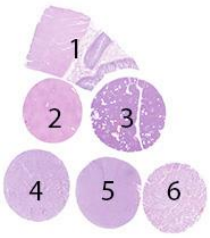
Assessment Run 52 2018
Vimentin (VIM)

Material
The slide to be stained for VIM comprised:



1. Colon, 2. Liver, 3. Pancreas, 4. Seminoma, 5. Malignant melanoma, 6. Renal cell carcinoma (RCC).

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing VIM staining as optimal included:

- An at least moderate, distinct cytoplasmic staining reaction of most endothelial cells, stromal cells, macrophages, and lymphocytes.
- An at least weak to moderate, distinct cytoplasmic staining reaction of virtually all endothelial and Kupffer cells of the sinusoids in the liver.
- An at least weak, distinct cytoplasmic staining reaction of the vast majority of epithelial cells of exocrine acini in the pancreas.
- A strong, distinct cytoplasmic staining reaction of virtually all neoplastic cells of the malignant melanoma and the seminoma (dot-like and/or complete cytoplasmic staining reaction).
- An at least moderate, distinct cytoplasmic staining reaction of virtually all neoplastic cells of the RCC.
- No staining reaction of epithelial cells in the colon and of hepatocytes in the liver.

Participation

Number of laboratories registered for VIM, run 52	318
Number of laboratories returning slides	308 (97%)

Results
308 laboratories participated in this assessment. 229 (74%) of these achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining were:
- Use of proteolytic pre-treatment
- Inefficient HIER (too short time or use of a non-alkaline buffer)
- Too low concentration of the primary antibody
- Use of less sensitive detection systems
- Unexplained technical issues

Performance history
This was the third NordiQC assessment of VIM. The overall pass rate decreased compared to previous run 30 (see Table 2).

Table 2. **Proportion of sufficient results for VIM in the three NordiQC runs performed**

	Run 12 2004	Run 30 2010	Run 52 2018
Participants, n=	79	164	308
Sufficient results	94%	83%	74%

Conclusion
The mAb clones **V9** and **3B4** and the rmAb clone **SP20** are all robust monoclonal antibodies for demonstration of VIM. HIER (preferable in an alkaline buffer), careful calibration of the primary Ab and application of a sensitive 3-step polymer/multimer based detection system were the most important parameters for an optimal performance. In this assessment, the RTU systems from Leica (PA0640) and Agilent/Dako (IR/GA630) both provided a high proportion of sufficient and optimal results and was superior compared to laboratory develop assays (including modifications of the RTU system from Roche/Ventana (790-2917) based on the same clone, V9.
Liver, colon and pancreas are recommended as positive and negative tissue controls for VIM. In the liver, virtually all Kupffer cells must show an at least moderate and distinct cytoplasmic staining reaction, while endothelial cells of the sinusoids must display an at least weak staining reaction. In the colon, endothelial cells of large vessels and stromal cells (e.g. fibroblasts and lymphocytes) must show a strong and distinct

cytoplasmic staining reaction, while intraepithelial T-cells must at least display a moderate staining intensity. In the pancreas, epithelial cells of exocrine acini must show a weak but distinct cytoplasmic staining reaction. Epithelial cells of the colon mucosa and hepatocytes in the liver must be negative.

Table 1. **Antibodies and assessment marks for VIM, run 52**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **V9**	57 10 6 3 2 2 1 1 1 1	Agilent/Dako Leica/Novocastra BioGenex Cell Marque GeneMed Linaris Diagnostic Biosystems Zymed/Invitrogen Zytomed Systems Thermo S/ Neomarkers	32	23	18	11	65%	83%
mAb clone **3B4**	29	Agilent/Dako	10	13	2	4	79%	100%
mAb clone **SRL33**	2	Leica/Novocastra	0	0	1	1	-	-
mAb clone **BS13**	1	Nordic Biosite	0	1	0	0	-	-
rmAb clone **SP20**	2 2 1	Cell Marque Thermo S./Neomarkers Diagnostic Biosystems	2	2	0	1	-	-
Ready-To-Use antibodies								
mAb clone **V9 IR630**	31	Agilent/Dako	27	1	3	0	90%	95%
mAb clone **V9 IR630**[3]	5	Agilent/Dako	5	0	0	0	-	-
mAb clone **V9 GA630**	29	Agilent/Dako	23	2	4	0	86%	100%
mAb clone **V9 GA630**[3]	2	Agilent/Dako	1	0	1	0	-	-
mAb clone **V9 790-2917**	100	Roche/Ventana	21	51	19	9	72%	78%
mAb clone **V9 347M-10**	2	Cell Marque	0	1	1	0	-	-
mAb clone **V9 PA0640**	7	Leica/Novocastra	5	2	0	0	100%	100%
mAb clone **V9 PA0640**[3]	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone **V9 KIT-0019**	1	Maixin	1	0	0	0	-	-
mAb clone **V9 8336-C010**	1	Sakura FineTek	1	0	0	0	-	-
mAb clone **V9 AM074-10M**	1	BioGenex	1	0	0	0	-	-
mAb clone **V9 ILM52311 R25**	1	Immunologic	0	0	0	1	-	-
mAb clone **3B4 760-2512**	3	Roche/Ventana	2	0	0	1	-	-
rmAb clone **SP20 347R-18**	1	Cell Marque	0	0	0	1	-	-
rmAb clone **SP20 MAD-000326QD**	2	Master Diagnostica	2	0	0	0	-	-
Total	308		133	96	49	30	-	
Proportion			43%	31%	16%	10%	74%	

1) Proportion of sufficient stains (optimal or good). 2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of VIM, Run 52
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **V9**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (10/11)*, Cell Conditioning 1 (CC1; Ventana) (6/37), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (10/16), Tris-EDTA/EGTA pH9 (2/4), Bond Epitope Retrieval Solution 1 (BERS1; Leica) (1/3), DBS Montage Citrate solution (1/1) or Citrate buffer pH 6 (2/5) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 33 of 40 (83%) laboratories produced a sufficient staining reaction (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **3B4**: Protocols with optimal results were all based on HIER using TRS pH 9 (3-in-1) (Dako) (3/7), CC1 (Ventana) (4/13), BERS2 (Leica) (2/5) or BERS1(Leica) (1/2) as retrieval buffer. The mAb was typically diluted in the range of 1:100-1:600 depending on the total sensitivity of the protocol employed. Using these protocol settings, 18 of 18 (100%) laboratories produced a sufficient staining reaction (optimal or good).

rmAb clone **SP20**: Protocols with optimal results were based on HIER using CC1 (Ventana) (2/3). The mAb was diluted 1:200 using either UltraView with amplification (Ventana) or OptiView (Ventana) as detection systems.

Table 3. **Proportion of optimal results for VIM for the most commonly used antibodies as concentrates on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark 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 **V9**	3/3**	0/1	1/1	0/1	5/17 (29%)	-	7/11 (64%)	1/2
rmAb clone **3B4**	1/4	-	1/2	-	4/10 (60%)	-	1/1	1/1

* 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 **V9**, product no. **PA0640**, Leica, Bond III/MAX:
Protocols with optimal results were typically based on HIER using BERS1 (efficient heating time 10-20 min. at 93-97°C), 15-20 min. incubation of the primary Ab and Bond Refined (DS9800) as detection system. Using these protocol settings, 5 of 5 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **V9**, product no. **IR630**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) as detection systems. Using these protocol settings, 21 of 22 (95%) laboratories produced a sufficient staining result (all assessed as optimal).

mAb clone **V9**, product no. **GA630**, Dako, Omnis:
Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (efficient heating time 30 min. at 97°C), 12-23 min. incubation of the primary Ab and EnVision FLEX (GV800/GV823) as detection systems. Using these protocol settings, 20 of 20 (100%) laboratories produced a sufficient staining result (all assessed as optimal).

mAb clone **V9**, product no. **790-2917**, Ventana, BenchMark XT, GX, ULTRA:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min. at 95-100°C), 16-32 min. incubation of the primary Ab and UltraView (760-500) with or without amplification kit (760-080) or OptiView (760-700) with or without amplification (760-099/860-099) as detection systems. Using these protocol settings, 51 of 65 (78%) laboratories produced a sufficient staining result (optimal or good).

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for VIM for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Leica BOND MAX/III mAb V9 **PA0640**	3/3	2/3	4/4	3/4
Dako AS mAb V9 **IR630**	92% (11/12)	92% (11/12)	88% (15/17)	82% (14/17)
Dako Omnis mAb V9 **GA630**	100% (16/16)	100% (16/16)	64% (7/11)	45% (5/11)
VMS Ultra/XT/GX mAb V9 **790-2917**	1/1	0/1	72% (71/99)	21% (21/99)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit – only protocols performed on the specified vendor IHC stainer were included.

Comments

In this NordiQC assessment for VIM, the overall predominant 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 100% of the insufficient results (79 of 79). Virtually all participating laboratories were able to stain VIM in cells with high-level expression as the neoplastic cells of the malignant melanoma and normal lymphocytes in lamina propria of the colon mucosa. Demonstration of VIM in exocrine acini of the pancreas, endothelial and Kupffer cells of liver sinusoids and neoplastic cells of the RCC was more challenging and could only be demonstrated when appropriate and sensitive protocol settings were applied.

The mAb clone V9 was the most widely used antibody for demonstration of VIM (see Table 1). Used as a concentrate within a laboratory developed (LD) assay, the mAb clone V9 gave an overall pass rate of 65% (55 of 84). Optimal results could be obtained on all four main IHC platforms - Omnis (Dako), Autostainer (Dako), Bond (Leica) and BenchMark (Ventana)(see Table 3). The most important protocol settings for optimal performance were use of efficient HIER in an alkaline buffer (88%, 28 of 32) and a relative high concentration of the primary Ab (average working dilution of 1:1,164, range 1:100-to 1:7,000). In addition, the majority of labs (69%, 22 of 32) applied a sensitive 3-step polymer/multimer (e.g. OptiView or Bond Refine) as detection system. For protocols assessed as insufficient (borderline or poor), the average working dilution was 1:4,872 (range 1:50 to 1:100,000) and proportion of labs applying HIER in alkaline buffer and a 3-step polymer/multimer detection system, decreased to 72% (20 of 28) and 54% (15 of 28), respectively.

The mAb 3B4 used within a LD-assay gave an overall pass rate of 79% (23 of 29) of which 34% (10 of 29) were assessed as optimal. The mAb 3B4 also provided optimal results on the four main IHC platforms (see Table 3). HIER, preferable in an alkaline buffer, careful calibration of the primary Ab in relation to a 2- or 3-step multimer/polymer detection system (e.g. UltraView or OptiView) were the main prerequisite for optimal performance.

Comparing pass rates for both mAb 3B4 and mAb V9 within LD-assays, in relation to the use of HIER in either an alkaline or acidic buffer (applying all protocol settings), the pass rate was 74% (70 of 94) for labs using an alkaline buffer, whereas, the pass rate was only 47% (8 of 17) for labs using an acidic buffer. Also, the proportion of optimal results was significant higher when applying an alkaline buffer compared to an acidic buffer, 39% (37 of 94) and 28% (5 of 17), respectively. Two protocols were based on enzymatic pre-treatment - both were assessed as poor.

61% (187 of 308) of the laboratories used a Ready-To-Use (RTU) system for VIM.
In this assessment, the RTU systems PA0640 (Leica), IR630 (Dako) or GA630 (Dako) based on mAb clone V9 was the most successful assays for demonstration of VIM (see Table 1). Grouped together, the pass rate was 90% (60 of 67) of which 82 % (55 of 67) were optimal. Both vendor recommended, and laboratory modified protocol settings could be used to obtain an optimal result, although there was a tendency towards better performance following the recommendations given by the respective

manufacturers (see Table 4). The RTU system GA630 (Omnis, Dako) demonstrated superior performance as all (16 of 16) protocols were assessed as optimal.

The Ventana RTU system (790-2917) for the BenchMark IHC platform based on mAb clone V9 was applied by 32% (100 of 308) of the participants and provided a lower pass rate of 72% (72 of 100) compared to the RTU systems described above. 21% (21 of 100) of the protocols were assessed as optimal. One lab followed the recommendations from the vendor strictly: HIER in CC1 for 64 min., 16 min. incubation time in primary Ab and used the biotin-based iView as the detection system (see Table 4). Using these protocol settings, the slide was assessed as good. All other labs modified their protocol settings, typically adjusting HIER time, prolonging incubation in primary Ab in combination with the use of a multimer-based detection systems such as UltraView or OptiView with or without amplification. The information provided in the spec sheet of the RTU product is outdated and needs to be revised - supporting laboratories with valid data and an optimized RTU product.

This was the third assessment of VIM in NordiQC (see Table 2). A pass rate of 74% was obtained, which is a decline compared to 83% in run 30, 2010. In the previous run 30, tonsil was recommended as control for demonstration of VIM. In this assessment, the inclusion of the liver and pancreas, both containing cellular structures expressing low level of VIM (see controls), challenged many laboratories and may have accounted for the decline in overall pass rate. Sufficient results could be obtained with a variety of protocol settings as long as the primary antibody concentration was carefully calibrated correspondingly to the chosen sensitivity of the whole assay.

Controls

According to the new guidelines provided by the International Ad Hoc Expert Committee (Appl Immunohistochem Mol Morphol. 2015 Jan;23(1):1-18.); liver, colon and pancreas are recommended as controls.

In liver, the protocol must be calibrated to give an intense staining reaction of virtually all Kupffer cells, whereas, endothelial cells of the sinusoids must display an at least weak intensity. Liver cells should be negative.

In the colon, dispersed intraepithelial T-cells must show an at least moderate staining reaction. Endothelial cells of large vessels and stromal cells (e.g. fibroblasts and lymphocytes) must display a strong but distinct cytoplasmic staining intensity. Epithelial cells of the colon mucosa should be negative.

In the pancreas, the vast majority of epithelial cells of exocrine acini should display a weak to strong predominantly cytoplasmic staining reaction.

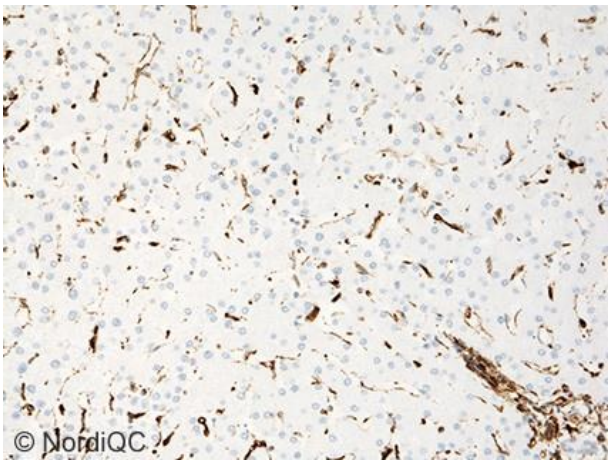


Fig. 1a (x200)
Optimal VIM staining of liver using the mAb clone 3B4, optimally calibrated, HIER in BERS2 pH 9 (Leica) and Bond Refine (Leica) as detection system.
The Kupffer cells show a moderate to strong, distinct cytoplasmic staining reaction, whereas the endothelial cells of the sinusoids display weak staining intensity.
Same protocol used in Figs. 2a - 6a.

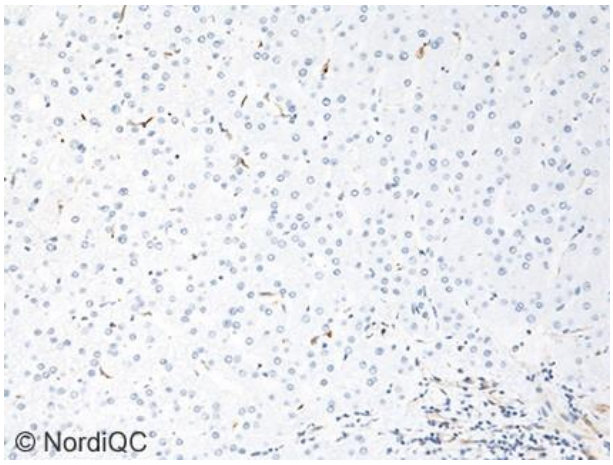


Fig. 1b (x200)
Insufficient VIM staining of liver using the mAb clone 3B4, too diluted, less efficient HIER in BERS1 pH 6 and Bond Refine (Leica) as detection system– same field as in Fig. 1a.
Only scattered Kupffer cells display a too weak staining intensity and the endothelial cells of the sinusoids are completely negative (compare Figs.1a - 6b). Same protocol used in Figs. 2b - 6b.

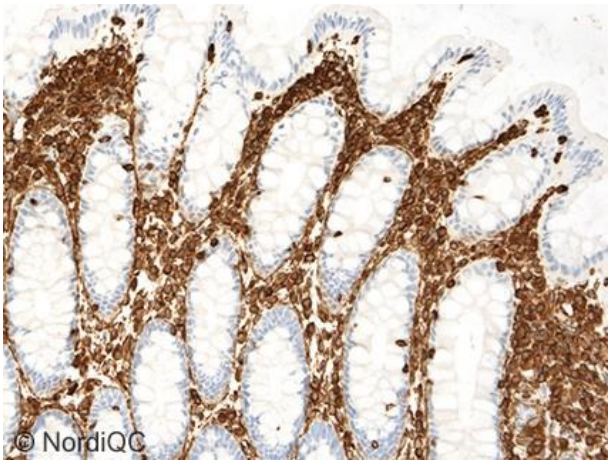


Fig. 2a (x200)
Optimal VIM staining of colon using same protocol as in Fig. 1a. Intraepithelial T-cells show an at least moderate and distinct cytoplasmic staining reaction. All stromal cells of lamina propria (mostly lymphocytes) display strong staining intensity, whereas epithelial cells of the colon mucosa are negative.

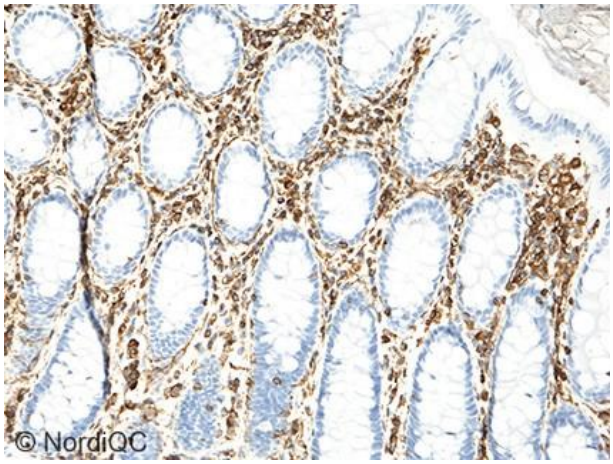


Fig. 2b (x200)
Insufficient VIM staining of colon using same protocol as in Fig. 1b – same field as in Fig. 2a.
The staining intensity and proportion of positive intraepithelial T-cells is significantly reduced. Also, stromal cells in lamina propria displays weaker intensity compared to the expected level seen in Fig. 2a.

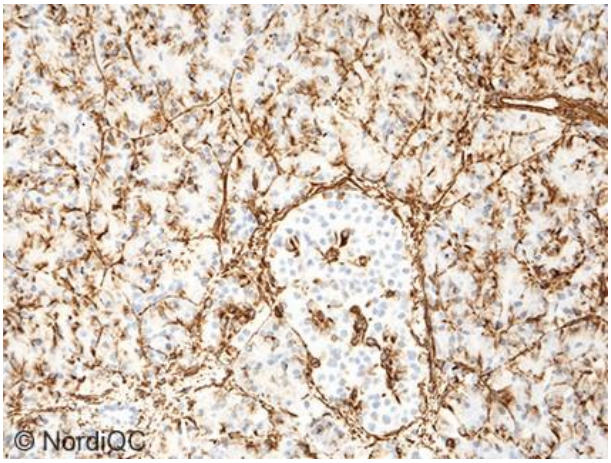


Fig. 3a (x200)
Optimal VIM staining of pancreas using same protocol as in Figs. 1a and 2a. The vast majority of epithelial cells of exocrine acini show an at least weak but distinct cytoplasmic staining reaction (baso-lateral expression pattern).

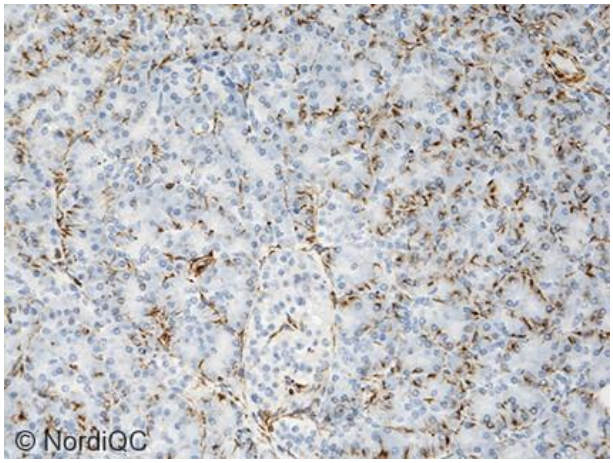


Fig. 3b (x200)
Insufficient VIM staining of pancreas using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The epithelial cells of exocrine acini are false negative. Only stroma cells display a weak staining intensity.

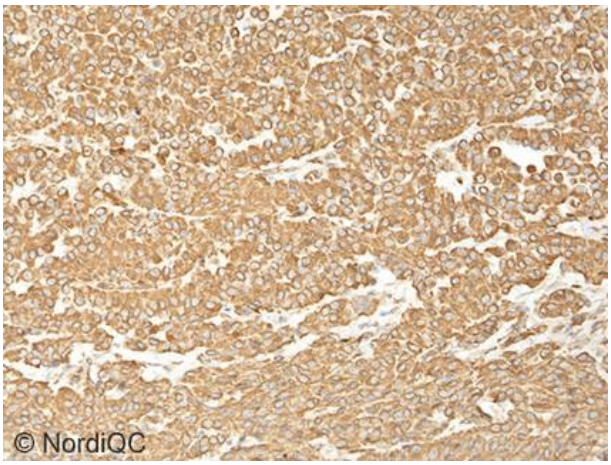


Fig. 4a (x200)
Optimal VIM staining of the malignant melanoma using same protocol as in Figs. 1a – 3a. All neoplastic cells show a strong and distinct cytoplasmic staining reaction.

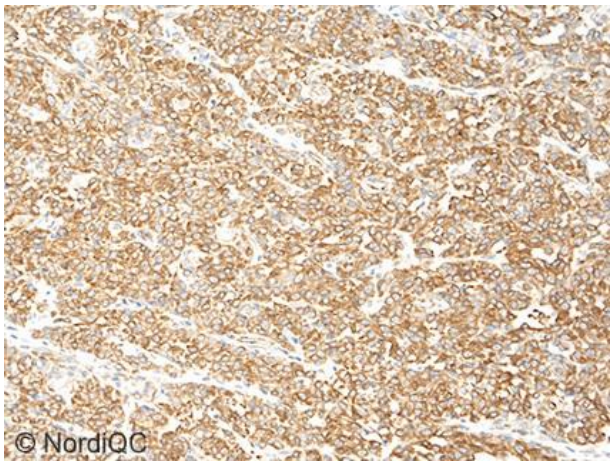


Fig. 4b (x200)
VIM staining of the malignant melanoma using same protocol as in Figs. 1b - 3b. The staining intensity of the neoplastic cells is comparable to the result obtained in Fig. 4a. However, compare with Fig. 5a-6b, emphasizing the importance of calibrating the protocol in relation to critical quality staining indicators (see Fig. 1a-3b) – same field as in Fig. 4a.

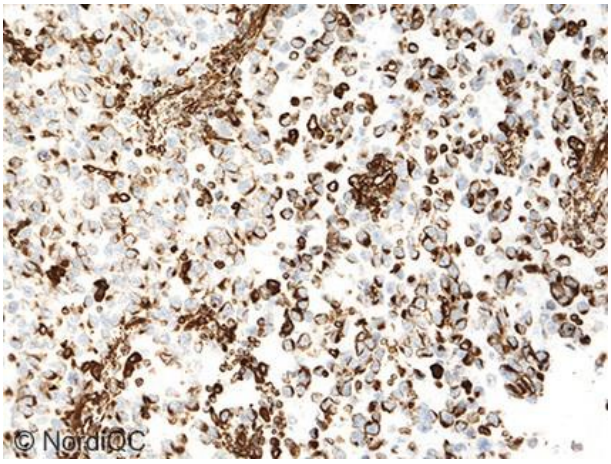


Fig. 5a (x200)
Optimal VIM staining of the seminoma using same protocol as in Figs. 1a – 4a. Virtually all the neoplastic cells show a strong and distinct cytoplasmic staining reaction (dot-like and/or complete cytoplasmic staining pattern).

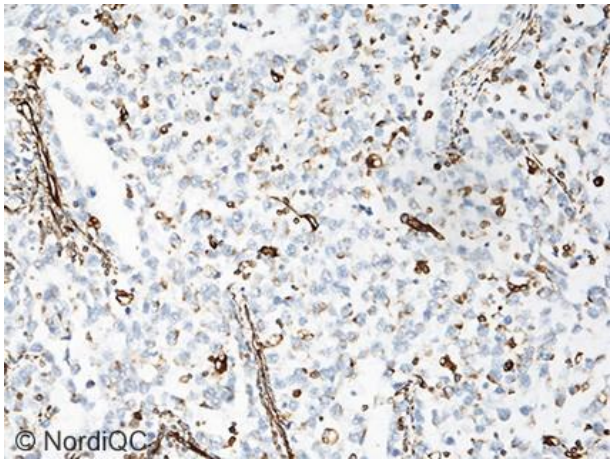


Fig. 5b (x200)
Insufficient VIM staining of the seminoma using same protocol as in Figs. 1b - 4b. The neoplastic cells only display a faint dot-like staining reaction or are completely negative – same field as in Fig. 5a.

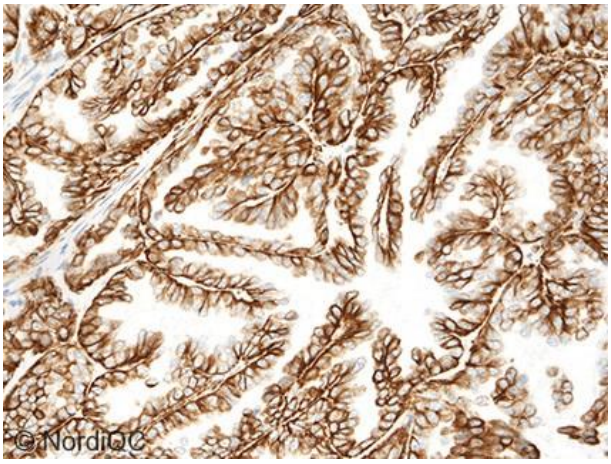


Fig. 6a (x200)
Optimal VIM staining of the RCC using same protocol as in Figs. 1a – 5a. All the neoplastic cells show a strong and distinct cytoplasmic staining reaction

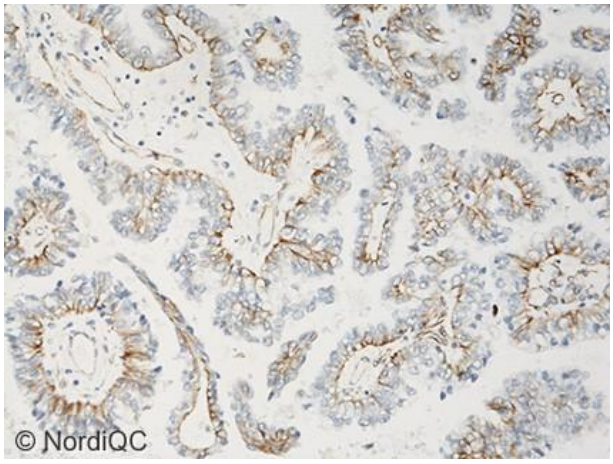


Fig. 6b (x200)
Insufficient VIM staining of the RCC using same protocol as in Figs. 1b - 5b. The neoplastic cells display too weak staining intensity or are completely negative.

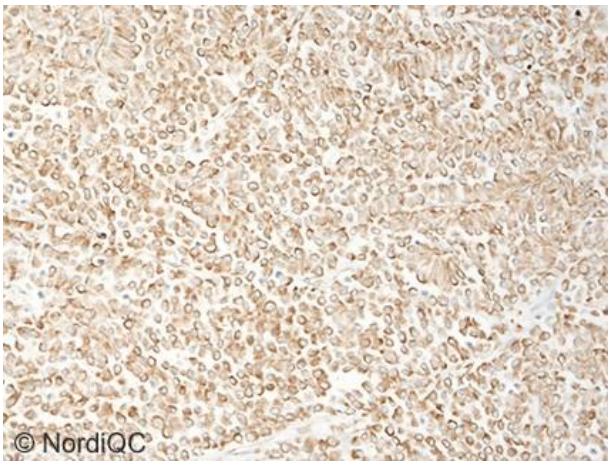


Fig. 7a
Insufficient VIM staining of the malignant melanoma using a protocol based on proteolytic pre-treatment. Although the vast majority of neoplastic cells show a moderate and distinct cytoplasmic staining reaction (compare with Fig. 4a), the protocol provided too low sensitivity – same protocol used in Fig. 7b.

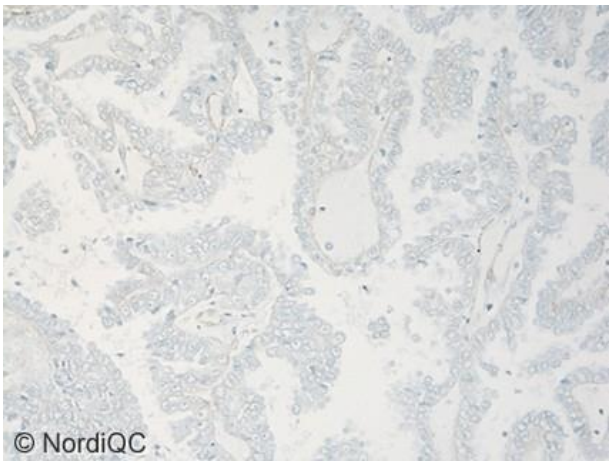


Fig. 7b (x200)
Insufficient VIM staining of the renal cell carcinoma using the same protocol as in Fig. 7a. All the neoplastic cells are false negative – compare with optimal results in Fig. 6a. Enzymatic pre-treatment should not be used for demonstration of VIM, as this antigen retrieval procedure is difficult to control, often causing extraction of the antigen of interest or impairing morphology.

MB/LE/MV/RR 05.04.2018