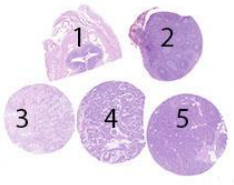
Assessment Run 57 2019

Mothers against Decapentaplegic Homolog 4 Drosophila (SMAD4)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests performed by the NordiQC participants for SMAD4 discriminating ductal pancreatic adenocarcinoma displaying loss of the protein from reactive conditions (e.g. chronic pancreatitis) or in metastatic settings (unknown primary tumor). Loss of SMAD4 expression is caused by inactivation of the SMAD4 gene due to sporadic mutations, which is most commonly seen in pancreatic adenocarcinomas (approximately 60%) but also in colorectal adenocarcinomas (approximately 10-20%).
Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for SMAD4 (see below).

Material
The slide to be stained for SMAD4 comprised:



1. Appendix, 2. Tonsil, 3. Pancreas*, 4. Colon adenocarcinoma,
5. Pancreatic adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing SMAD4 staining as optimal included:

- A strong, predominantly nuclear staining reaction of virtually all basal squamous epithelial cells in the surface epithelium of the tonsil. The staining intensity is reduced with maturation and the superficial squamous epithelial cells were typically negative or only faintly demonstrated.
- A moderate to strong, nuclear but also cytoplasmic staining reaction of epithelial cells in the proliferative compartment of the crypts in the appendix. The luminal epithelial cells were typically negative or only faintly demonstrated.
- An at least moderate, nuclear but also cytoplasmic staining reaction of virtually all other cells types e.g. normal acinar cells of the pancreas, lymphocytes in the tonsil and the neoplastic cells of the colon adenocarcinoma.
- No staining reaction of the neoplastic cells (loss of SMAD4) in foci's of the well-differentiated pancreatic ductal carcinoma (tissue core 3)* and the vast majority of neoplastic cells in the pancreatic adenocarcinoma (tissue core 5). Normal pancreatic glands and stroma cells intermingling between the neoplastic cells should display an at least moderate staining intensity (intern positive control).

*The pancreas presented regions with a well-differentiated pancreatic ductal adenocarcinoma showing loss of SMAD4.

Participation

Number of laboratories registered for SMAD4, run 57	66
Number of laboratories returning slides	52 (79%)

Results
52 laboratories participated in this assessment. 22 (42%) 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:
- Less successful performance of mAb clone B-8 on the fully automated platforms from Dako (Omnis) and Ventana (Benchmark).
- Less successful performance of rmAb clones SP306 and RBT-SMAD4.
- Insufficient Heat Induced Epitope Retrieval (HIER) – too short time or use of an acidic HIER buffer
- Too low concentration of the primary Ab
- Use of a less sensitive detection system
- Unexplained technical issues

Performance history
This was the first NordiQC assessment of SMAD4. The pass rate was low (42%).

Conclusion
The mAb clone **B-8** and the rmAb clones **EP618Y**, **D3R4N** could all be used to obtain an optimal staining result. The mAb clone B-8 is sensitive to the chosen platform and none of the protocols performed on the fully automated platforms Omnis (Dako) and BenchMark (Ventana) could provide an optimal result. For users of the Omnis (Dako) or BenchMark (Ventana) platform, the rmAb clones EP618Y and D3R4N are better alternatives. The rmAb clone EP618Y provided the highest proportion of sufficient and optimal results. Used within a Laboratory Developed (LD) assay, efficient HIER in an alkaline buffer, carefully calibration of the primary Ab and use of a sensitive 3-step multimer/polymer detection systems were the most important parameters for an optimal staining result.

Tonsil is recommendable as external tissue control for SMAD4: The vast majority of basal squamous epithelial cells in the surface epithelium must show a strong, predominantly nuclear staining reaction, whereas superficial squamous epithelial cells typically should be negative or only faintly demonstrated. Virtually all other cells e.g. lymphocytes must show an at least moderate, predominantly nuclear but also cytoplasmic staining reaction. In addition, a tumor (pancreas or colon) with loss of SMAD4 due to inactivation of the gene could be included, in which stromal cells (internal tissue control) should display the required staining reaction - at least moderate intensity. However, for SMAD4 it has to be emphasized that internal positive tissue controls being e.g. normal stromal cells adjacent to the neoplastic cells are preferred to external controls. An observed intact nuclear expression of SMAD4 proteins in the internal normal cells together with loss of SMAD4 proteins in the neoplastic cells is diagnostic essential[1]

Table 1. **Antibodies and assessment marks for SMAD4, run 57**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **B-8**	17 2 1	Santa Cruz Zeta Corp GeneTex	4	3	3	10	35%	80%
mAb clone **BSB-63**	3	BioSB	0	1	1	1	-	-
mAb clone **JM56**[3]	1	Leica	0	0	0	1	-	-
rmAb clone **EP618Y**	15 1	Abcam Epitomics	10	2	2	2	75%	77%
rmAb clone **D3R4N**	2	Cell Signaling	1	0	1	0	-	-
rmAb clone **RBT-SMAD4**	5	BioSB	0	0	3	2	-	-
rmAb clone **SP306**	2 1	Zytomed Systems Abcam	0	0	2	1	-	-
pAb **PA5-35330**	1	Thermo F. Scientific	0	0	0	1	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Total	52		16	6	12	18	-	
Proportion			31%	11%	23%	35%	42%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Antibody discontinued by the vendor

Detailed analysis of SMAD4, Run 57
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **B-8**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/4)*, Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/2) or Tris-EDTA / EGTA pH 9 (1/2) as retrieval buffers. 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, 80% (4/5) of the laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP618Y**: Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (Dako) (4/5), CC1 (Ventana) (3/6) or BERS2 (Leica) (2/2) as retrieval buffer. The mAb was diluted in the range of 1:100-1:1,400. Using these protocol settings, 77% (10/13) of the laboratories produced a

sufficient staining result. One laboratory obtained an optimal result applying combined antigen retrieval based on HIER in CC1 followed by enzymatic digestion with protease 3 (Ventana).

rmAb clone **D3R4N**: One protocol with an optimal result was based on HIER using TRS pH 9 (3-in-1) (Dako) as retrieval buffer. The mAb was diluted 1:125 and Envision Flex+ with an additional amplification step (mouse linker) as the detection system.

Table 3. **Proportion of optimal results for SMAD4 for the most commonly used antibodies as concentrate on the four 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
mAb clone **B-8**	2/3[**]	-	0/1	-	0/6	-	1/1	-
rmAb clone **EP618Y**	1/2	-	3/3	-	4/7 57%	-	2/2	-

[*] 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 for SMAD4, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells and structures expected to be demonstrated. This pattern was observed in 60% (24/40) of the insufficient results. The remaining insufficient results were characterized by poor signal-to-noise ratio and/or false positive staining reaction, often in combination with too weak staining reaction compromising the interpretation. Virtually all participating laboratories were able to demonstrate SMAD4 in high-level antigen expressing cells as basal squamous epithelial cells of the tonsil, whereas the diagnostic important demonstration of SMAD4 in stromal cells and remnants of normal pancreatic acini (internal positive tissue controls) intermingling between neoplastic cells of the pancreatic adenocarcinoma (tissue core 5) and the well-differentiated ductal pancreatic adenocarcinoma (tissue core 3), both displaying loss of SMAD4, was more challenging and only seen when appropriate protocol settings were applied.

All protocols were based on concentrated antibody formats and used within LD assays. The mAb clone B-8 and the rmAb clone EP618Y were the most widely used antibodies for the demonstration of SMAD4. The pass rate for mAb clone B-8 was only 35 % (7/20) and 20% (4/20) of the protocols received an optimal mark. For this clone, the most prevalent cause of an insufficient result was related to the use on the fully automated platforms Omnis (Dako) and BenchMark (Ventana). The proportion of sufficient results was only 11% (1/9) of which none was assessed as optimal It is known from NordiQC reference laboratories that the mAb clone B-8 is difficult (or impossible) to optimize on these platforms and thus, it is advisable to substitute this clone with a more robust primary Ab e.g. rmAb clones EP618Y or D3R4N. As shown in Table 1, and for laboratories using the mAb clone B-8 with optimal protocol settings (efficient HIER and appropriate antibody titer), the overall proportion of sufficient results increased dramatically, to 80% versus 35%, when the Ab was applied on the platforms Autostainer (Dako) or BOND (Leica). In the remaining insufficient results, protocols were typically based on less sensitive protocol parameters as insufficient HIER (in acidic buffer and too short efficient HIER time) or too diluted primary Ab. These parameters were either applied alone or in combination at the same time.

31% (16/52) of the laboratories used the rmAb clone EP618Y and as shown in Table 3, optimal results could be obtained on the most common platforms including the fully automated instruments from Dako (Omnis) and Ventana (Benchmark). For optimal performance, all (10/10) laboratories applied HIER in an alkaline buffer e.g. CC1(Ventana) or TRS pH9 (3-in-1) (Dako), used the antibody in the dilution range 1:100-1:1,400 and applied a 3-step multimer/polymer detection system as OptiView (Ventana) or Envision Flex+ (Dako). For the four protocols assessed as insufficient (see Table 1), 75% (3/4) were based on 2-step multimer/polymer detection systems.

Other frequently used primary Abs for detection of SMAD4 were the rmAb clones SP306 and RBT-SMAD4. None of the protocols (8/8) based on these Abs could provide a sufficient result. The insufficient results were typically characterized by a too weak staining intensity, an aberrant granular deposit of the reaction product and/or a false positive staining result and often seen simultaneously. The protocols seemed difficult to calibrate, providing an adequate signal-to-noise ratio, as the required staining intensity of the internal positive tissue controls (stromal cells and remnants of normal pancreatic glands) often were associated with a false positive staining reaction in the neoplastic cells of the pancreatic carcinomas.

This was the first assessment of SMAD4 in NordiQC providing an overall disappointing low pass rate of 42% (22/52). The marker is challenging and the most important parameters influencing the final outcome in negative direction was: 1) Use of the mAb clone B-8 on the fully automated instruments Omnis (Dako) and Benchmark (Ventana), 2) Use of the rmAb clones SP306 and RBT-SMAD4, often proving false positive results, 3) Use of protocol settings providing too low sensitivity e.g. insufficient HIER (acidic buffer or too short time), too low concentration of the primary Ab or application of a 2-step multimer/polymer detection system.
Laboratories should apply an Ab that work on the in-house IHC platform, calibrate the protocols correctly and stain according to the expected antigen level of the recommended control material (see below).

Controls
Tonsil is recommendable as external positive tissue control for SMAD4: The basal squamous epithelial cells in the surface epithelium must show a strong, predominantly nuclear staining reaction, whereas squamous epithelial cells in the superficial layer typically are negative or only faintly demonstrated. Virtually all other cells e.g. lymphocytes should display an at least moderate staining intensity. Advisable, a tumor (pancreas or colon) displaying loss of SMAD4 due to inactivation of the gene could be included, in which stromal cells (internal control) must show an at least moderate staining intensity. However, for SMAD4 it has to be emphasized that internal positive tissue controls being e.g. normal stromal cells adjacent to the neoplastic cells are preferred to external controls. An observed intact nuclear expression of SMAD4 proteins in the internal normal cells together with loss of SMAD4 proteins in the neoplastic cells is diagnostic essential[1]

[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.

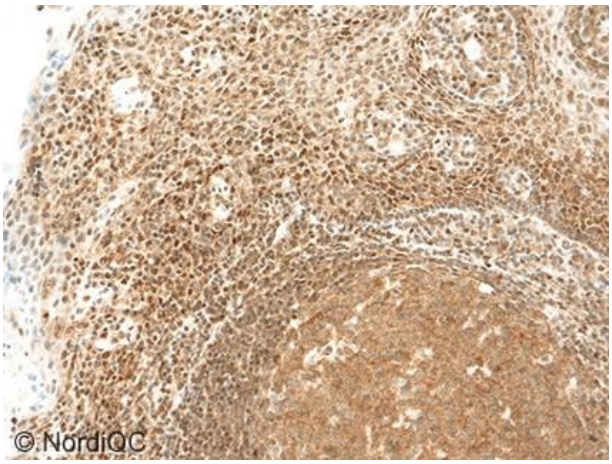


Fig. 1a (x200)
Optimal SMAD4 staining of the tonsil using the rmAb EP618Y optimally calibrated, HIER in an alkaline buffer (CC1, Ventana) and OptiView (Ventana) as the detection system - same protocol used in Figs. 2a-4a. The vast majority of basal squamous epithelial cells show a strong, predominantly nuclear staining reaction, whereas squamous epithelial cells in the superficial layer of the surface epithelium are negative. Lymphocytes display a moderate nuclear but also cytoplasmic staining reaction.

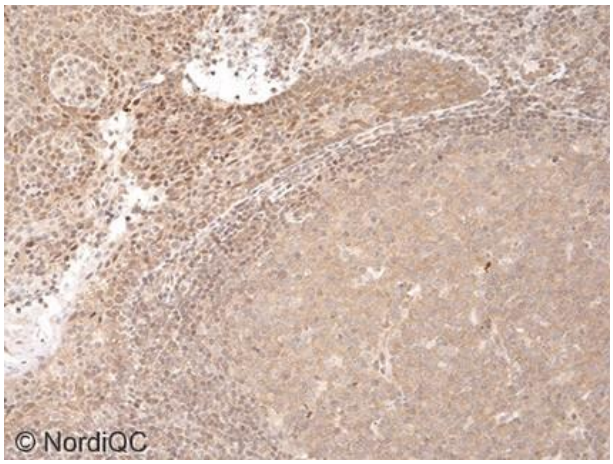


Fig. 1b (x200)
SMAD4 staining of the tonsil using an insufficient protocol based on the rmAb EP618Y with a protocol providing too low sensitivity, HIER in CC1 and UltraView (Ventana) as the detection system - same protocol used in Figs. 2b-4b. Although the squamous epithelial cells are stained, the intensity is reduced, and the reaction product is less distinct which is critical in relation to tumors displaying loss of SMAD4– compare Fig. 4b.

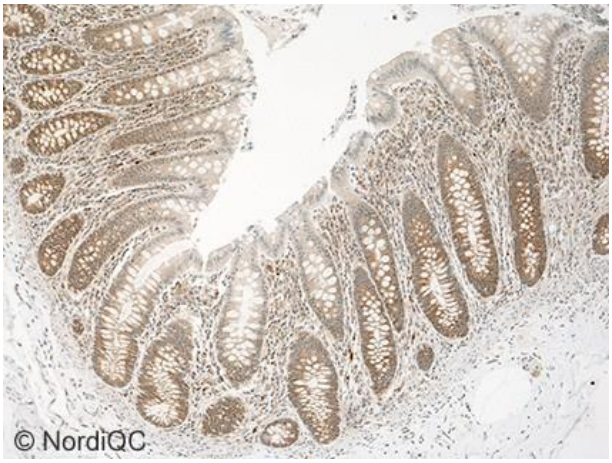


Fig. 2a (x100)
Optimal SMAD4 staining of the appendix using same protocol as in Fig. 1a. The epithelial cells lining the basal compartment (proliferative zone) of the crypts show a moderate nuclear but also cytoplasmic staining reaction, whereas the epithelial cells of the luminal compartment are negative or only faintly demonstrated.

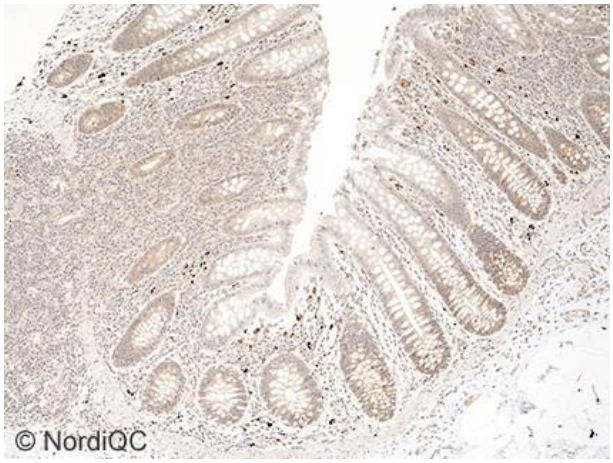


Fig. 2b (x100)
SMAD4 staining of the appendix using same insufficient protocol as in Fig. 1b. The epithelial cells in the basal compartment of the crypts display too weak staining intensity - compare with Fig. 2a.

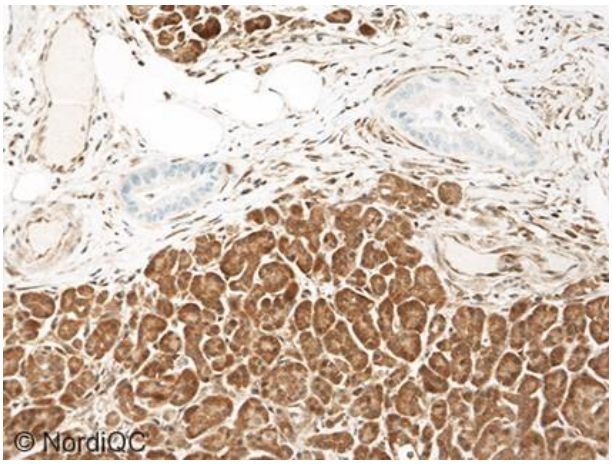


Fig. 3a (x200)
Optimal SMAD4 staining of the pancreas (tissue core 3) displaying regions of a high differentiated ductal pancreatic adenocarcinoma - same protocol as in Figs. 1a-2a. Virtually all normal glands and stromal cells (intern controls) show a moderate to strong nuclear but also cytoplasmic staining reaction, whereas the neoplastic cells are negative (loss of SMAD4).

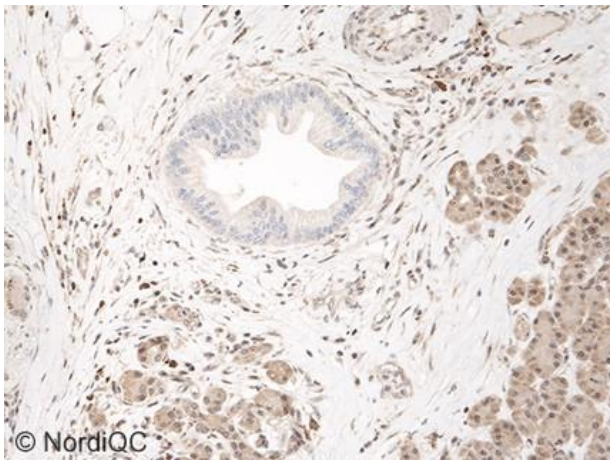


Fig. 3b (x200)
SMAD4 staining of the same tumor/tissue core as in Fig. 3a - same insufficient protocol as in Fig. 1b-2b. Although the staining provided the expected reaction pattern, staining intensity is reduced and critical in relation to interpretation in more challenging cases – compare Fig 4a-4b.

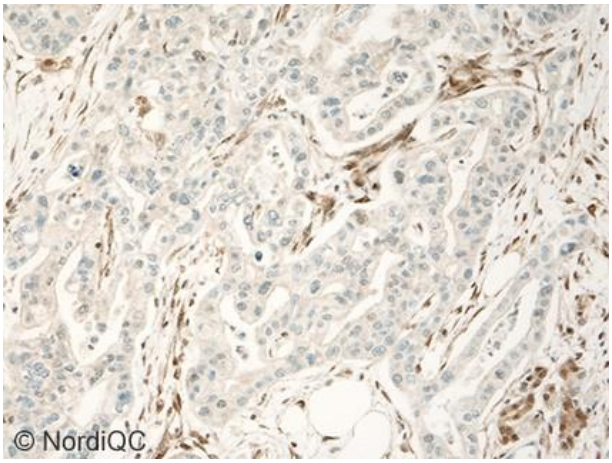


Fig. 4a (x200)
Optimal SMAD4 staining of the pancreas adenocarcinoma (tissue core 5) using same protocol as in Figs. 1a-3a. Virtually all neoplastic cells are negative for SMAD4, and importantly, the stromal cells (internal control) display a moderate nuclear and cytoplasmic staining reaction.

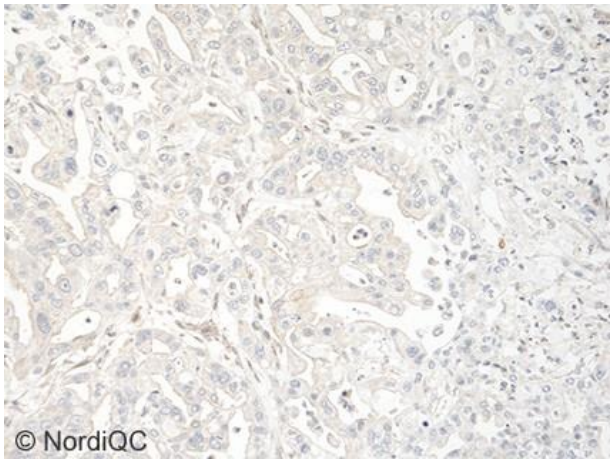


Fig. 4b (x200)
Insufficient SMAD4 staining of the pancreas adenocarcinoma (tissue core 5) using same protocol as in Figs. 1b-3b. The neoplastic cells are as expected negative for SMAD4, but the interpretation is compromised due to the false negative staining of the internal control (stromal cells).

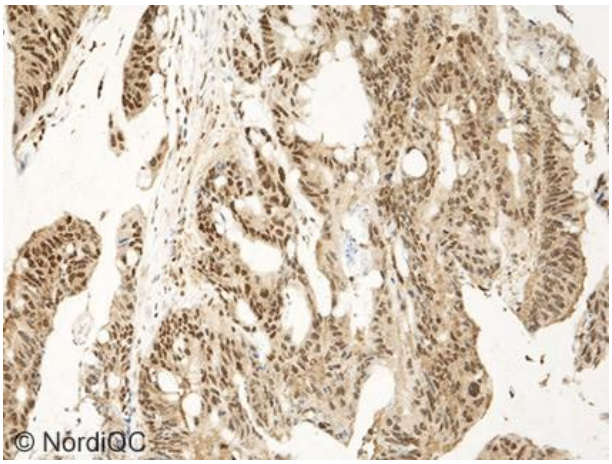


Fig. 5a (x200)
Optimal SMAD4 staining of the colon adenocarcinoma using the mAb B-8 optimally calibrated on the Autostainer, HIER in TRS pH 9 (Dako) and Envision Flex+ (Dako) as the detection system - same protocol used in Fig. 6a. Virtually all neoplastic cells display a moderate to strong and distinct nuclear/cytoplasmic staining reaction.

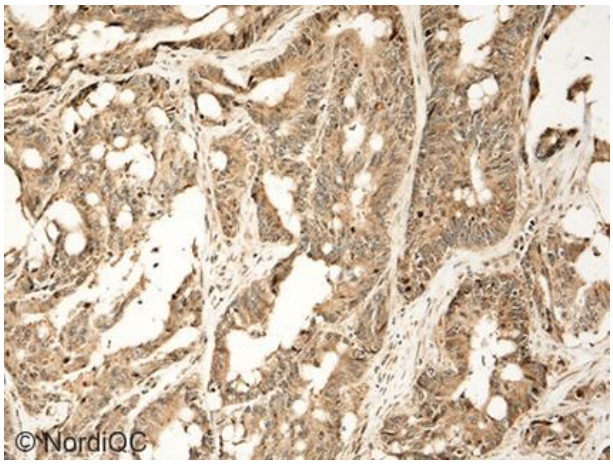


Fig. 5b (x400)
Insufficient SMAD4 staining of the colon adenocarcinoma using the mAb B-8 on the BenchMark (Ventana), primary Ab diluted 1:25, efficient HIER in CC1 and OptiView (Ventana) as the detection system - same protocol used in Fig. 6b. Although applying highly sensitive protocol settings, only background reaction is seen and virtually none of the tumor cells displayed the expected nuclear staining reaction as shown in Fig. 5 a. This primary Ab provided insufficient results on the fully automated instruments Omnis (Dako) and Benchmark (Ventana), typically characterized by too weak reactions poor signal-to noise ratio or both simultaneously.

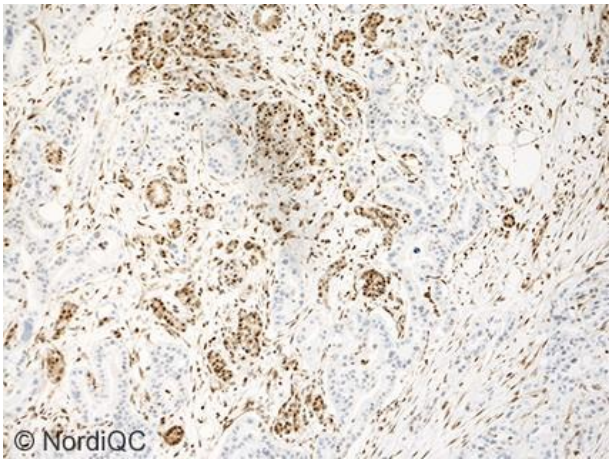


Fig. 6a (x100)
Optimal SMAD4 staining of the pancreas adenocarcinoma (tissue core 5) using the same protocol as in Fig. 5a. The protocol provided the expected staining pattern (in all cores) and displayed superior contrast between the neoplastic cells (loss of SMAD4) and the intern positive controls (stromal cells and remnants of normal pancreatic acini/glands).

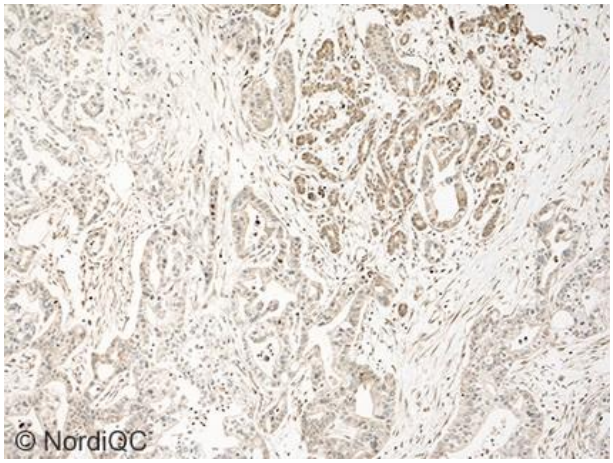


Fig. 6b (x100)
Insufficient SMAD4 staining of the pancreas adenocarcinoma (tissue core 5) using the same protocol as in Fig. 5b. The staining reaction is characterized by a dubious reaction pattern compromising interpretation – compare with Fig. 6a It was observed that the mAb B-8 could only provide optimal and sufficient results on Autostainer/Dako and BOND/Leica platforms with more gentle washing procedures compared to the Omnis (Dako) and BenchMark (Ventana) platform. For these two platforms, the rmAbs EP618 or D3R4N are better alternatives.

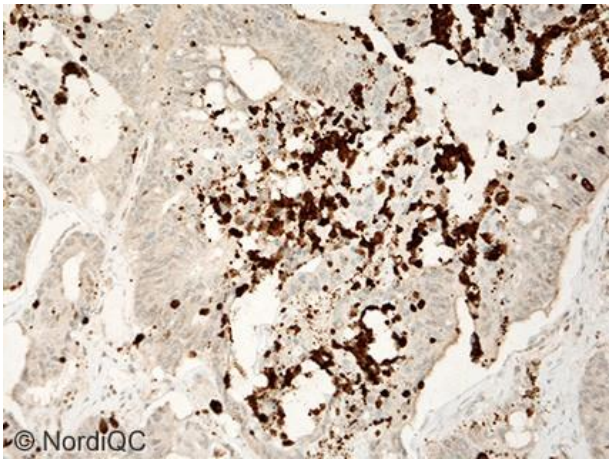


Fig. 7a (x200)
Insufficient SMAD4 staining of the colon adenocarcinoma based on a protocol using the rmAb SP306. The neoplastic cells are false negative, and an aberrant granular deposit of the reaction product is seen. This Ab, but also the rmAb RBT-SMAD4, often provided too weak and/or false positive staining result of e.g. the pancreas adenocarcinoma (tissue core 5) – see Fig. 7b.

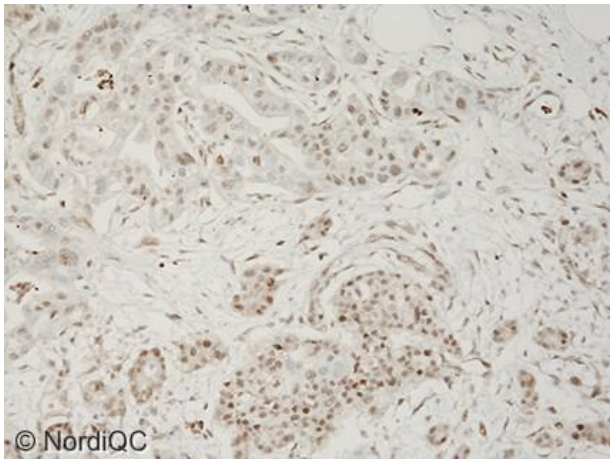


Fig. 7b (x200)
Insufficient SMAD4 staining of the pancreas adenocarcinoma (tissue core 5) using the same protocol as in Fig. 7a. The neoplastic cells are false positive. Protocols based on the rmAbs SP306 and RBT-SMAD seem difficult to calibrate, providing the required analytical sensitivity and specificity needed for SMAD4 IHC.

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