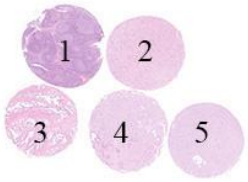
Assessment Run 65 2022

α-methylacyl-CoA racemase (AMACR, P504S)

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for AMACR, typically used in the diagnostic work-up of differentiation of normal prostate glands and prostate intraepithelial neoplasia (PIN) from prostate adenocarcinomas. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for AMACR (see below).

Material
The slide to be stained for AMACR comprised:



1. Tonsil, 2. Kidney, 3. Prostate hyperplasia, 4. Prostate adenocarcinoma, 5. Prostate adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a AMACR staining as optimal included:

- A moderate to strong, distinct granular cytoplasmic staining reaction of epithelial cells lining the renal proximal tubules.
- An at least weak, distinct granular cytoplasmic staining reaction in most of the epithelial cells lining the renal distal tubules.
- A strong, distinct granular cytoplasmic staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma, tissue core no. 4.
- An at least weak to moderate distinct granular cytoplasmic staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma, tissue core no. 5.
- 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. However, in case of PIN cocktails, the expected reaction for the other abs was accepted e.g. p63 in squamous epithelial cells and lymphocytes etc.

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 65	356
Number of laboratories returning slides	334 (94%)

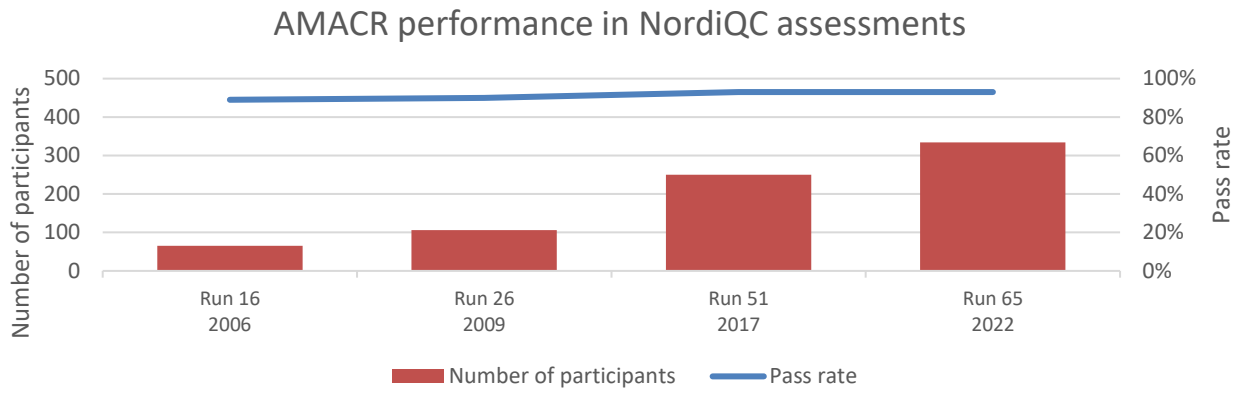
Results
At the date of assessment, 94% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

334 laboratories participated in this assessment and 93% 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 reactions were:
- Too low concentration of the primary antibody
- Insufficient HIER (inappropriate buffer and/or too short efficient heating time)
- Too high concentration of the primary antibody

Performance history
This was the fourth NordiQC assessment of AMACR. A significant increase in participants was observed with a similar pass-rate compared to the previous run (see Graph 1), which primarily is due to the access and use of robust primary antibodies and well calibrated Ready-To-Use (RTU) systems (see Table 1).

Graph 1. **Proportion of sufficient results for AMACR in the four NordiQC runs performed**



Conclusion
The rmAb clones **13H4** and **SP116** were the most widely used and 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 moderate to 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 65**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
rmAb clone **13H4**	86 13 5 2 1 2 1 1 1 1 3 1	Agilent/Dako Cell Marque Zeta Corporation Biologo Thermo/NeoMarkers BioSB BioGenex BioZol DBS Epredia Nordic MUbio Immunologic	85	23	7	2	92%	73%
mAb clone **EPMU1**	4	Leica Biosystems	1	3	-	-	-	-
mAb clone **IHC504**	1	GenemeMe	1	-	-	-	-	-
pAb P504S **ACA200**	3	Biocare Medical	3	-	-	-	-	-
PIN-Cocktails[3]								
pAb **P504s** + mAb clone **4A4**	1	Zytomed Systems	-	1	-	-	-	-
Conc total	126		90	27	7	2	93%	72%
Ready-To-Use antibodies							Suff.[1]	OR.[2]
rmAb clone **SP116 790-6011**[3]	7	Ventana/Roche	4	3	-	-	100%	57%
rmAb clone **SP116 790-6011**[4]	61	Ventana/Roche	47	12	2	-	97%	77%
rmAb clone **13H4 IS/IR060**[3]	11	Dako/Agilent	9	2	-	-	100%	82%
rmAb clone **13H4 IS/IR060**[4]	27	Dako/Agilent	15	12	-	-	100%	56%
rmAb clone **13H4 GA060**[3]	34	Dako/Agilent	30	4	-	-	100%	88%

rmAb clone **13H4** **GA060**[4]	32	Dako/Agilent	29	1	2	-	94%	91%
mAb clone **EPMU1** **PA0210**[3]	3	Leica Biosystems	1	1	1	-	-	-
mAb clone **EPMU1** **PA0210**[4]	7	Leica Biosystems	3	2	2	-	71%	43%
rmAb clone **13H4**[3]	2	Sakura	2	-	-	-	-	-
rmAb clone **13H4** **504R-10-ASR**	3	Cell Marque	3	-	-	-	-	-
rmAb clone **13H4** **MAD-000305QD**	5	Master Diagnostica	2	-	2	1	40%	40%
rmAb clone **13H4** **BSB 5059**	2	Bio SB	2	-	-	-	-	-
rmAb clone **13H4** **OPAA3024**	1	Biocare Medical	-	-	1	-	-	-
rmAb clone **13H4** **AN449-5M**	1	BioGenex	1	-	-	-	-	-
rmAb clone **13H4** **HAR078**	1	PathnSitu	-	1	-	-	-	-
mAb clone **325A9D5** **PA051**	1	Abcarta	1	-	-	-	-	-
mAb clone **C7H4** **CAM-0201**	1	Celnovte	-	-	1	-	-	-
pAb **P504S** **PP/APA200**	1	Biocare Medical	1	-	-	-	-	-
pAb **P504S** **RBG002**	1	Zytomed System	-	-	1	-	-	-
Unknown Clone **P504S** **PA200AA**	1	Fa. Medac	1	-	-	-	-	-
PIN-Cocktails[3]								
rmAb clone **13H4** + mAb clone **4A4** **PIN001-G**	1	Biozol	1	-	-	-	-	-
rmAb clone **13H4** + mAb clone **4A4** + mAb clone **34BE12** **PIN002-G**	1	Biologo	1	-	-	-	-	-
rmAb clone **13H4** + mAb clone **A4A** **MAD-000485**	1	Vitro/Master Diagnostica	1	-	-	-	-	-
mAb clone **XM26** + mAb clone **LL002** + mAb clone **4A4 +** pAb **P504S** **API 225DS AA**	1	Biocare Medical	1	-	-	-	-	-
mAb clone **LL002** + pAb **P504S** **PPM201H**	2	Biocare Medical	1	1	-	-	-	-
RTU total	208		156	39	12	1	94%	75%
Total	334		246	66	19	3	-	
Proportion			74%	20%	5%	1%	93%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Detailed analysis of AMACR, Run 65
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) (3/6)*, TRS pH 9 (Dako/Agilent) (7/8), Cell Conditioning 1 (CC1, Ventana/Roche) (52/71), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (20/26), Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/1), Citrate pH 6 (1/1) or Diagnostic Biosystems EDTA buffer pH 8,0 (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 102 of 110 (93%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

Table 2. **Proportion of optimal results for AMACR for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibody	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT / Ultra**		**Leica Biosystems 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 clone **13H4**	3/5** (60%)	0/1	7/8 (88%)	-	49/68 (72%)	-	19/23 (83%)	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
rmAb clone **SP116,** product no. **790-6011,** Ventana/Roche, Benchmark Ultra/XT:
Protocols with optimal results were based on HIER using CC1 (efficient heating time 32-64 min. at 95-100°C), 16-36 min. incubation of the primary Ab and OptiView (760-700), UltraView DAB (760-500) and UltraView Universal Alkaline Phosphatase Red (760-501) as detection system. Using these protocol settings, 61 of 63 (97%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **13H4**, product no. **IS/IR060**, Dako/Agilent/, Autostainer Link/Classic:
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.

rmAb clone **13H4**, product no. **GA060**, Dako/Agilent, Omnis:
Protocols with optimal results were based on HIER using TRS High pH 9 (GV804) (efficient heating time 20-30 min. at 97°C), 10-30 min. incubation of the primary Ab and EnVision FLEX or EnVision FLEX+ (GV800 + GV809) as the detection system. Using these protocol settings, 59 of 60 (98%) laboratories produced a sufficient staining result.

Table 3 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 according to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **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
VMS Ultra/XT rmA**b SP116 790-6011**	100% (7/7)	57% (4/7)	97% (59/61)	77% (47/61)
Dako AS rmAb **13H4 IS/IR060**	100% (11/11)	82% (9/11)	100% (10/10)	90% (9/10)
Dako Omnis rmAb **13H4 GA050**	100% (34/34)	88% (30/34)	96% (25/26)	96% (25/26)

* 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, 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 60% (12 of 20) of the insufficient results. 35% (7/20) of the insufficient staining results was due to a false positive reaction in the apical compartment of the cytoplasm of the benign epithelial cells in the prostate hyperplasia (see Fig. 6b).
Most participating laboratories were able to demonstrate AMACR in neoplastic cells of the prostate adenocarcinoma, tissue core no. 4, whereas the demonstration of AMACR in the distal tubules of the kidney and in the neoplastic cells of the prostate adenocarcinoma, tissue core no. 5, was more challenging and only seen with appropriate protocol settings.

38% (126 of 334) 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 92% (108 of 117) and 73% (85 of 117) optimal. The rmAb 13H4 could be used to obtain optimal staining results on all four of the main IHC platforms 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/Roche or FLEX, Dako/Agilent). However, the general performance of the assays was improved by applying 3-step multimer/polymer detection systems (e.g. OptiView, Ventana/Roche or FLEX+ (Dako/Agilent) enhancing the demonstration of low level expressing antigens and providing superior signal-to-noise ratio (see Figs. 1a+b-4a+b).

Four laboratories used the mAb EPMU1 within a LD assay on a Bond platform (Leica Biosystems) and all producing a sufficient result but only one marked as optimal. The protocol with optimal result was based on HIER using BERS2 for 30 min., an Ab titer of 1:300, incubation for 15 min. in combination with a 3-step polymer detection system (Refine). The other protocols assessed as good were characterized by a poor-signal-to-noise ratio and most likely caused by a higher titer of 1:100-200 and a longer incubation time for the primary Ab (20 min.), and hereby inducing an excessive background reaction.

The polyclonal P504S Ab from Biocare Medical was used by 3 laboratories, all stained on the Ventana Benchmark platform and all with optimal result. The protocols were based on a dilution factor of 1:50-200, incubation time of 32-44 min., HIER in CC1 for 36-64 min. and UltraView DAB as detection system

62% (208 of 334) of the laboratories used Ready-To-Use (RTU) systems for detection of AMACR including PIN-cocktails. In run 51 the number of laboratories using RTU systems was only 40% with most participants using the Dako/Agilent RTU systems based on the rmAb clone 13H4 from. In this assessment the number of participants using the RTU system based on rmAb clone SP116 from Ventana/Roche has increased from only 6% in run 51 to now 20%.

As shown in Table 3, only 10% used the Ventana/Roche RTU product by the vendor recommended protocol settings applying HIER in CC1 for 64 min., 16 min. incubation time of the primary Ab in combination with UltraView Alkaline Phosphatase Red (760-501) as detection system and all laboratories received a sufficient mark of which, 57% (4/7) being optimal.
It was observed that using the basic vendor recommended protocol settings on HIER and primary Ab incubation time but using OptiView as detection system was found very successful as 100% (n=26) of the protocols based on these settings gave an optimal result. Overall OptiView with DAB as chromogen facilitated the demonstration of AMACR in low expressing structures and being slightly superior to the Alkaline Phosphatase Red system (Figs. 5a+b).

The Dako/Agilent IS/IR060 product based on rmAb clone 13H4 produced for the Autostainer Link 48 and Classic was used by 38 laboratories. 17 laboratories applied the product on other IHC platforms. As shown in Table 3 all of the 21 laboratories using the RTU product on the intended platform received a sufficient mark, 86% optimal (18/21). The high pass rate for the RTU product for both off-label use on non-Autostainer platform (100% sufficient) and within intended use do underline the robustness of the clone. In general, it must be emphasized that modifications of vendor recommended protocol settings for RTU systems including migration of the RTU Abs to another platform than the intended, require a meticulous validation process by the end-users. As seen in this assessment, modifications can be successful but potentially also generate aberrant results and therefore must be carefully monitored.

The Dako/Agilent GA060 product also based on rmAb clone 13H4 produced for the Dako Omnis platform was used by 66 laboratories. 6 used the product on other IHC platforms. Using the vendor recommendations with the EnVision FLEX+ protocol and HIER with TRS high for 30 min. and primary Ab incubation for 10 min., 100% (34/34) of the laboratories received a sufficient result, 88% (30/34) optimal. 26 participants applied a laboratory modified protocol increasing the Ab incubation time to 15-30 min. with and without linker (FLEX) and 96% (25/26) with a sufficient result and all optimal.
Similar to the corresponding RTU format for Autostainer, the product seemed to be robust despite being applied within different settings (see Figs 6a.I-II).

The Leica Biosystems PA0210 RTU system based on mAb clone EPMU1 was used by 9 participants on the intended Bond IHC platform. The vendor recommended protocol based on HIER in BERS2 for 20 min., 15 min. incubation time of primary Ab and Refine as detection system was used by 3 participants, but with mixed result as shown in Table 1. However, the recommended protocol is based on peroxidase blocking after the primary Ab. Unfortunately, the data entry for protocol submission does not entail this information and hence no conclusion on impact on this step can be generated.
Overall, the clone EPMU1 both as RTU and as concentrated format was found less successful as typically a poor signal-to-noise or excessive background reaction was observed.

This was the fourth NordiQC assessment of AMACR (see Graph 1). A pass rate of 93% was obtained which is equal to the last assessment in run 51, 2017, even though the number of participants has increased with 84 new laboratories. From the latest run a big increase in laboratories using the RTU products was observed going from 40% in run 51 to 60% in this run. Among the different clones available most were able to produce a sufficient result both as concentrated formats (93%) and as RTU products (94%).
The high pass rates were supported by adequate vendor protocol recommendations for the RTU products providing optimal results on the intended IHC platforms. However also laboratory modified changes as especially seen for the Ventana/Roche RTU system changing the recommended detection system was found very successful.
The mAb clone EPMU1 form Leica Biosystems was the least successful clone, although able to obtain optimal results. With this clone very careful calibration was necessary to avoid poor-signal-to-noise ratio or background. It still has to be investigated if endogenous peroxidase blocking before or after the primary Ab incubation step will affect the performance of this clone.

Controls
Kidney is recommended as positive tissue control for AMACR: Virtually all epithelial cells of the proximal tubules must show a moderate to strong and distinct granular cytoplasmic staining, whereas epithelial cells of the distal tubules must display a weak granular cytoplasmic staining reaction in the main part of the tubules. Normal prostate is recommended as negative tissue control for AMACR: The epithelial cells must be negative or only show a focal staining reaction.

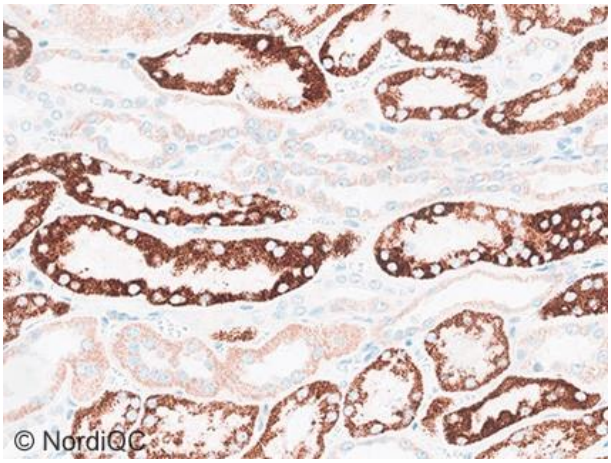


Fig. 1a (x100)
Optimal staining reaction for AMACR of kidney using the rmAb 13H4 as concentrate on the Ventana Benchmark, HIER in an alkaline buffer (CC1 pH 8,5) and a 3-step multimer detection system (Optiview, Ventana/Roche) - same protocol used in Figs. 2a-4a.
The epithelial cells of the proximal tubules show a strong granular cytoplasmic staining, whereas the epithelial cells of distal tubules display a weak granular cytoplasmic staining reaction.

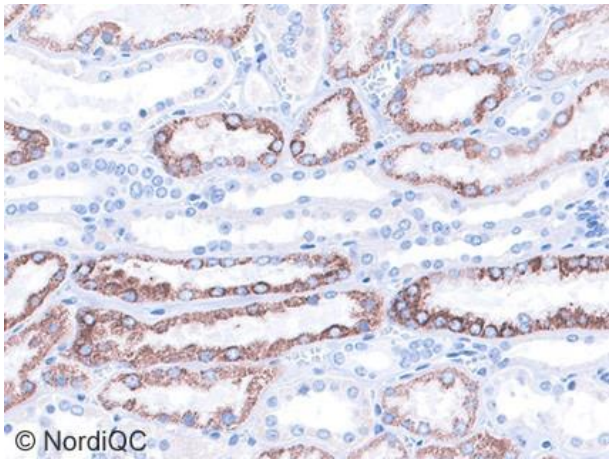


Fig. 1b (x100)
Insufficient staining reaction for AMACR of kidney using the rmAb 13H4 as concentrate (too diluted) on the Ventana Benchmark, HIER in an alkaline buffer (CC1 pH 8,5) and a 3-step multimer detection system (Optiview, Ventana/Roche) - same protocol used in Figs. 2b-4b.
The intensity of the staining reaction is significantly reduced, and the epithelial cells of distal tubules are false negative - compare with Fig. 1a (same field).

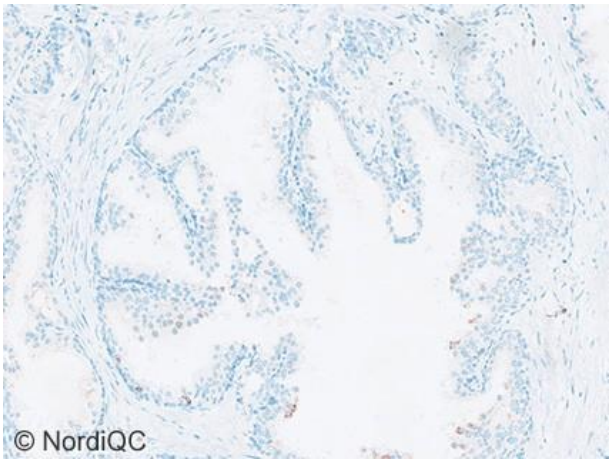


Fig. 2a (x100)
Optimal staining reaction for AMACR of the normal/hyperplastic prostate using the same protocol as in Fig. 1a. The epithelial cells of the glands are negative or only focally, display a weak granular cytoplasmic staining reaction.

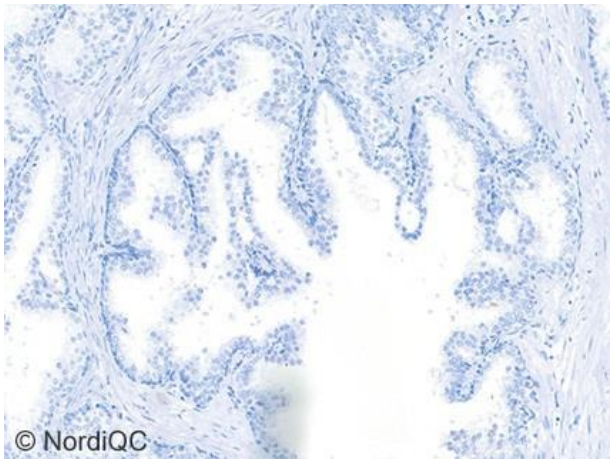


Fig. 2b (x100)
Staining reaction for AMACR of the normal/hyperplastic prostate using the same insufficient protocol as in Fig. 1b. Although the staining pattern is as expected the protocol provided too low analytical sensitivity - compare with with Figs. 1a-1b, 3a-3b and 4a-4b.

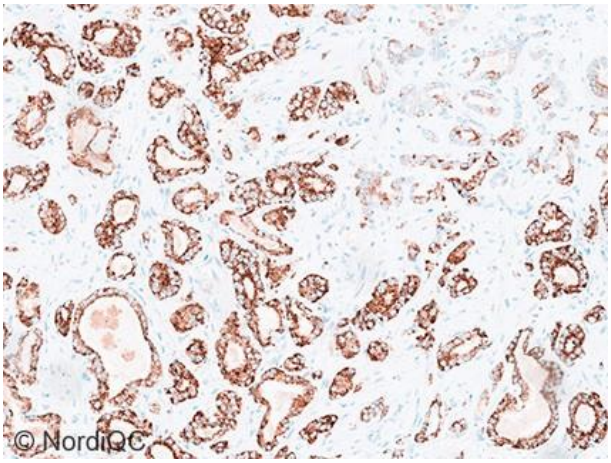


Fig. 3a (x100)
Optimal staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 4) using same protocol as in Figs. 1a - 4a. The vast majority of neoplastic cells displays a strong, distinct granular staining reaction. The entire cytoplasmic compartment in the neoplastic cells are stained.

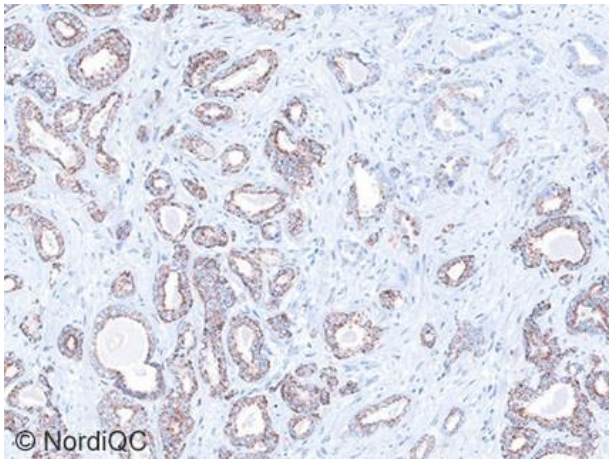


Fig. 3b (x100)
Insufficient staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 4) using same protocol as in Figs. 1b - 4b. Although the majority of the neoplastic glands are stained, the intensity is too weak and the staining pattern is in many cases primarily restricted to the apical compartment of the cytoplasm in the neoplastic cells - compare with Fig. 3a (same field).

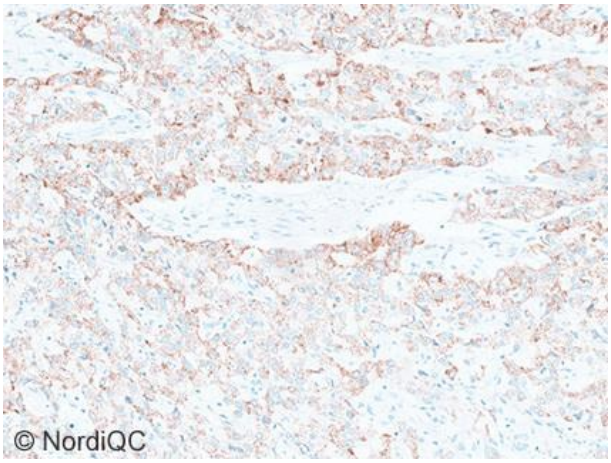


Fig. 4a (x100)
Optimal staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 5) using same protocol as in Figs. 1a - 4a. The vast majority of neoplastic cells displays a weak to moderate, distinct granular staining reaction of the cytoplasm.

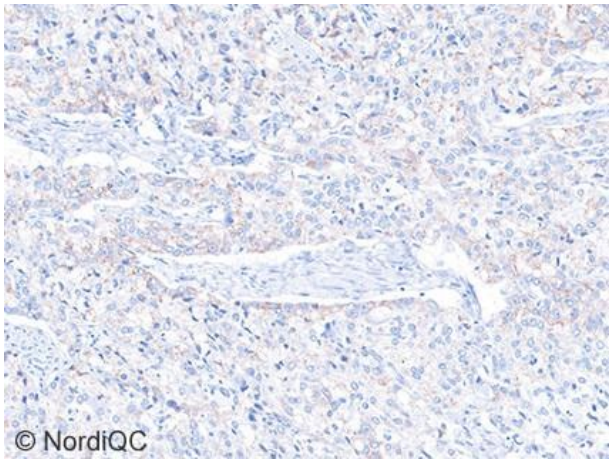


Fig. 4b (x100)
Insufficient staining reaction for AMACR of the prostate adenocarcinoma (tissue core no 5) using same protocol as in Figs. 1b - 4b. Only few neoplastic cells show a diffuse and faint cytoplasmic staining reaction. The strong counterstaining also impacts the interpretation - compare with Fig. 4a (same field).

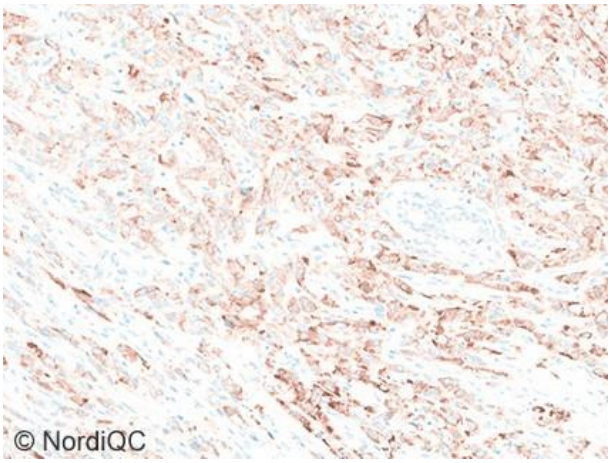


Fig. 5a (x100)
Optimal staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 5) using the RTU rmAb SP116 by vendor recommended settings but with a 3-step multimer detection system (Optiview, Ventana/Roche). The vast majority of neoplastic cells display a weak to moderate, distinct granular staining reaction of the cytoplasm. Compare with Figs. 4a-b and 5b.

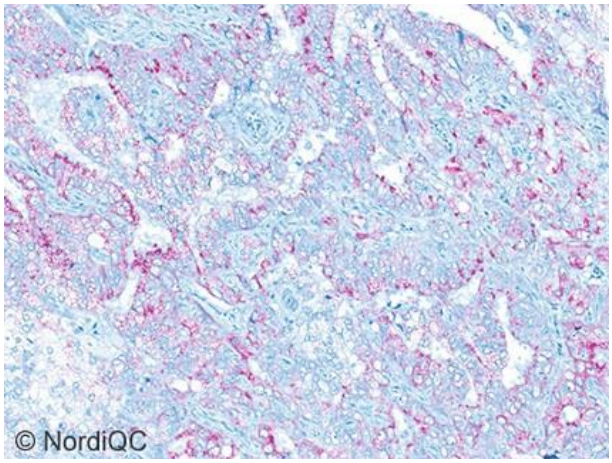


Fig. 5b (x100)
Sufficient (marked as good) staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 5) using the RTU rmAb SP116 with vendor recommended protocol settings and UltraView Alkaline phospahase as detection system. The vast majority of neoplastic cells displays a distinct granular staining reaction within the cytoplasm but reduced compared to the level seen in Fig. 5a. In addition, the enhanced intensity of counterstain complicates the scoring of AMACR in the weakly stained neoplastic cells. – Compare to Figs. 4a-b and 5a.

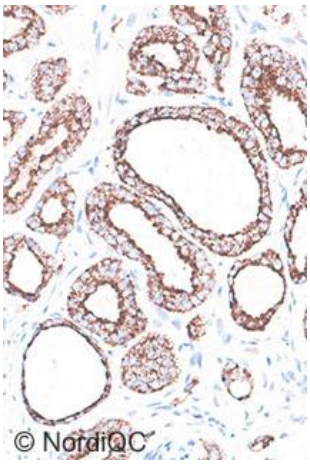


Fig. 6a.I (x200)

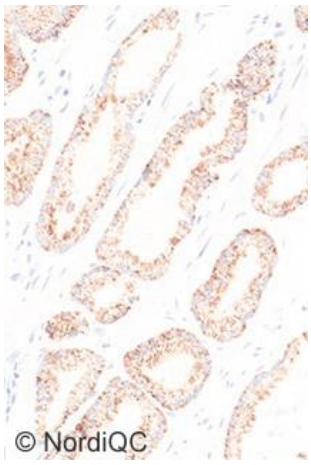


Fig 6a.II (x200)
Optimal staining reaction for AMACR of the prostate adenocarcinoma (tissue core no. 4) by the rmAb clone 13H4 GA060 for the Dako Omnis by a LD protocol for the RTU (Fig. 6a.I) and with vendor recommended settings (Fig. 6a.II). In Fig. 6a.I the Ab incubation was increased to 20 min. as the only change to the protocol. – Compare to Fig. 1a-1b.

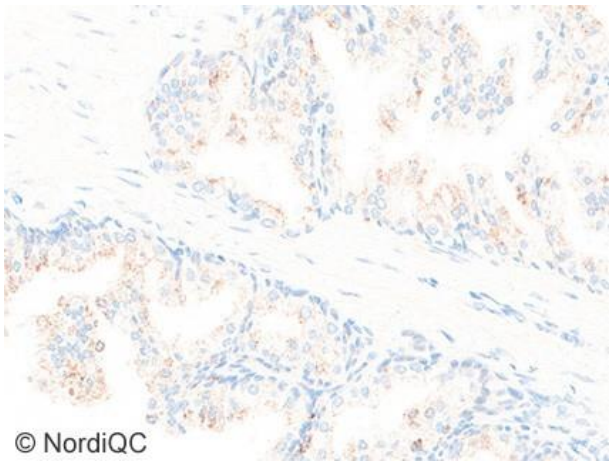


Fig. 6b (x200)
Insufficient staining reaction for AMACR of the prostate hyperplasia using the mAb clone EPMU1 (PA0210) on the Leica Bond platform with protocol F, HIER in an alkaline buffer (BERS2 pH 9,0) and a 3-step polymer detection system (Bond Refine, Leica Biosystem). A significant increased number of benign epithelial cells show a distinct granular cytoplasmic staining reaction compromising the scoring and final interpretation for diagnosis. – Compare to Figs. 2a-b.

TJ/LE/SN 16.06.2022