Assessment Run 51 2017
# α-methylacyl-CoA racemase (AMACR, P504S)

Material
The slide to be stained for AMACR comprised:

1. Colon, 2. Kidney, 3. Tonsil, 4. Prostate intraepithelial neoplasia (PIN),
5. Prostate hyperplasia, 6. Prostate adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a AMACR staining as optimal included:

- A strong, distinct granular cytoplasmic staining reaction of epithelial cells
  lining both the basal and luminal compartment of the crypt epithelium in the colon.
- A strong, distinct granular cytoplasmic staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma.
- A strong, distinct granular cytoplasmic staining reaction of epithelial cells lining the renal proximal tubules.
- An at least weak, distinct granular cytoplasmic staining reaction of epithelial cells lining the renal distal tubules.
- An at least moderate, distinct granular cytoplasmic staining reaction of virtually all neoplastic cells in the PIN.
- A negative or only focal, weak granular cytoplasmic staining reaction of epithelial cells in the hyperplastic prostate glands.
- No staining of other cells including lymphocytes, macrophages and squamous epithelial cells in the tonsil.
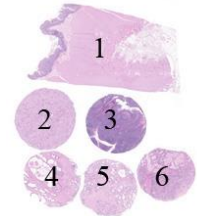
In this run, and for participants using PIN-cocktails (e.g. P63+AMACR/P504s), only the specific reactions for AMACR were assessed.

Participation

Number of laboratories registered for AMACR, run 51	266
Number of laboratories returning slides	250 (94%)

Results
250 laboratories participated in this assessment. 233 (93%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and the assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Insufficient HIER (inappropriate buffer and/or too short efficient heating time)
- Use of less sensitive detection systems

Performance history
This was the third NordiQC assessment of AMACR. The overall pass rate was high and comparable to the results obtained in the previous assessments for AMACR (see Table 2).

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

	Run 16 2006	Run 26 2009	Run 51 2017
Participants, n=	65	106	250
Sufficient results	89%	90%	93%

Conclusion
The rmAb clones **13H4** and **SP116,** and the mAb clone **EPMU1,** and the pAb **p504S/CP200** were all robust Abs for demonstration of AMACR. Irrespective of the primary Ab applied, efficient HIER preferable in an alkaline buffer and careful calibration of the primary Ab, in combination with a sensitive IHC system (3-step polymer/multimer system), were the main prerequisites for an optimal staining result.
The combination of kidney and normal/hyperplastic prostate was found to be the most reliable positive and negative tissue controls: In kidney, epithelial cells of proximal tubules must display a strong distinct

granular cytoplasmic staining, whereas epithelial cells of the distal tubules must show a weak, but distinct granular cytoplasmic staining reaction. In the normal/hyperplastic prostate, epithelial cells of the glands must be negative or only focally, display a weak granular reaction pattern.

Table 1. **Antibodies and assessment marks for AMACR, run 51**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
rmAb clone **13H4**	102 13 8 6 3 2 1 1	Agilent/Dako Cell Marque Zeta Corporation Biologo Immunologic Thermo/NeoMarkers BioSB Diagnostic Biosystem	78	51	7	0	95%	96%
mAb clone **EPMU1**	8	Leica/Novocastra	2	4	1	1	75%	100%
mAb clone **BS2**	1	Nordic Biosite	0	0	1	0	-	-
pAb P504S **CP200**	4	Biocare Medical	4	0	0	0	-	-
PIN-Cocktails[3]								
rmAb clone **13H4** + mAb clone **4A4** + mAb clone **D5/16 B4**	1	Agilent/Dako	0	1	0	0	-	-
pAb **P504s** + mAb clone **4A4**	1	Zytomed Systems	0	1	0	0	-	-
Ready-To-Use antibodies								
rmAb clone **13H4 IS/IR060**	26	Agilent/Dako	13	13	0	0	100%	100%
rmAb clone **13H4**[4] **IS/IR060**	10	Agilent/Dako	6	4	0	0	100%	-
rmAb clone **13H4 GA060**	22	Agilent/Dako	14	7	1	0	95%	100%
rmAb clone **13H4**[5] **GA060**	4	Agilent/Dako	3	0	0	1	-	-
rmAb clone **13H4 MAD-000305QD**	5	Master Diagnostica	1	3	1	0	-	-
rmAb clone **13H4 504R-10-ASR**	4	Cell Marque	3	1	0	0	-	-
rmAb clone **13H4 AN449-5M**	2	Biogenex	1	0	1	0	-	-
rmAb clone **13H4 RMA-0546**	1	Maixin	1	0	0	0	-	-
rmAb clone **13H4 MON-RTU1167**	1	Monosan/Sanbio	1	0	0	0	-	-
rmAb clone **13H4 RM-9130-R7**	1	Thermo S./Neomarkers	0	0	1	0	-	-
rmAb clone **SP116 790-6011**	14	Roche/Ventana	5	8	1	0	93%	100%
mAb clone **EPMU1 PA0210**	2	Leica/Novocastra	1	0	0	1	-	-
pAb **P504S PP/APA200**	2	Biocare Medical	1	1	0	0	-	-
pAb **P504S RBG002**	1	Zytomed System	1	0	0	0	-	-
Unknown 8291-C010	1	Sakura Finetek USA	1	0	0	0	-	-
PIN-Cocktails[3]								
rmAb clone **13H4** + mAb clone **4A4 PIN001-G**	1	Biologo	0	1	0	0	-	-
rmAb clone **13H4** + mAb clone **4A4** + mAb clone **34BE12 PIN002-G**	1	Biologo	1	0	0	0	-	-
mAb clone **XM26** + mAb clone **LL002** + mAb clone **4A4** + pAb **P504S**	1	Biocare Medical	1	0	0	0	-	-

PPM225							
Total	250		138	95	14	3	-
Proportion			55%	38%	6%	1%	93%

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Only the specific reaction for AMACR was assessed.
4) RTU system developed for the Agilent/Dako semi-automatic system (Autostainer) but used by laboratories on different platforms (e.g. Leica BOND III/Max) or manually.
5) RTU system developed for the Agilent/Dako full-automated systems (Omnis) but used by laboratories on different platforms (e.g. Ventana Benchmark Ultra/XT).

Detailed analysis of AMACR, Run 51
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
rmAb **13H4**: Protocols with optimal results were based on heat induced epitope retrieval (HIER) using Target Retrieval Solution (TRS 3-in-1) pH 9 (Dako/Agilent) (8/11) *, TRS pH 9 (Dako/Agilent) (3/5), Cell Conditioning 1 (BenchMark, Ventana) (47/84), Bond Epitope Retrieval Solution 2 (Bond, Leica) (16/21), TRIS-EDTA/EGTA pH 9 (3/7) or Citrate pH 6.7 (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 100 of 104 (96%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb **EPMU1**: Protocols with optimal results were based on HIER using BERS2 (Leica) (2/6) as retrieval buffer and the mAb was diluted 1:50. Using these protocol settings, 2 of 2 (100%) laboratories produced a sufficient staining (optimal or good).

pAb **p504S, CP200**: Protocols with optimal results were based on HIER using CC1 (Ventana) (4/4). The pAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed.

Table 3. **Proportion of optimal results for AMACR for the most commonly used antibody as concentrate on the 3 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
rmAb **13H4**	9/11** (82%)	-	1/2	-	40/71 (56%)	-	13/16 (81%)	-

* 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
rmAb clone **13H4**, product no. **IS/IR060**, Agilent/Dako, Autostainer:
Protocols with optimal results were based on HIER using TRS High pH 9 (K8004 or S2375) (efficient heating time 10-40 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX or Flex+ (K8000/K8002 + K8009) as detection systems. Using these protocol settings, 21 of 21 (100%) laboratories produced a sufficient staining result. One protocol with an optimal result was based on no pre-treatment at all, incubated with primary Ab for 30 min and used EnVision FLEX (K8000) as detection system.

rmAb clone **13H4**, product no. **GA060**, Agilent/Dako, Omnis:
Protocols with optimal results were based on HIER using TRS High pH 9 (GV804) (efficient heating time 24-30 min. at 97°C), 10-30 min. incubation of the primary Ab and EnVision FLEX+ (GV800 + GV809) as the detection system. Using these protocol settings, 20 of 20 (100%) laboratories produced a sufficient staining result. One protocol with an optimal result was based on no pre-treatment at all, incubated with primary Ab for 30 min. and used EnVision FLEX (GV800/823) as detection system.

rmAb clone **SP116,** product no. **790-6011,** Ventana, Benchmark Ultra/XT:
Protocols with optimal results were based on HIER using CC1 (efficient heating time 56-64 min. at 95-100°C), 16-32 min. incubation of the primary Ab and OptiView (760-700) as detection system. Using these protocol settings, 5 of 5 (100%) laboratories produced an optimal staining result.

mAb clone **EPMU1,** product no. **PA0210,** Leica/Novocastra, BOND III/BOND MAX:
One protocol with an optimal result was based on HIER using Epitope retrieval solution pH 9/RE7119-CE (efficient heating time 15 min. at 120°C), 15 min. incubation of the primary Ab and BOND Refine (DS9800) as detection system.

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 were included.

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

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS rmAb **IS/IR060**	100% (8/8)	63% (5/8)	100% (15/15)	53% (8/15)
Dako Omnis rmAb **GA050**	1/1	1/1	94% (17/18)	67% (12/18)
VMS Ultra/XT rmAb **790-6011**	2/2	0/2	92% (11/12)	42% (5/12)

* 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 AMACR assessments, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 88% (15 of 17) of the insufficient results. Most participating laboratories were able to demonstrate AMACR in neoplastic cells of the prostate adenocarcinoma, whereas the demonstration of AMACR in the distal tubules of the kidney and in the neoplastic cells of the PIN was more challenging and only seen with appropriate protocol settings. For the remaining insufficient results, the staining patterns were characterized by false positive staining reaction due to contamination with smooth muscle cell marker (e.g. actin) compromising interpretation.

60% (151 of 250) of the laboratories used concentrated Ab format within laboratory developed (LD) assays for AMACR including PIN-cocktails. The rmAb 13H4 was the most widely used Ab and used, within a LD assay, the rmAb 13H4 gave an overall pass rate of 95% (129 of 136) and 57% (78 of 136) optimal. The rmAb 13H4 could be used to obtain optimal staining results on the respective automatic platforms from the three major vendors as shown in Table 3.
HIER, preferable in an alkaline buffer in combination with a careful calibration of the primary Ab seem to be the most critical parameters for a sufficient and optimal result. In this run, optimal results could be obtained by using a 2-step multimer/polymer detection system (e.g. UltraView/Ventana or Flex/Dako). However, the general performance of the assays was significant improved by applying a 3-step multimer/polymer detection system (e.g. OptiView with or without amplification/Ventana or Flex+/Dako). Using optimal protocol settings for the rmAb 13H4 as concentrate and dividing protocols into groups based on the assessment score, 68% (44 of 65) and 32% (21 of 65) of protocols with optimal results applied a 3-step multimer/polymer or a 2-step multimer/polymer detection system, respectively. In comparison, for protocols assessed as good, the proportion of laboratories using a 3-step multimer/polymer declined to 45% (18 of 40), whereas the number of assays based on 2-step multimer/polymer detection system increased to 55% (22 of 40). None of the laboratories obtaining an insufficient result used a 3- step multimer/polymer detections system.

Eight laboratories used the mAb EPMU1 within a LD assay and 75% (6 of 8) produced a sufficient result of which 25% (2 of 8) were assessed as optimal. The mAb was mainly applied on the Leica BOND platforms, BOND-III/MAX (6 of 8), giving an overall pass rate of 83% (5 of 6) of which two were assessed as optimal. All used HIER in BERS2 as retrieval buffer and Bond Refine as detection system. The two optimal protocols used the primary Ab in relative high concentration (1:50), whereas the protocols assessed as good or borderline used lower concentrations (dilution range of 1:200-1:800). The two remaining assays were performed on the Benchmark Ultra (Ventana), using HIER in CC1 and UltraView with or without amplification as detection systems. One of these was assessed as sufficient (good).

All protocols (4 of 4) based on the pAb P504S, CP200 were assessed as optimal. All were stained on Ventana platforms (Bencmark Ultra or XT) using HIER in CC1 as retrieval buffer (efficient HIER time 36-64 min. at 95-100°C), dilution range 1:50-1:200 of the primary Ab and OptiView or UltraView as detection system.

40% (99 of 250) of the laboratories used Ready-To-Use (RTU) systems for detection of AMACR including PIN-cocktails. In general, the RTU systems from the two major vendors (Agilent/Dako and Roche/Ventana) all gave a high proportion of sufficient and optimal results. Both vendor and laboratory modified protocol settings could be used to produce a sufficient result. Following protocol recommendations, as provided by the respective vendors, a pass rate of 100% was obtained (see Table 4).

The RTU systems IS/IR/GA060 based on rmAb clone 13H4 (Dako) was used by 48 laboratories. An overall pass rate of 98% (47 of 48) was seen and 56% (27 of 48) were optimal. In this assessment, the RTU system IS/IR060 (Autostainer) provided the highest proportion of sufficient results (100%, 26 of 26) of which 50% (13 of 26) were optimal. The RTU system GA060 (Omnis) provided a slightly lower pass rate of 95% (21 of 22) of which 64% (14 of 22) were optimal. The vendor recommended protocol settings is based on HIER in TRS pH 9 (30 min. at 97°C), incubation of the primary Ab for 17.5 min. and Flex+ (GV800/823 + GV821) as detection system. Only one laboratory followed this recommendation (assessed as optimal). Most laboratories (17 of 18) modified the assays, typically adjusting incubation time in primary Ab or reducing efficient HIER time in TRS pH 9. One protocol assessed as insufficient, used HIER in TRS pH 6.1 (30 min. at 97°C) and reduced incubation time of the primary Ab to 15 min. The RTU systems IS/IR/GA060 were used off-label (other automatic platforms or manually) by 13 laboratories and all were assessed as sufficient (see Table 1).

The Ventana RTU system based on rmAb clone SP116 (790-6011), developed for the BenchMark IHC platforms, was used by 14 participants. An overall pass rate of 93% (13 of 14) was seen and 36% (5 of 14) were optimal. Optimal results could only be obtained by using laboratory modified protocol settings, typically prolonging incubation time in primary Ab, performing HIER in CC1 for 56-64 min. at 95°C and applying OptiView as the detection system. If the stainings were performed, according to the official protocol recommendations provided by Ventana (HIER in CC1 64 min. at 95°C, 16 min. incubation with primary Ab and UltraView (760-501) as the detection system), none of two submitted protocols provided optimal results – both assessed as good. Using all protocol settings, and UltraView with or without amplification as detection systems, the pass rate was 100% (3 of 3) but none were assessed as optimal. One protocol was assessed as insufficient despite using protocol settings similar to assays providing sufficient results.

In total, five laboratories used PIN-cocktails, both within LD assays and as RTU-formats, for detection of AMACR. All were assessed as sufficient and 40% (2 of 5) produced an optimal result.

This was the third NordiQC assessment of AMACR (see Table 2). A pass rate of 93% was obtained which is a minor improvement to 90% in run 26, 2009. The accessibility of robust clones (e.g. rmAb 13H4, rmAb SP116, pAb CP200 and mAb EPMU1), use of efficient HIER preferable in alkaline buffer and careful calibration of the primary Ab, accounts for the overall high pass rate in this run.

Controls
Kidney is recommended as positive tissue control for AMACR: Virtually all epithelial cells of the proximal tubules must show a strong and distinct granular cytoplasmic staining, whereas epithelial cells of the distal tubules must display a weak granular cytoplasmic staining reaction. Normal prostate is recommended as negative tissue control for AMACR: The epithelial cells must be negative or only show a focal staining reaction.
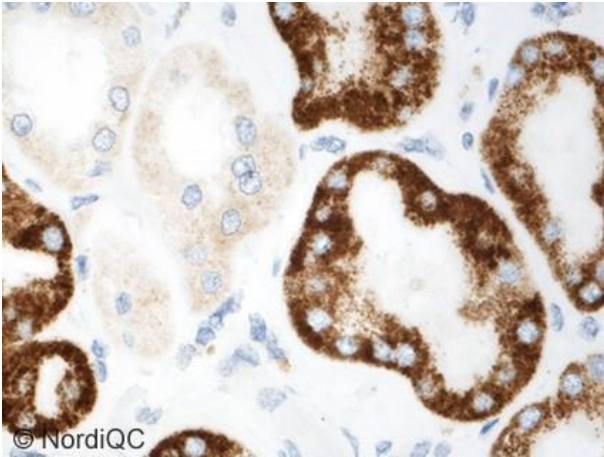
Fig. 1a (x600)
Optimal staining reaction for AMACR of kidney using the rmAb 13H4 as concentrate on the Omnis, HIER in an alkaline buffer (TRS pH 9) and a 3-step polymer based detection system (FLEX+, Dako) - same protocol used in Figs. 2a-5a. The epithelial cells of the proximal tubules show a strong granular cytoplasmic staining, whereas the distal tubules only display a weak granular cytoplasmic staining reaction.
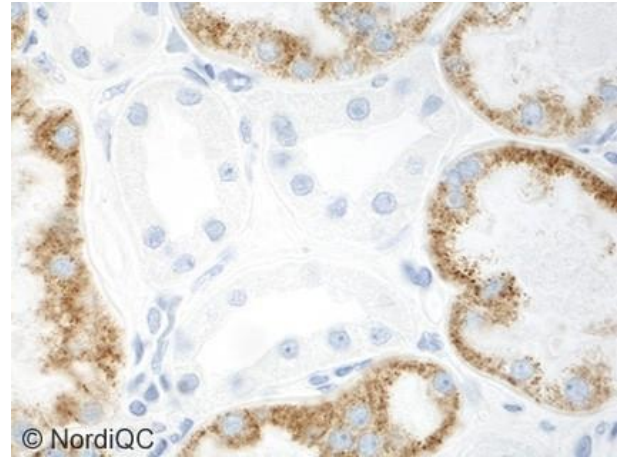

Fig. 1b (x600)
Insufficient staining reaction for AMACR of kidney using the rmAb 13H4 as concentrate (too diluted) on the Omnis, HIER in an alkaline buffer (TRS pH 9) and the less sensitive 2-step polymer based detection system (FLEX, Dako) - same protocol used in Figs. 2b-5b. The intensity of the staining reaction is significantly reduced, and the distal tubules are false negative - compare with Fig. 1a (same field).
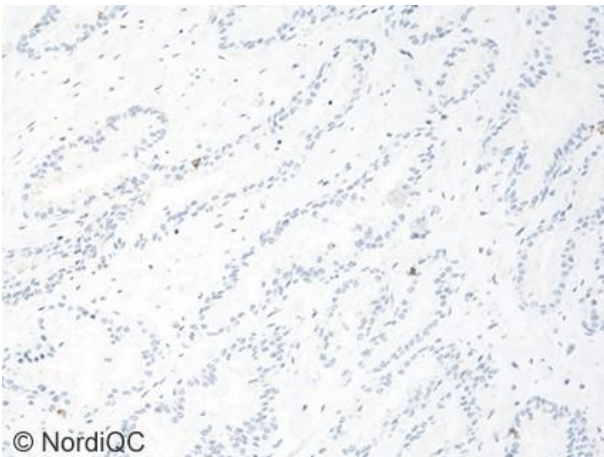
Fig. 2a (x200)
Optimal staining reaction for AMACR of the normal/hyperplastic prostate using the same protocol as in Fig. 1a. The epithelial cells of the glands are negative or only focally, displays a weak granular cytoplasmic staining reaction.
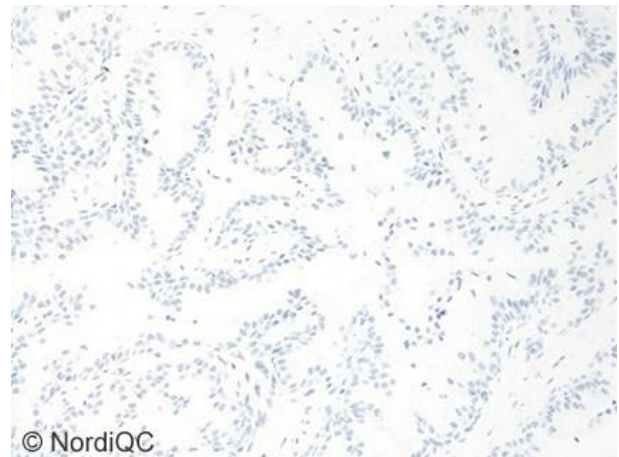

Fig. 2b (x200)
Insufficient staining reaction for AMACR of the normal/hyperplastic prostate using same protocol as in Fig. 1b. Although the staining pattern is as expected the protocol provided too low sensitivity - compare with Fig. 2a (same field), but also with Figs. 1a-1b, 3a-3b, 4a-4b and 5a-5b.
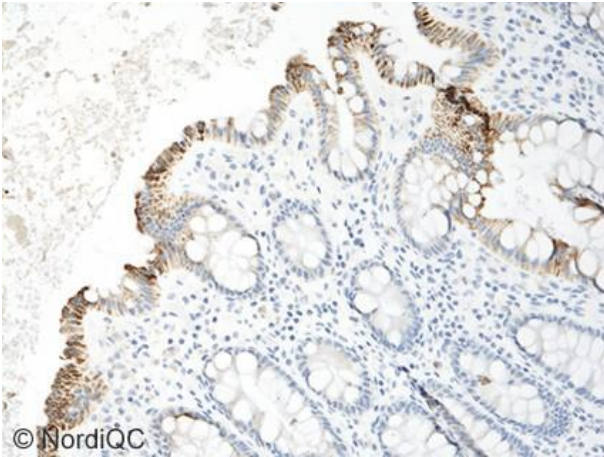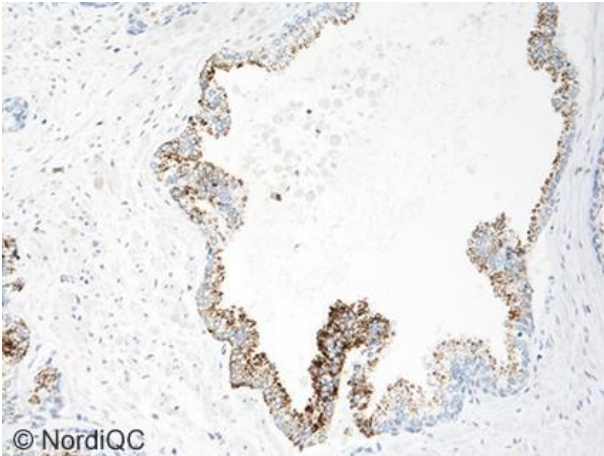
Fig. 3a (x200)
Optimal staining reaction for AMACR of colon using same protocol as in Figs. 1a - 2a. The vast majority of luminal epithelial cells display a strong, distinct granular cytoplasmic staining reaction. The same staining pattern was also seen in the basal compartment of the colon crypts.
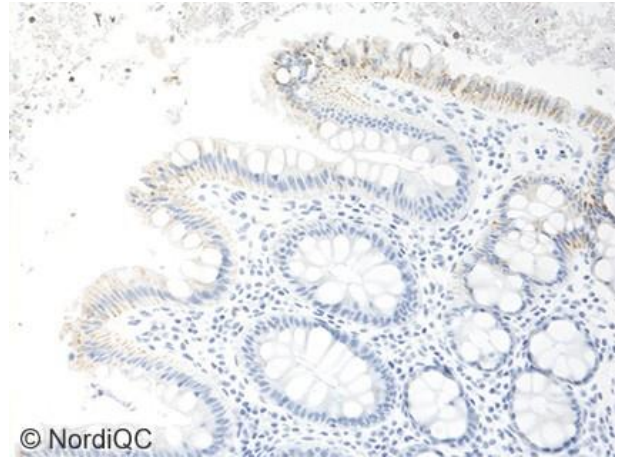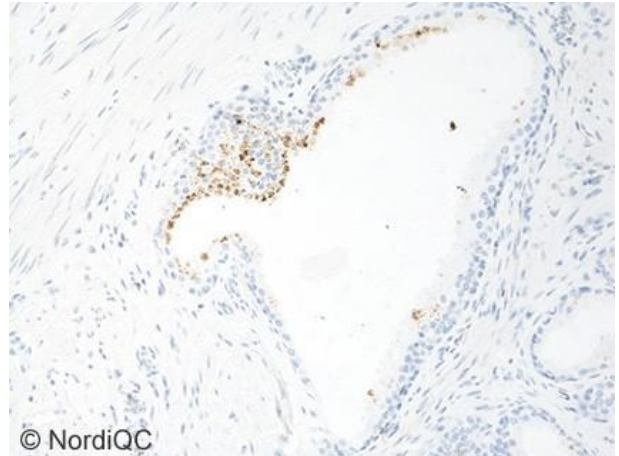

Fig. 3b (x200)
Insufficient staining reaction for AMACR of the colon using same protocol as in Figs. 1b - 2b. The luminal epithelial cells show a faint to weak staining reaction and the proportion of stained cells is reduced - compare with Fig. 3a (same field).

Fig. 4a (x200)
Optimal staining reaction for AMACR of the PIN using same protocol as in Figs. 1a - 3a. The majority of neoplastic cells displays a moderate to strong, distinct granular staining reaction.

Fig. 4b (x200)
Insufficient staining reaction for AMACR of the PIN using same protocol as in Figs. 1b - 3b. The proportion of stained neoplastic cells is reduced, and intensity is too weak - compare with Fig. 4a (same field).
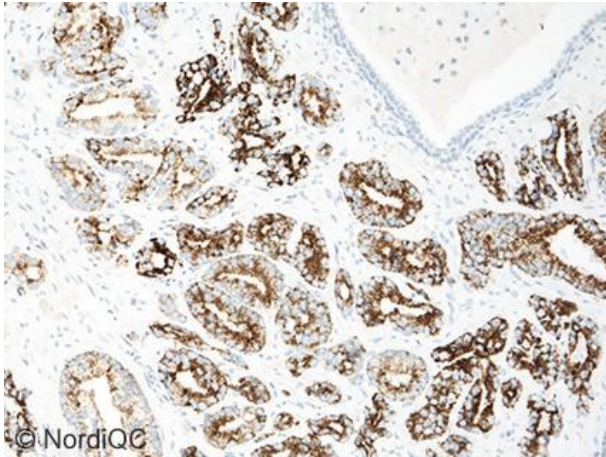
Fig. 5a (x200)
Optimal staining reaction for AMACR of the prostate adenocarcinoma using same protocol as in Figs. 1a - 4a. The vast majority of neoplastic cells displays a moderate to strong, distinct granular staining reaction. Both the basolateral and the apical compartment of the cytoplasm in the neoplastic cells are stained.
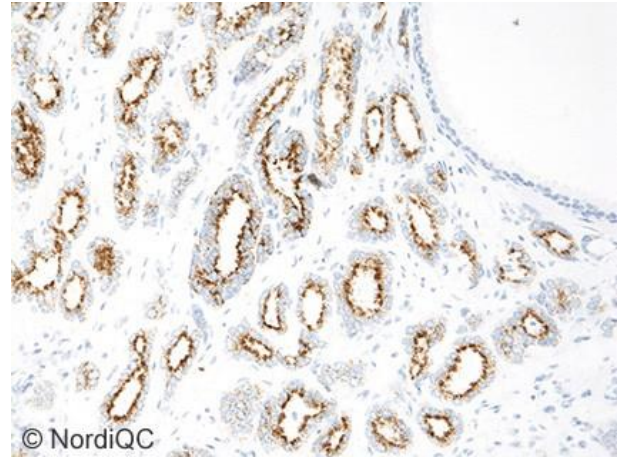

Fig. 5b (x200)
Insufficient staining reaction for AMACR of the prostate adenocarcinoma using same protocol as in Figs. 1b - 4b. Although the majority of the neoplastic glands are stained, intensity is too weak and the staining pattern is primarily restricted to the apical compartment of the cytoplasm in the neoplastic cells - compare with Fig. 5a (same field).
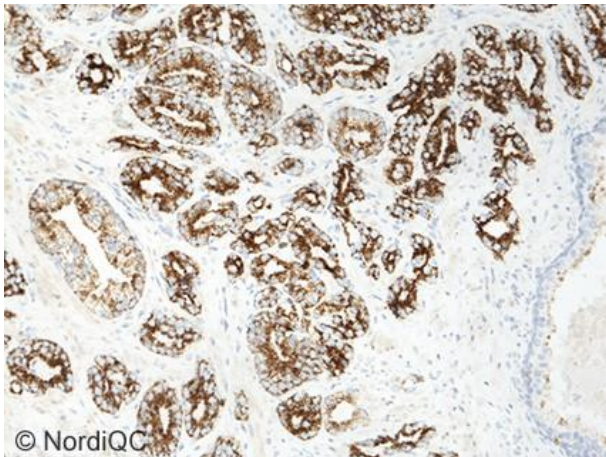

Fig. 6a (x200)
Optimal staining reaction for AMACR of the prostate adenocarcinoma using the rmAb 13H4 as RTU-system (GA060, Omnis, Dako) applying protocol settings as recommended by the vendor: HIER in an alkaline buffer (TRS pH 9) and a 3-step polymer based detection system (FLEX+, Dako). Staining pattern is similar to the reactions obtained in in Fig. 5a - also compare with Fig. 6b.
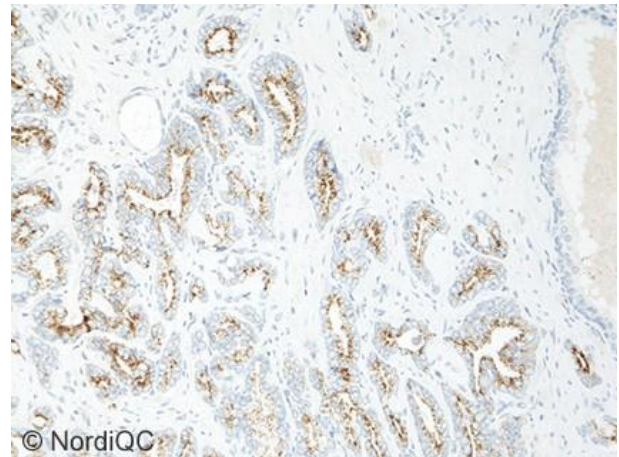

Fig. 6b (x200)
Insufficient staining reaction for AMACR of the prostate adenocarcinoma using the rmAb 13H4 as RTU-system (GA060, Omnis, Dako) with exactly the same protocol settings as in Fig. 6a, except for performing HIER in TRS pH 6.1 and reducing incubation time of the primary Ab. The staining intensity is too weak, and the staining pattern is similar to the reactions obtained in in Fig. 5b - also compare with Fig. 6a.

MB/LE/MV/RR 29.11.2017