainly having availability to low affinity BAP1 Abs requiring highly sensitive detection systems based on tyramide amplification seem to compromise the reproducibility as just minor fluctuations in the protocol performance will affect the precision of the results. This was especially observed for mAb clone C-4 being by far the most used Ab for detecting BAP1 with IHC. Although an optimal result can be obtained on all of the main fully-automatic stainer platforms, it is proving to be challenging to optimize and the same protocol settings did not produce the same staining result between different laboratories. Finding the balance of high signal to low noise is difficult, however focusing on sufficient HIER in an alkaline buffer, using a sensitive 3- or 4-step detection system and carefully calibrating the primary antibody concentration with possibly trying out different antibody diluents are the fundament to adequate staining results.
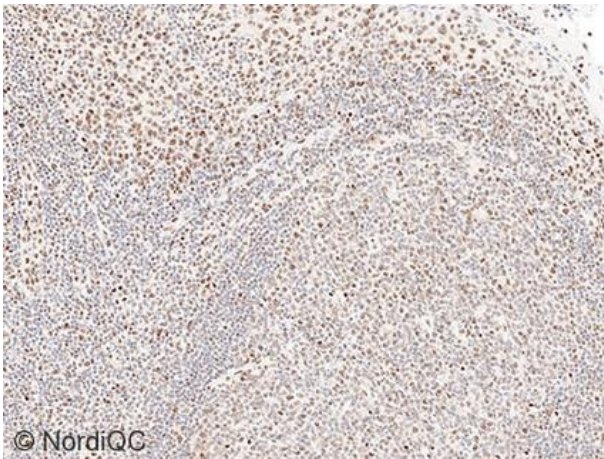
Fig. 1a
Optimal BAP1 staining of the tonsil, tissue core no. 2, using the mAb clone C-4 - diluted, 1:50 (incubation time 32 min.), epitope retrieval using HIER in CC1 (32 min.), a 3-step multimer based detection system (OptiView) with tyramide amplification (OptiView Amplification) and performed on BenchMark (Ventana/Roche). All germinal centre lymphocytes and squamous epithelial cells show a moderate to strong nuclear staining reaction, whereas an at least weak to moderate nuclear staining reaction is seen in most mantle zone and interfollicular lymphocytes. Same protocol used in Figs. 2a-4a.
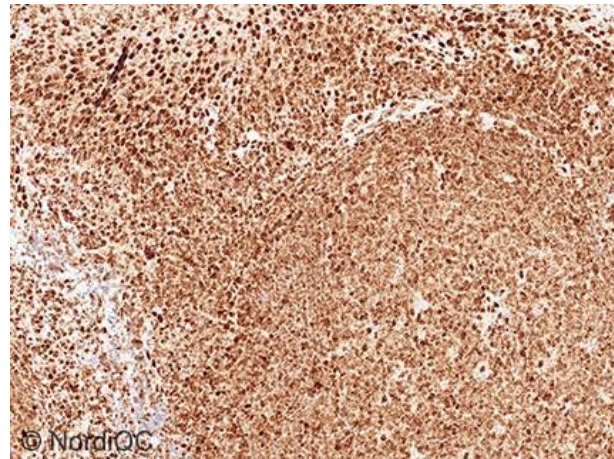
Fig. 1b
Insufficient BAP1 staining of tonsil using a very similar protocol to Fig. 1a based on the mAb clone C-4 diluted 1:50 (32 min.), HIER in CC1 for 40 min. and OptiView with OptiView Amplification Kit as detection system. Although all cells exhibit a moderate to strong nuclear staining reaction, it is not distinctly restricted to the nuclei as an extensive reaction is seen in the cytoplasmic compartment and it is virtually impossible to separate the individual cells in the lymphoid follicle – compare with Fig. 1a, same tissue. Same protocol used in Figs. 2b-4b.
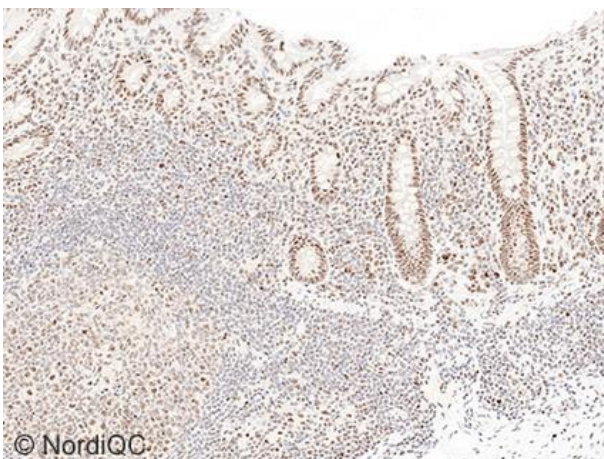

Fig. 2a
Optimal BAP1 staining of appendix using same protocol as in Fig. 1a. Virtually all epithelial cells display a moderate to strong nuclear staining reaction, and the vast majority of lymphocytes/stromal cells show a weak to moderate nuclear staining reaction.
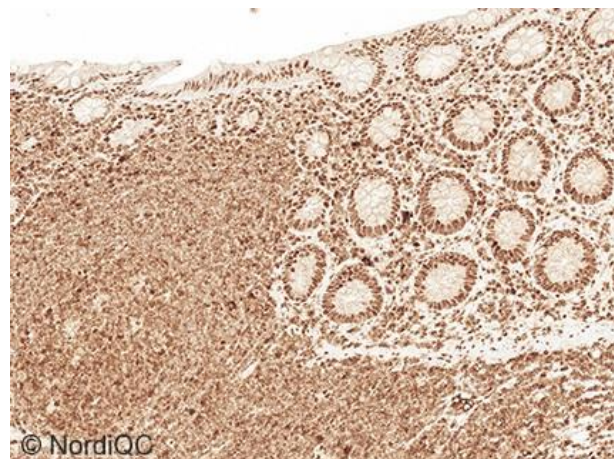

Fig. 2b
Insufficient BAP1 staining of the appendix using same protocol as in Fig. 1b. Similar to Fig. 1b, separation of cells is hindered due to a prominent aberrant granular cytoplasmic staining reaction most likely caused by the tyramide-based amplification of the signal – compare with Fig. 2a, same tissue.

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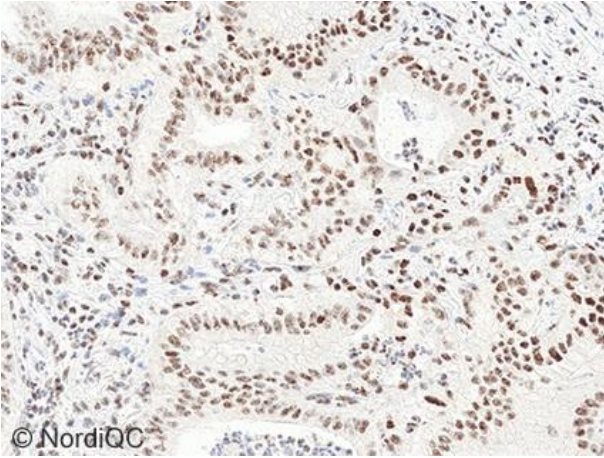
Page **6** of **8**

Fig. 3a
Optimal BAP1 staining of the lung adenocarcinoma, tissue core no. 3, using same protocol as in Figs. 1a-2a. The vast majority of the neoplastic and stomal cells display a moderate to strong nuclear staining reaction.
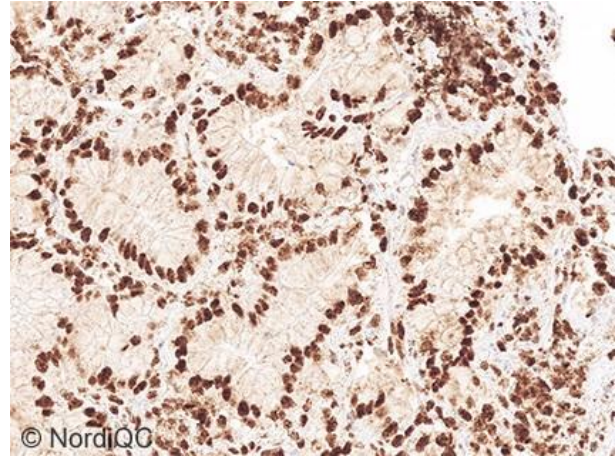

Fig. 3b
Insufficient BAP1 staining of the lung adenocarcinoma, tissue core no. 3, using same protocol as in Figs. 1b-2b. The neoplastic cells show a strong nuclear staining reaction together with a moderate cytoplasmic and membranous staining reaction – compare with Fig. 3a, same tissue.
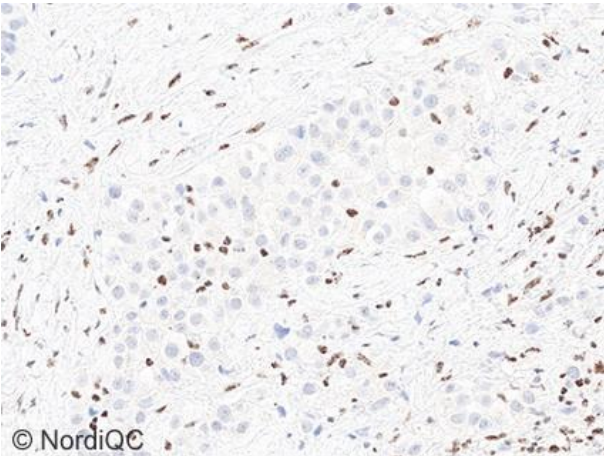

Fig. 4a
Optimal BAP1 staining of the malignant mesothelioma, tissue core no. 4, using same protocol as in Figs. 1a–3a. All neoplastic cells are negative, whereas stromal cells show a distinct, moderate to strong nuclear staining reaction serving as internal positive tissue control verifying the loss of BAP1 expression in the tumor.
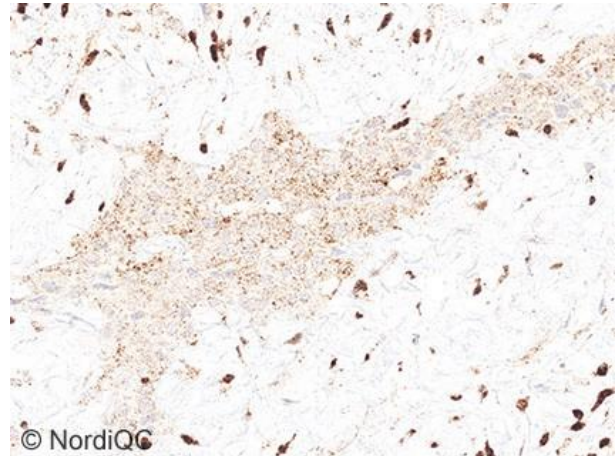

Fig. 4b
Insufficient BAP1 staining of the malignant mesothelioma, tissue core no. 4, using same protocol as in Figs. 1b – 3b. The stromal cells display a strong nuclear staining reaction, whereas specifically the tumor cells harbor a distinct mostly cytoplasmic but also nuclear aberrant granular staining reaction complicating the interpretation – compare with Fig. 4a, same tissue.
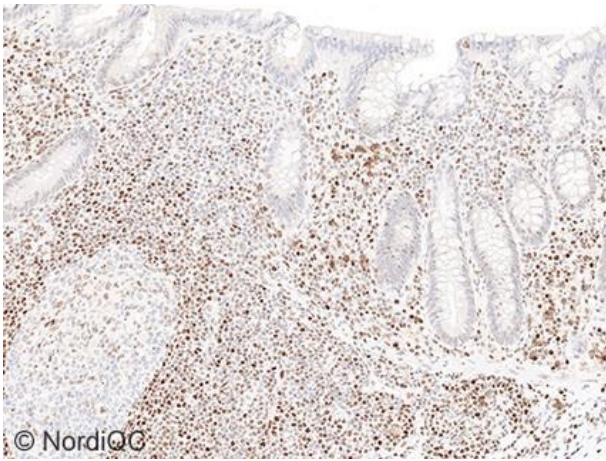
Fig. 5a
Insufficient BAP1 staining of the appendix, tissue core no. 4, using a polyclonal antibody. Epithelial cells and germinal center B-cells are aberrantly negative or only faintly stained while mantle zone B-cells and stromal cells together with lymphocytes in lamina propria exhibit a moderate to strong nuclear staining reaction.
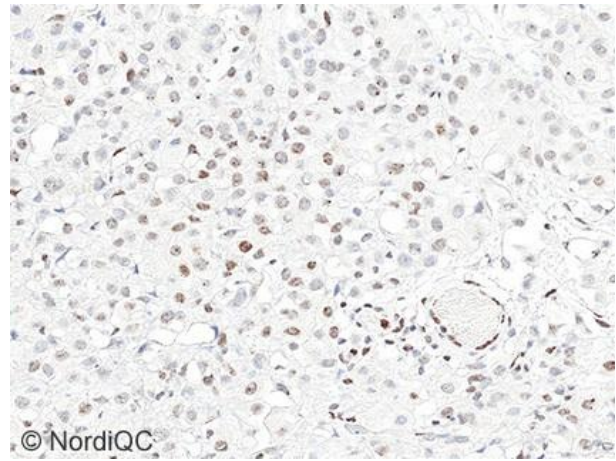

Fig. 5b
False positive BAP1 staining of the malignant mesothelioma, tissue core no. 4, using same protocol as in Fig. 5a. Distinct positive nuclear staining reaction can be seen in both the neoplastic and stromal cells. Similar staining pattern was seen with most polyclonal BAP1 antibodies used in this assessment and thus compromising the ability to demonstrate loss of BAP1 protein caused by gene mutations.

BT/LE/SN 04.07.2024

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Page **8** of **8**