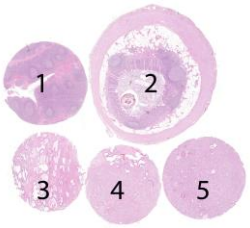
Assessment Run 50 2017

ERG (Ets-Related Gene)

Material
The slide to be stained for ERG comprised:



1. Tonsil, 2. Appendix, 3. Prostate with hyperplasia, 4-5. Prostate adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing ERG staining as optimal included:

- A moderate to strong nuclear staining reaction of virtually all endothelial cells in all specimens.
- An at least weak to moderate nuclear staining reaction of the majority of neoplastic cells in the prostate adenocarcinoma, tissue core no. 4
- A moderate to strong nuclear staining reaction of virtually all neoplastic cells in the prostate adenocarcinoma, tissue core no. 5.
- No staining of epithelial cells in appendix and the prostate with hyperplasia.
- A weak to moderate nuclear staining reaction for rmAb clones EP111 and EPR3864 in peripheral T-cells and mantle zone B-cells was expected and accepted.

Participation

Number of laboratories registered for ERG, run 50	137
Number of laboratories returning slides	130 (95%)

Results
130 laboratories participated in this assessment. 87 (67%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibody
- Insufficient HIER - use of non-alkaline HIER buffers
- Too low concentration of the primary antibody
- Low sensitivity detection systems

Performance history
This was the first NordiQC assessment of ERG and a pass rate of 67% was observed.

Table 2. **Proportion of sufficient results for ERG in the first NordiQC run performed**

	Run 50 2017
Participants, n=	130
Sufficient results	67%

Conclusion
In this first NordiQC assessment of ERG, the rmAb clones **EP111** and **EPR3894** were the two most successful antibodies. Optimal results were obtained both within laboratory developed (LD) assays on the main IHC platforms and as Ready-To-Use systems (Dako and Ventana).
Within LD assays, efficient HIER in an alkaline buffer and use of a sensitive and specific 3-step polymer / multimer based detection system gave the highest proportion of optimal results.
The Dako RTU systems based on rmAb clone EP111 for Autostainer and Omnis were most successful and provided a pass rate of 100% when using the IHC assays as "plug-and-play".
Appendix can be recommended as positive and negative tissue control for ERG. Virtually all endothelial cells must show a moderate to strong nuclear staining reaction. A weak cytoplasmic staining reaction in endothelial cells with high ERG expression must be accepted, whereas epithelial cells and muscle cells must be negative. In addition, for the rmAb clones EP111 and EPR3894, tonsil can be highly recommended, as these two Abs also label lymphocytes. Peripheral T-cells and mantle zone B-cells display a low level staining reaction. It was observed that in the optimal staining results in the prostate specimens (being the primary focus in this assessment), the lymphocytes showed a weak nuclear staining reaction being identified even at low magnification (5x).

Table 1. **Antibodies and assessment marks for ERG, run 50**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **9FY**	8 1 1	Biocare Thermo/Neomarkers Zytomed	0	0	2	8	-	-
rmAb clone **EP111**	20 8 1	Agilent/Dako Cell Marque BioSB	7	7	11	4	48%	53%
rmAb clone **EPR3864**	8 2	Abcam/Epitomics Zeta Corporation	6	2	1	1	80%	100%
Ready-To-Use antibodies								
mAb clone **9FY PM421AA**	3	Biocare	0	1	2	0	-	-
mAb clone **9FY MAD-000609QD**	2	Master Diagnostika	0	1	0	1		
rmAb clone **EP111 IR659**	18	Agilent/Dako	14	3	1	0	94%	100%
rmAb clones **EP111 GA659**	12	Agilent/Dako	9	2	0	1	92%	100%
rmAb clone **EP111 434R**	3	Cell Marque	1	2	0	0	-	-
rmAb clone **EP111 AN782**	1	Biogenex	1	0	0	0	-	-
rmAb clone **EP111 RMPD034**	1	Epitomics	1	0	0		-	-
rmAb clone **EP111 RMA-0748**	1	Maixin	1	0	0	0	-	-
rmAb clone **EPR3864 790-4576**	40	Roche/Ventana	9	20	9	2	73%	74%
Total	130		49	38	26	17	-	
Proportion			38%	29%	20%	13%	67%	

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

Detailed analysis of ERG, Run 50

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

rmAb clone **EP111**: Protocols with optimal results were based on HIER using Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/4)*, Cell Conditioning 1 (CC1, Ventana) (5/16) or Tris-EDTA pH 9 (1/2) as retrieval buffer. The rmAb was diluted in the range of 1:25-1:100. Using these protocol settings, 8 of 16 (50%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EPR3864**: Protocols with optimal results were based on HIER using CC1 (Ventana) (2/4), BERS2 (Leica) (3/3) or Target Retrieval Solution, High pH (Dako) (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:100-1:400. Using these protocol settings, 7 of 7 (100%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for ERG for the most commonly used antibody concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer Link / Classic**		**Dako/Agilent 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 clone **EP111**	0/1**	-	0/2	-	5/12 (42%)	-	1/3	-

* 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 **EP111** product no. **IR659**, Dako Autostainer Link:
Protocols with optimal results were based on HIER in PT-Link using Target Retrieval Solution (TRS) pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) +/- Rabbit Linker (K8009) as detection systems.
Using these protocol settings, 13 of 13 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP111**, product no. **GA659**, Dako Omnis:
Protocols with optimal results were based on HIER using TRS High pH 9 (efficient heating time 24-30 min. at 97°C), 25-30 min. incubation of the primary Ab and EnvisionFLEX (GV800) + Rabbit Linker GV809 as detection system.
Using these protocol settings, 9 of 9 (100%) laboratories produced a sufficient staining result.

rmAb clone **EPR3864**, product no. **790-4576,** Roche/ Ventana BenchMark Ultra / XT:
Protocols with optimal results were based on HIER using Cell Conditioning 1 (efficient heating time 32-64 min.) and 16-44 min. incubation of the primary Ab. UltraView (760-500) or OptiView (760-700) +/- amplification kit were used as detection systems.
Using these protocol settings, 28 of 38 (74%) laboratories produced a sufficient staining result.

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

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS48 rmAb EP111 **IR659**	100% (8/8)	100% (8/8)	100% (6/6)	83% (5/6)
Dako Omnis rmAb EP111 **GA659**	100% (9/9)	100% (9/9)	0% (0/2)	0% (0/2)
VMS Ultra / XT rmAb EPR3864 **790-4576**	60% (3/5)	0% (0/5)	74% (26/35)	23% (8/35)

* 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 first NordiQC assessment of ERG, the prevalent feature of the insufficient staining results was either a generally too weak or completely false negative reaction of cells expected to be demonstrated.
Too weak or false negative staining reaction was seen in 95% of the insufficient results (41 of 43). The majority of the laboratories were able to demonstrate ERG in endothelial cells and neoplastic cells in the prostate adenocarcinoma, tissue core no. 5, whereas the prostate adenocarcinoma, tissue core no. 4, was more challenging and displayed a lower level of ERG expression. Too weak staining was most frequently caused by use of protocol settings providing too low technical sensitivity for an otherwise successful primary Ab or use of a less successful primary Ab as mAb clone 9FY.
The remaining insufficient results were characterized by a poor signal-to-noise ratio.
In the assessment, mainly the ERG expression in endothelial cells and the prostate specimens were encountered. The rmAb clones EP111 and EPR3894 also labels lymphocytes, whereas lymphocytes are negative with mAb clone 9FY (and mAb clone UMAB77, Origene – internal NordiQC data).

38% (49 of 130) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for ERG. The rmAb clone EP111 was the most widely used antibody and provided a modest pass rate of 48%. Use of optimal protocol settings (HIER buffer and appropriate titre) only improved the pass rate to 53%, as seen in Table 1. Detailed protocol analysis to identify additional parameters important to secure a sufficient and optimal staining result for rmAb clone EP111 did not reveal any pattern. In the analysis, focus was addressed on HIER time, primary Ab incubation time, Ab diluent and choice of detection system and none of these was found to be influential for a sufficient versus insufficient result.
In contrast the rmAb clone EPR3894 was found most successful within a LD assay for ERG. Using an appropriate titre of the primary Ab (range 1:100-1:400) and HIER in an alkaline buffer, a pass rate of 100% was seen. In combination with a 3-step polymer/multimer based detection system, 83% was optimal.
The mAb clone 9FY was used by 10 laboratories as a concentrate. Despite applying similar protocol settings (e.g. HIER and detection systems) as for the rmAbs clones EP111 and EPR3894, all produced insufficient staining results (borderline or poor) as shown in Table 1.

Ready-To-Use (RTU) antibodies were used by 62% (81 of 130) of the laboratories. The Ventana RTU system based on rmAb clone EPR3894 (790-4576) was the most widely used RTU system applied by 40 laboratories (1 laboratory used 790-4576 on Leica Bond system). An overall pass rate of 73% was seen and 20% were optimal.
Only 13% of the laboratories (5 of 39) followed the Ventana/Roche recommended protocol settings for 790-4576 (see Table 4) and a pass rate of 60% was seen, none achieved optimal mark.
In contrast, 87% of the laboratories (34 of 39) used laboratory modified protocol settings resulting in a slightly improved pass rate of 74% and 23% optimal. The predominant successful modification was use of a 3-step detection system (OptiView) opposed to the recommended 2-step system (UltraView). In addition prolonged HIER and/or use of longer incubation time of the primary Ab than the recommended 16 min. was also frequently used with success.

The Dako RTU system IR659 for Autostainer based on the rmAb EP111 provided a pass rate of 100% and 100% optimal using the vendor recommended protocols settings (HIER in TRS High for 20 min., 20 min. incubation of the primary Ab, and EnVision FLEX+ as detection kit). Laboratory modified protocol settings such as reduced HIER and/or prolonged primary Ab incubation time could also provide optimal results (see Table 4).
The corresponding Dako RTU system GA659 for Omnis also provided a pass rate of 100% and 100% optimal using the RTU system as "plug-and-play" following the recommended protocol settings (HIER in TRS High for 30 min., 25 min. incubation of the primary Ab, and EnVision FLEX+ as detection kit).

Controls
Appendix can be recommended as positive and negative tissue control for ERG irrespective of the Ab applied. Virtually all endothelial cells must show a moderate to strong nuclear staining reaction. A weak cytoplasmic staining reaction in endothelial cells with high ERG expression must be accepted, whereas epithelial cells and muscle cells must be negative.
As appendix does not contain any reliable structures with low level ERG expression, supplemental positive tissues / cells may be required. This is needed to identify and monitor an adequately calibrated protocol for the purpose to demonstrate ERG in neoplastic tissues with reduced ERG expression as seen in this assessment for the prostate adenocarcinoma, tissue core no 4. For this purpose tonsil is very valuable for the rmAb clones EP111 and EPR3894 as these Abs also label lymphocytes. Peripheral T-cells and mantle zone B-cells express a low level staining reaction and it was observed that in the optimal staining results in the prostate specimens, the staining in lymphocytes could be identified even at low magnification (5x).

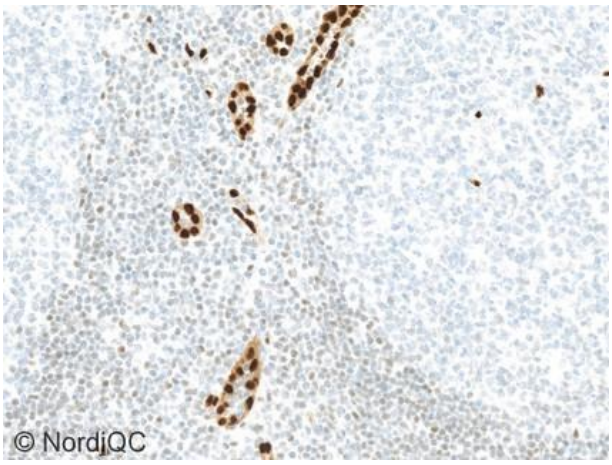


Fig. 1a
Optimal staining for ERG of tonsil using the rmAb clone EP111 within a laboratory developed assay optimally calibrated, using HIER in an alkaline buffer and a 3-step multimer based detection system, OptiView Ventana. Virtually all endothelial cells show a strong nuclear staining reaction, while mantle zone B-cells and interfollicular lymphocytes show a weak but distinct nuclear staining reaction. .
Also compare with Figs. 2a – 4a, same protocol.

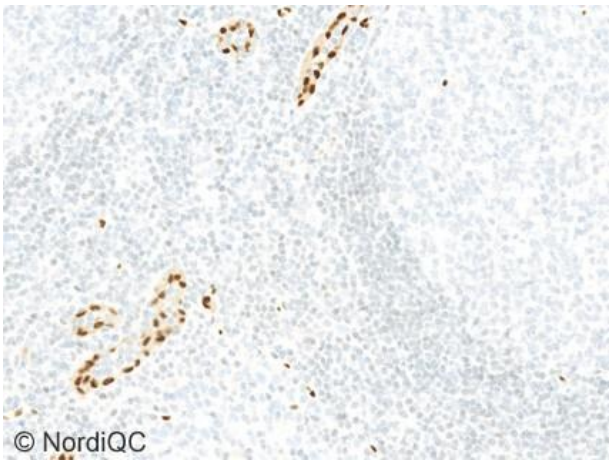


Fig. 1b
Insufficient staining for ERG of tonsil using the rmAb clone EP111 within a laboratory developed assay providing a too low analytical sensitivity. A too low titre of the primary antibody and the use of a less sensitive 2-step multimer based system, UltraView Ventana, were the main causes for the insufficient result.
Only endothelial cells with high level ERG expression are distinctively demonstrated, while lymphocytes show an equivocal staining reaction
Also compare with Figs. 2b – 4b, same protocol.

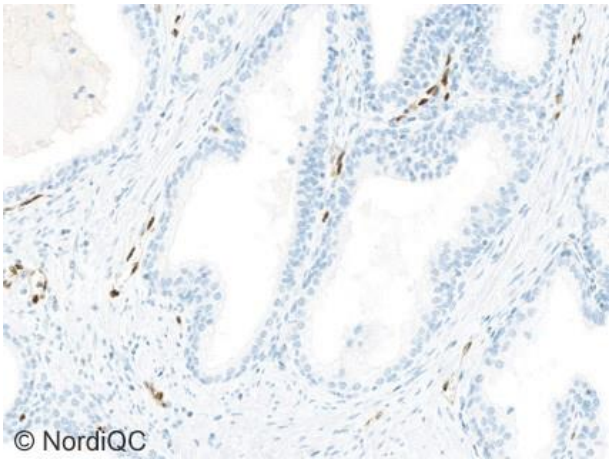


Fig. 2a
Optimal ERG staining of the prostate hyperplasia using same protocol as in Fig. 1a.
A distinct nuclear staining reaction in endothelial cells is seen, while the epithelial cells of prostate glands are negative.
A high signal-to-noise ratio is observed.

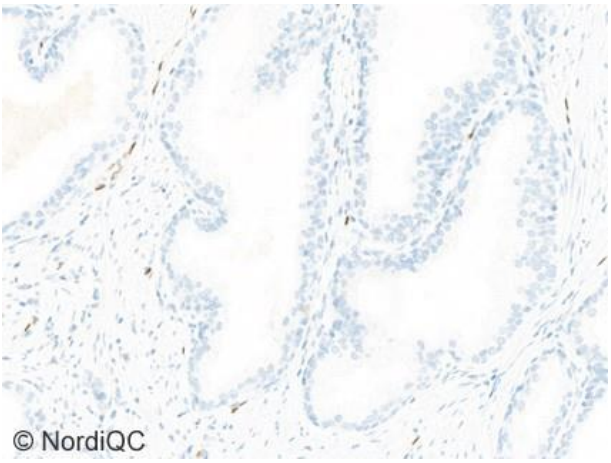


Fig. 2a
Insufficient ERG staining of the prostate hyperplasias using same protocol as in Fig. 1b.
The intensity and proportion of endothelial cells demonstrated is significantly reduced compared to the level expected and shown in Fig. 2a – same field.

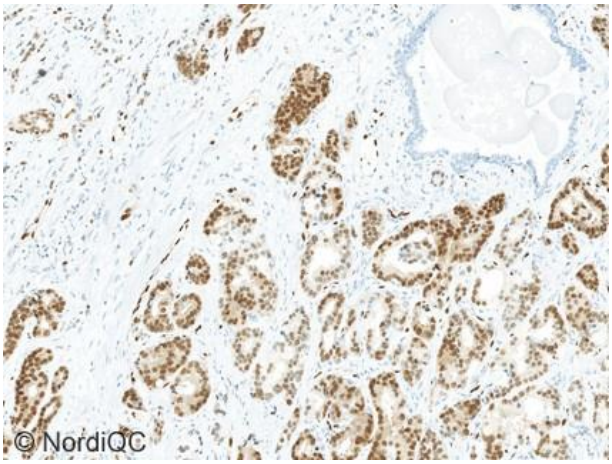


Fig. 3a
Optimal ERG staining of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a.
A moderate to strong and distinct nuclear staining reaction is seen in virtually all neoplastic cells. A weak cytoplasmic staining reaction is seen, but in general, a high signal-to-noise ratio is observed.

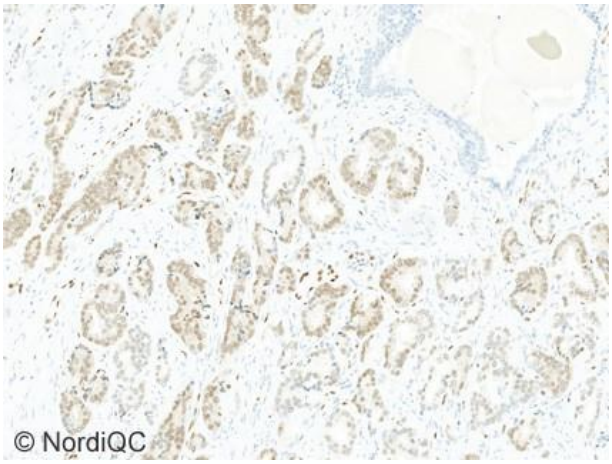


Fig. 3b
ERG staining of the prostate adenocarcinoma, tissue core no. 5, using same insufficient protocol as in Figs. 1b and 2b.
The majority of neoplastic cells are demonstrated, however also compare with Fig. 4b.
The two adenocarcinomas showed different levels of ERG expression and only the tumour with high level expression is distinctively demonstrated.

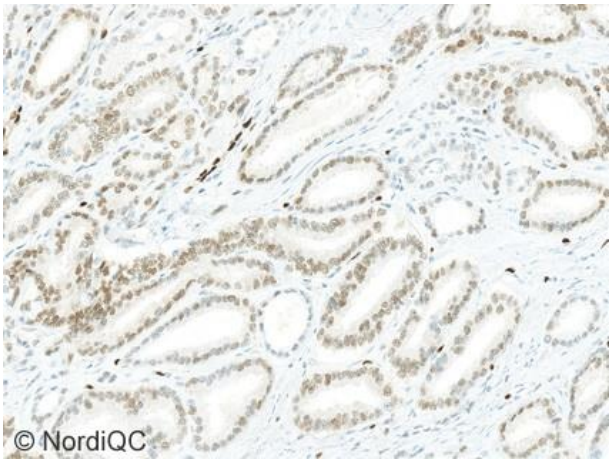


Fig. 4a
Optimal ERG staining of the prostate adenocarcinoma, tissue core no. 4, using same protocol as in Figs. 1a - 3a.
A weak to moderate, distinct nuclear staining reaction is seen in the majority of neoplastic cells.

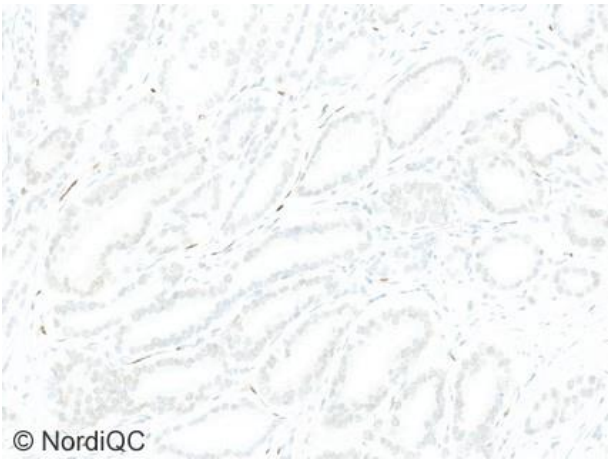


Fig. 4b
Insufficient ERG staining of the prostate adenocarcinoma, tissue core no. 4, using same protocol as in Figs. 1b – 3b.
Only the endothelial cells show a distinct nuclear staining reaction, while the neoplastic cells virtually are negative.

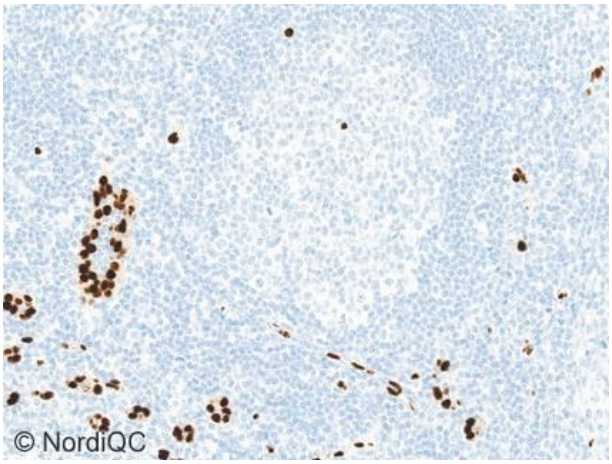


Fig. 5a
Staining for ERG of tonsil using the mAb clone 9FY within a laboratory developed test using HIER in an alkaline buffer and a 3-step polymer based detection system.
This antibody does not react with lymphocytes, whereas an intense nuclear staining reaction in endothelial cells is seen.
However despite this intense staining reaction an insufficient result in the prostate adenocarcinomas was seen – see also Fig. 5b, same protocol.
14 of 15 protocols based on mAb clone 9FY provided an insufficient result and only one sufficient result assessed as good.

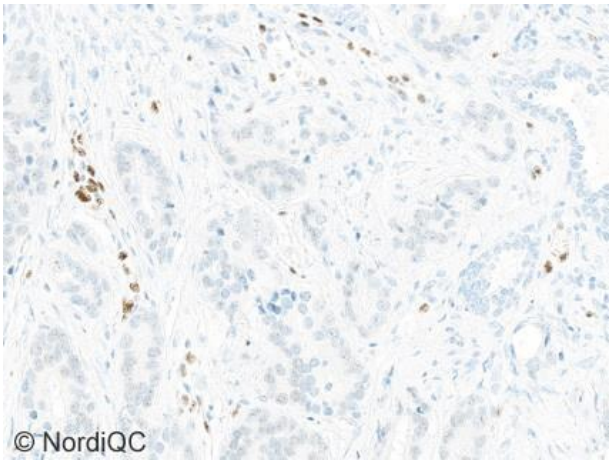


Fig. 5b
Insufficient ERG staining of prostate adenocarcinoma, tissue core no. 5 with high level ERG expression using same protocol as in Fig. 5a.
Only the endothelial cells show a distinct nuclear staining reaction, while virtually all the neoplastic cells are negative.
Compare with Fig. 3a showing the level expected.

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