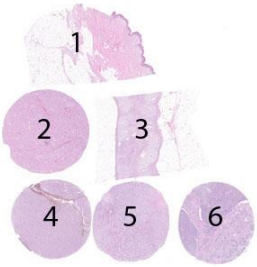
Assessment Run 49 2017

Melan-A (MLA)

Material
The slide to be stained for MLA comprised:



1. Skin, 2. Kidney, 3. Adrenal gland, 4-5. Malignant melanoma, 6. Granulosa cell tumour.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a MLA staining as optimal included:

- A moderate to strong, distinct cytoplasmic staining reaction of virtually all melanocytes in skin.
- A moderate to strong, distinct granular cytoplasmic staining reaction of virtually all adrenal cortical epithelial cells (only mAb clone A103).
- A moderate to strong, distinct cytoplasmic staining reaction of the vast majority of neoplastic cells in the malignant melanoma tissue core no 4.
- An at least weak to moderate, distinct cytoplasmic staining reaction of the majority of neoplastic cells in the malignant melanoma tissue core no. 5.
- An at least weak to moderate granular cytoplasmic staining reaction of the majority of neoplastic cells in the granulosa cell tumour (only mAb clone A103).
- No or only minimal staining reaction in the kidney.

Participation

Number of laboratories registered for MLA, run 49	273
Number of laboratories returning slides	255 (93%)

Results
255 laboratories participated in this assessment, 60% 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:
- Low sensitivity detection systems
- Too short efficient HIER time
- Too low concentration of the primary antibody
- Less successful performance of the mAb clone A103 on Ventana BenchMark and Dako Omnis platforms

Performance history
This was the sixth NordiQC assessment of MLA. A small decrease in pass rate was observed (see table 2).

Table 2. **Proportion of sufficient results for MLA in the last six NordiQC runs performed**

	Run 16 2006	Run 20 2007	Run 24 2008	Run 31 2011	Run 42 2014	Run 49 2017
Participants, n=	79	90	115	165	198	273
Sufficient results	32%	48%	50%	66%	68%	60%

The decreased pass rate (60%) in this run may be explained by participation of many new laboratories. Compared to Run 42 in 2014, a total of 75 new laboratories participated in the current run.

Conclusion
The mAb clones **A103**, **M2-7C10**, **BS52** and the rmAb clone **EP43** can all be used to obtain optimal staining for MLA. The mAb clone **A103** was the most frequently used antibody for MLA. Irrespectively of the Ab applied, efficient HIER, preferable in an alkaline buffer, in combination with a sensitive and specific IHC system were the main prerequisites for optimal performance.
In this assessment, the Ready-To-Use system for MLA from Dako (IR633/IS633) applied according to the vendor recommended protocol settings provided the highest proportion of sufficient and optimal results. In the current run, the mAb A103 RTU (IR633/IS633) was also applied to the Dako Omnis platform but resulted in a low pass rate. Only 13% (2 of 15) achieved sufficient results.

The recommended protocol settings for the Ventana mAb A103 MLA RTU system (790-2990) is based on the use of UltraView-AP as detection system. This results in a very low pass rate of 7%, compared to laboratories using UltraView-AP with amplification giving a pass rate of 100%.
The recently introduced rmAb clone EP43 showed promising performance as optimal results were seen on both the Ventana Benchmark and Dako Omnis platforms. mAb A103 has on these platforms in both this assessment and internal NordiQC evaluations showed an inferior performance.

Adrenal gland is a recommendable positive tissue control for MLA when using the mAb clone A103. A moderate to strong granular cytoplasmic staining reaction must be seen in virtually all epithelial cells throughout the adrenal cortex. For the rest of the MLA clones (without cross reactivity towards steroid producing cells) normal skin and melanomas with low MLA expression must be used as positive tissue controls. In normal skin, virtually all melanocytes should show a strong positive cytoplasmic reaction and weak to moderate reaction in the melanocytic dendrites in most melanocytes (see Fig. 8). Kidney is recommended as negative tissue control. No staining should be seen in the epithelial cells of tubules. Scattered epithelial cells may show a granular staining reaction caused by lipofuscin.

Table 1. **Antibodies and assessment marks for MLA, Run 49**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **A103**	73 18 2 7 1 1 4	Dako/Agilent Leica/Novocastra Monosan Cell Marque Immunologic Zeta Corp. Thermo Scientific	26	31	32	17	54%	60%
mAb clone **M2-7C10**	1 1	Cell Marque Zytomed	1	1	0	0	-	-
mAb clone cocktail **M2-7C10+M2-9E3**	2 3	NeoMarkers Biocare	1	2	2	0	-	-
mAb clone cocktail **HMB45+MC-7310 +M2-9E3+T311**	4	Biocare	2	1	0	1	-	-
mAb clone cocktail **A103+M2-7C10+ M2-9E3**	1	Life technologies	0	0	1	0	-	-
mAb clone **BS52**	1	Nordic Biosite	1	0	0	0	-	-
rmAb **EP43**	3 1	Epitomics Cell Marque	4	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **A103, IR633/IS633**	57	Dako/Agilent	22	15	18	2	65%	94%
mAb clone **A103 790-2990**	60	Ventana/Roche	20	17	16	7	62%	90%
mAb clone **A103, PA0233**	5	Leica/Novocastra	1	2	2	0	-	-
mAb clone **A103, 281M-87/281M-88**	4	Cell Marque	1	1	2	0	-	-
mAb clone **A103, API3114**	1	Biocare	1	0	0	0	-	-
mAb clone **A103, PDM153**	1	Diagnostic BioSystems	0	0	1	0	-	-
mAb clone **A103, MAB-0275**	1	maixin	1	0	0	0	-	-
mAb clone **M2-7C10+M2-9E2 MAD-001767QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone cocktail **HMB45+A103+T311 790-4677**	1	Ventana/Roche	0	0	1	0	-	-

mAb clone cocktail **HMB45+A103+T311 790-4677**	1	Cell Marque	0	0	1	0	-	-
Total	255		81	71	76	27	-	
Proportion			32%	28%	30%	10%	60%	

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

Detailed analysis of MLA, Run 49
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **A103**: Protocols with optimal results were all based on HIER using Cell Conditioning 1 (CC1, Ventana) (9/54)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (10/17), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (4/15), Tris-EDTA/EGTA pH 9 (2/6) or Tris-HCl pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 50 of 83 (60%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **M2-7C10**: One protocol with an optimal result was based on HIER in a pressure cocker using Citrate pH 6 as retrieval buffer. The mAb was diluted 1:30 and a 3-step polymer detection kit from Zytomed was used in a manual setup.

mAb clone cocktail **M2-7C10+M2-9E3**: 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 1:200 and a 3-step polymer detection kit, EnVision Flex+ (Dako) was used on the Dako Autostainer Link.

mAb clone cocktail **HMB45 + MC-7C10+M2-9E3 + T311**: Protocols with optimal results were based on HIER using Tris-EDTA/EGTA pH 9 (1/1) or Bond Epitope Retrieval Solution 1 (Bond, Leica) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50–1:500. Using these protocol settings, 2 of 4 (50%) laboratories produced an optimal staining.

mAb clone **BS52**: One protocol with an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 as retrieval buffer. The mAb was diluted 1:100 and a 2-step polymer detection kit, BioSite Histo Plus HRP Polymer anti-Mouse (Nordic Biosite) was used on the Thermo Autostainer platform.

rmAb clone **EP43**: Protocols with optimal results were based on HIER using TRS High pH (Dako) (3/3) or CC1 (Ventana) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:25–1:100. Using these protocol settings, 4 of 4 (100%) laboratories produced an optimal staining.

Table 3. **Proportion of optimal results for MLA for the most commonly used antibody as concentrate on the 4 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 **A103**	9/16** (56%)	-	0/5 (0%)	-	9/54 (17%)	-	4/15 (27%)	0/2
rmAb clone **EP43**	-	-	3/3	-	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 **A103**, product no. **790-2990**, Ventana/Roche, BenchMark GX / XL / ULTRA:
Protocols with optimal results were typically based on 36-64 min. HIER using Cell Conditioning 1 (CC1, Ventana), 16-52 min. incubation of the primary Ab and UltraView (Ventana 760-500) with amplification (760-080), OptiView (Ventana 760-700) or OptiView (Ventana 760-700) with amplification (760-099 / 860-099) or UltraView Universal Alkaline Phosphatase Red Detection Kit (760-501) with amplification (760-080) as detection system. Using these protocol settings, 27 of 30 (90%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **A103**, product no. **IR633/IS633,** Dako/Agilent, Autostainer Classic/Link:
Protocols with optimal results were all based on HIER in PT-Link using Target Retrieval Solution pH 9 (3-in-1), 20 - 30 min. incubation of the primary Ab and EnVision Flex (K8000) or Envision Flex+ (K8002) as detection system. Using these protocol settings, 30 of 32 (94%) laboratories produced a sufficient staining (optimal or good).

mAb clone **A103,** product.no. **PA0233**, Novocastra/Leica, BOND III/MAX:
One protocol with an optimal result was based on HIER using Bond Epitope Retrieval Solution pH 6, 15 min. incubation of the primary Ab and BOND Polymer Refine Detection (DS9800) as detection system with prolonged incubation time in the polymer (30 min.).

mAb clone **A103** product no. **API 3114**, Biocare, intelliPATH:
One protocol with an optimal result was based on HIER using Diva Decloaker pH 6.2 in a Pressure Cooker (efficient heating time 15 min. at 110°C), 30 min. incubation of the primary Ab and MACH4 Universal HRP-Polymer (M4U534) as detection system.

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.

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

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT mAb A103 **790-2990**	0% (0/2)	0% (0/2)	64% (37/58)	34% (20/58)
Dako AS mAb A103 **IR633/IS633**	100% (16/16)	69% (11/16)	88% (14/16)	50% (8/16)

* 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 integrated.

Comments
In this assessment and in concordance with the previous NordiQC assessments for MLA, the prevalent feature of an insufficient staining was a general too weak or false negative staining reaction of cells and structures expected to be demonstrated. This was observed in 94% of the insufficient results (97 of 103) and was almost (94 of 97) restricted to protocols based on the mAb clone A103 either on its own or in combination with other clones. In general, almost all laboratories could detect MLA in normal skin and the malignant melanoma, tissue core no. 4, whereas demonstration of MLA in the malignant melanoma, tissue core no. 5, and the granulosa cell tumour (A103 clone only) was much more challenging and required an optimally calibrated protocol.

48% (123 of 255) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for MLA. The mAb clone A103 was the most widely used antibody, as seen in table 1. Optimal results could be obtained on the three most widely used IHC platforms. However, as previously observed, the performance of the mAb clone A103 seemed to be related to the IHC platform (see table 3). If the Ab was applied on the Dako Autostainer (diluted in the range of 1:20-1:200 and HIER in an alkaline buffer), 56% of the laboratories obtained an optimal staining result. In comparison, only 17% of the laboratories using the same clone and similar protocol settings on the Ventana BenchMark platform obtained an optimal result. A similar trend was seen on the Dako Omnis platform where no laboratories obtained an optimal result. Several parameters could have contributed to this difference e.g. sensitivity of the detection systems used and/or other reagents used. Furthermore, it is well known, that the staining reaction of clone A103 in granulosa cell tumours and other steroid producing cells/structures is related to a cross reaction of the clone to an unknown antigen. However, the cross reaction of clone A103 in steroid producing cells and tumours (adrenal cortical and non-epithelial tumours) is widely used in diagnostics and thus the staining performance in these cells is included in the overall assessment of MLA in NordiQC. Laboratories obtaining sufficient results with mAb clone A103 on the Ventana Benchmark platform all used UltraView-HRP or UltraView-AP both with amplification or OptiView-HRP as detection system in combination with an average diluting factor of 1:41, an average incubation time of 38 min. and an average heating time in CC1 for 62 min. Compared to the laboratories that did not pass the average dilution factor was 1:115, incubation time 31 min. and heating time 46 min. These data indicates that the optimization focus for mAb clone A103 must be titre, sufficient incubation time to secure robust binding of the Ab to all

antigenic sites, efficient HIER and use of a sensitive detection system. All these precautions in addition to the platform dependence might explain the low proportion of optimal results for this Ab.

The recently introduced rmAb clone EP43 showed very promising results. Four laboratories used this new clone and all obtained optimal results. Although the rmAb clone EP43 shows no cross reaction to steroid producing cells, data from this assessment and internal NordiQC validation tests indicates, that EP43 might be the most sensitive marker for MLA (see Fig. 7) in melanomas.

52% (132 of 255) of the laboratories used Abs in Ready-To-Use formats. The most widely used RTU systems for MLA were Ventana **790-2990** and Dako **IR633/IS633**. Both are based on the clone mAb A103. Unlike previous assessments, the overall pass rate was quite similar for the two RTU systems. The Dako IR633/IS633 system had an overall pass rate of 65% (37 of 57 laboratories) compared to a pass rate of 62% (37 of 60) for the Ventana 790-2990 system. In Run 42, 2014, the pass rates were 91% and 56% respectively. An explanation for the decline in the overall pass rate for the Dako system could be the recent introduction of the Dako Omnis immunostainer, which was not in use in 2014. In the present run, 15 laboratories used the IR633/IS633 system on a Dako Omnis and only 2 laboratories passed. It is important to stress that the IR633/IS633 system has no officially recommended protocol settings for the Dako Omnis (Fig. 1 – Fig. 4). Recommended protocol settings from Dako are only available for the Dako Autostainer. Only 56% of the laboratories (32 of 57) used the recommended stainer platform. 50% of these laboratories (16 of 32) used the IR633/IS633 system according to the recommended protocol settings, and they all passed, with 69% (11 of 16) being optimal (table 4). The remaining 16 laboratories using laboratory modified protocol settings on the Dako Autostainer had a pass rate of 88%.
60 laboratories used the mAb A103 as RTU system (790-2990) from Ventana, but only two laboratories used the recommended protocol settings and both got insufficient results (see table 4). The recommended protocol settings are based on the use of UltraView-AP without amplification as detection system, but among 13 laboratories using this detection system, the pass rate was only 7% (1 of 13) and none achieved optimal mark. In contrast, 12 laboratories using UltraView-AP with amplification, 16-32 min. incubation of the primary Ab and HIER in CC1 for 36-64 min., all achieved optimal marks (100%). These data strongly support that Ventana/Roche needs to revise the recommended protocol settings for the RTU system 790-2990 from UltraView-AP to UltraView-AP with amplification (see Fig. 5 – Fig. 6). With similar incubation times and HIER settings, smaller proportions of optimal marks could also be achieved with both UltraView-HRP with amplification (40%) and OptiView with or without amplification (31%).

Controls
Adrenal gland is a recommendable positive tissue control for MLA when using the mAb clone A103.
A moderate to strong granular cytoplasmic staining reaction must be seen in virtually all epithelial cells throughout the adrenal cortex. However, this reaction pattern can only be applied when a non-biotin based detection system is used, as adrenal cortical cells are rich on endogenous biotin and a false positive staining reaction will thus mimic the specific reaction and eliminate the potential as a reliable positive control. For the rest of the MLA clones (without cross reactivity towards steroid producing cells) normal skin and melanomas with low MLA expression must be used as positive controls. In normal skin, virtually all melanocytes should show a strong positive reaction in the cytoplasm and weak to moderate reaction in the melanocytic dendrites in most melanocytes (see Fig. 8). Kidney is recommended as negative tissue control. No staining should be seen in the epithelial cells of tubules. Scattered epithelial cells may show a granular staining reaction caused by lipofuscin.

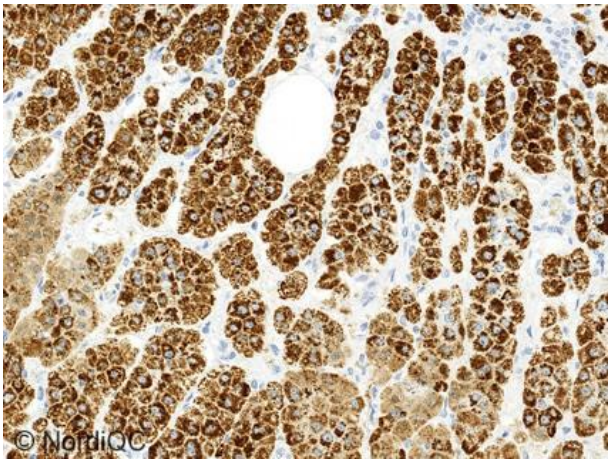


Fig. 1a (x200)
Optimal MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (Dako IR633/IS633) with an incubation time of 20 min., HIER in TRS High pH 9 for 10 min., 2-step polymer based detection kit (EnVision Flex) and performed on Autostainer Link, Dako. Virtually all cortical epithelial cells show a moderate to strong, distinct, granular cytoplasmic staining reaction. No background reaction is seen.
Also compare with Figs. 2a – 4a, same protocol.

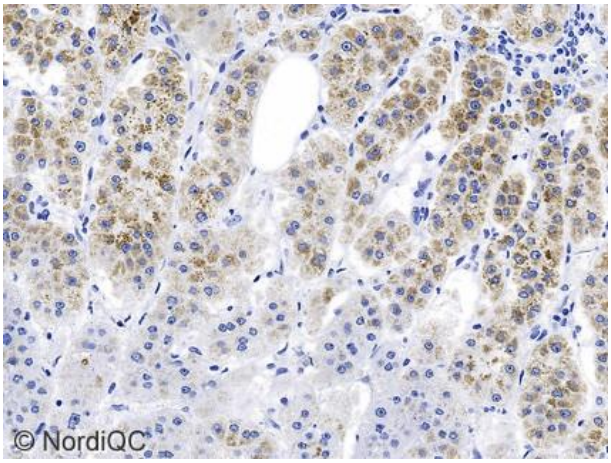


Fig. 1b (x200)
MLA staining of the adrenal gland using an insufficient protocol. Using the mAb clone A103 in a RTU format (Dako IR633/IS633) in similar settings as in Fig. 1a, but on the Dako OMNIS instrument. The majority of cortical epithelial cells are demonstrated, but the intensity is significantly reduced. Compare with Fig. 1a. – same field. Also compare with Figs. 2b - 4b – same protocol.

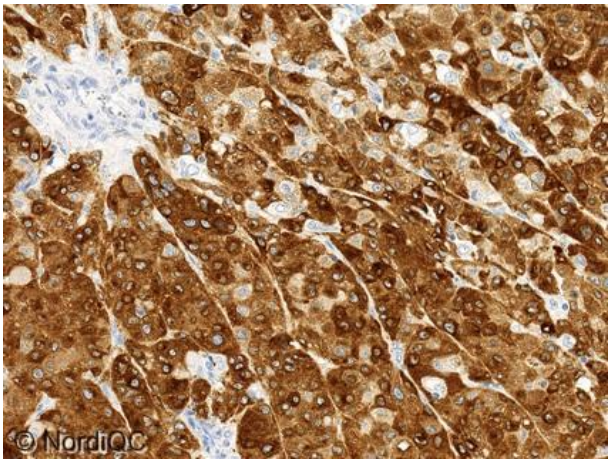


Fig. 2a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 4 (high-level expressor), using same protocol as in Fig. 1a. All the neoplastic cells show a moderate to strong cytoplasmic staining reaction.
No background reaction is seen.

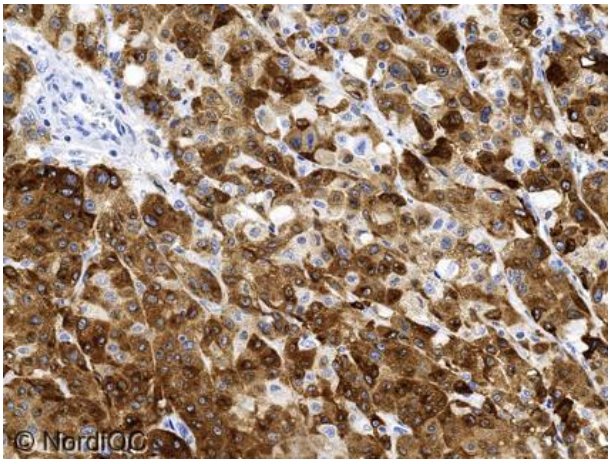


Fig. 2b (x200)
MLA staining of the malignant melanoma, tissue core no. 4 (high-level expressor), using same protocol as in Fig. 1b – same field as in Fig. 2b. The majority of neoplastic cells are demonstrated, but the intensity is slightly reduced compared to Fig. 2a.
However, compare with Fig. 3b and 4b, same protocol.

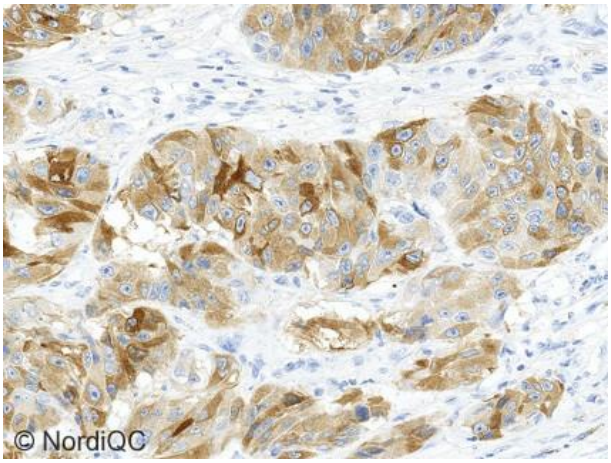


Fig. 3a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using same protocol as in Figs. 1a and 2a.
The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction.
No background reaction is seen.

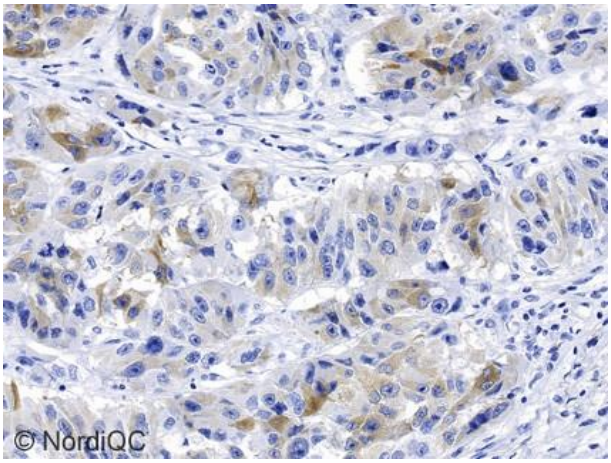


Fig. 3b (x200)
Insufficient MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using the same protocol as in Figs. 1b and 2b.
Only a few scattered neoplastic cells display a very faint staining reaction. Compare with Fig. 3a – same field.

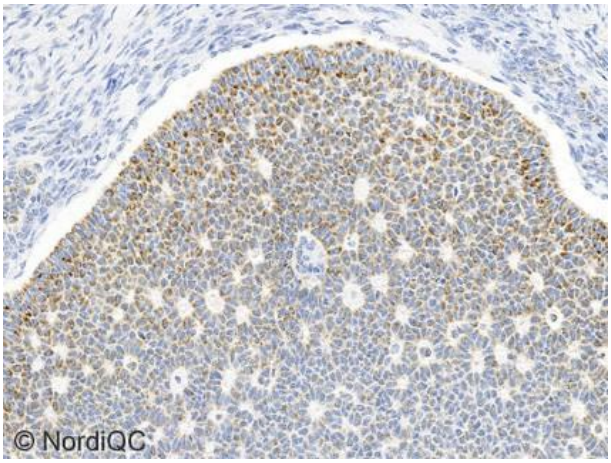


Fig. 4a (x200)
Optimal MLA staining of the granulosa cell tumor using same protocol as in Figs. 1a - 3a.
The majority of the neoplastic cells show a weak to moderate granular cytoplasmic staining reaction.
No background reaction is seen.

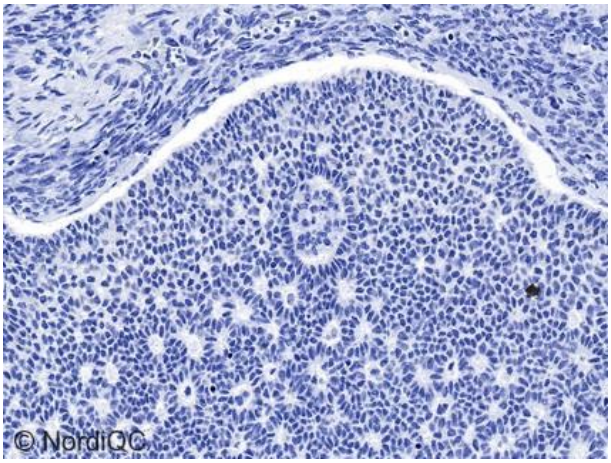


Fig. 4b (x200)
Insufficient MLA staining of the granulosa cell tumor using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
All the neoplastic cells are negative.

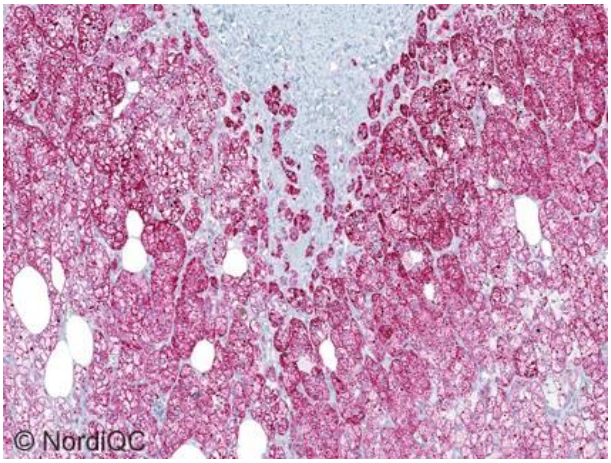


Fig. 5a (x100)
Optimal MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (790-2990) with an incubation time of 32 min., HIER in **CC1 for 64 min**., and **UltraView AP-RED (760-501) with amplification** as detection system and performed on the BenchMark Ultra. Virtually all cortical epithelial cells show a moderate to strong, distinct, granular cytoplasmic staining reaction.

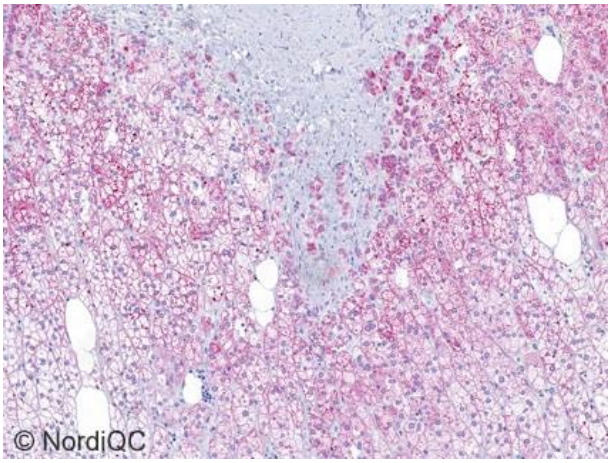


Fig. 5b (x100)
Insufficient MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (790-2990) with an incubation time of 32 min., HIER in **CC1 for 36 min**., and **UltraView AP-RED (760-501)** as detection system and performed at the BenchMark Ultra. The combination of relative short HIER and a detection system without amplification results in a significantly reduced intensity and proportion of cortical epithelial cells demonstrated.
Compare with Fig. 5a (same field)

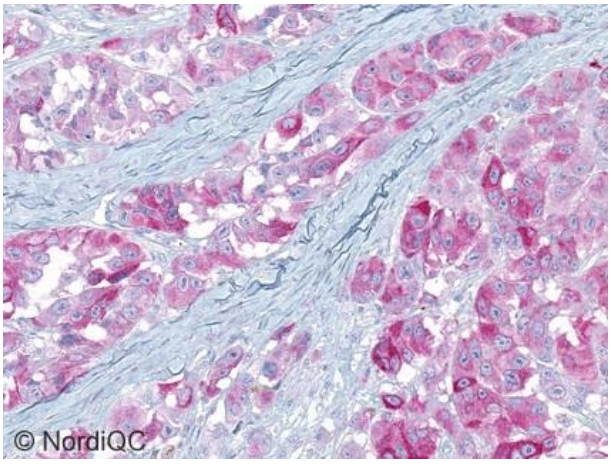


Fig. 6a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using same protocol as in Fig. 5a.
The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction.
No background reaction is seen.

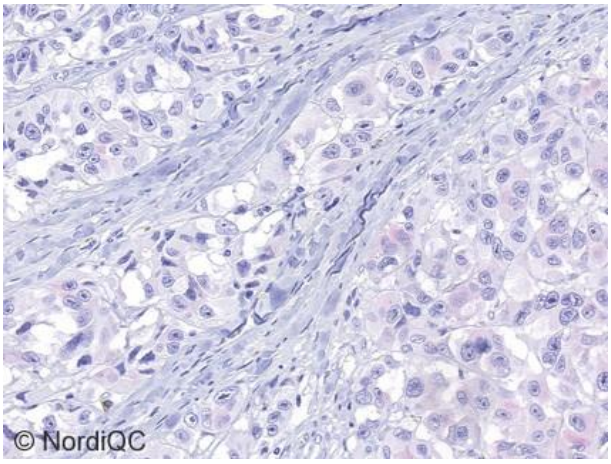


Fig. 6b (x200)
Insufficient MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using the same protocol as in Figs 5b.
The neoplastic cells are false negative.
Compare with Fig. 6a – same field.

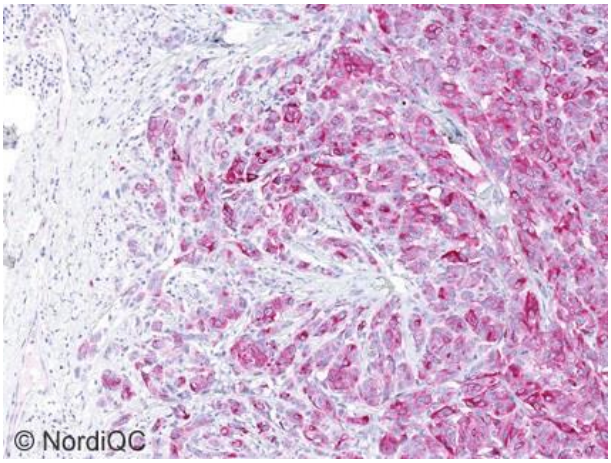


Fig. 7a (x100)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using mAb A103 by the same protocol as in Fig. 5a.
The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction. Although this reaction is the strongest seen (and assessed as optimal) with clone mAb A103, it is clearly weaker than the reaction obtained with clone rmAb EP43. Compare with Fig. 7b. (same field)

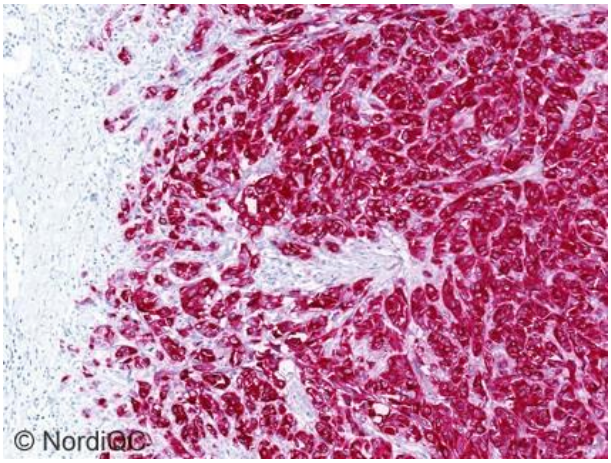


Fig. 7b (x100)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using rmAb EP43 in similar protocol settings as in Fig. 5a.
All neoplastic cells show a moderate to strong cytoplasmic staining reaction. No background reaction is seen.
In melanomas, rmAb EP43 in general seem to increase the analytical sensitivity compared to mAb A103. Compare with mAb A103 in Fig. 7a.

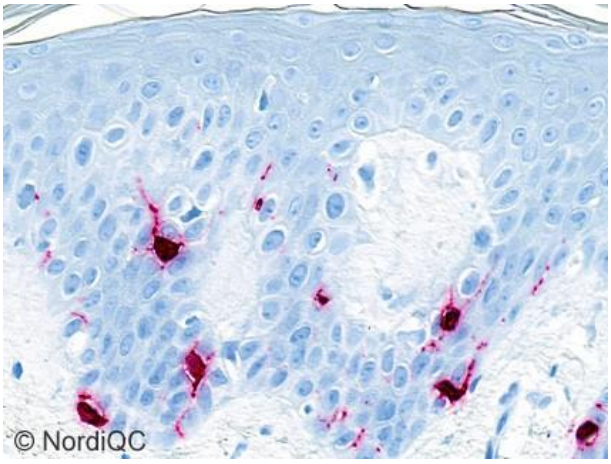


Fig. 8 (x400)
Optimal MLA staining in normal skin using rmAb EP43. All melanocytes show a strong cytoplasmic staining reaction. Melanocytic dendrites are weakly to moderately labelled. No background reaction is seen.

ON/SN/LE/RR 12.04.2017