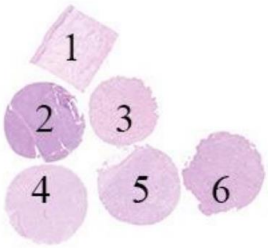
Assessment Run 63 2021
GATA3

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for GATA3, typically identifying urothelial and breast carcinomas in the diagnostic work-up of cancer of unknown primary (CUP) origin. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for GATA3 (see below).

Material
The slide to be stained for GATA3 comprised:



1. Uterine cervix 2. Tonsil 3. Kidney, 4. Breast carcinoma, 5. Urothelial carcinoma, 6. Non Small Cell Lung Carcinoma (NSCLC)

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a GATA3 staining as optimal included:

- A weak to moderate, distinct nuclear staining reaction in the majority of squamous epithelial cells situated in the basal and intermediate compartment of the surface epithelium in the uterine cervix.
- An at least moderate, distinct nuclear staining reaction of virtually all podocytes (renal glomeruli) and of epithelial cells in the collecting ducts of the kidney.
- An at least moderate, distinct nuclear staining reaction of virtually all T helper cells in all specimens, in particular Th2 cells in T-zones of the tonsil and dispersed T-cells in the NSCLC.
- A weak to strong, distinct nuclear staining reaction of the majority of neoplastic cells in the breast ductal carcinoma.
- An at least moderate, distinct nuclear staining reaction of the vast majority of neoplastic cells in the urothelial carcinoma.
- No staining reaction of the neoplastic cells in the NSCLC and of the squamous epithelial cells in the tonsil.

A weak cytoplasmic background was accepted in the tubuli of the kidney, as long as the interpretation was not compromised.

Participation

Number of laboratories registered for GATA3, run 63	349
Number of laboratories returning slides	320 (92%)

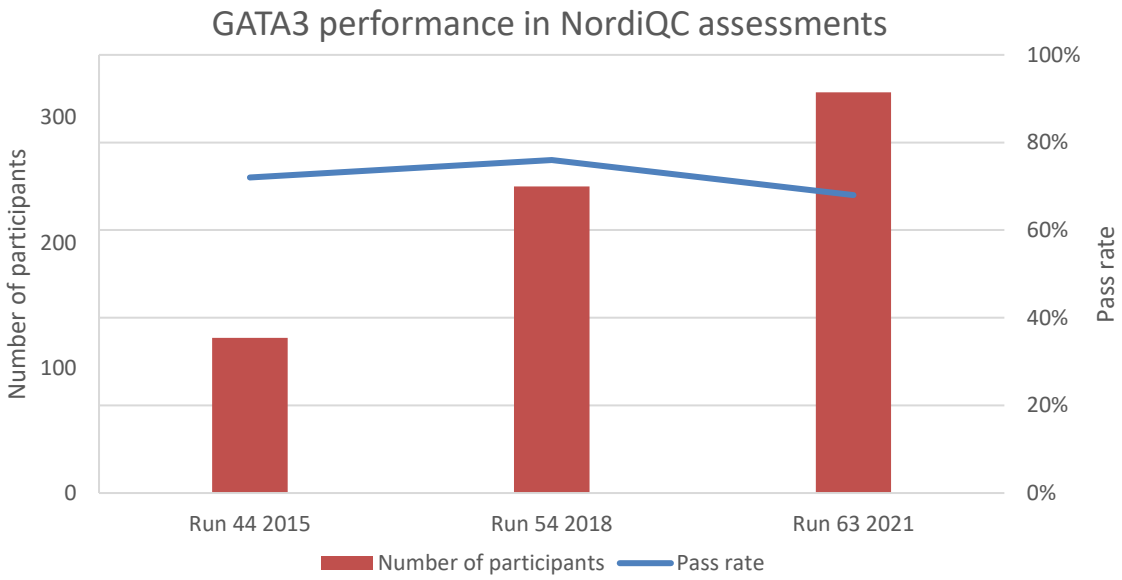
Results
At the date of assessment, 92% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

320 laboratories participated in this assessment and 68% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining were:
- Less successful primary antibodies (L50-823 concentrated format from Biocare)
- Use of less sensitive detection systems
- Too low concentration of the primary antibody
- Insufficient Heat Induced Epitope Retrieval (HIER, too short efficient heating time or use of a citrate-based buffer)

Performance history
This was the third NordiQC assessment of GATA3. A pass rate of 68% was observed, which was lower compared to the previous run 54, 2018.

Graph 1. **Proportion of sufficient results for GATA3 in the three NordiQC runs performed**



Conclusion
The mAb clone **L50-823** and the rmAb clone **EP368** could both be used for demonstration of GATA3. The vast majority of participants used the mAb clone L50-823 either within a laboratory developed (LD) assay or as a Ready-to-use (RTU) format. Used within a LD assay, optimal results could be obtained on all four main IHC systems (Dako Autostainer, Dako Omnis, Ventana BenchMark and Leica Bond). Efficient HIER, preferable in an alkaline buffer, careful calibration of the primary antibody and use of a 3-layer detection system were the most important prerequisites for optimal staining results.
The RTU system 760-4897 (Ventana/Roche) also based on the mAb clone **L50-823** provided a high proportion of sufficient and optimal results, especially if OptiView (760-700) was used as detection system.
Normal uterine cervix and tonsil are recommendable as positive and negative tissue controls for GATA3. In uterine cervix a weak to moderate staining reaction of squamous epithelial cells situated in the basal and intermediate layer of the surface epithelium must be seen whereas the superficial epithelial cells and stroma cells must be negative. In the tonsil the vast majority of T helper cells (Th2) in the T-zones must show an at least moderate but distinct nuclear staining reaction. No staining of B-cells should be seen.

Table 1. **Antibodies and assessment marks for GATA3, Run 63**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **L50-823**	88 24 4 3 3 2 2 1 1	Cell Marque Biocare BD Pharmingen Zytomed Systems Gennova Bio-SB Immunologic Anacrom DBS	31	40	33	24	56%	25%
rmAb clone **EP368**	5 1	Cell Marque Quartett	4	-	1	1	67%	67%
mAb clone **HG3-31**	2	Santa Cruz	-	-	-	2	-	-
rmAb clone **ZR65**	1	Zeta Corporation	-	-	1	-	-	-
Conc total	137		35	40	35	27	55%	26%
Ready-To-Use antibodies							Suff.[1]	OR.[2]
mAb clone **L50-823 760-4897**[3]	56	Ventana/Roche	36	12	8	-	86%	64%
mAb clone **L50-823 760-4897**[4]	67	Ventana/Roche	41	16	7	3	85%	61%
mAb clone **L50-823 390M-17,18,10**	42	Cell Marque	14	12	13	3	62%	33%
mAb clone **L50-823 PM 405AA**	12	BioCare Medical	5	3	2	2	67%	42%
mAb clone **L50-823 MAD-000632QD**	3 1	Master Diagnostica Vitro SA	1	2	1	-	-	-
mAb clone **L50-823 CGM-0130**	1	Celnovte	-	1	-	-	-	-
mAb clone **GATA3/6664 AMB89**	1	BioGenex	-	-	-	1	-	-
RTU total	183		97	46	31	9	78%	53%
Total	320		132	86	66	36		
Proportion			41%	27%	21%	11%	68%	

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 (indicated in percentage if ≥5 asessed protocols).

Detailed analysis of GATA3, Run 63

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb clone **L50-823**: Protocols with optimal results were all based on HIER in an alkaline buffer using Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (4/19)*, Cell Conditioning 1 (CC1, Ventana/Roche) (15/46), Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) (11/36) and TRS High (3-in-1) (Dako/Agilent) pH 9 (1/12) as retrieval buffer. The mAb was typically diluted in the range of 1:50-400 depending on the total sensitivity of the protocol employed. Using these protocol settings, 67 of 110 (61%) laboratories produced a sufficient result (optimal or good).

* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP368:** Protocols with optimal results were based on HIER using TRS pH 9 (2/2), TRS High (3-in-1) pH 9 (1/1) and TRIS-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:100-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 4 of 4 (100%) laboratories produced an optimal staining result.

Table 2. **Proportion of optimal results for GATA3 for the most commonly used antibodies 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	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **L50-823**	1/12** (8%)	0/1	11/36 (31%)	0/1	15/46 (33%)	0/1	4/19 (21%)	-
rmAb clone **EP368**	1/1	-	2/2	-	0/1	-	0/1	-

* 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 **L50-823**, product no. **760-4897**, Ventana, BenchMark XT, ULTRA:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 24-64 min.), 16-40 min. incubation of the primary Ab and UltraView with or without amplification (760-500/760-080) or OptiView with or without amplification (760-700/760-099) as detection system. Using these protocol settings, 90 of 100 (90%) laboratories produced a sufficient staining result.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU system. The performance was evaluated both as a "true" plug-and-play system 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 GATA3 for the most commonly used RTU IHC system**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT mAb **L50-823 760-4897**	86% (48/56)	64% (36/56)	86% (54/63)	65% (41/63)

* 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 third NordiQC assessment for GATA3, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of the cells expected to be demonstrated. This pattern was seen in 93% of the insufficient results (95 of 102 laboratories). Too weak staining result was characterized by a reduced staining reaction both in regard to the intensity and the proportion of cells expected to be demonstrated. The remaining insufficient results were caused by background staining or excessive counterstaining compromising interpretation (see Figs. 5a-5b).
Virtually all laboratories were able to demonstrate GATA3 in high-level antigen expressing cells, such as neoplastic cells of the urothelial carcinoma, the epithelial cells of the collecting ducts and podocytes in glomeruli of the kidney. However, demonstration of GATA3 in low-level antigen expressing cells as normal T helper cells (Th2) (all specimens), the neoplastic cells of the breast carcinoma or squamous epithelial cells situated in the basal and intermediate layer of the surface epithelium of the uterine cervix was more challenging and required optimally calibrated protocols.

137 laboratories used concentrated antibodies within LD-assays, providing a pass-rate of 55% (75/137) of which 26% (35/137) were assessed as optimal. Optimal results could be obtained using the mAb clone L50-823 or the rmAb clone EP368. However, the mAb clone L50-823 was by far the most applied antibody for demonstration of GATA3 and used by 97% (128/137) of the laboratories. As shown in Table 2, this antibody clone gave optimal results on all main IHC platforms, however with a relatively low proportion. The performance of the concentrated formats of mAb L50-823 was as in previous runs influenced by the company/distributor of the primary Ab. In this assessment, 18% (24 of 137) and 64% (88 of 137) of the laboratories purchased the Ab from Biocare or Cell Marque, respectively. Using similar protocol settings, and applying the mAb L50-823 from Biocare, the overall pass rate was only 33% (8 of 24) of which 8% (2 of 24) were assessed as optimal, whereas the proportion of sufficient results were 69% (61 of 88) of which 33% (29 of 88) were giving an optimal mark if laboratories used the primary Ab from Cell Marque. The discrepancy in performance observed between the two products of the mAb clone L50-853 is difficult to elucidate upon and can be related to different parameters. The inferior performance could be related to a

lower anti-GATA3 immunoglobulin fraction in the Biocare product compared to the product from Cell Marque or other manufacturing differences impeding the antibody affinity. In this aspect, the average dilution factor for a sufficient result was 1:77 and 1:162 for the Biocare and Cell Marque L50-823 product, respectively.
It was also observed that the Cell Marque L50-823 product provided an optimal result with all commercially available antibody diluents, whereas the Biocare L50-823 product required the use of a low pH diluent as Van Gogh or Renoir Red (Biocare). Overall it was observed that for participants using the concentrate from Biocare and diluting the primary Ab in either the Van Gogh pH 6.0 or Renoir Red pH 6.2 buffer, the pass rate was 80% (4 of 5) of which 40% (2 of 5) were optimal, whereas the pass rate was significantly lower, 21% (4 of 19), using other diluents from e.g., Dako/Agilent or Ventana/Roche and none (0/19) were assessed as optimal.

As mentioned in previous reports, parameters as efficient HIER in an alkaline buffer in combination with a careful calibration of the primary Ab was critical for optimal performance of mAb clone L50-823. In addition, the choice of detection system also impacted the overall performance of the assays. Using optimal protocol settings as described above, the pass rate for 2-step multimer/polymer detection systems (e.g., UltraView or EnvFlex) was 33% (4 of 12) of which only 8% (1 of 12) were assessed as optimal. In comparison, the pass rate was 63% (63 of 100) of which 30% (30 of 100) were optimal if a 3-step multimer/polymer detection system was applied (e.g., UltraView + Amplification, OptiView or EnvFlex+). The concentrated format of mAb clone L50-823 from Cell Marque were used by 9 laboratories applying UltraView with amp. as detection system, providing a pass rate of 44% (4/9). In comparison, 25 laboratories used OptiView as detection system and the proportion of sufficient results increased considerably to 76% (19 of 25), indicating OptiView was the superior choice compared to UltraView + amplification kit as 3-layer detection system on the Ventana platforms.

Data from Table 4 underlines the importance of using a 3-layer detection system applying the mAb clone L50-823 both as concentrates or as RTU formats.

Table 4. **Summarization of the proportion of sufficient and optimal marks using either 2- or 3-layer detection systems**.**

		2-layer detection system		3-layer detection system	
Antibodies	**n**	**Sufficient**	**Optimal**	**Sufficient**	**Optimal**
mAb conc **L50-823** Cell Marque	88	36% (4/11)	9% (1/11)	74% (57/77)	36% (28/77)
mAb conc **L50-823** Biocare Medical	24	(0/1)	(0/1)	26% (6/23)	9% (2/23)
mAb clone RTU **L50-823 760-4897*** Ventana/Roche	107	53% (18/34)	6% (2/34)	99% (84/85)	88% (75/85)
mAb clone RTU **L50-823 390M-17,18,10** Cell Marque	42	27% (4/15)	13% (2/15)	96% (26/27)	52% (14/27)
mAb clone RTU **L50-823 PM 405AA** Biocare Medical	12	(0/2)	(0/2)	80% (8/10)	50% (5/10)

*Only protocols performed on the intended IHC stainer device are included.
** regardless of the protocol settings applied e.g., HIER time and/or incubation time in the primary Ab (≥10 protocols assessed).

Using the mAb clone L50-853 within a LD-assay on the Bond III/MAX platforms (Leica Biosystems), 46% (5 of 11) of the insufficient results was caused by excessive cytoplasmic background staining and was primarily related to the use of too long incubation time and/or too high concentration of the primary Ab (see Fig. 6b).

Although the number of participants using the rmAb clone EP368 was low, the antibody provided a relative high proportion of optimal results (67%, 4 of 6) and as shown in Table 2, only on the platforms from Dako/Agilent. As for the mAb clone L50-853, on the rmAb clone EP368 require protocol settings providing high level of analytical sensitivity and specificity, such as usage of HIER in an alkaline buffer, a typical dilution range of 1:50-200 and use of a 3-step polymer detection system. In the two cases assessed as insufficient, the protocols were based on a too diluted primary Ab and/or the use of a 2-step

multimer/polymer detection system (UltraView and Bond Refine). Bond Refine (Leica Biosystems) act by nature as a 2-step polymer detection system if the host of the primary Ab is produced in a rabbit (e.g., clone EP368), and thus, the antibody titer must be calibrated according to the total sensitivity of the protocol employed.

The mAb clone HG3-31 (Santa Cruz) displayed a poor performance due to false negative staining reaction (see Table 1). This was also described in previous runs for GATA3. Although the Ab was used with similar protocol settings, e.g. HIER, detection systems etc., as for the mAb clone L50-823, the protocols provided too low analytical sensitivity. Therefore, laboratories should substitute this clone with one of the more robust Abs providing optimal results (mAb L50-823 or rmAb EP368).

RTU formats were used by 57% (182/320) of the laboratories providing a pass-rate of 78%, 53% being optimal. The only "true" RTU system was the product 760-4897 from Ventana/Roche based on the mAb clone L50-823 and obtained among all other analysis, both LD-assays and RTU formats, the highest pass-rate in the assessment (see Table 1).
According to the instruction giving by the vendor, both UltraView and OptiView can be used as detection systems. Applying vendor recommended protocol settings based on OptiView, HIER in CC1 for 32 min. and incubation in the primary Ab for 32 min., the pass-rate was 100%, 92% being optimal. However, and using the vendor recommendations based on UltraView, HIER in CC1 for 64 min. and incubation in the primary ab for 32 min., the proportion of sufficient results declined significantly to 52% (9/17) and no optimal results was achieved. As shown in Table 3, 63 of the laboratories applied laboratory modified protocol settings typically adjusting HIER time, incubation time in the primary Ab and/or choice of detection system, giving nearly identical results compared to vendor recommended protocol settings. Four laboratories used this clone on a non-intended platform with mixed results. In general, the choice of detection system was very important as mentioned above and for the RTU system 760-4897, the proportion of optimal results was considerably higher using a 3-layer multimer detection system instead of a 2-step multimer detection system, 88% and 6%, respectively, regardless of other protocol settings applied e.g., HIER time in CC1 and/or incubation time in the primary Ab.

The Ready-to-Use products from Cell Marque (mAb clone L50-823, product no. 390M-17, -18, -10) and Biocare (mAb clone L50-823, product no. PM 405AA) had very similar pass rates, and the challenges regarding choice of titre, diluent and as such inferior performance for the concentrated format from Biocare was not seen for the corresponding RTU product. Both RTU products could produce optimal results on the four main IHC platforms. However, these two RTU formats are developed and validated by "third-party" IHC provider and not within a Ready-To-Use system and thus, laboratories are obligated to optimize protocols and validate assay performance in relation to the applied in-house platform(s) and more importantly, to relevant clinical samples displaying a broad spectrum of antigen densities for GATA3 and to critical staining indicators as described for the controls.

This was the third assessment of GATA3 in NordiQC (see Graph 1). The pass rate declined in this run compared to the latest run 54, 2018. The number of participants increased with 31% and most of these new users applied a RTU format. The RTU system 760-4897 (Ventana/Roche) based on the mAb clone L50-853 (developed for the BenchMark platforms), provided the highest proportion of sufficient results for demonstration of GATA3 and was the only assay applied as an "true plug and play" system in this assessment. The reduced pass rate in this run was in particular related to the LD assays based on the concentrated format of mAb clone L50-8023 and extended use of less successful generic RTU formats not being developed and validated as a final RTU system including indications of protocol, platform, purpose and expected performance.
In this assessment the included breast carcinoma was a triple negative breast tumor harboring lower expression level of GATA3 compared to the ductal breast carcinoma (high expressing level of GATA3) used in the previous run. Combined with the tonsil and uterine cervix these three tissue samples were critical indicators of the analytical sensitivity of the protocol applied (see Figs. 1a-3a). Kidney is less useful as an indicator of an optimally calibrated protocol as it might not unravel lack of analytical sensitivity due to the high level of GATA3 seen in both podocytes and epithelial cells of the collecting ducts.

Controls
Uterine cervix and tonsil are recommended as positive and negative tissue controls for GATA3. In uterine cervix a weak to moderate staining reaction in the majority of the basal and intermediate squamous epithelial cells must be seen whereas the superficial epithelial cells and stroma cells must be negative.
In the tonsil the vast majority of T helper cells (Th2) in the T-zones must show an at least moderate but distinct nuclear staining reaction. No staining of B-cells should be seen.

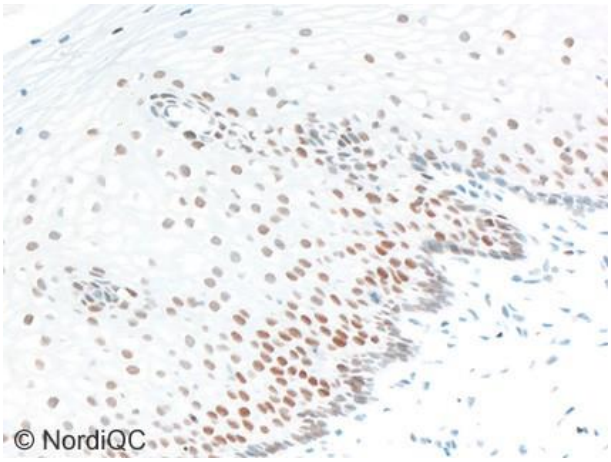


Fig. 1a (x200)
Optimal GATA3 staining of the uterine cervix using the RTU system 760-4897 (Ventana/Roche), based on the mAb clone L50-823, applying vendor recommended protocol settings and OptiView as detection system. The squamous epithelial cells in the basal and intermediate layer of the surface epithelium display a weak to moderate, but distinct nuclear staining reaction, whereas the nuclei of superficial layers and stroma cells are negative. Same protocol settings as in Figs. 2a-5a.

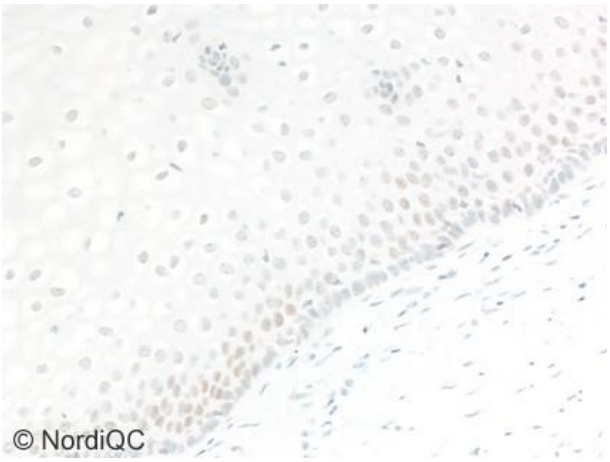


Fig. 1b (x200)
Insufficient GATA3 staining of the uterine cervix using the same RTU system as in Fig. 1a, but with the vendor recommended protocol settings based on UltraView as the detection system. The proportion and intensity of cells expected to be demonstrated is significantly reduced, displaying only faint or false negative staining reaction. Same protocol settings as in Figs. 2b-5b. Compare with Fig. 1a

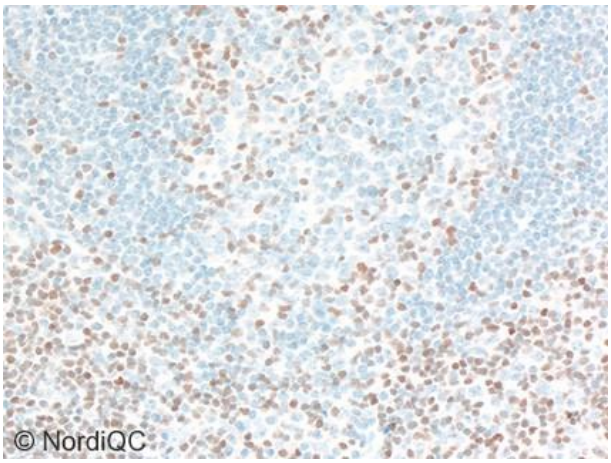


Fig. 2a (x200)
Optimal GATA3 staining of the tonsil using the same protocol as in Figs. 1a-5a. The vast majority of T helper cells (Th2) display a moderate but distinct nuclear staining reaction, whereas the B-cells are negative.

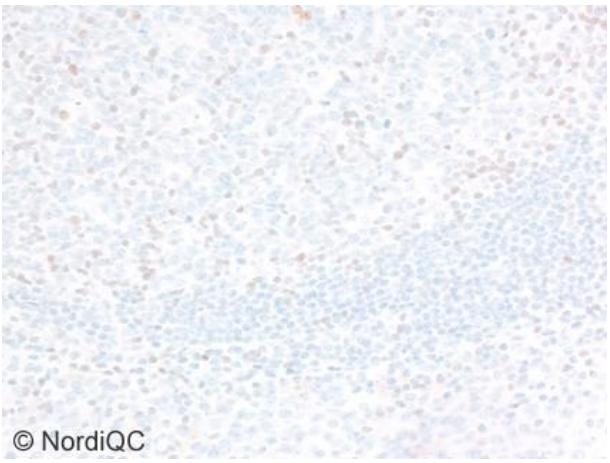


Fig. 2b (x200)
Insufficient GATA3 staining of the tonsil using the same protocol as in Figs. 1b-5b. The vast majority of T helper cells (Th2) are false negative and only a fraction of germinal centre T-cells are weakly demonstrated – compare with Fig. 2a.

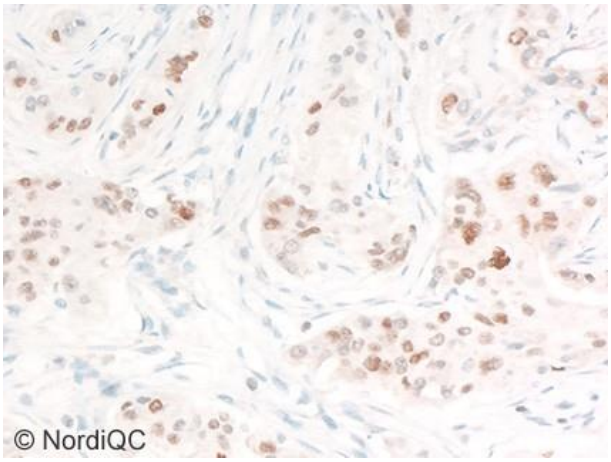


Fig. 3a (x200)
Optimal GATA3 staining of the breast carcinoma using same protocol settings as in Figs. 1a-5a.
A weak to strong nuclear staining reaction of virtually all neoplastic cells are seen.

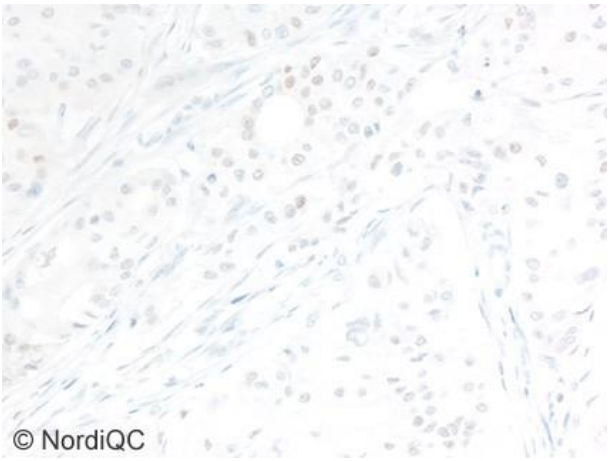


Fig. 3b (x200)
Insufficient GATA3 staining of the breast carcinoma using the same protocol settings as in Figs. 1b-5b. The vast majority of neoplastic cells are false negative and only few are weakly positive – compare with Fig. 3a

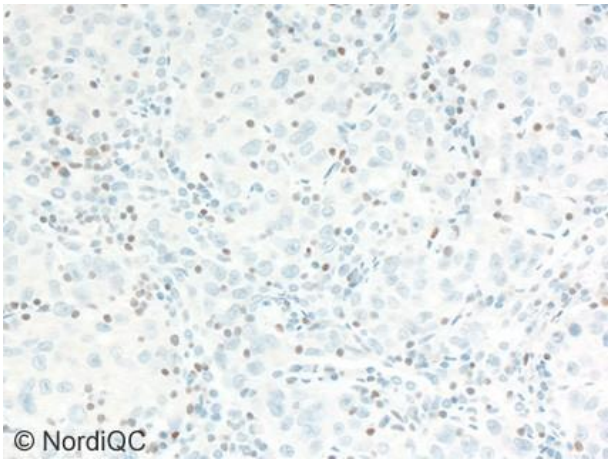


Fig. 4a (x200)
Optimal GATA3 staining of the NSCLC using same protocol settings as in Figs. 1a-3a. All neoplastic cells are negative, and nuclei of the stromal T-cells are moderately positive.

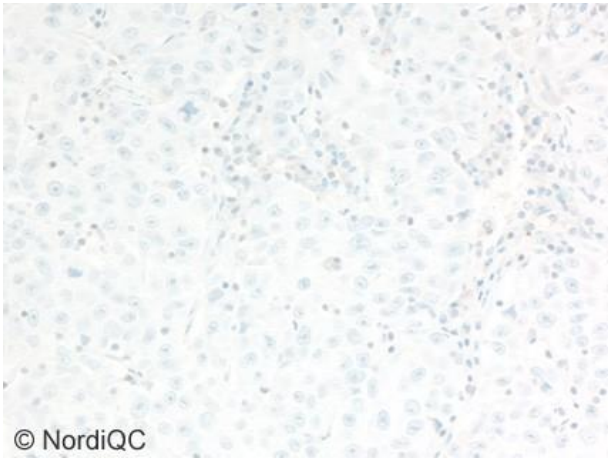


Fig. 4b (x200)
Insufficient GATA3 staining of the NSCLC using the same protocol as in Figs. 1b-3b. All neoplastic cells are negative as expected, but virtually all T-cells intermingling between the tumor cells are false negative – compare with Fig. 4a.

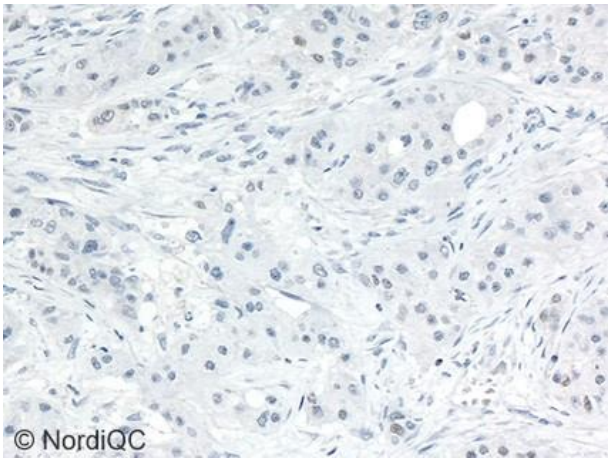


Fig. 5a (x200)
Insufficient staining of the breast carcinoma using the concentrate from Cell Marque, based on the mAb clone L50-823, on the Autostainer (Dako/Agilent) and EnvisionFlex+ as the detection system.
Interpretation is difficult due to too weak specific staining reaction in combination with an excessive counterstaining, risking misdiagnosis in the diagnostic work of CUP – compare with Fig. 3a.

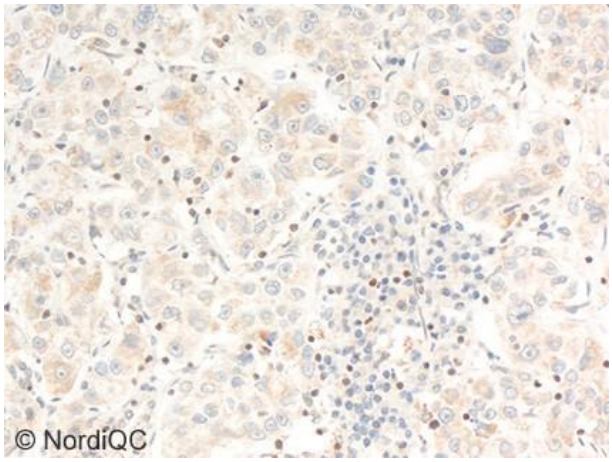


Fig. 5b (x200)
Insufficient GATA3 staining the NSCLC using the mAb clone L50-853 within a LD-assay and on the Bond III platform (Leica Biosystems). Bond Refine was used as the detection system. An aberrant granulated cytoplasmic reaction of the neoplastic cells is displayed and mainly caused by extended incubation time in primary Ab (25 min.). This aberrant staining pattern was seen in all tissue cores – compare with Fig. 4a.

TJ/SN/LE/MB 09.12.2021