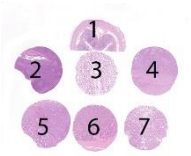
Assessment Run 58 2020

Special AT-rich sequence-binding protein 2 (SATB2)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for SATB2, identifying and characterizing colorectal carcinomas and neuroendocrine tumours in the diagnostic workup for carcinoma of unknown origin. Relevant clinical tissues, both normal and neoplastic, were selected displaying a broad spectrum of antigen densities for SATB2 (see below).

Material
The slide to be stained for SATB2 comprised:



1. Appendix, 2. Tonsil, 3. Testis, 4-5. Colon adenocarcinoma, 6. Colon neuroendocrine tumour, 7. Ovarian mucinous adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a SATB2 staining as optimal included:

- A strong, distinct nuclear staining reaction of virtually all epithelial cells of the appendix, and a weak to moderate but distinct nuclear staining reaction in dispersed ganglion cells of the plexuses of Auerbach (myenteric) and Meissner.
- A weak to moderate, distinct nuclear staining reaction of a subset of interfollicular lymphocytes of the tonsil.
- An at least weak to moderate, distinct nuclear staining reaction of dispersed germ cells (primarily spermatocytes) in seminiferous tubules of the testis.
- An at least moderate, distinct nuclear staining reaction of virtually all neoplastic cells of the neuroendocrine tumour and the colon adenocarcinoma, tissue core 4.
- A strong, distinct nuclear staining reaction of all neoplastic cells in the colon adenocarcinoma, tissue core 5.
- No staining reaction of neoplastic cells in the ovarian mucinous adenocarcinoma or other cellular structures, including smooth muscle cells of lamina muscularis propria of the appendix and the vast majority of lymphocytes in the tonsil.

Participation

Number of laboratories registered for SATB2, run 58	119
Number of laboratories returning slides	105 (88%)

Results
105 laboratories participated in this assessment. 61 (58%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and the assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Less successful primary antibodies e.g. mAbs CL0276 and SATBA4B10, rmAb EPNCIR130A and pAbs
- Insufficient HIER (use of acidic buffer)
- Less sensitive detection systems
- Unexplained technical issues

Performance history

This was the first NordiQC assessment of SATB2. The overall pass rate of 58% was relatively low (see Table 2).

Table 2. **Proportion of sufficient results for SATB2 in the first NordiQC run performed**

	Run 58 2020
Participants, n=	105
Sufficient results	58%

Conclusion

The mAb clone OTI5H7 and the rmAb clones EP281, SP281 and ZR167 could all provide an optimal result for the demonstration of SATB2. HIER in alkaline buffer, precise calibration of the primary Ab and use of a 3-step polymer or multimer based detection system were the main prerequisites for an optimal result. Protocols based on the mAbs CL0276, CL0320 and SATBA4B10, the rmAb EPNCIR130A and the pAbs (HPA001042 and Ab68885) produced inferior results, typically providing poor-signal-to-noise ratio, false positive and/or false negative results. Appendix, tonsil and testis are recommended tissue controls for SATB2. In appendix, virtually all epithelial cells must show a strong nuclear staining reaction, whereas the ganglion cells of nerve plexus should display a weak to moderate nuclear reaction. In tonsil, a subset of interfollicular lymphocytes must display a weak to moderate nuclear staining reaction. In testis, dispersed germ cells in the seminiferous tubules should at least show a weak to moderate but distinct nuclear staining reaction. No staining reaction should be seen in other cellular structures.

Table 1. **Antibodies and assessment marks for SATB2, run 58**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **CL0276**	5 2 1	Atlas Antibodies Sigma Aldrich Novus Biologicals	0	0	0	8	0%	0%
mAb clone **CL0320**	1	Atlas Antibodies	0	0	1	0	-	-
mAb clone **SATBA4B10**	3 2 2	Abcam Santa Cruz Zytomed Systems	0	0	2	5	0%	0%
mAb clone **OTI5H7**	1	ZSBio	1	0	0	0	-	-
rmAb clone **EP281**	30 12 1 1 1 1	Epitomics Cell Marque Immunologic BioSB Biocare Medical Unknown	22	14	4	6	78%	82%
rmAb clone **SP281**	4 1	Abcam Spring Bioscience	2	1	1	1	60%	40%
rmAb clone **ZR167**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **EPNCIR130A**	5	Abcam	0	0	0	5	0%	0%
pAb **HPA001042**	5	Sigma Aldrich	0	0	2	3	0%	0%
pAb **Ab69995**	1	Abcam	0	0	0	1	-	-
Ready-To-Use antibodies							Suff.[1]	OR[2]
rmAb clone **EP281 384R-17/18**	19	Cell Marque	7	10	1	1	89%	37%
rmAb clone **EP281 PR/HAR239**	2	PathnSitu	2	0	0	0	-	-
rmAb clone **EP281 API3225**	1	Biocare Medical	0	1	0	0	-	-
rmAb clone **EP281 MAD-000747QD**	1	Máster Diagnostica	0	0	1	0	-	-
rmAb clone **EP281 BSB3199**	2	BioSB	0	0	0	2	-	-
Total	105		35	26	12	32	-	
Proportion			33%	25%	11%	31%	58%	

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

Detailed analysis of SATB2, Run 58
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
rmAb **EP281**: Protocols with optimal results were all based on Heat Induced Epitope Retrieval (HIER) using an alkaline buffer as Cell Conditioning 1 (CC1) (Ventana) (21/32)* or Target Retrieval Solution (TRS) High pH (3-in-1) (Dako) (1/9) as retrieval buffer. The rmAb was typically diluted in the range of 1:25–1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 28 of 34 (82%) laboratories produced a sufficient staining (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

rmAb **SP281**: Two protocols with optimal results were based on HIER using CC1 (Ventana) as retrieval buffer. The mAb was diluted in range 1:150-1:250 and OptiView (Ventana) was used as detection system

rmAb **ZR167**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer. The mAb was diluted 1:200 and OptiView (Ventana) was used as detection system.

mAb **OTI5H7**: One protocol with an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (BERS2) (Leica) as retrieval buffer. The mAb was diluted 1:100 and BOND Refine (Leica) was used as detection system

Table 3. **Proportion of optimal results for SATB2 for the most commonly used antibody as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link/Classic**		**Dako Omnis**		**Ventana BenchMark GX /XT/ Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
rmAb clone **EP281**	0/6** (0%)	0/1	1/3	-	18/25 (72%)	-	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).

Comments
In this first NordiQC assessment of SATB2, the prevalent feature of an insufficient result was either a generally too weak staining reaction of cells expected to be demonstrated and/or a poor signal-to-noise ratio/false positive staining reaction compromising interpretation. Too weak or false negative staining reaction was seen in 68% of the insufficient results (30 of 44). The majority of all laboratories were able to stain SATB2 in epithelial cells of the appendix and neoplastic cells of the colon adenocarcinoma (tissue core 5), whereas demonstration of SATB2 in germ cells (primarily spermatocytes) in seminiferous tubules of the testis and neoplastic cells of the neuroendocrine tumour and colon adenocarcinoma (tissue core 4) was more challenging and required a carefully calibrated protocol. In 27% (12 of 44) of the insufficient results, a combination of both a too weak/false negative result and a poor signal-to-noise ratio/false positive staining result was seen. In the remaining insufficient protocols, a poor signal-to-noise ratio and/or false positive staining reaction was observed.

The overall pass rate was significantly affected by the use of less successful primary antibodies, and in particular related to the mAbs CL0276, CL0320 and SATBA4B10, the rmAb EPNCIR130A and the pAbs HPA001042 and Ab68885 accounting for 61% (27/44) of the insufficient results (see Table 1). Depending on the primary antibody applied, these antibodies could be allocated into different subgroups based on the abnormal/atypical staining pattern:

1) False negative staining result was observed for the mAb CL0320 and the rmAB EPNCIR130A.
2) False positive and false negative staining results were observed for the mAbs CL0276 and SATBA4B10.
3) Aberrant cytoplasmic staining reaction in combination with both false positive and false negative staining results was observed for the pAb HPA001042.
4) False negative result in combination with an aberrant distinct cytoplasmic staining reaction was observed for the pAb Ab69995.

Representative illustrations of the problems can be seen in Figs.7a-9b.

Although the number of protocols submitted by the participants was low, e.g. one each for mAb clone CL0320 and pAb Ab69995, it seems unlikely that extensive protocol optimization can improve the staining reaction to an acceptable level comparable to the result obtained by the rmAbs EP281 and SP281. The

combination of both a reduced analytical sensitivity providing false negative results and coexisting aberrant false positive reaction patterns complicates the optimization process, and thus, laboratories are recommended to substitute these unsuccessful clones with more robust antibodies as the rmAbs EP281 or SP281.

rmAb clone EP281 was the most widely used antibody for the demonstration of SATB2 accounting for 68% (71/105) of all protocols. Used as a concentrate within a laboratory developed (LD) assay, the rmAb clone EP281 gave an overall pass rate of 78% (36/46). As shown in Table 3, a high proportion of optimal results could be obtained on the fully automated IHC platform Benchmark (Ventana), whereas the proportion of optimal results was significant lower on the platforms Autostainer and Omnis (Dako). The most common causes of an insufficient staining result were use of a protocol with too low analytical sensitivity, typically applying inefficient HIER (e.g. use of an inappropriate low pH buffer), too diluted primary Ab but more importantly, use of a less sensitive detection system. Virtually all protocols assessed as optimal (21/22) were based on HIER in alkaline buffer and a 3-step multimer/polymer detection system. The vast majority of these protocols, 86% (19/22), were performed on the BenchMark platform (Ventana). Use of efficient HIER in CC1 (32-64 min. at 96-100°C), an optimally calibrated antibody (see range above) and applying OptiView (Ventana) as detection system, provided an overall pass rate of 94% (17/18). Thus, assays based on these protocol settings are very robust and recommendable for detection of SATB2. One result assessed as insufficient, was challenged by technical issues on the Benchmark platform. Only 10% (1/10) of the protocols performed on the semi-automated platform Autostainer or the fully automated platform Omnis (both Dako) were assessed as optimal (see Table 3). The protocol providing an optimal result was based on HIER in TRS pH 9 (3-in-1), the primary antibody was diluted 1:100 and Flex+ with an additional linker step (mouse) was applied as detection system. For the two protocols performed on the BOND platform (Leica), both assessed as good, the Leica detection system, Refine, acts by nature as a 2-step polymer system for rabbit primary antibodies and only enhances the analytical sensitivity for mouse primary antibodies. In summary, choice of detection system influenced the overall performance of the assays and using, otherwise optimal protocol settings as HIER in an alkaline buffer and a dilution of the primary Ab in the range of 1:25-200, 71% (5/7) of protocols based on a 2-step detection system as e.g. EnVision Flex (Dako) provided insufficient results. Therefore, it is advisable to use 3-step polymer/detection systems in combination with efficient HIER in alkaline buffer and carefully calibrated the primary antibody to provide an IHC protocol that is able to demonstrate SATB2 in cellular structures with both low- and high-level SATB2 expression (see controls).

Optimal results could also be obtained with mAb OTI5H7 and the rmAbs EP281 and ZR167. Protocols with optimal results were all based on similar successful settings as for EP281 applying efficient HIER in an alkaline buffer and a 3-step polymer/multimer detection system.

In this assessment, no Ready-To-Use (RTU) antibodies including corresponding systems were available from the three major vendors Ventana, Dako and Leica (see Table 1). All RTU formats were based on rmAb EP281 and the majority laboratories (19/25) used the product from Cell Marque, 384R-17/18. All protocols (10/10) applying this RTU product on the Benchmark platform (Ventana) were assessed as sufficient of which 60% (6/10) were optimal. The protocols with optimal results were typically based on HIER in CC1 (Ventana) (efficient heating time 24-32 min. at 96-100°C), 4-24 min. incubation of the primary Ab and OptiView (760-700) as detection system. Using these protocol settings, 8 of 8 (100%) laboratories produced a sufficient result.
In the remaining protocols, the pass rate was 78% (7/9) of which only 11% (1/9) were assessed as optimal. The two insufficient protocols, using otherwise highly sensitive protocol settings, applied Flex (Dako) as the detection system. The single protocol providing an optimal result was stained on the Omnis and protocol settings were based on HIER in TRS pH 9 (30 min.), 30 min. incubation time in primary Ab and Flex+ as the detection system.

This was the first assessment of SATB2 in NordiQC and a pass rate of 58% was obtained (see Table 2). The most important parameters influencing the final result in negative direction was:

1) Use of less successful primary antibodies, the mAbs CL0276, CL0320 and SATBA4B10, the rmAb EPNCIR130A and pAbs HPA001042 and Ab68885, typically providing false negative result often in combination with poor-signal-to-noise and/or false positive staining results. In total, 26% (27/105) of the protocols were based on one of these unsuccessful antibodies.
2) The use of protocols with a level of too low analytical sensitivity primarily related to use of a low sensitive 2-step polymer/multimer detection system e.g. Flex (Dako).

In total, 68% (71/105) of the protocols were based on the rmAb EP281, and using all protocol settings, 50% (11/22) were assessed as sufficient applying a 2-step polymer/multimer detection system of which only 14% (3/22) were giving an optimal mark. In comparison, using a 3-step polymer/multimer detection

system, 92% (45/49) of the protocols gave a sufficient result of which 57% (28/49) were assessed as optimal. Noteworthy, protocols based on rmAb EP281 performed on BenchMark platform (Ventana) were observed to be most successful providing a pass rate of 96% (43/45) and 64% (29/45) optimal. Importantly, laboratories should use a robust Ab, calibrate the protocols correctly and stain according to the expected antigen level of the recommended control material (see below).

Controls

Appendix, testis and tonsil are recommendable as positive and negative tissue controls. In appendix, virtually all epithelial cells must show a strong nuclear staining reaction, whereas dispersed ganglion cells of nerve plexuses should display a weak to moderate nuclear staining reaction. No staining reaction should be seen in other cellular structures including smooth muscle cells (lamina muscularis propria) of the appendix. In tonsil, a subset of interfollicular lymphocytes must display a weak to moderate nuclear staining reaction, whereas the vast majority of lymphocytes should be negative. In testis, dispersed germ cells of seminiferous tubules should at least display a weak to moderate, distinct nuclear staining reaction.

Fig. 1a (x200)
Optimal staining for SATB2 of the appendix using the rmAb EP281 as a concentrate (1:100), HIER in TRS pH 9 (3-in-1) and Flex+ (Dako) with an additional mouse linker step as detection system - same protocol used in Figs. 2a - 6a. Virtually all epithelial cells show a distinct and strong nuclear staining reaction.

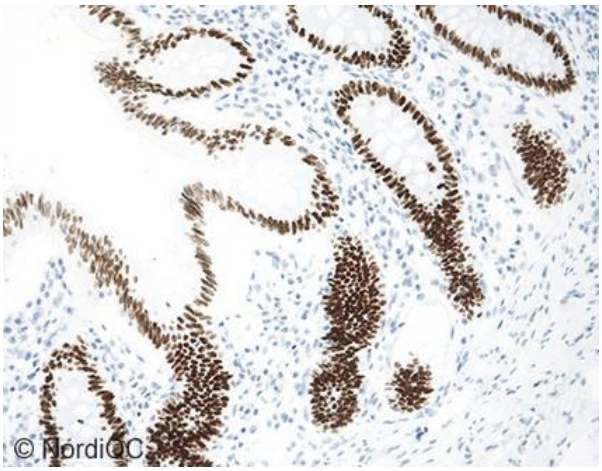


Fig. 1b (x200)
Insufficient staining for SATB2 of the appendix using the rmAb EP281 as a concentrate (1:50), HIER TRS pH 9 (3-in-1) and Flex (Dako) as detection system - same protocol used in Figs. 2b – 6b. Although the staining intensity is comparable to the result obtained in Fig. 1a, the protocol provided too low analytical sensitivity due to the use of a less sensitive detection system - compare with Figs. 2a-6b. This emphasizes the need to identify and apply a recommendable tissue control with a low and critical SATB2 level expression as e.g. testis– see Figs. 3a and 3b.

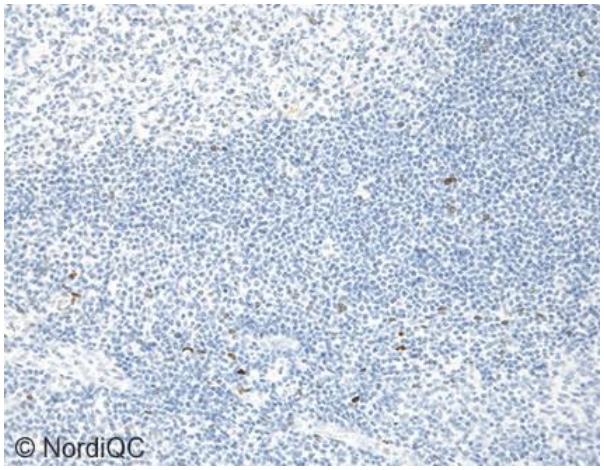


Fig. 2a (x200)
Optimal staining for SATB2 of the tonsil using same protocol as in Fig. 1a. A subset of lymphocytes, primarily situated in the T-zones, display a weak to moderate but

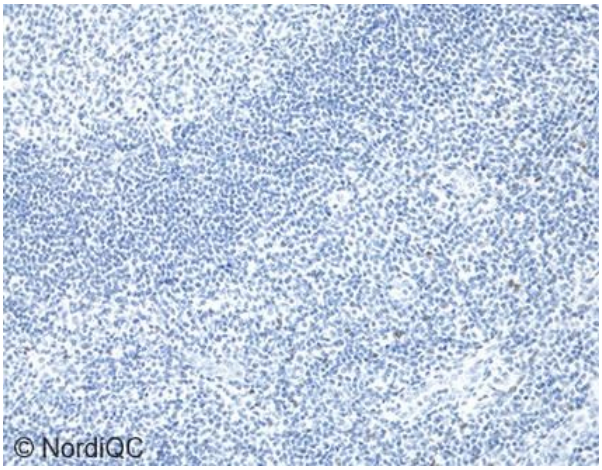


Fig. 2b (x200)
Insufficient staining for SATB2 of the tonsil using same protocol as in Fig 1b. The staining intensity is too weak, and proportion of positive lymphocytes is significantly reduced - compare with Fig. 2a.

distinct nuclear staining reaction.

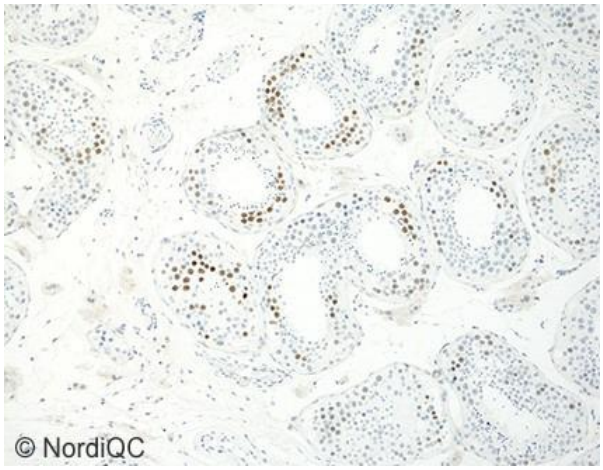


Fig. 3a (x200)
Optimal staining for SATB2 of testis using same protocol as in Figs. 1a and 2a. Dispersed germ cells, primarily spermatocytes, in seminiferous tubules display a weak to strong nuclear staining reaction, whereas the majority of spermatogonia (basal compartment) are negative or only faintly demonstrated.

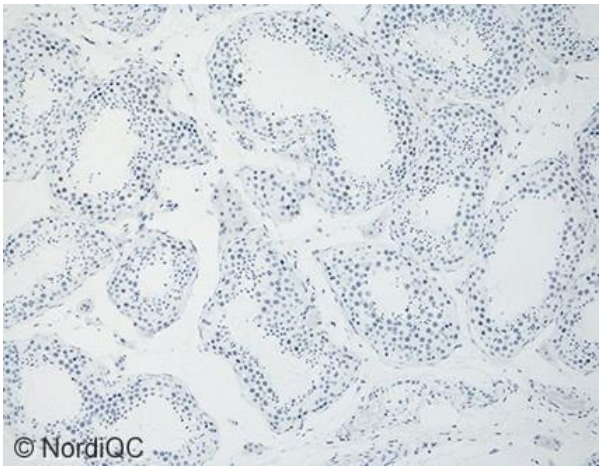


Fig. 3b (x200)
Insufficient staining for SATB2 of testis using same protocol as in Figs. 1b and 2b. The germ cells are false negative or only show faint nuclear staining reaction - compare with Fig. 3a.

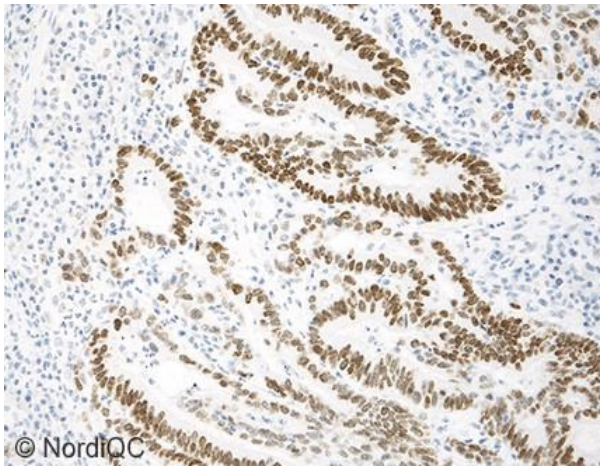


Fig. 4a (x200)
Optimal staining for SATB2 of the colon adenocarcinoma, tissue core 4, using same protocol as in Figs. 1a - 3a. The vast majority of neoplastic cells display a moderate to strong nuclear staining reaction.

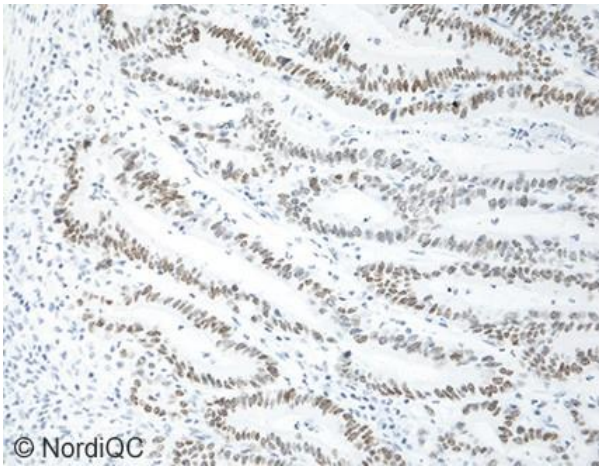


Fig. 4b (x200)
Staining for SATB2 of the colon adenocarcinoma, tissue core 4, using same insufficient protocol as in Figs. 1b -3b. The proportion and intensity of positive neoplastic cells is significantly reduced - compare with Fig. 4a.

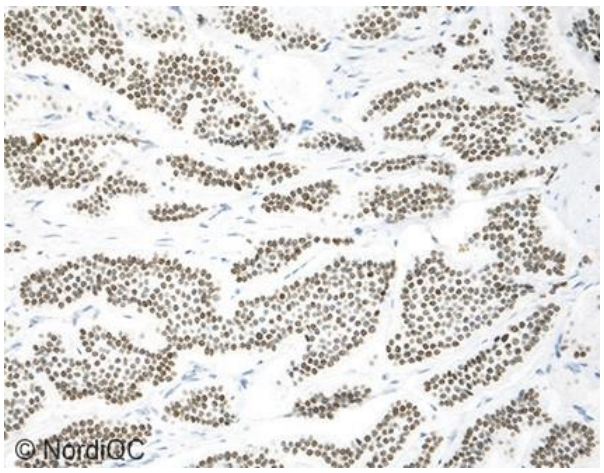


Fig. 5a (x200)
Optimal staining for SATB2 of the colon neuroendocrine tumour using same protocol as in Figs. 1a - 4a. Virtually all neoplastic cells show a moderate to strong, but distinct nuclear staining reaction.

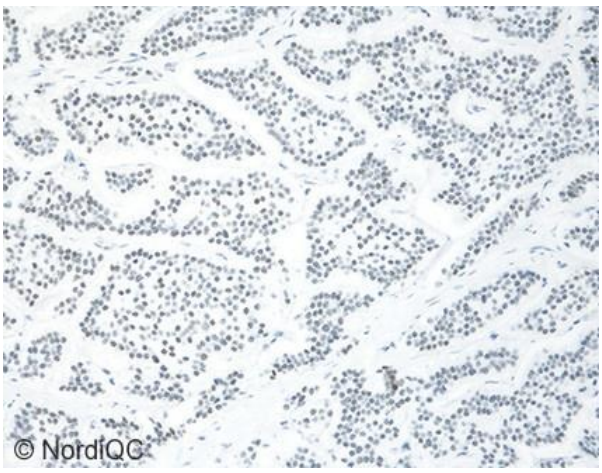


Fig. 5b (x200)
Insufficient staining for SATB2 of the colon neuroendocrine tumour using same protocol as in Figs.1b - 4b. A significant proportion of neoplastic cells are completely negative or only faintly demonstrated - compare with Fig. 5a.

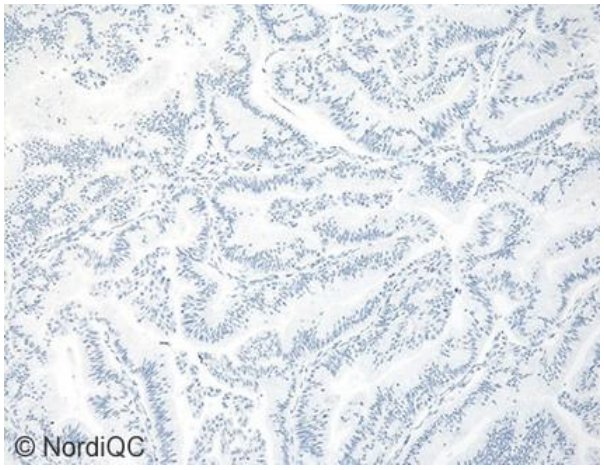


Fig. 6a (x100)
Optimal staining for SATB2 of the ovarian mucinous adenocarcinoma using same protocol as in Figs. 1a - 5a. All neoplastic cells are negative.

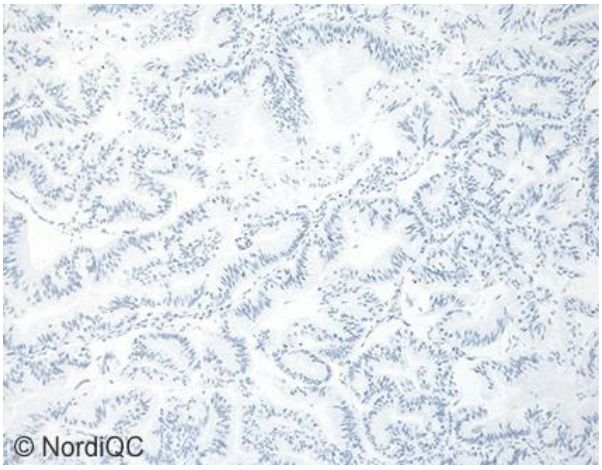


Fig. 6b (x100)
Staining for SATB2 of the ovarian mucinous adenocarcinoma using same insufficient protocol as in Figs. 1b - 5b. Although the protocol gave the expected reaction pattern, the result is not reliable as the applied protocol settings provided too weak and false negative staining results in several of the tissue cores included and expected to be positive in this assessment (see above).

Fig. 7a (x100)
Insufficient staining for SATB2 of the appendix using the mAb CL0246 within a LD assay – same protocol in Figs. 7a-7d. The primary Ab gave the expected reaction pattern of epithelial cells, but also provided both false negative and positive staining results. In the appendix, false positive staining was seen in smooth muscle cells of lamina muscularis propria. The mAb clone SATBA4B10 provided similar reaction patterns - see description in comments above.

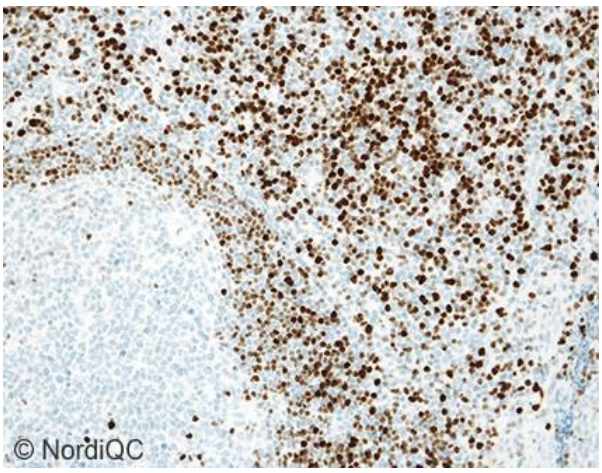


Fig. 7b (x200)
Insufficient staining for SATB2 of tonsil using same assay as in Fig. 7a. A false positive staining reaction of the vast majority of mantle zone B-cells and of interfollicular lymphocytes is seen - compare with optimal protocol in Fig. 2a.

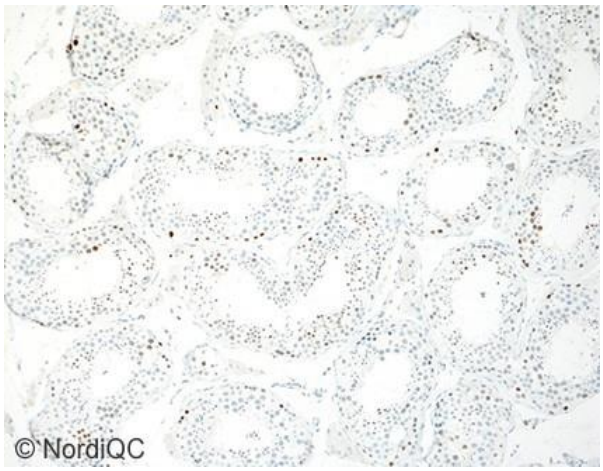


Fig. 7c (x200)
Insufficient staining for SATB2 of testis using same assay as in Figs. 7a-7b. Although dispersed germ cells are positive, the staining showed an aberrant and inverted reaction pattern in which the majority of spermatocytes of seminiferous tubules are false negative or only faintly demonstrated, whereas a subset of spermatogonia (basal compartment) display a moderate to strong nuclear reaction - compare with Fig. 3a.

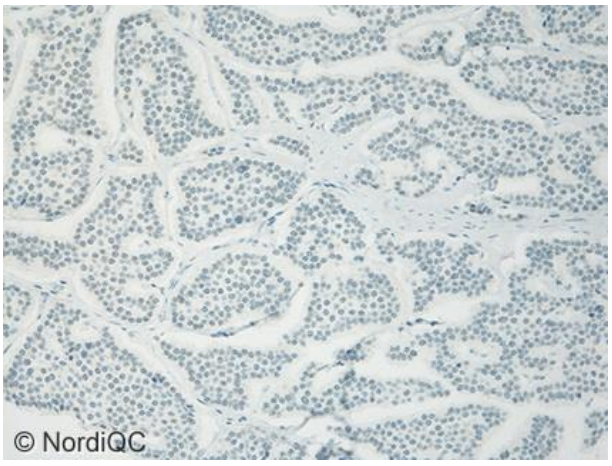


Fig. 7d (x200)
Insufficient staining for SATB2 of colon neuroendocrine tumour using same assay as in Figs. 7a-7c. The neoplastic cells are false negative – compare with the optimal result in Fig. 5a. The mAb clone CL0276, but also mAb clone SATBA4B10, provided inferior results and laboratories were challenged by primary antibodies that from a technical perspective are impossible to optimize to an appropriate level of analytical sensitivity and specificity.

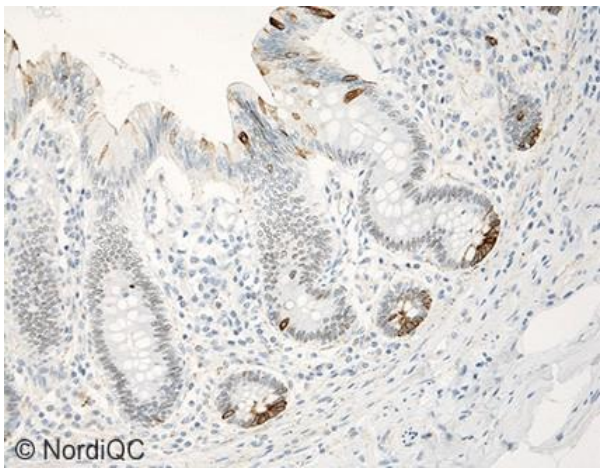


Fig. 8a (x200)
Insufficient staining for SATB2 of the appendix using the pAb Ab69995 within a LD assay – same protocol in Fig. 8b. Virtually all epithelial cells show a too weak nuclear staining reaction and at the same time an aberrant but distinct cytoplasmic reaction of scattered epithelial cells in the basal and luminal compartment of the epithelium - compare with the optimal result in Fig. 1a.

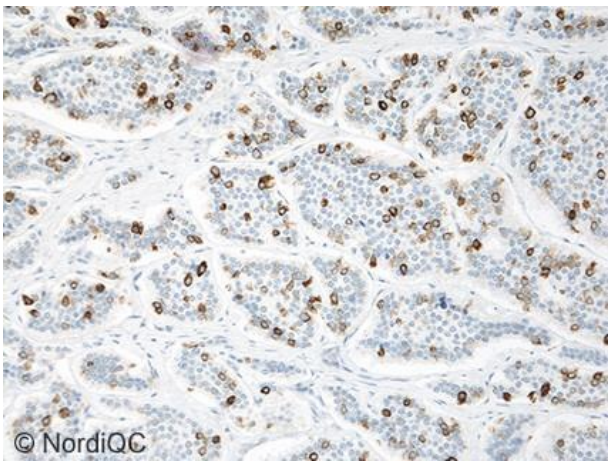


Fig. 8b (x200)
Insufficient staining for SATB2 of colon neuroendocrine tumour using same assay as in Fig. 8a. The neoplastic cells are false negative and dispersed tumour cells display an aberrant cytoplasmic staining reaction – compare with the optimal result in Fig. 5a. In addition to this atypical staining pattern, the antibody provided false negative results in all cores expected to be positive except for the colon adenocarcinoma tissue core 5.

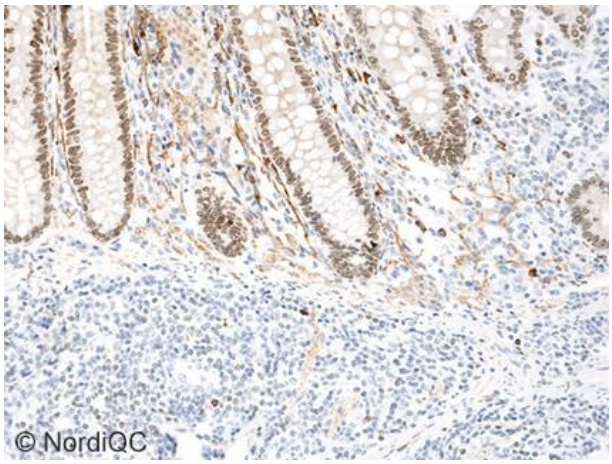


Fig. 9a (x200)
Insufficient staining for SATB2 of the appendix using the pAb HPA001042 within a LD assay – same protocol in Fig. 9b. Virtually all epithelial cells show a weak to moderate nuclear staining reaction but nerve cells in lamina propria mucosa display an aberrant cytoplasmic reaction. In addition to this abnormal staining pattern, which also could be seen in other cellular structures (see Fig. 9b), the antibody provided both false positive and false negative staining results of e.g. mantle zone B-cells of the tonsil and neoplastic cells of the neuroendocrine tumour, respectively. Also, compare with optimal result in Fig. 1a.

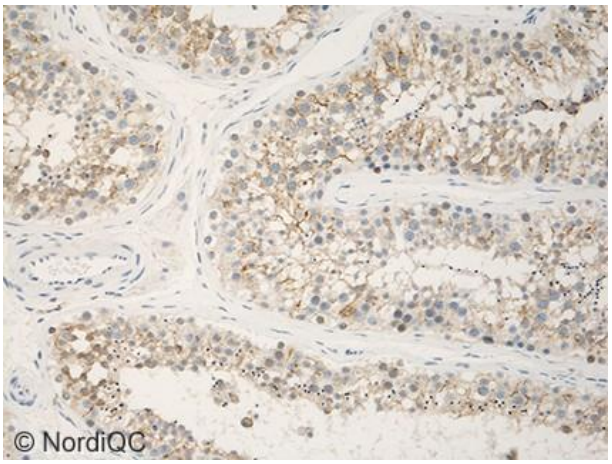


Fig. 9b (x200)
Insufficient staining for SATB2 of testis using same assay as in Fig. 9a. The germ cells are completely negative and show an aberrant cytoplasmic staining reaction – compare with optimal result in Fig. 3a. This antibody, but also the mAbs CL0276, CL0320, SATBA4B10, the rmAb EPNCIR130A and the pAbs (HPA001042 and Ab68885) provided inferior results – all displaying atypical staining patterns. All laboratories using one of these antibodies are advised to substitute the primary antibody with a more robust clone e.g. EP281 or SP281, and recalibrate protocol settings according to the expected reaction pattern described for the tissue controls.

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