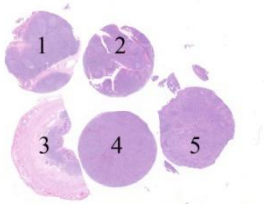
## Assessment Run 41 2014

# B-cell specific activator protein (BSAP, PAX5)

**Material**
The slide to be stained for BSAP comprised:




1. Tonsil, 24h fixation, 2. Tonsil, 48h fixation, 3. Appendix, 4. Diffuse large B-cell lymphoma (DLBCL), 5. Hodgkin Lymphoma, classical.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a BSAP staining as optimal included:

- A distinct moderate to strong nuclear staining reaction of virtually all mantle zone B-cells, germinal centre B-cells and interfollicular peripheral B-cells in the tonsils and appendix.
- A moderate to strong, nuclear staining reaction of virtually all neoplastic cells of the DLBCL.
- An at least weak but distinct nuclear staining reaction of the vast majority of Hodgkin and Reed-Sternberg cells in the Hodgkin lymphoma.
- No staining reaction of other cells, including T-cells, squamous epithelial cells of the tonsils and columnar epithelial cells of the appendix.

A weak, cytoplasmic staining reaction in cells with a strong nuclear staining reaction was accepted.

**Participation**

| | |
|---|---|
| Number of laboratories registered for BSAP, run 41 | 158 |
| Number of laboratories returning slides | 150 (95%) |

**Results**
150 laboratories participated in this assessment. Of these, 126 (84%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- too low concentration of the primary antibody
- false positive staining reaction, most likely due to antibody contamination (rmAb clone SP34)
- use of low sensitivity detection systems

**Performance history**
This was the 2nd NordiQC assessment of BSAP. An increased pass rate was seen compared to run 18, 2010 (see table 2).

Table 2. **Proportion of sufficient results for BSAP in the two NordiQC runs performed**

| | Run 28 2010 | Run 41 2014 |
|---|---|---|
| Participants, n= | 86 | 150 |
| Sufficient results | 67% | 84% |

**Conclusion**
The mAbs clones **24**, **1EW**, **DAK-Pax5** and the rmAb clone **SP34** could all be used to obtain optimal staining results for BSAP. Irrespective of the clone applied, efficient HIER, use of sensitive detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result. The Ready-To-Use systems for BSAP from Dako, Leica and Ventana provided the highest proportion of sufficient and optimal results.
Tonsil and appendix are recommended as positive and negative tissue controls: Virtually all B-cells, except plasma cells, must show an as strong as possible nuclear staining reaction (a weak cytoplasmic staining reaction must be accepted). No staining reaction must be seen in T-cells, squamous epithelial cells of the tonsil or columnar epithelial cells.

Table 1. **Antibodies and assessment marks for BSAP, run 41**

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | Suff. OPS[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **1EW** | 9 | Leica/Novocastra | 4 | 3 | 2 | 0 | 78% | 100% |
| mAb clone **24** | 20 | BD Biosciences | 8 | 7 | 3 | 2 | 75% | 88% |
| mAb clone **BC/24** | 4 | Biocare | 1 | 2 | 1 | 0 | - | - |
| mAb clone **DAK-Pax5** | 23 | Dako | 11 | 8 | 4 | 0 | 83% | 84% |
| rmAb clone **3852-1** | 1 | Abcam | 1 | 0 | 0 | 0 | - | - |
| rmAb clone **SP34** | 9<br>4<br>2 | Cell Marque<br>Spring Biosciences<br>Thermo/NeoMarkers | 4 | 6 | 5 | 0 | 71% | 86% |
| pAb **ILP46318** | 1 | Immunologic | 0 | 1 | 0 | 0 | - | - |
| pAb **RB-9406** | 5 | Thermo/NeoMarkers | 0 | 2 | 3 | 0 | - | - |
| pAb **RBK008** | 1 | Zytomed | 0 | 1 | 0 | 0 | - | - |
| Ready-To-Use antibodies | | | | | | | | |
| mAb clone **1EW PA0552** | 4 | Leica/Novocastra | 3 | 1 | 0 | 0 | - | - |
| mAb clone **BC/24 PM207** | 1 | Biocare | 0 | 1 | 0 | 0 | - | - |
| mAb clone **DAK-Pax5 IS/IR650** | 21 | Dako | 20 | 0 | 1 | 0 | 95% | 95% |
| mAb clone **DAK-Pax5 GA650** | 5 | Dako | 5 | 0 | 0 | 0 | 100% | 100% |
| rmAb clone **BV6 RMPD027** | 1 | Diagnostic Biosystems | 0 | 0 | 1 | 0 | - | - |
| rmAb clone **SP34 790-4420** | 37 | Ventana | 23 | 12 | 2 | 0 | 95% | 94% |
| rmAb clone **SP34 312R-18** | 1 | Cell Marque | 0 | 1 | 0 | 0 | - | - |
| pAb **MAD-005661QD** | 1 | Master Diagnostica | 1 | 0 | 0 | 0 | | |
| Total | 150 | | 81 | 45 | 22 | 2 | - | |
| Proportion | | | 54% | 30% | 15% | 1% | 84% | |

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

## Detailed analysis of BSAP, Run 41

The following protocol parameters were central to obtain optimal staining:

### Concentrated antibodies

mAb clone **1EW**: Protocols with optimal results were all based on HIER using Bond Epitope Retrieval Solution 2 (BERS2; Leica) (3/6)* or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:40 depending on the total sensitivity of the protocol employed. Using these protocol settings 4 of 4 (100%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **24**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/5), TRS pH 9 (Dako) (3/4), Tris-EDTA/EGTA pH 9 (3/5) or Citrate pH 6 (1/3) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 14 of 16 (88%) laboratories produced a sufficient staining result.

mAb clone **BC/24**: One protocol with an optimal result was based on HIER using BERS2 (Leica) (1/2) as retrieval buffer. The dilution factor was unknown.

mAb clone **DAK-Pax5**: Protocols with optimal results were all based on HIER using TRS pH 9 (3-in-1) (Dako) (3/4), TRS pH 9 (Dako) (3/3), TRS pH 6.1 (Dako) (1/1), Cell Conditioning 1 (CC1; Ventana) (2/10), Tris-EDTA/EGTA pH 9 (1/1) or Citrate pH 6 (1/2) as retrieval buffer. The mAb was diluted in the range of 1:10-1:150 depending on the total sensitivity of the protocol employed. Using these protocol settings 16 of 19 (84%) laboratories produced a sufficient staining result.

rmAb clone **SP34**: Protocols with optimal results were all based on HIER using CC1 (Ventana) (4/10) as retrieval buffer. The mAb was diluted in the range of 1:50-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 of 7 (86%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for BSAP for the two most commonly used antibodies as concentrate on the 3 main IHC systems***

| Concentrated antibodies | **Dako Autostainer Link / Classic** | | **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 **24** | 4/7** (57%) | - | 0/4 | - | 0/1 | - |
| mAb clone **DAK-Pax5** | 5/6** (83%) | 2/2 | 1/8 (13%) | - | 0/1 | - |

* 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 **1EW**, product no. PA0552, Leica, Bond-max/Bond-III:
Protocols with optimal results were all based on HIER using BERS 2 pH 9 (Bond, Leica) (efficient heating time 20 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 3 of 4 (75%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DAK-Pax5**, product no. **IS650/IR650**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 6.1, TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 95-99°C) and 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 20 of 21 (95%) laboratories produced a sufficient staining result.

mAb clone **DAK-Pax5**, product no. **GV650**, Dako, OMNIS:
Protocols with optimal results were typically based on HIER using TRS pH 6.1, TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 30 min. at 97°C) and 20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (GV800/GV800+GV821) as detection system. Using these protocol settings 5 of 5 (100%) laboratories produced an optimal staining.

pAb product no. **790-4420**, Ventana, BenchMark XT/Ultra:
Protocol with optimal result was based typically on HIER using Cell Conditioning 1 (efficient heating time 32-64 min.) and 16-64 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings 33 of 35 (94%) laboratories produced a sufficient staining result.

**Comments**
In this second NordiQC assessment for BSAP, the prevalent features of an insufficient staining result was a generally too weak or completely false negative staining reaction of the cells expected to be demonstrated. This was observed in 63% of the insufficient results (15 of 24). Virtually all laboratories were able to demonstrate BSAP in high-level antigen expressing cells, e.g., normal peripheral B-cells in tonsils and the neoplastic cells of the diffuse large B-cell lymphoma. In contrast, BSAP in low-level antigen expressing cells (e.g. Hodgkin and Reed-Sternberg cells) could only be demonstrated with an optimally calibrated protocol. Too low concentration of the primary Ab and/or usage of detection systems with low sensitivity typically were the main parameters providing a too weak staining reaction for BSAP.

The selection of the clone must also be tailored to the IHC system used: Two NordiQC assessments and internal NordiQC studies have revealed inferior performance of the mAb clone 24 on the BenchMark IHC system (Ventana) compared to the Autostainer system (Dako/LabVision). Several parameters may have contributed to this difference e.g. washing conditions, affinity of the primary antibody, sensitivity of the detection systems used and/or impact of other reagents.

Analogous to the mAb clone 24, performance of the mAb clone 1EW is affected by endogenous peroxidase blocking. If this step is conducted just prior to incubation of the primary Ab, detrimental effects on the staining quality are seen (ref. NordiQC internal study and Leica and Abcam datasheet for clone 1EW). As a consequence, the blocking step must be performed after incubation of the primary Ab.

In the remaining 35% of the insufficient results, poor signal-to-noise ratio and/or false positive staining reaction were seen. A diffuse background staining was typically caused by a too high concentration of the primary Ab and mainly seen for the pAb RB-9406 (NeoMarkers/Thermo) and the mAb clone DAK-Pax5 (Dako).

The otherwise well-performing concentrated format of the rmAb clone SP34 (Cell Marque, Spring and NeoMarkers/Thermo) occasionally gave an aberrant staining pattern, where appendiceal epithelium in addition to BSAP positive tissues were stained.
This staining pattern was seen in 5 laboratories using concentrates of the rmAB SP34 from the above mentioned three different vendors and is most likely caused by contamination of the concentrates with another Ab (eg. cytokeratin 20)
All 5 protocols were evaluated as insufficient (borderline) as further investigation and restaining are required by the laboratories to verify the staining result for BSAP and troubleshoot the problem. At present, no data are available concerning the extent of the contamination, but this will be followed up by correspondence to the respective vendors by NordiQC.
In this assessment, the Ready-To-Use (RTU) systems from Dako, Leica and Ventana based on the mAb clone DAK-Pax5, mAb clone 1EW and rmAb SP34, respectively, provided a higher pass rate and proportion of optimal results compared to in-house validated protocols using same clones as a concentrate (see table 1).
Optimal results for the RTU formats were typically obtained by using the official protocol recommendations given by the respective companies. Laboratory modified protocol settings (typically adjusting HIER, incubation time of the primary Ab and/or choice of detection system) could also provide sufficient and optimal result.

It is difficult to conclude on the specific causes for the improvement of the pass rate in this run, as many laboratories participated for the first time and many laboratories have changed their IHC systems compared to the previous run in 2010. However, the availability and extended use of high quality and robust RTU systems for BSAP seems to be one of the central elements. In run 28, 2010 11% of the participants (9 of 86) used one of the above mentioned RTU systems from Dako, Leica and Ventana. In this run 44% (66 of 150) of the participants used a RTU system from one of these three vendors and grouped together a pass rate of 97% was obtained.

**Controls**
Tonsil and appendix are recommended as positive and negative tissue controls for BSAP. In the tonsil the protocol must be calibrated to provide a distinct and strong nuclear staining reaction in virtually all mantle zone B-cells, germinal centre B-cells and interfollicular peripheral B-cells. In appendix dispersed B-cells in lamina propria must be clearly identified. A weak cytoplasmic staining reaction in B-cells must be accepted.
No staining reaction must be seen in other cells including T-cells, epithelial cells of the tonsil and appendix. As a supplement to tonsil and appendix, especially in the technical calibration phase, it is recommended to verify the protocol on Hodgkin lymphoma, classical subtype.

Figures, see page 5-6.


Fig. 1a (X200)
Optimal BSAP staining of the tonsil using the rmAb clone SP34, using HIER in an alkaline buffer (CC1 pH 8.5, Ventana) and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone and germinal centre B-cells show a strong nuclear staining reaction. A weak cytoplasmic staining reaction is seen in cells with strong nuclear reaction, but no general background staining is observed – also compare with Figs. 2a - 4a, same protocol.

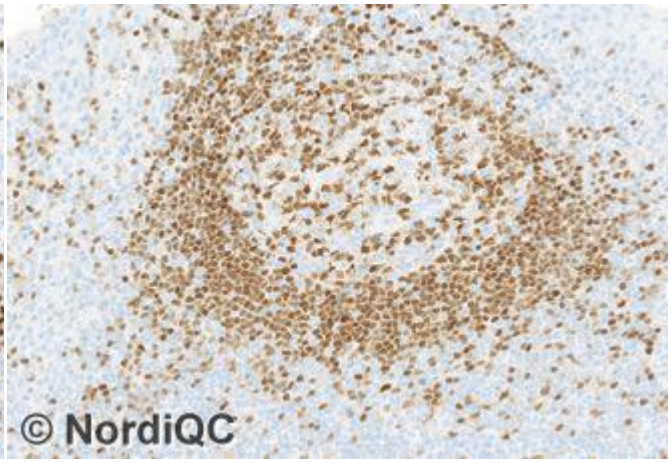



Fig. 1b (X200)
BSAP staining of the tonsil using an insufficient protocol based on the mAb clone DAK-pax5. The protocol provided a too low sensitivity most likely due to a too low titre of the primary Ab and use of a 2-step multimer based detection system (UltraView, Ventana) - same field as in Fig. 1a. The majority of mantle zone and germinal centre B-cells are demonstrated, but the intensity is significantly reduced – also compare with Figs. 2b & 3b, same protocol.

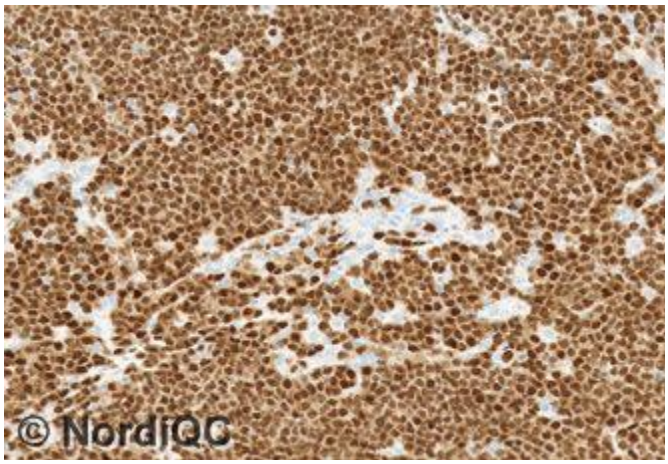



Fig. 2a (X200)
Optimal BSAP staining of the diffuse large B-cell lymphoma using same protocol as in Fig. 1a. Virtually all neoplastic cells show a strong nuclear staining reaction. A weak cytoplasmic staining reaction is seen, but no general background staining is observed.

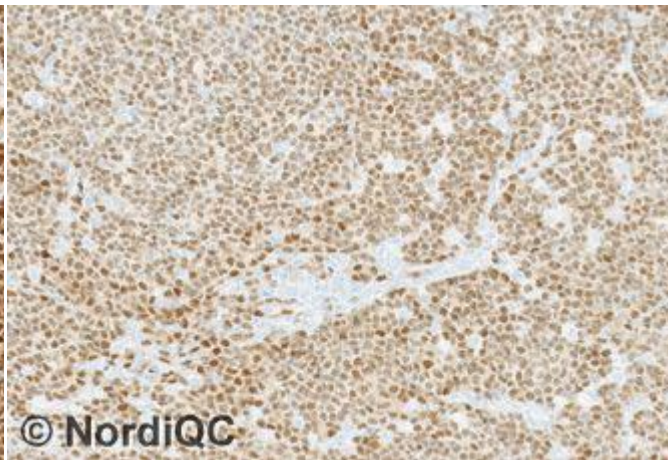



Fig. 2b (X200)
BSAP staining of the diffuse large B-cell lymphoma using same protocol as in Fig. 1b - same field as in Fig 2a. The vast majority of neoplastic cells are demonstrated, though the intensity expected is reduced. However also compare with Fig. 3b, same protocol.

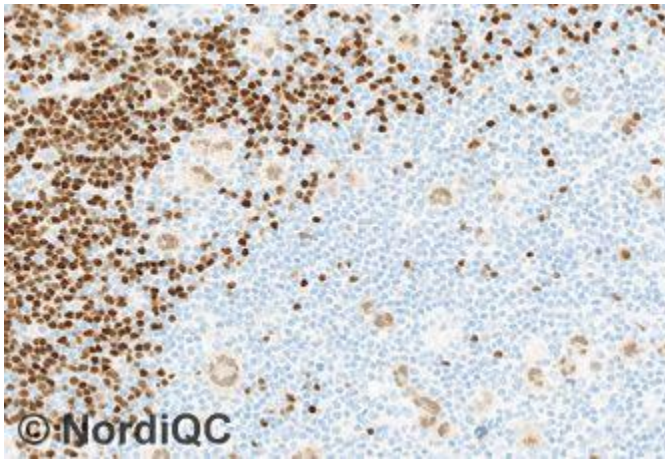



Fig. 3a (X200)
Optimal BSAP staining of the Hodgkin lymphoma using same protocol as in Figs. 1a & 2a. The peripheral B-cells show a strong nuclear staining reaction, whereas Hodgkin and Reed-Sternberg cells show a weak but distinct nuclear staining reaction.

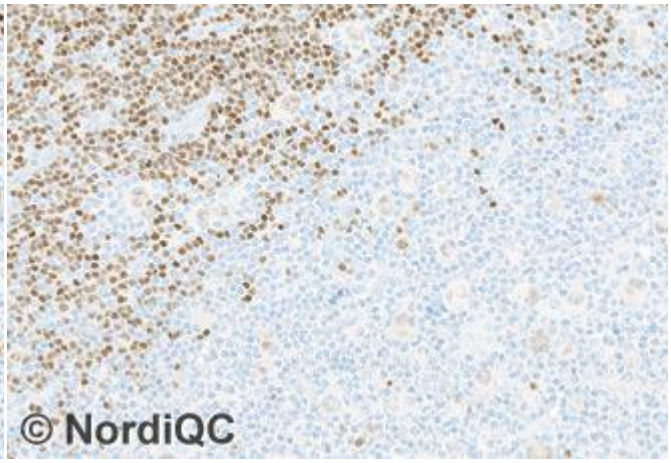



Fig. 3b (X200)
Insufficient BSAP staining of the Hodgkin lymphoma using same protocol as in Figs. 1b & 2b. – same field as in Fig. 3a. Only the peripheral B-cells are demonstrated, while the Hodgkin and Reed-Sternberg cells virtually are negative.

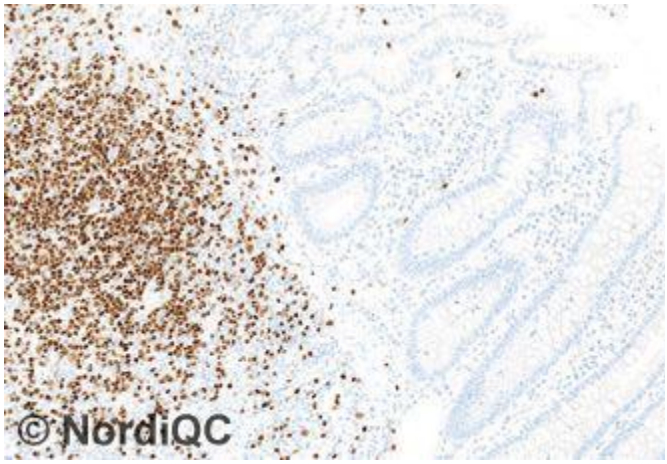



Fig.4a (X200)
Optimal BSAP staining of the appendix using same protocol as in Figs. 1a - 3a. The peripheral B-cells show a strong nuclear staining reaction, while the epithelial cells are negative.

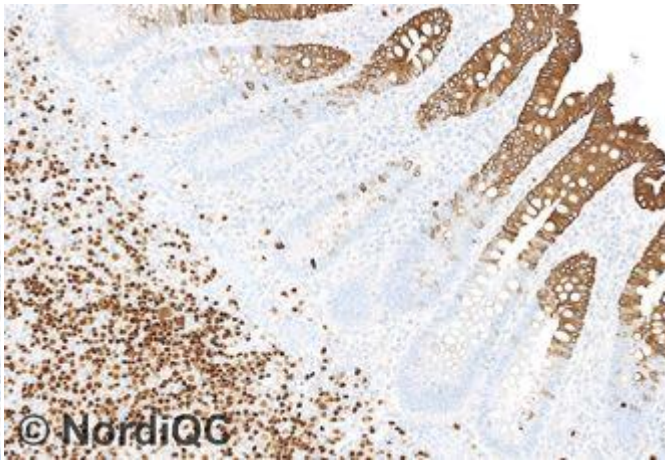



Fig. 4b (X200)
Aberrant BSAP staining of the appendix. In addition to the expected staining result for BSAP of the B-cells, the epithelial cells display a staining reaction corresponding to CK20. This aberrant staining result was frequently seen, when the rmAb clone SP34 was used as a concentrate and most likely caused by a contamination of the raw material of the clone. The staining reaction was seen in products from all companies providing the clone as a concentrate (see table 1).

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