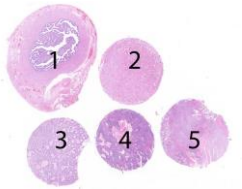
Assessment Run 43 2015

Wilms' tumour-1 protein (WT1)

Material
The slide to be stained for WT1 comprised:



1. Fallopian tube, 2. Kidney, 3. Lung adenocarcinoma, 4. Serous ovarian carcinoma, 5. Mesothelioma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a WT1 staining as optimal included:

- A strong and distinct nuclear staining of virtually all epithelial and smooth muscle cells in the fallopian tube
- A strong and distinct nuclear staining of virtually all neoplastic cells in the ovarian serous carcinoma
- A strong and distinct nuclear staining of virtually all neoplastic cells in the mesothelioma
- A moderate to strong nuclear staining of podocytes and parietal epithelial cells in kidney
- No staining of the lung adenocarcinoma or the renal tubules

A cytoplasmic staining reaction in a variety of cells, e.g. endothelial, smooth muscle, striated muscle and plasma cells, was expected and accepted for the mmAb clone 6F-H2. In the majority of cells, a distinct dot-like staining of nucleoli was seen and accepted for the rmAb clone EP122.

Participation

Number of laboratories registered for WT1, run 43	243
Number of laboratories returning slides	220 (91%)

Results
220 laboratories participated in this assessment. 179 (81%) 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:
- Less successful performance of the mmAb 6F-H2 on the Ventana BenchMark platform
- Use of less sensitive detection systems
- Use of too short HIER pretreatment
- Too low concentration of the primary antibody

Performance history
This was the third NordiQC assessment of WT1. A minor decrease in the pass rate was seen compared to run 28 in 2010 (see table 2).

Table 2. **Proportion of sufficient results for WT1 in the three NordiQC runs performed**

	Run 15 2005	Run 28 2010	Run 43 2015
Participants, n=	24	96	220
Sufficient results	38%	83%	81%

Conclusion
The mmAbs clones **6F-H2**, **WT49** and the rmAb **EP122** are all recommendable antibodies for demonstrating WT1. Using the two most widely used WT1 antibodies (clone 6F-H2 and WT49), HIER in an alkaline buffer for at least 20 min (or at least 48 min for Ventana BenchMark users) and a sensitive and specific 3-step polymer/multimer based detection system gave the highest proportion of optimal results. A combined HIER–protease retrieval protocol was introduced in this WT1 assessment. At the Ventana BenchMark platform this retrieval protocol provided a significant improvement in the signal-to-noise ratio for the mmAb clone 6F-H2.

The concentration of the primary antibody must be carefully calibrated. The overall proportion of insufficient results for mmAb 6F-H2 as concentrate were 36% (16 of 45) at the Ventana platform compared to only 10% (4 of 39) on non-Ventana platforms, suggesting that the mmAb clone 6F-H2 is more difficult to optimise on the Ventana platform compared to other platforms. Compared to the concentrates, the Ready-To-Use systems for WT1 from Dako and Leica provided the highest proportion of sufficient and optimal results. Depending on the clone and retrieval procedures, both fallopian tube and kidney can be used as positive tissue controls for WT1. In fallopian tube a strong, distinct nuclear staining reaction of the majority of the epithelial cells and smooth muscle cells must be seen. In kidney a strong, distinct nuclear staining in podocytes and the parietal epithelial cells of Bowman's capsule should be seen, whereas the epithelial cells of the tubules should be negative.

Table 1. **Antibodies and assessment marks for WT1, run 43**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mmAb clone **6F-H2**	70 7 2 1 1 1 1 1	Dako Cell Marque Immunologic BioSB Genemed Novous Thermo Fisher Zeta	23	41	17	3	76%	81%
mmAb clone **WT49**	20 1	Leica/Novocastra Monosan	9	8	2	2	81%	88%
rmAb clone **EP122**	1	Epitomics	0	1	0	0	-	-
pAb, **C-19**	1	Santa Cruz	0	0	1	0	-	-
pAb, **RB-9267-P1**	1	Thermo Fisher	0	0	0	1	-	-
Ready-To-Use antibodies								
mmAb clone **6F-H2 IR055/IS055**	51	Dako	40	8	2	1	94%	100%
mmAb clone **6F-H2 760-4397**	45	Ventana/Cell Marque	4	33	5	3	82%	97%
mmAb clone **6F-H2 348M-98**	3	Cell Marque	0	2	1	0	**-**	**-**
mmAb clone **6F-H2 PM258**	1	BioCare	0	0	1	0	-	-
mmAb clone **6F-H2 MAD-005671QD**	1	Master Diagnostica	0	1	0	0	-	-
mmAb clone **6F-H2 MON-RTU1210**	1	Monosan	0	0	1	0	-	-
mmAb clone **WT49 PA0562**	8	Leica/Novocastra	5	2	1	0	88%	100%
mmAb clone **MX012 MAB-0678**	1	Maixin	0	1	0	0	-	-
rmAb clone **EP122 AN828-5M**	1	Biogenex	1	0	0	0	-	-
Total	220		82	97	31	10	-	
Proportion			37%	44%	14%	5%	81%	

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

Detailed analysis of WT1, Run 43
The following protocol parameters were central to obtain an optimal staining:

Concentrated Antibodies
mmAb clone **6F-H2**: Protocols with optimal results were based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution pH 9 (3-in-1) (Dako) (5/6)*, Cell Conditioning 1 (Ventana) (3/38), Bond Epitope Retrieval Solution 2 (Leica) (4/7), Tris-EDTA/EGTA pH 9 (4/8), Target Retrieval Solution pH

9 (Dako) (4/9) or Borg Decloaker pH 9,5 (BioCare) (1/2) as retrieval buffer. Optimal results were also obtained using a combination of HIER in Cell Conditioning 1 (Ventana) and Protease 3 pretreatment (Ventana) (2/4). The mAb was typically diluted in the range of 1:40-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings 56 of 69 (81%) laboratories produced a sufficient staining result (optimal or good).
*(number of optimal results/number of laboratories using this buffer)

mAb clone **WT49**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Bond Epitope Retrieval Solution 2 (Leica) (4/8), Target Retrieval Solution pH 9 (3-in-1) (Dako) (3/5) or Cell Conditioning 1 (Ventana) (2/4) as retrieval buffer. The mAb was diluted in the range of 1:10-1:25 depending on the total sensitivity of the protocol employed. Using these protocol settings 14 of 16 (88%) laboratories produced a sufficient staining result.

Table 3 summarizes the overall proportion of optimal staining results for the most frequently used concentrated antibodies on the three most commonly used IHC stainer platforms.

Table 3. **Proportion of optimal results for WT1 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic / Omnis		Ventana BenchMark XT / Ultra			Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC1 pH 8.5 + Protease 3	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **6F-H2**	9/13 (69%)**	-	3/38 (8%)	2/4	0/1	4/6 (67%)	-
mAb clone **WT49**	3/6 (50%)	-	2/4	-	-	4/7 (57%)	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use (RTU) Antibodies and corresponding systems
mmAb clone **6F-H2** product no. IR055/IS055, Dako, Autostainer+/Autostainer Link/Omnis:
Protocols with optimal results were typically based on HIER using Target Retrieval Solution pH 9 (Dako), Target Retrieval Solution pH 9 (3-in-1) (Dako), Tris-EDTA/EGTA pH 9 or TRS pH 6.1 (Dako) (efficient heating time 10-30 min. at 95°C – 100°C) and 15-30 min incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002/GV800) as detection system. Using these protocol settings 45 of 45 (100%) laboratories produced a sufficient staining result (optimal or good).

mmAb clone **6F-H2** product no. 760-4397, Ventana/Cell Marque, Ventana Benchmark XT/Ultra:
Protocols with optimal results were typically based on either 1) 56-64 min. HIER using Cell Conditioning 1 (Ventana), 32 min. incubation of the primary Ab and OptiView or UltraView (Ventana 760-700 or 760-500) as detection system or 2) Combined retrieval using HIER in Cell Conditioning 1 followed by proteolytic treatment in Protease 3 (Ventana) for 4 min at 36°C, 8 min. incubation of the primary Ab and modified OptiView detection system with amplification (4 min. + 4 min.). The modification uses the onboard dilution option for both the link Ab and the labeled multimer on the BenchMark stainer. Using these protocol settings 32 of 33 (97%) laboratories produced a sufficient staining result (optimal or good).

mmAb clone **WT49** product no. PA0562, Leica/Novocastra, Leica Bond-III/Bond-max:
Protocols with optimal results were all based on HIER using BERS 2 pH 9 (Bond, Leica) (efficient heating time 20-40 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 7 of 7 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP122** product no. AN828-5M, BioGenex, BioGenex i6000:
One protocol with an optimal result was based on HIER using Citrate pH 6 (efficient heating time 10 min. at 100°C) and 60 min incubation of the primary Ab and Super Sensitive Polymer (BioGenex) as detection system.

Comments
In concordance with the previous assessments for WT1 in NordiQC (run 15, 2005 and run 28, 2010) the prevalent feature of the insufficient results was a too weak or completely false negative nuclear staining reaction of the cells expected to be demonstrated. This was observed in 78% of the insufficient results (32 of 41). The remaining 9 cases (22%) showed a general poor signal-to-noise ratio due to excessive background staining often in combination with a too weak specific staining.

The majority of the participating laboratories were able to demonstrate WT1 in epithelial and smooth muscle cells of the fallopian tube, podocytes and parietal epithelial cells in kidney and neoplastic cells of the mesothelioma, whereas the demonstration of WT1 in neoplastic cells of the ovarian serous carcinoma was more challenging and only seen with appropriate protocol settings (Fig. 1a. to Fig. 4b.). In spite of this, both fallopian tube and kidney can be used as positive tissue controls provided that virtually all epithelial and smooth muscle cells of the fallopian tube and podocytes and parietal epithelial cells in kidney exhibit strong and distinct nuclear staining reaction.

Optimal demonstration of WT1 could be achieved with the mmAbs clones 6F-H2, WT49 and the rmAb EP122 (Table 1). Three slightly different staining patterns were observed (Fig. 1a – Fig. 7b). In the optimally calibrated HIER-based protocols, the mmAb WT49 gave a strong and exclusively nuclear staining reaction in epithelial and smooth muscle cells of the fallopian tube, neoplastic cells of the ovarian serous carcinoma, neoplastic cells of the mesothelioma and in podocytes and parietal epithelial cells in kidney (Fig. 1a – Fig. 4b). A similar nuclear staining pattern was seen with rmAb EP122, but with the addition of a distinct staining of the nucleoli in the majority of cells (Fig. 7a and 7b). The nature of this aberrant staining reaction is unknown, but as it didn't interfere with the reading of the specific nuclear WT1 reaction, no downgrading was done on that basis. With mmAb clone 6F-H2 in an optimally calibrated protocol based on HIER, strong nuclear staining was seen in all the expected cells. In addition to the nuclear staining reaction, clone 6F-H2 also provided cytoplasmic staining reaction in plasma cells, smooth muscle cells, skeletal muscle cells and endothelial cells. This staining pattern was expected and accepted, but in some cases it complicates the interpretation e.g. the nuclear staining in the parietal epithelia cells of the Bowman capsule is hard to recognize due to a strong cytoplasmic reaction (Fig. 5b and Fig. 6b). The recommended protocol from Dako (the most used vendor) for the mmAb clone 6F-H2 as a concentrate is based on proteolytic pretreatment in pepsin. Using proteolytic pretreatment, no cytoplasmic reaction in endothelial and muscle cells was seen. However, in this and previous assessments (run 15, 2005 and run 28, 2010), no optimal staining reaction was obtained, when proteolytic pretreatment was used. This was primarily due to a too weak intensity and reduced proportion of positive cells. In the present assessment, combination of HIER at high pH and short/weak proteolytic pretreatment was introduced for the mmAb clone 6F-H2. In the optimally calibrated protocols, this modification results in a staining pattern showing a strong nuclear positive staining reaction virtually without any cytoplasmic staining reaction in endothelial and muscle cells (Fig. 5a and Fig. 6a).

Optimal results could be achieved on the three main staining platforms, when the mmAbs clone 6F-H2 and WT49 were used as concentrates (see table 3). For both clones, careful calibration of the titre of the primary Ab, efficient HIER and use of a highly sensitive detection system were the main protocol prerequisites for optimal results. Typically, mmAb clone WT49 was used in 3-step detection systems (polymer or multimer) at low titer (1:10 – 1:25) with an incubation time of 20-60 min. after efficient HIER at alkaline pH (20 min for ER2 pH 9.0 (Leica) or 20 min for TRS pH 9.0 (Dako) or 56 min for CC1 pH 8.5 (Ventana)). The importance of careful calibration of the titre and incubation time is illustrated in Fig. 1a – Fig. 4b. Significant difference in the performance of the mmAb clone 6F-H2 was seen in relation to the IHC platform used (se table 3). If clone 6F-H2 was applied on either the Dako Autostainer system or the Leica Bond system, used in the range of 1:40-1:400, HIER in an alkaline buffer, 65% of the laboratories obtained an optimal staining result. In comparison, only 8% of the laboratories using the same clone and similar protocol settings on the Ventana BenchMark platform obtained an optimal result. Furthermore, the overall proportion of insufficient stains for mAb 6F-H2 concentrates were 36% (16 of 45) on the Ventana platform compared to only 10% (4 of 39) on non-Ventana platforms. The reasons for these discrepancies are currently not known, but they suggests, that optimising protocols for the mAb clone 6F-H2 is more difficult on the Ventana BenchMark platform than on the other two platforms. An important observation in the current assessment was that 4 laboratories using the mmAb clone 6F-H2 on the Ventana BenchMark platform, introduced a combined retrieval procedure not previously seen in WT1 assessments. The combination is based on HIER in CC1 pH 8.5 (Ventana) for 32 min followed by proteolytic treatment in Protease 3 (Ventana) for 4 min. This dramatically improves the signal to noise ratio, by virtually eliminating the cytoplasmic staining of endothelial cells and muscle cells (Fig. 5a – Fig. 6b). All four laboratories passed, two of them receiving optimal marks.

The three major Ready-To-Use systems based on mmAbs WT49 (Leica) and 6F-H2 (Dako and Ventana) all performed better than protocols based on the corresponding concentrates. The Dako 6F-H2 and Leica WT49 RTU systems had pass rates of 94% and 88% respectively compared to 76% and 81% in the concentrated formats. Moreover both systems (Dako and Leica) reached an encouraging pass rate of 100% when optimal protocol settings were applied (see table 1). Similar findings were seen with the Ventana 6F-H2 RTU system, showing a pass rate of 82% compared to 76% in the concentrated format. Although the Ventana 6F-H2 RTU pass rate improves to 97% when optimal protocol settings were applied

(see table 1), some noteworthy differences exist compared to the Dako RTU. In the Dako 6F-H2 RTU system optimal mark was seen in 82% (38 of 45). In contrast only 12% (4 of 33) were optimal using the Ventana 6F-H2 RTU system. The reasons for these differences are unclear, but similar findings were observed with the concentrated format of mAb clone 6F-H2. Although the numbers are small, it should be noted that three laboratories using the Ventana 6F-H2 RTU system and the combined retrieval procedure (HIER-Protease as previously described) all provided sufficient stains, and two received optimal marks.

Controls

Fallopian tube and kidney are both recommendable as positive tissue controls when mmAb WT49 and rmAb EP122 are used for WT1 detection. In Fallopian tube, the protocol must be calibrated to provide a strong, distinct nuclear staining in virtually all epithelial and smooth muscle cells. In kidney, optimally calibrated protocols must show a strong, distinct nuclear staining in podocytes and parietal epithelial cells of Bowman's capsule. Epithelial cells of the tubules should not display any nuclear or cytoplasmic staining. When mAb clone 6F-H2 is used with HIER as sole retrieval method, the resulting cytoplasmic staining reaction in endothelial and muscle cells makes kidney less useful as positive tissue. On the other hand, if mAb clone 6F-H2 is used with combined HIER-Protease retrieval, the cytoplasmic staining reaction is virtually eliminated and consequently kidney can be very useful as positive tissue control.

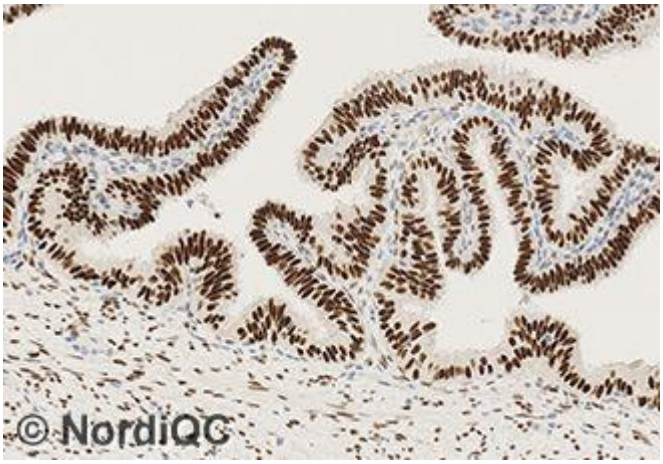


Fig. 1a
Optimal WT1 staining of the Fallopian tube using the mmAb clone WT49 (Leica) diluted 1:10 and with an incubation time of 25 min. after HIER in an alkaline buffer (BERS2, Leica) using a 3-step polymer system (Refine, Bond, Leica) and performed on the Bond III. A strong, distinct nuclear staining of virtually all epithelial cells and muscle cells is seen (same protocol used in Figs. 1a. - 4a.). Compare with Fig. 1b.

Fig. 1b
Insufficient WT1 staining of the Fallopian tube using the mmAb clone WT49 (Leica) diluted 1:25 and with an incubation time of 15 min. after HIER in an alkaline buffer (BERS2, Leica) using a 3-step polymer system (Refine, Bond, Leica) and performed on the Bond Max. The combination of a low titer and short incubation time results in insufficient staining. Only a moderate nuclear staining of the epithelial cells and muscle cells is seen. Compare with Fig. 1a. – same field. Also compare with Figs. 2b, 3b and 4b – same protocol.

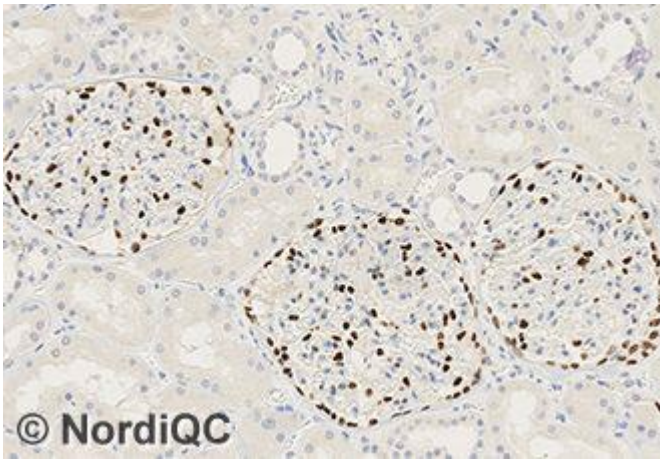


Fig. 2a
Optimal WT1 staining of the kidney using the same protocol as in Fig. 1a. A strong, distinct nuclear staining of the podocytes and the epithelial cells lining the Bowman capsule is seen. Compare with Fig. 2b.

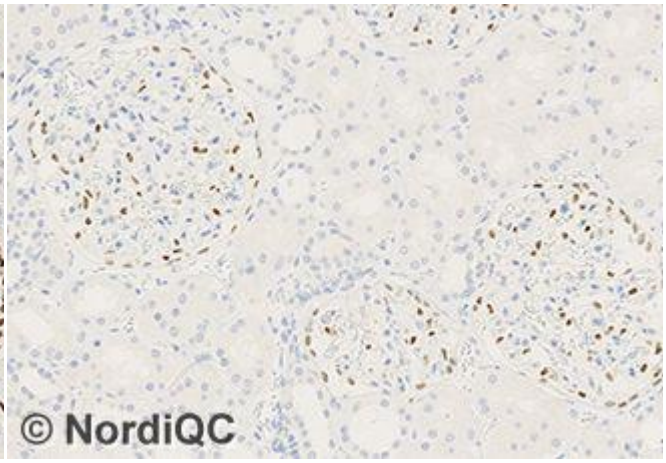


Fig. 2b
Insufficient WT1 staining of the kidney using the same protocol as in Fig. 1b. Only a weak nuclear staining of the podocytes and the epithelial cells lining the Bowman capsule is seen. Compare with Fig. 2a. - same field.

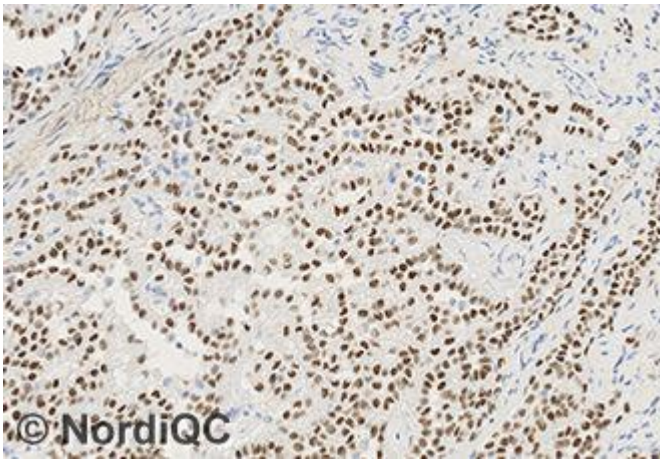


Fig. 3a
Optimal WT1 staining of the mesothelioma using the same protocol as in Figs. 1a & 2a. A strong, nuclear staining is seen in virtually all the neoplastic cells of the mesothelioma. Compare with Fig. 3b.

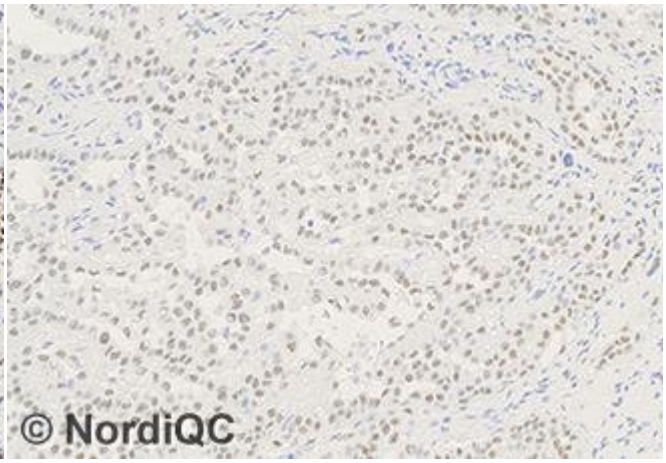


Fig. 3b
Insufficient WT1 staining of the mesothelioma using the same protocol as in Figs. 1b & 2b. The majority of neoplastic cells display only a moderate to weak nuclear staining reaction. Compare with Fig. 3a. – same field.

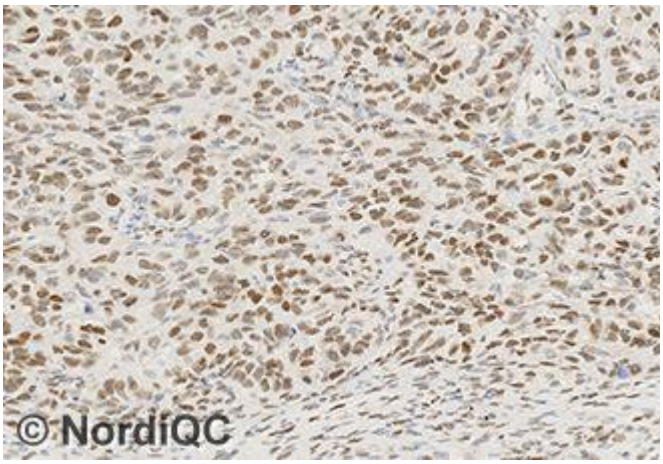


Fig. 4a
Optimal WT1 staining in the serous ovarian carcinoma using the same protocol as in Figs. 1a, 2a & 3a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. Compare with Fig 4b.

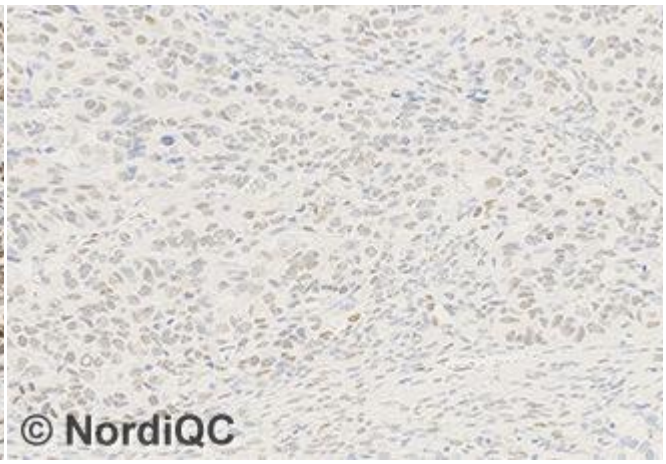


Fig. 4b
Insufficient WT1 staining in the serous ovarian carcinoma using the same protocol as in Figs. 1b, 2b & 3b. Only faint nuclear staining is seen and only in a minor fraction of the neoplastic cells. Compare with Fig. 4a. – same field.

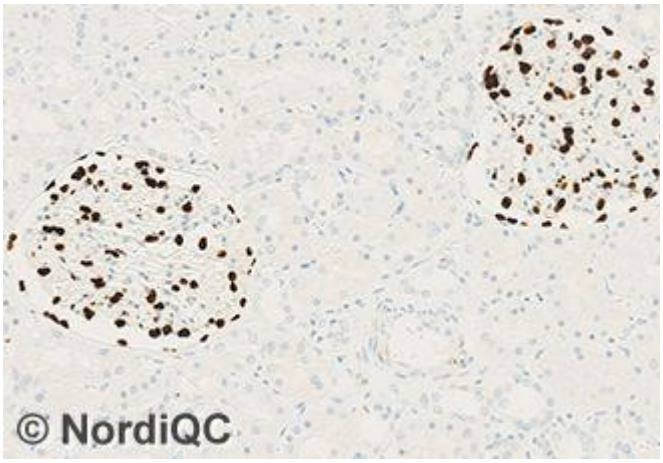


Fig. 5a
Optimal WT1 staining of the kidney using the mmAb 6F-H2 (Ventana/Cell Marque, RTU) with HIER in an alkaline buffer (CC1, Ventana) followed by protease treatment in Protease 3 (Ventana) using a 3-step multimer system (OptiView, Ventana) and performed on the BenchMark Ultra. A strong, distinct nuclear staining of the podocytes and the epithelial cells lining the Bowman capsule is seen. No cytoplasmic staining of endothelial and muscle cells is seen. Compare with Fig. 5b.

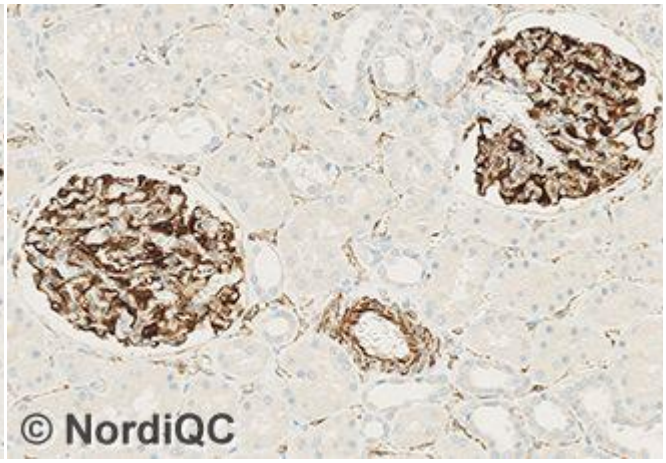


Fig. 5b
Good WT1 staining of the kidney using the mmAb 6F-H2 (Ventana/Cell Marque, RTU) with HIER in an alkaline buffer (CC1, Ventana) using a 3-step multimer system (OptiView, Ventana) and performed on the BenchMark Ultra. A moderate nuclear staining of the podocytes and the epithelial cells lining the Bowman capsule is seen. Moderate cytoplasmic staining of endothelial and muscle cells is also seen, making interpretation more challenging. Compare with Fig. 5a. - same field.

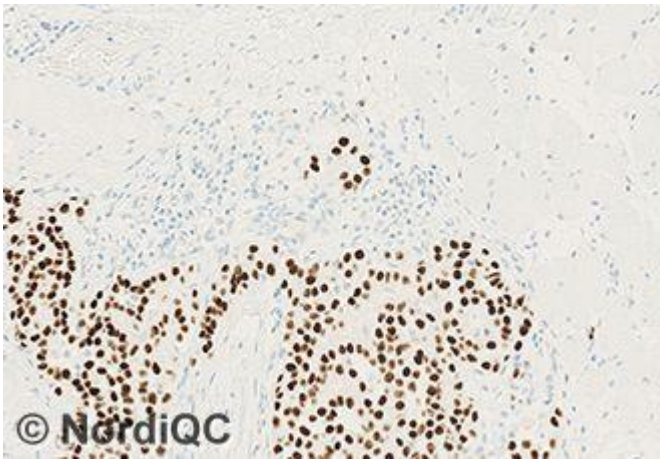


Fig. 6a
Optimal WT1 staining in the mesothelioma using the same protocol as in Figs. 5a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No cytoplasmic reaction is seen. Compare with Fig 6b.

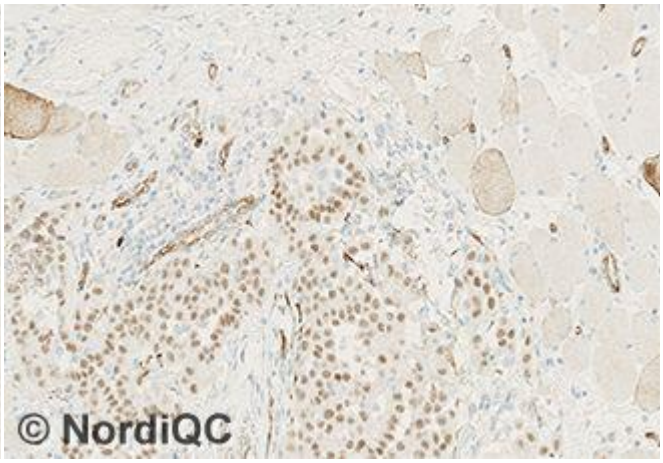


Fig. 6b
Good WT1 staining in the mesothelioma using the same protocol as in Fig. 5b. The majority of the neoplastic cells show a moderate nuclear staining reaction. A moderate cytoplasmic reaction is seen in the endothelial cells and smooth muscle cells. A minor proportion of skeletal muscle cells exhibit weak to moderate cytoplasmic reaction. Compare with Fig. 6a - same field.

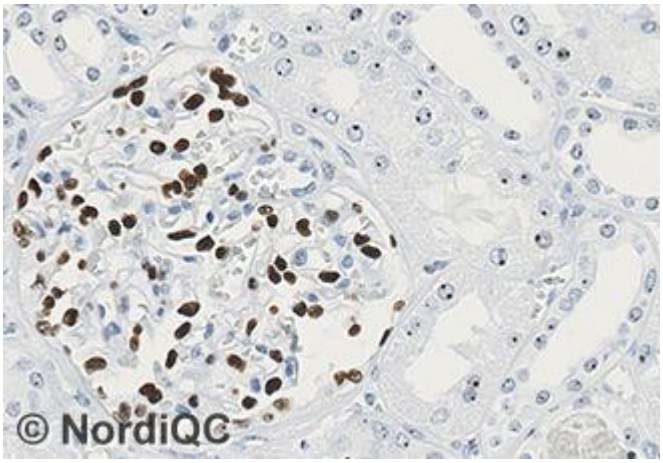


Fig. 7a
Optimal WT1 staining of the kidney using the rmAb clone EP122 (BioGenex, RTU) after HIER in an citrate buffer, pH 6 using a 3-step polymer system (Super Sensitive, BioGenex) and performed on the i6000 (BioGenex). No cytoplasmic reaction, but distinct staining of nucleoli in the majority of tubular epithelial cells are seen. Compare with Fig. 2a and Fig. 5a.

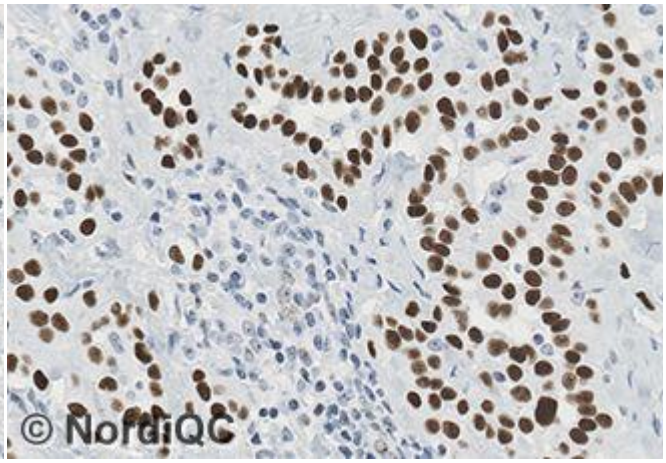


Fig. 7b
Optimal WT1 staining in the mesothelioma using the same protocol as in Fig. 7a. Virtually all the neoplastic cells show a strong nuclear staining reaction. No cytoplasmic reaction, but weak staining of nucleoli is seen in many stromal cells.

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