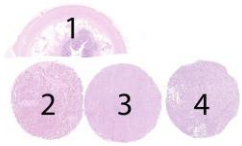
Assessment Run 48 2016

Desmin (DES)

Material
The slide to be stained for DES comprised:

1. Appendix, 2. Placenta, 3. Leiomyoma, 4. Rhabdomyosarcoma.



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing DES staining as optimal included:

- A moderate to strong, distinct cytoplasmic staining reaction of virtually all smooth muscle cells in lamina muscularis mucosae and muscularis propria of the appendix
- An at least weak to moderate cytoplasmic staining reaction in most smooth muscle cells of vessels in all specimens tested
- An at least weak cytoplasmic staining reaction of myofibroblasts lining the appendiceal epithelial cells
- A moderate to strong, distinct cytoplasmic staining reaction of virtually all neoplastic cells in the leiomyoma.
- An at least weak to moderate cytoplasmic staining reaction of the majority of neoplastic cells in the rhabdomyosarcoma.
- No staining reaction of the appendiceal epithelial cells and the cytotrophoblastic and syncytiotrophoblastic cells in the placenta

Participation

Number of laboratories registered for DES, run 48	300
Number of laboratories returning slides	275 (92%)

Results
275 laboratories participated in this assessment. 240 (87%) labs achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Poor performance of mAb clone D33 on the Dako Omnis platform.
- Omission of HIER
- Proteolytic pre-treatment for mAb clone DE-R-11
- Too low concentration of the primary antibody

Performance history
This was the fourth NordiQC assessment of DES. The pass rate seems very stable in the runs performed, as shown in table 2, despite a major number of new participants.

Table 2. **Proportion of sufficient results for DES in the four NordiQC runs performed**

	Run 5 2001	Run 21 2007	Run 35 2012	Run 47 2016
Participants, n=	42	119	194	275
Sufficient results	86%	80%	85%	87%

Conclusion
The widely used mAb clones **D33** and **DE-R-11** and many newly introduced Abs, as clone **BS21,** could all be used to obtain an optimal result for DES. For all clones, HIER was found to be preferable as epitope retrieval providing higher sensitivity compared to the use of enzymatic pre-treatment (or no pre-treatment).
The performance of mAb clone D33 seems to be influenced by the stainer platform as a significantly reduced proportion of sufficient and optimal results was observed on the Dako Omnis platform compared to e.g. Dako Autostainer or Leica Bond.
Appendix is recommendable as positive and negative tissue control for DES. Virtually all smooth muscle cells in lamina muscularis mucosae and muscularis propria must show a moderate to strong cytoplasmic

staining reaction, while an at least weak to moderate staining reaction must be seen in dispersed smooth muscle cells in the vessels and in myofibroblasts lining the epithelial cells.
No staining reaction must be seen in the appendiceal epithelial cells.

Table 1. **Antibodies and assessment marks for DES, run 48**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BS21**	4	Nordic Biosite	4	0	0	0	-	-
mAb clone **D9**	1	MP Biomedicals	1	0	0	0	-	-
mAb clone **D33**	95 3 3 2 2 2 2 1	Agilent/Dako Biogenex Thermo/Neomarkers Biocare Genemed Monosan Zytomed Immunologic	49	45	14	2	85%	90%
mAb clone **DE-R-11**	13	Leica/Novocastra	7	5	1	0	92%	100%
mAb clone **ZC18**	1	Invitrogen/Zymed	0	0	1	0	-	-
rmAb clone **EP15**	1	Cell Marque	1	0	0	0	-	-
pAb **RB-9014-P**	1	Thermo/Neomarkers	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **D33 PM036**	1	Biocare	1	0	0	0	-	-
mAb clone **D33 AM072**	2	Biogenex	1	1	0	0	-	-
mAb clone **D33 243M**	2	Cell Marque	0	2	0	0	-	-
mAb **D33 IR606/IS606**	47	Agilent/Dako	23	14	5	5	79%	97%
mAb **D33 PMD006**	1	Diagnostic Biosystems	0	1	0	0	-	-
mAb clone **D33 E057**	2	Linaris	0	2	0	0	-	-
mAb clone **D33 MAD-001011QD**	2	Master Diagnostica	0	2	0	0	-	-
mAb clone **D33 kit-0023**	1	Maixin	0	1	0	0	-	-
mAb clone **DE-R-11 PA0032**	10	Leica/Novocastra	8	2	0	0	100%	100%
mAb clone **DE-R-11 760-2513**	74	Roche/Ventana	34	33	6	1	91%	95%
rmAb clone **EP15 243R**	1	Cell Marque	1	0	0	0	-	-
pAb **MAD-583-QD-7**	1	Master Diagnostica	0	1	0	0	-	-
Total	275		131	109	27	8	-	
Proportion			48%	39%	10%	3%	87%	

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

Detailed analysis of DES, Run 48

The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies

mAb clone **BS21**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (3/3)* or Tris-EDTA pH9 (1/1) as retrieval buffer and the mAb was diluted in the range of 1:50-1:150. Using these protocol settings, 4 of 4 (100%) laboratories produced an optimal staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **D9**: One protocol with an optimal result was based on HIER for 32 min. using Cell Conditioning 1 (CC1, Ventana) (1/1) as retrieval buffer. The mAb was diluted 1:50 using UltraView as detection system and performed on the Ventana BenchMark XT.

mAb clone **D33**: Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (Dako) (11/26), CC1 (Ventana) (19/48), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (12/14), BERS1 (Leica) (1/1), Diva Decloaker pH 6.2 (Biocare) (2/4) or Tris-EDTA pH 9 (4/8) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:200. Using these protocol settings, 90 of 100 (90%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DE-R-11**: Protocols with optimal results were based on HIER using CC1 (Ventana) (4/7) or BERS2 (Leica) (3/4) as retrieval buffer. The mAb was diluted in the range of 1:50-1:100. Using these protocol settings, 12 of 12 (100%) laboratories produced a sufficient staining result.

rmAb clone **EP15**: One protocol with an optimal result was based on HIER for 32 min. using CC1 (Ventana) (1/1) as retrieval buffer. The rmAb was diluted 1:200 using OptiView as detection system and performed on the Ventana BenchMark GX.

pAb **RB-9014-P**: One protocol with an optimal result was based on HIER in PT module using Citrate pH 6 (1/1) as retrieval buffer. The pAb was diluted 1:800 using a 2-step polymer based detection kit (Immunologic) and performed on Autostainer, Thermo.

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

Concentrated antibodies	**Dako Autostainer / Omnis***		**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 **D33**	11/21** (52%)AS 0/5 Omnis	0/1 AS	19/45 (42%)	-	12/13 (92%)	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 **D33**, product no. **PM036**, Biocare, IntelliPATH:
One protocol with an optimal result was based on HIER using Diva decloaker pH 6.2 in a Pressure Cooker, 45 min. incubation of the primary Ab and MACH 4 Universal HRP-Polymer (MRH534) as detection system.

mAb clone **D33**, product no. **IR606/IS606**, 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 95-99°C), 20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 32 of 33 (97%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **DE-R-11** product no. **PA0032**, Leica/Novocastra, BOND III/MAX:
Protocols with optimal results were typically based on HIER using BERS2 (Bond Leica) (efficient heating time 20 min. at 99-100°C), 15-20 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 6 of 6 (100%) produced a sufficient staining result.

mAb clone **DE-R-11**, product no. **760-2513**, Roche/Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 16-98 min.) and 8-40 min. incubation of the primary Ab. UltraView (760-500) or OptiView (760-700) were used as detection systems. 4 used a combined pre-treatment using HIER in CC1 (efficient heating time 16-32 min.) followed by proteolysis in P3 for 4-8 min. Using these protocol settings, 53 of 56 (95%) laboratories produced a sufficient staining result.

Table 4 summarises 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, whereas e.g. Dako RTU Ab formats applied on a Ventana stainer device were excluded.

Table 4. **Comparison of pass rates for vendor recommended and laboratory modified RTU protocols**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS48 mAb D33 **IR606/IS606**	100% (15/15)	80% (12/15)	93% (13/14)	57% (8/14)
Leica BOND mAb DE-R-11 **PA0032**	100% (6/6)	100% (6/6)	1/1	0/1
VMS Ultra/XT mAb DE-R-11 **760-2513**	78% (14/18)	0% (0/18)	95% (53/56)	61% (34/56)

* 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 are included.

Comments

In this assessment and in concordance with the previous NordiQC assessments for DES, the prevalent feature of an insufficient staining was a too weak or false negative staining reaction of the cells expected to be demonstrated. Too weak or false negative staining reaction was seen in 97 % of the insufficient results (34 of 35). Virtually all the laboratories were able to demonstrate DES in high-level antigen expressing structures such as smooth muscle cells in muscularis propria in the appendix, whereas demonstration of DES in neoplastic cells in both the leiomyoma and rhabdomyosarcoma was more challenging and required an optimally calibrated protocol.

48% (131 of 275) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for DES. The mAb clone D33 was the most widely used antibody and provided optimal results on all three main IHC platforms from Leica, Ventana and Dako (Autostainer) as listed in table 3. Used as a concentrate within a LD assay, mAb clone D33 gave an overall pass rate of 85% (94 of 110) of which 45% were optimal (see table 1). It was observed, that the pass rate and especially the proportion of optimal results was highly influenced by the sensitivity of the detections systems used. If a 2-step polymer or multimer based detection system e.g., EnVision Flex (Dako) or UltraView (Ventana) was used, 82% of the laboratories (53 of 64) obtained a sufficient staining result of which 23% (15 of 64) were assessed as optimal. If a more sensitive 3-step polymer or multimer based detection system as e.g., EnVision Flex+, Bond Refine (Leica) or OptiView was used, 89% of the laboratories (41 of 46) produced a sufficient staining result and 74% were optimal.
In addition, the titre of mAb D33 must be carefully calibrated to provide an IHC protocol, which is able to demonstrate DES in structures with both low-level and high-level expression, as the different neoplasias included in the circulated material. In this assessment the Median Dilution Value (MDV) for optimal results was 1:80 (range 1:20-1:200), whereas a MDV of 1:194 (range 1:50-1:500) was seen in protocols with insufficient results.

mAb clone D33 seemed to provide an inferior performance on Dako Omnis platform compared to Dako Autostainer platform. If mAb clone D33 was applied within a LD assay using optimal protocol settings as listed above and performed on Dako Autostainer a pass rate of 100% (21 of 21 laboratories) was seen of which 52% were optimal. On Dako Omnis the pass rate was 40% (2 of 5 laboratories) with no optimal, despite fully comparable protocol settings were applied for both systems.

Also many other Abs as mAb clone DE-R-11 and BS21 provided optimal results within a LD assay. For all Abs, efficient HIER, preferable in an alkaline buffer, in combination with a sensitive detection system were the main prerequisites for an optimal result.

Ready-To-Use (RTU) antibodies were used by 52% (144 of 275) of the laboratories.
The Ventana RTU system based on mAb clone DE-R-11 (760-2513) was the most widely used RTU system applied by 74 laboratories. An overall pass rate of 91% was seen and 46% were optimal.
Optimal results could only be obtained by use of laboratory modified protocol setting using HIER in CC1 as single retrieval method or a combined method using HIER in CC1 followed by proteolysis in P3. If the protocols were performed accordingly to the recommendations provided by Ventana, using Proteolysis in P1, none of 18 protocols provided an optimal result and only a pass rate of 78% was obtained. Laboratory modified protocol settings provided a pass rate of 95% and 61% were optimal.

The Leica RTU system also based on mAb clone DE-R-11 (PA0032) provided an overall pass rate of 100%.

All 6 laboratories applying the protocol settings given by Leica, using HIER in ER2 for 20 min., 15 min. incubation of the primary Ab and Refine as detection system, produced optimal results.
For the Dako RTU system based on mAb clone D33 (IR606/IS606), an overall pass rate of 79% was observed. If protocols were performed according to the recommendations provided by Dako using HIER in TRS High for 20 min., 20 min. incubation of the primary Ab with FLEX as detection system and staining performed on the Autostainer, a pass rate of 100% (15 of 15) was obtained of which 80% were optimal.
8 laboratories used the RTU format on Omnis by protocol settings similar to the Dako recommendations for Autostainer but modified to "Omnis RTU" settings using HIER for 30 min. in TRS High pH and 20 min. incubation of the primary Ab and polymer. A pass rate of 25% was seen, none was optimal. In addition, four other laboratories used FLEX+ as detection system. Two of these were as assessed as "Good", while the other two were insufficient.

Controls
In this assessment and as observed in the previous runs for DES, appendix is recommendable as positive and negative tissue control. Virtually all the smooth muscle cells in the lamina muscularis mucosae and muscularis propria must show a moderate to strong cytoplasmic staining reaction, while an at least weak to moderate staining reaction must be seen in most smooth muscle cells in the vessels and in dispersed myofibroblasts lining the epithelial cells.
No staining reaction must be seen in the appendiceal epithelial cells.
Tonsil can be used primarily as supplementary negative tissue control. No staining reaction must be seen in lymphocytes and epithelial cells.
The recommendations of the above mentioned tissue controls for IHC are concordant to the guidelines published by the International Ad Hoc Expert Committee[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.

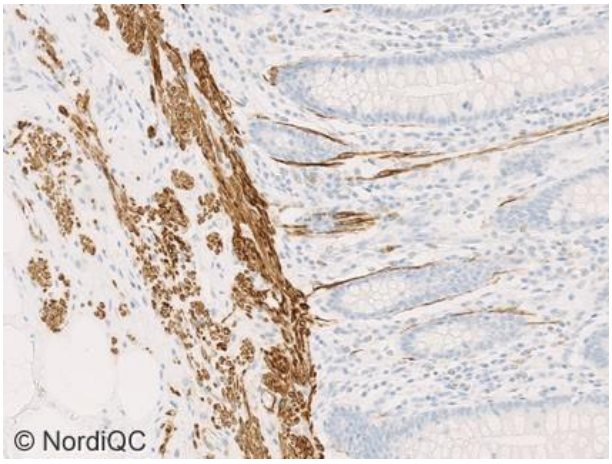


Fig. 1a
Optimal staining for DES of the appendix using the mAb clone DE-R-11 as Ready-To-Use format, 760-2513 Ventana, with HIER in CC1 and OptiView as detection system.
The protocol is modified by the laboratory using HIER as pre-treatment compared to the recommendation given by the producer suggesting proteolysis (Figs. 1b-5b).
The smooth muscle cells of lamina muscularis propria and myofibroblasts lining the epithelial cells show a moderate to strong staining reaction.
No background staining is seen.
Also compare with Figs. 2a - 5a – same protocol.

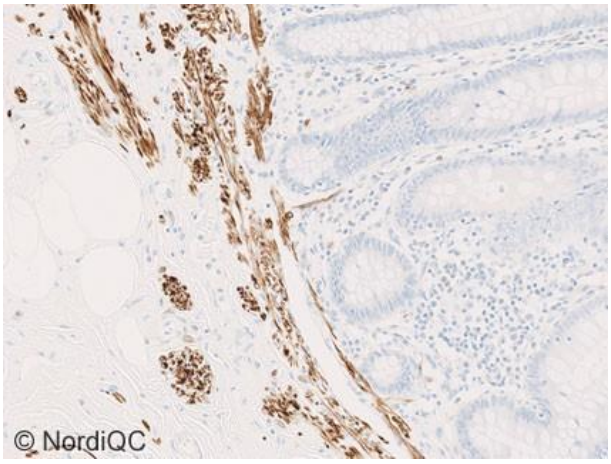


Fig. 1b
Insufficient staining for DES of the appendix using the mAb clone DE-R-11 as Ready-To-Use format, 760-2413 Ventana, with the protocol settings recommended by the producer suggesting proteolysis in P1 as pre-treatment – same field as in Fig. 1a.
The smooth muscle cells of lamina muscularis propria are demonstrated, while the myofibroblasts with low level DES expression are unstained.
Also compare with Figs. 2b - 5b – same protocol.

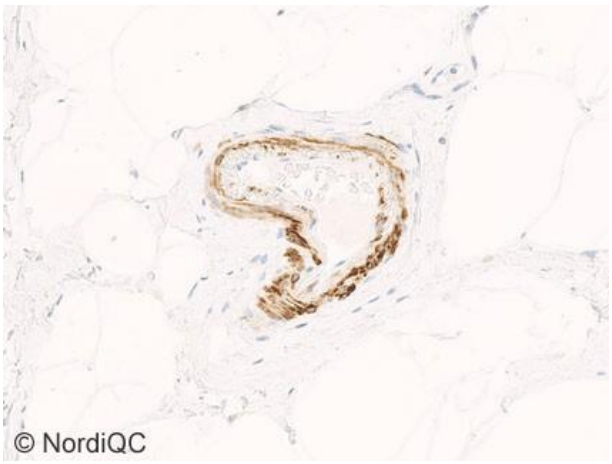


Fig. 2a
Optimal staining for DES of the appendix using the same protocol as in Fig. 1a.
The smooth muscle cells in large and small vessels are distinctively demonstrated.

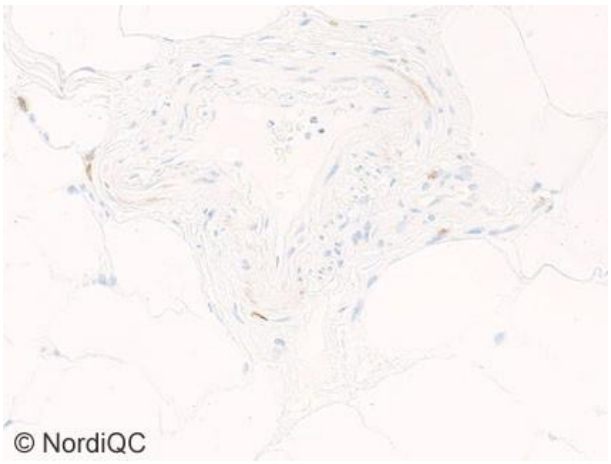


Fig. 2b
Insufficient staining for DES of the appendix using the same protocol as in Fig. 1b - same field as in Fig. 2a.
The smooth muscle cells of the vessels are false negative.

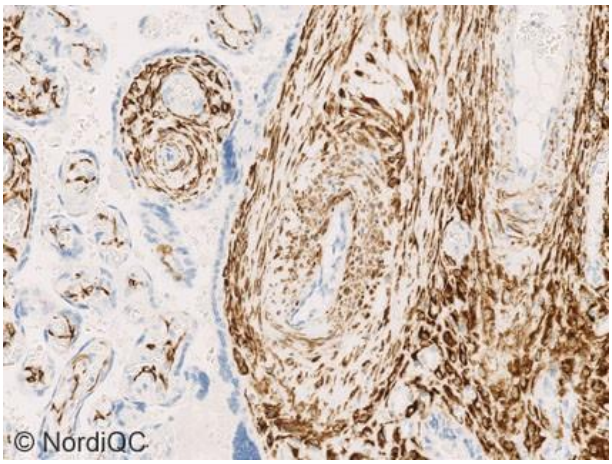


Fig. 3a
Optimal staining for DES of the placenta using the same protocol as in Figs. 1a and 2a.
The vast majority of smooth muscle in vessels in the stromal compartment of villi show a moderate to strong cytoplasmic staining reaction.

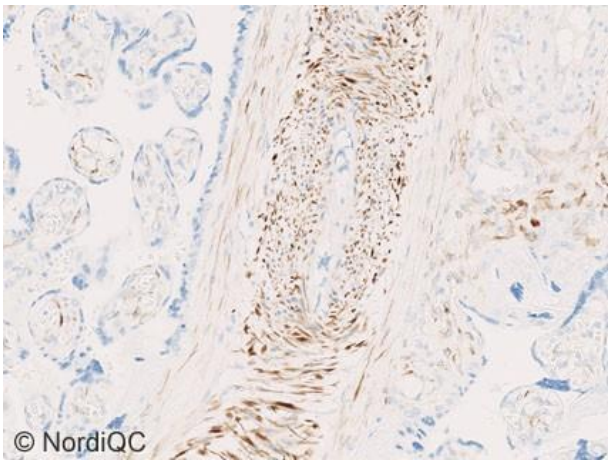


Fig. 3b
Insufficient staining for DES of the placenta using the same protocol as in Figs. 1b and 2b - same field as Fig. 3a.
A significantly reduced intensity and proportion of positive cells is seen compared to the result expected and shown in Fig. 3a.

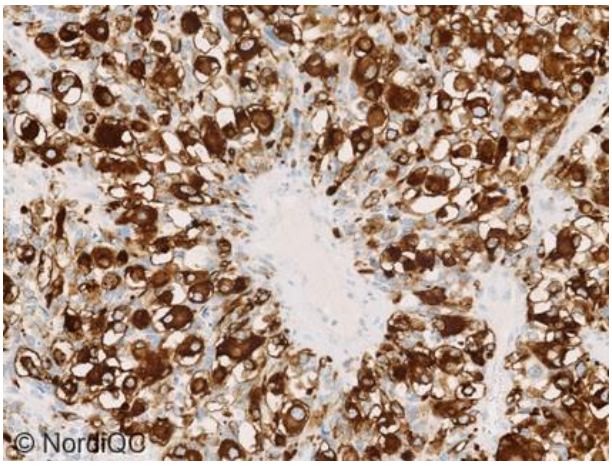


Fig. 4a
Optimal staining for DES of the rhabdomyosarcoma using same protocol as in Figs. 1a - 3a.
The vast majority of neoplastic cells show a moderate to strong and distinct cytoplasmic staining reaction.

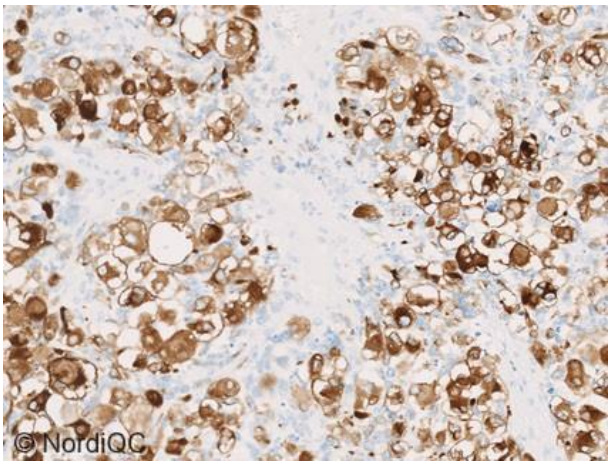


Fig. 4b
Staining for DES of the rhabdomyosarcoma using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
A reduced intensity and proportion of positive cells is seen compared to the result obtained in Fig. 4a.
Also compare with Fig. 5b – same protocol.

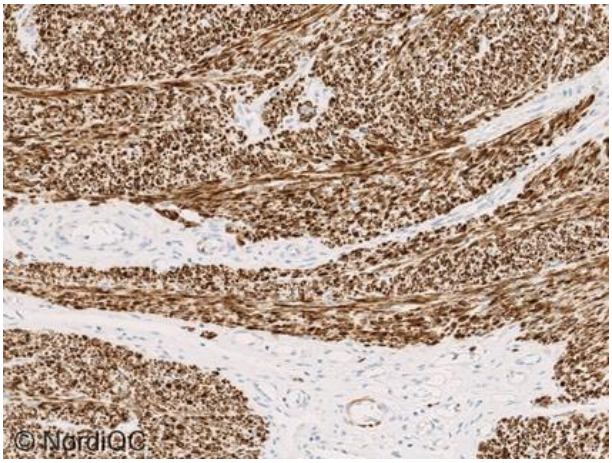


Fig. 5a
Optimal staining for DES of the leiomyoma using the same protocol as in Figs. 1a - 4a.
Virtually all the neoplastic cells show a moderate to strong cytoplasmic staining reaction. Also note the smooth muscle cells in normal vessels (center, bottom) show a distinct staining reaction.

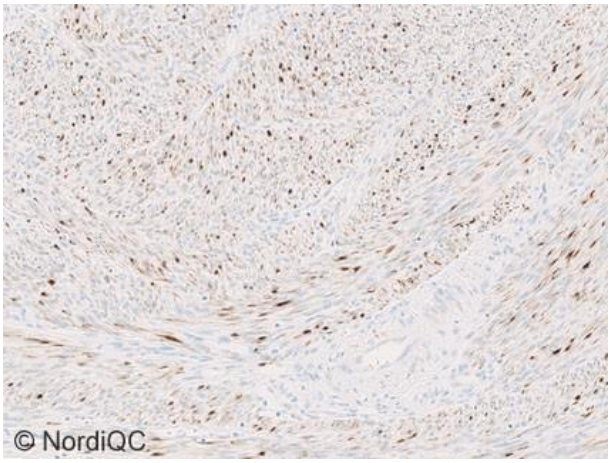


Fig. 5b
Insufficient staining for DES of the leiomyoma using the same protocol as in Figs. 1b - 4b - same field as Fig. 5a.
Only dispersed neoplastic cells show a weak staining reaction, whereas the smooth muscle cells of the normal vessels are negative.

SN/LE/MV/RR 19.11.2016