Lung tumours

Optimization of antibodies, selection, protocols and controls

NQC Workshop 2016

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Lung tumours: Antibodies, protocols and controls

NordiQC

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Lung markers in NordiQC assessments:

- Napsin A (NQC in 2015)
- TTF-1 (NQC in 2016)
- p63 (NQC in 2014)
- p40 (NQC in 2015)
- SYP (NQC in 2015)
- Iu-ALK (NQC in 2015)
- WT1 (NQC in 2015)
- CEA (NQC in 2016)
- Calretinin (NQC in 2015)
- CGA (NQC in 2016)
- Podoplanin (NQC in 2012)
- CD56 (NQC in 2013)

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ANNUAL REVIEW ISSUE

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg[1,2] · Søren Nielsen[1]

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
   a. Poor antibodies[a]
   b. Less robust antibodies[b]
   c. Poorly calibrated RTUs
   d. Stainer platform dependent antibodies
2. Insufficiently calibrated antibody dilutions (20 %)
3. Insufficient or erroneous epitope retrieval (27 %)
4. Error-prone or less sensitive visualization systems[c] (19 %)
5 Other (17 %)
   a. Heat-induced impaired morphology
   b. Proteolysis induced impaired morphology
   c. Drying out phenomena
   d. Stainer platform-dependant protocol issues
   e. Excessive counterstaining impairing interpretation

Lung tumours: Antibodies, protocols and controls

Target	High scoring clones*	Low scoring clones*
Napsin A	mmAb: IP64 and MRQ-60	pAb: 760-4446 and 352A-7x
TTF1	mmAb: SPT24 and SP141	mmAb: 8G7G3/1
p63	mmAb: DAK-p63 and 4A4	mmAb: 7JUL
p40	mmAb: BC28 and rmAb: ZR8	Many pAbs
SYP	mmAb: 27G12, rmAb MRQ-40 and DAK-SYNAP	mmAb: SY38
Iu-ALK	rmAb: D5F3, mmAb: 5A4	mmAb: ALK1
WT1	mmAb: WT49 and 6F-H2	
CEA	mmAb: CEA31 and COL-1	mmAb: TF3H8-1 and II-7
CGA	pAb: A0430§ / IR502§, mmAb: LK2H10	rmAb: SP12, mmAb DAK-A3
Calretinin	rmAb: SP65, pAb 18-0211	rmAb: SP11
Podoplanin	mmAb: D2-40	mmAb: D2-40 #
CD56	rmAb: MRQ-42, mmAb: CD564 and 123C3	mmAb: 123C3 #

Ventana platform § Products discontinued * on the basis of the assessments in NordiQC

Recommended protocols from NordiQC assessment schemes

Among protocols shown to give *optimal* staining results, one or more are selected to cover a spectrum of laboratories, antibodies, protocols and platforms.

Only the latest recommended protocols for each antibody/clone/epitope are listed here. Previously recommended protocols are not listed any longer but may be obtained from NordiQC on request.

Laboratories producing optimal stains are named in the protocols to encourage direct communication. If a laboratory wishes to remain anonymous, this must be specified when protocols are submitted.

mAb = mouse monoclonal antibody, rmAb = rabbit monoclonal antibody, pAb = polyclonal antibody

Protocols Run 44 (July 2015) and B19 (April 2015)

Epitope	Antibody	Platform			
		Dako	Leica	Ventana	Other
ASMA	mAb 1A4	ASMA-run45	ASMA-run45	ASMA-run45	ASMA-run45
	mAb asm-1	-	ASMA-run45	-	-
	rmAb EP188	-	-	ASMA-run45	-
CD4	mAb 1F6	CD4-run45	CD4-run45	CD4-run45	-
	mAb 4B12	CD4-run45	-	-	CD4-run45
	rmAb EP204	CD4-run45	-	-	-
	rmAb EPR6855	CD4-run45	-	-	-
	rmAb SP35	CD4-run45	-	CD4-run45	-
DOG1	mAb K9	DOG1-run45	DOG1-run45	DOG1-run45	-
	rmAb SP31	DOG1-run45	-	DOG1-run45	-
GATA3	mAb L50-823	GATA3-run45	GATA3-run45	GATA3-run45	GATA3-run45
Napsin A	mAb BS10	-	-	-	NapsinA-run45
	rmAb EPR6252	NapsinA-run45	-		
	mAb IP64	NapsinA-run45	NapsinA-run45	NapsinA-run45	NapsinA-run45
	rmAb KCG1.1	NapsinA-run45	-	-	-
	mAb MRQ-60	NapsinA-run45	-	-	-
	mAb TMU-Ad02	-	-	NapsinA-run45	-
p40	mAb BC28	p40-run45	p40-run45	p40-run45	-
	rmAb ZR8	p40-run45	-	p40-run45	p40-run45

Nordic Immunohistochemical Quality Control

Institute of Pathology, Aalborg University Hospital, Ladegaardsgade 3, P.O.Box 561, DK-9100 Aalborg, Denmark

Recommended protocol for Napsin A (IP64)

Obtained in General Module, run 44

Primary antibody	
Clone	IP64
Producer	Leica/Novocastra
Product no. (Lot no.)	NCL-L-Napsin A (6026035)
Dilution	1:50
Diluent buffer and additive(s)	Antibody Diluent K8016, Dako
Incubation time / temperature	30 min./RT
Epitope retrieval, proteolysis	None
Proteolysis enzyme	None
Proteolysis time	None
Epitope retrieval, HIER	
Device	PT module
Buffer, pH	Target Retrieval Solution pH 9 (3-in-1), Dako
Warm-up / heating max / resting time	97°C
Maximum heating temperature	8 min./20 min./18 min. (Start 85°)
Visualization system	
Method	3-step polymer conjugate
Producer, product no.	Dako, K8012
Incubation time / temperature	20 min. + 30 min./RT
Chromogen	
Type	DAB
Producer, product no.	Dako, K8012
Incubation time / temperature	10 min./RT
Enhancement, type	
Immunostainer	
Type	Autostainer Link 48, Dako

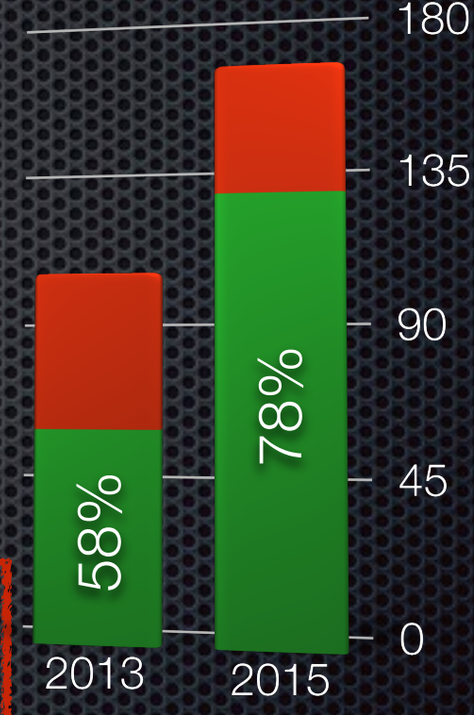
Lung tumours: Antibodies, protocols and controls

Napsin A / RUN 44 2015

Pass: 78 %

Table 1. **Antibodies and assessment marks for Napsin A, run 44**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **IP64**	86	Leica/Novocastra	39	39	6	2	91%	92%
mAb clone **MRQ-60**	8	Cell Marque	3	4	1	0	88%	100%
mAb, clone **TMU-Ad02**	4 3	Biocare IBL	1	2	4	0	43%	-
rmAb clone **KCG1.1**	2 2 1 1	Zytomed Diagnostic Biosystems Abcam Acris	1	5	0	0	100%	-
rmAb clone **BC15**	1	Zytomed	1	0	0	0	-	-
mAb, clone **BS10**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **EPR6252**	1	Abcam	1	0	0	0	-	-
pAb **352A-7x**	8	Cell Marque	0	1	1	6	13%	-
Ready-To-Use antibodies								
mAb clone **MRQ-60 760-4867**	18	Ventana/Cell Marque	1	16	1	0	84%	-
mAb clone **MRQ-60 352M-98**	3	Cell Marque	0	3	0	0	-	-
mAb clone **MRQ-60 MAD-000633QD**	3	Master Diagnostica	0	3	0	0	-	-
rmAb clone **BC15 API 3043**	1	Biocare	0	0	1	0	-	-
mAb clone **IP64 AM701-5M**	1	BioGenex	0	0	1	0	-	-
mAb clone **IP64 ZM-0473**	1	ZSGB-BIO	0	1	0	0	-	-
rmAb clone **EP205 352R-18**	1	Cell Marque	1	0	0	0	-	-
mAb clone **MX015 MAB-0704**	1	Maixin	0	1	0	0	-	-
pAb **760-4446**	12	Ventana/Cell Marque	0	1	0	11	8%	-
pAb **PPM428DS**	1	Biocare	0	0	0	1	-	-
pAb **MP-394-DS6**	1	Menapath	0	0	0	1	-	-
pAb **RAB-0639**	1	Maxim	0	1	0	0	-	-
Total	162		49	77	15	21	-	
Proportion			30%	48%	9%	13%	78%	



Cross reactivity!

Lung tumours: Antibodies, protocols and controls

Napsin A / RUN 44 2015

The mAbs clones IP64, MRQ-60, TMU-Ad02, BS10 and the rmAbs KCG1.1, EP205, EPR6252, BC15 are all recommendable Abs for demonstrating Napsin A.

Selected clones	Retrieval	Dilution range
mmAb IP64	HIER, High pH*	1:50 - 1:400
mmAb MRQ-60	HIER, High pH	1:500 - 1:800

** HIER, pH6 can be used if a sensitive 3-step polymer/multimer detection system is used.*

Table 3. **Proportion of optimal results for Napsin A using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autost. Link / Classic, Omnis		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **IP64**	10/16 (63%)**	1/5 (20%)	17/35 (49%)	1/1	2/8 (25%)	4/12 (33%)
mAb clone **MRQ-60**	3/4	-	0/1	-	-	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.

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

Lung tumours: Antibodies, protocols and controls

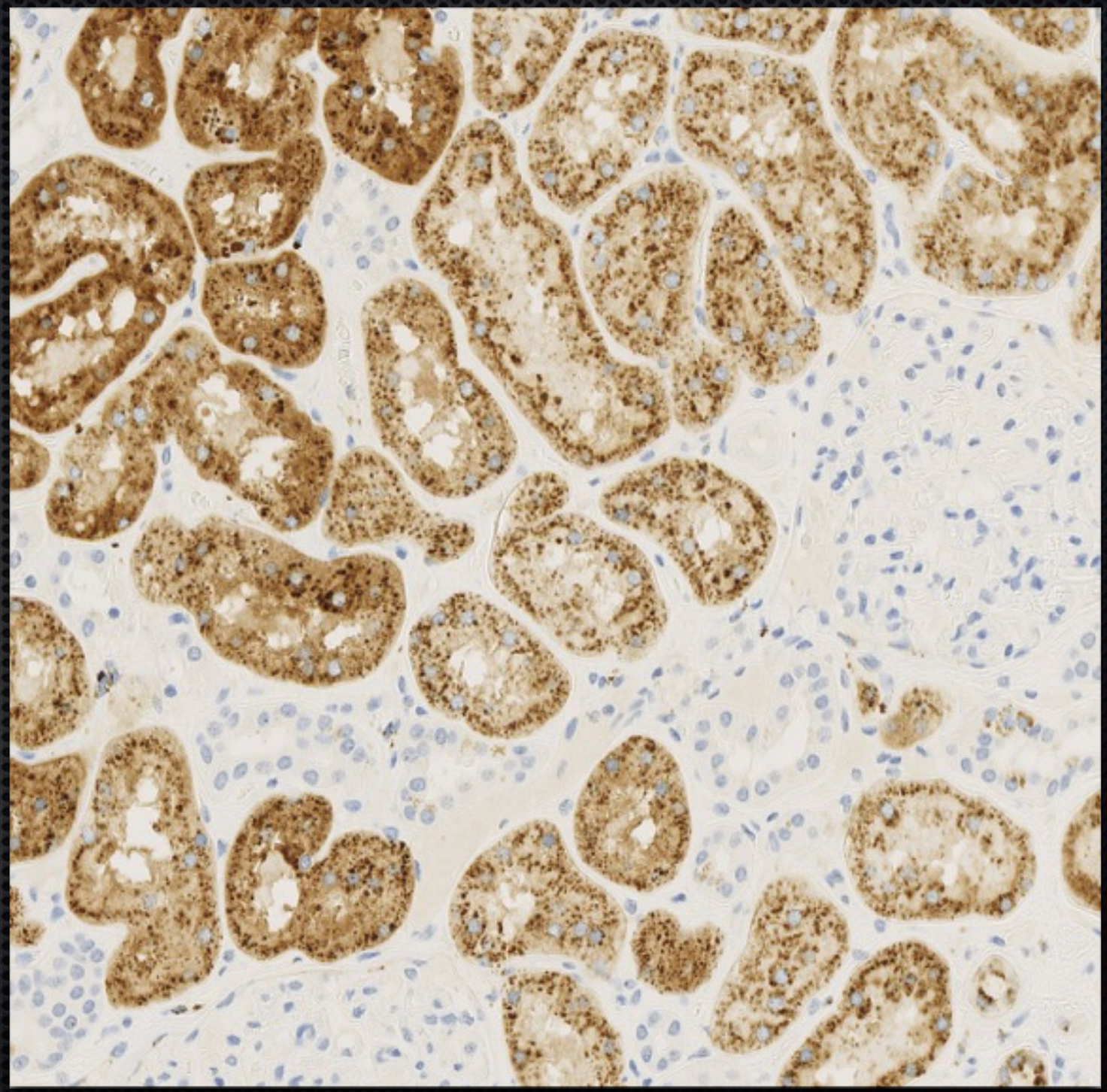
Napsin A / RUN 44 2015

Controls

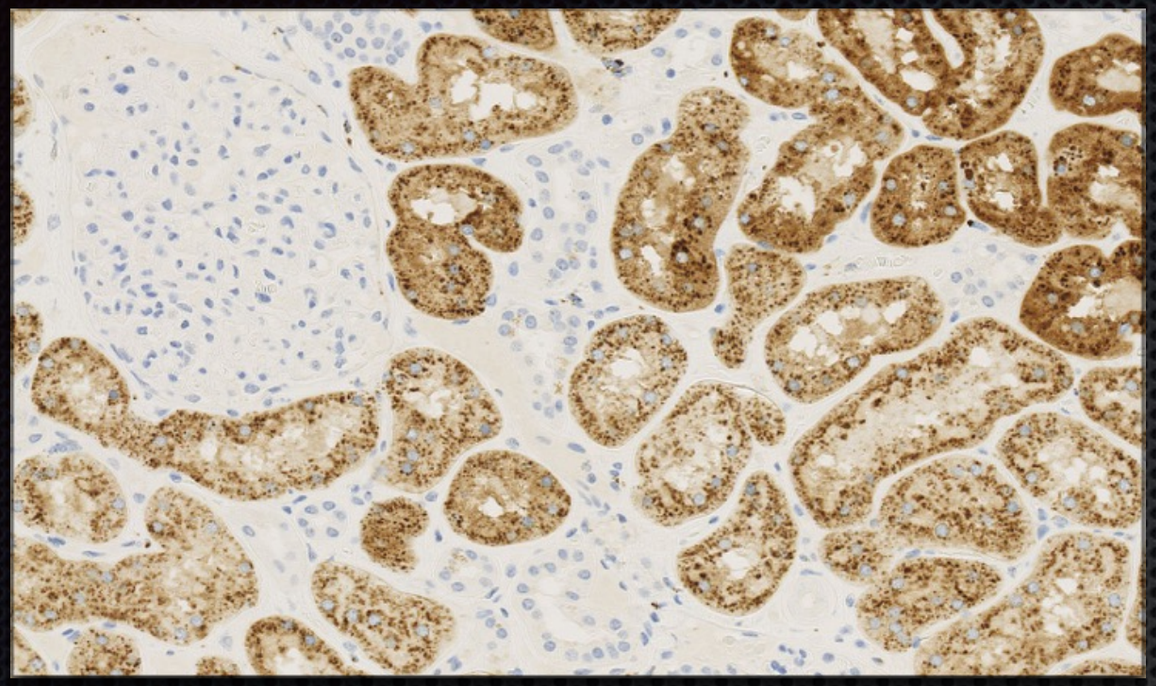
<u>Positive</u>: Kidney

* Virtually all epithelial cells of the proximal tubules must show an at least moderate, distinct granular cytoplasmic staining reaction.

<u>Negative</u>: Colon

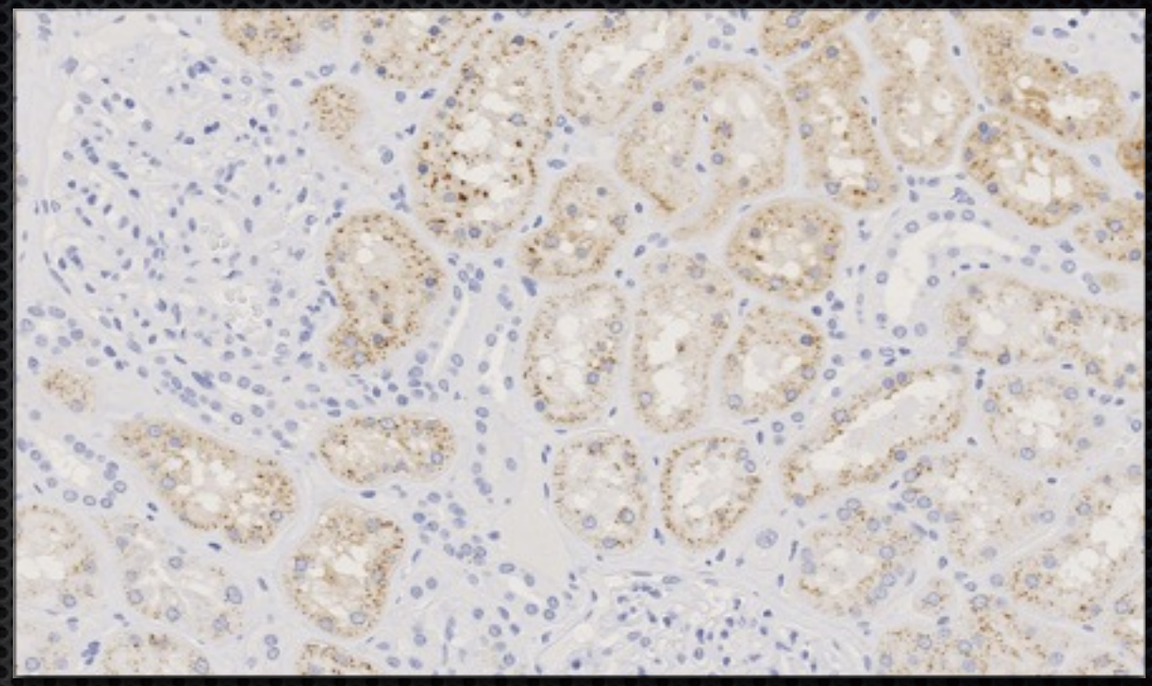


Napsin A / RUN 44 2015



Optimal-IP64-1/100-HIER/pH9-BOND

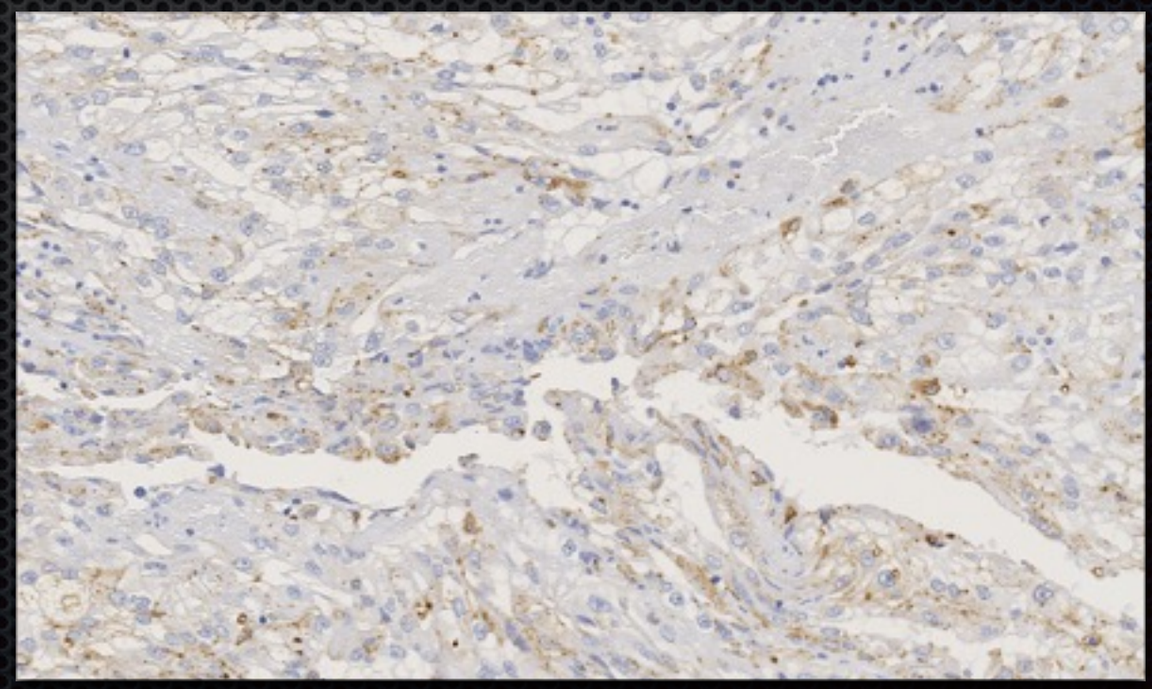
Kidney



Insuff.-IP64-1/400-HIER/pH6-BOND

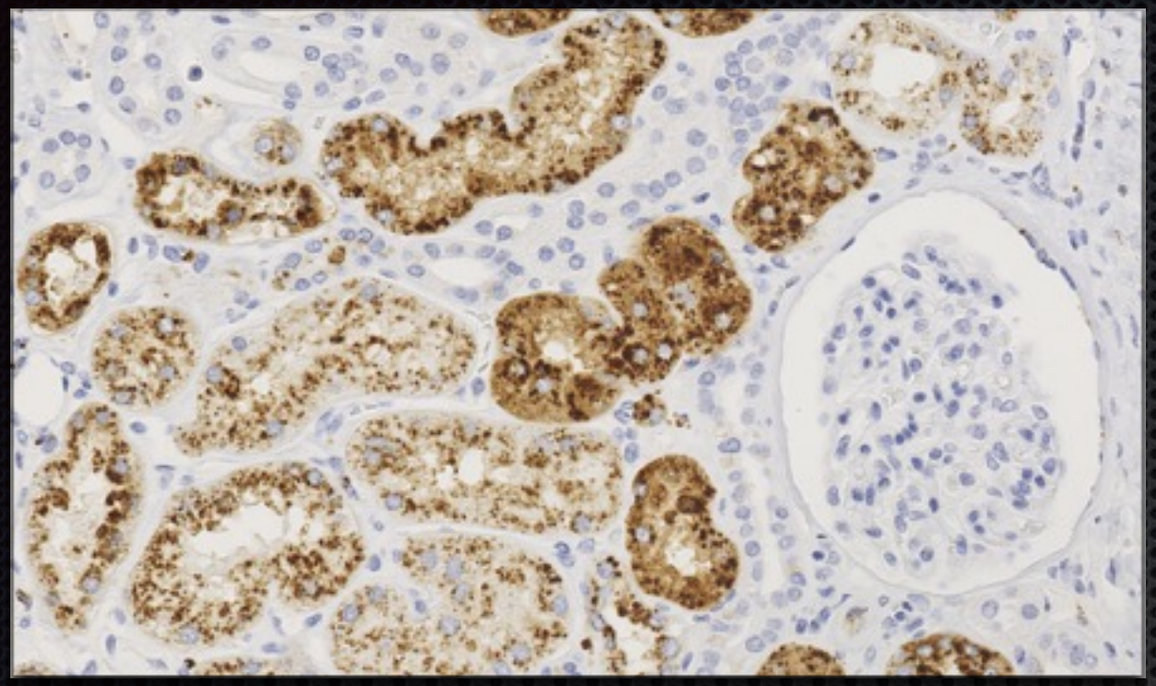
Optimal-IP64-1/100-HIER/pH9-BOND

RCC



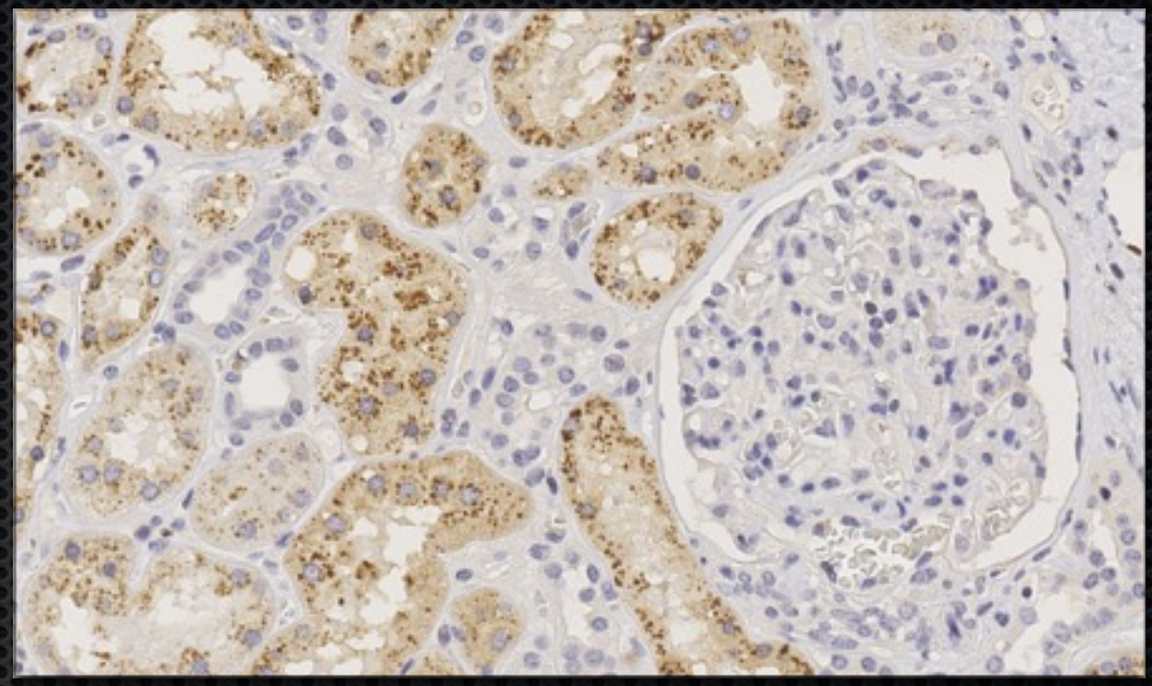
Insuff.-IP64-1/400-HIER/pH6-BOND

Napsin A / RUN 44 2015

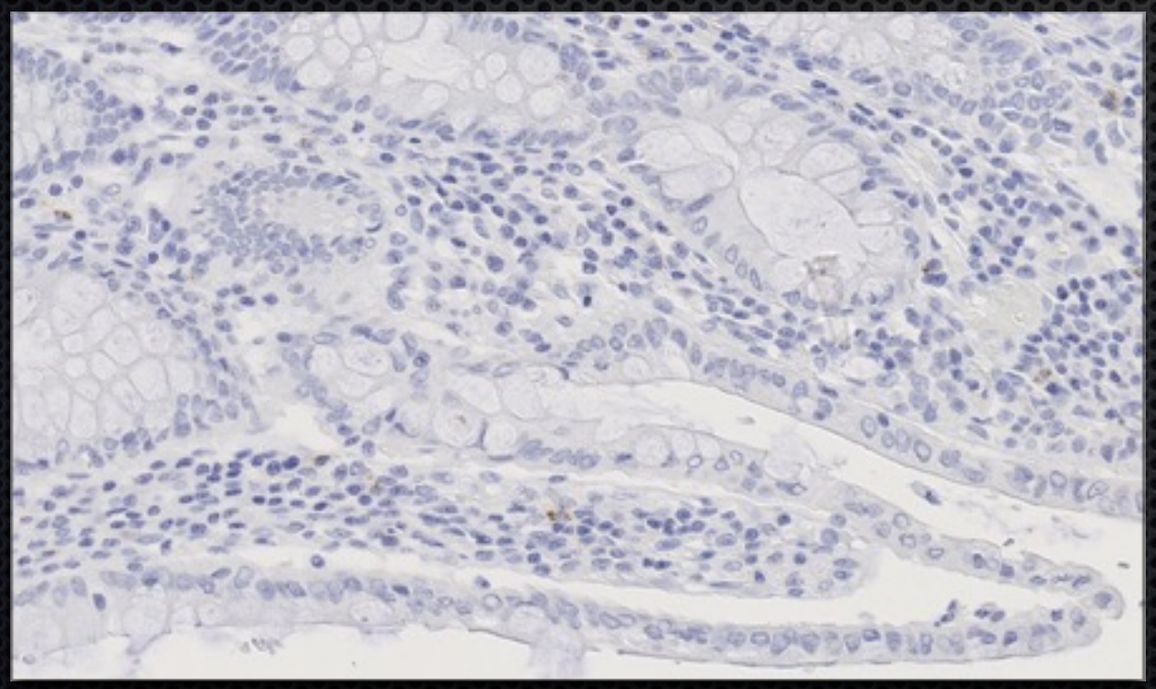


Optimal-IP64-1/100-HIER/pH8.5-VBM

Kidney



Insuff-pAb760-4446-HIER/pH8.5-VBM



Optimal-IP64-1/100-HIER/pH8.5-VBM

Colon

Insuff-pAb760-4446-HIER/pH8.5-VBM

Lung tumours: Antibodies, protocols and controls

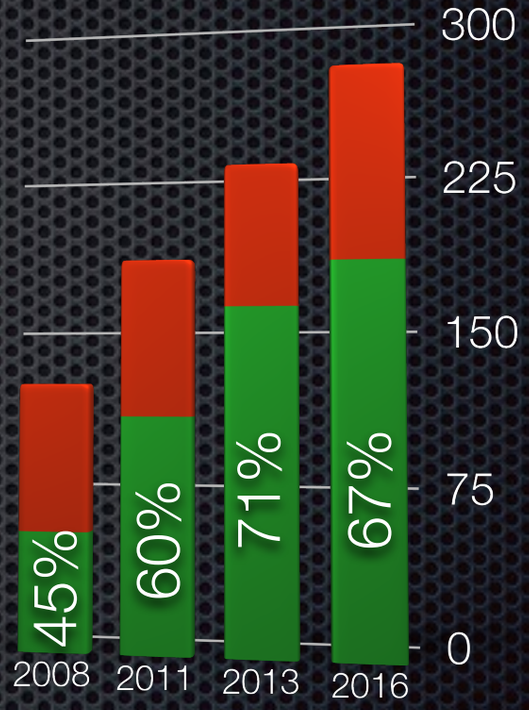
TTF1 / RUN 47 2016

Pass: 67 %

Table 1. **Antibodies and assessment marks for TTF1, run 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **8G7G3/1**	15 3 6 1	Dako/Agilent Thermo/NeoMarkers Cell Marque Zeta Corp.	0	0	14	11	0%	-
mAb clone **SPT24**	120 9 5 2	Leica/Novocastra Monosan Immunologic BioCare	76	43	14	3	88%	89%
rmAb clone **EP229**	1	Cell Marque	1	0	0	0	-	-
rmAb clone **SP141**	1	Spring Bioscience	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **8G7G3/1 790-4398**	16	Ventana/Roche	0	1	7	8	7%	-
mAb clone **8G7G3/1 IR056**	31	Dako/Agilent	0	1	24	6	3%	-
rmAb clone **SP141 790-4756**	50	Ventana/Roche	30	17	3	0	94%	94%
mAb clone **SPT24 PA0364**	8	Leica/Novocastra	7	1	0	0	100%	100%
mAb clone **SPT24 MAD-000486QD**	2	Master Diagnostica SL	2	0	0	0	-	-
mAb clone **SPT24 API 3126**	1	BioCare	1	0	0	0	-	-
mAb clone **MX011 MAB-0677**	1	Maixin	1	0	0	0	-	-
Total	272		118	64	62	28	-	
Proportion			43%	24%	23%	10%	67%	

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



Lung tumours: Antibodies, protocols and controls

TTF1 / RUN 47 2016

Recommendable clones	Retrieval	Dilution range
mAb SPT24	HIER, High pH*	1:20 - 1:400
rmAb SP141	HIER, High pH, CC1	RTU

** HIER, pH6 can be used if a sensitive 3-step polymer/multimer detection system is used.*

Table 4. **The overall pass rate in the last 4 runs for the mmAb clones SPT24, 8G7G3/1 and the rmAb clone SP141**

	SPT24 All protocol settings		**SP141* All protocol settings**		**8G7G3/1 All protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal	Sufficient	Optimal
Participants	90% (429/479)	64% (308/479)	94% 59/63	65% 41/63	7% (17/259)	0% (0/259)

* Because rmAb clone SP141 is only recently introduced, data represents Run 39 and 46 only

Table 3. **Proportion of optimal results for TTF1 for the mAb clone SPT24 as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic / OMNIS**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **SPT24**	30/40** (75%)	2/2	24/58 (41%)	-	11/15 (73%)	2/2

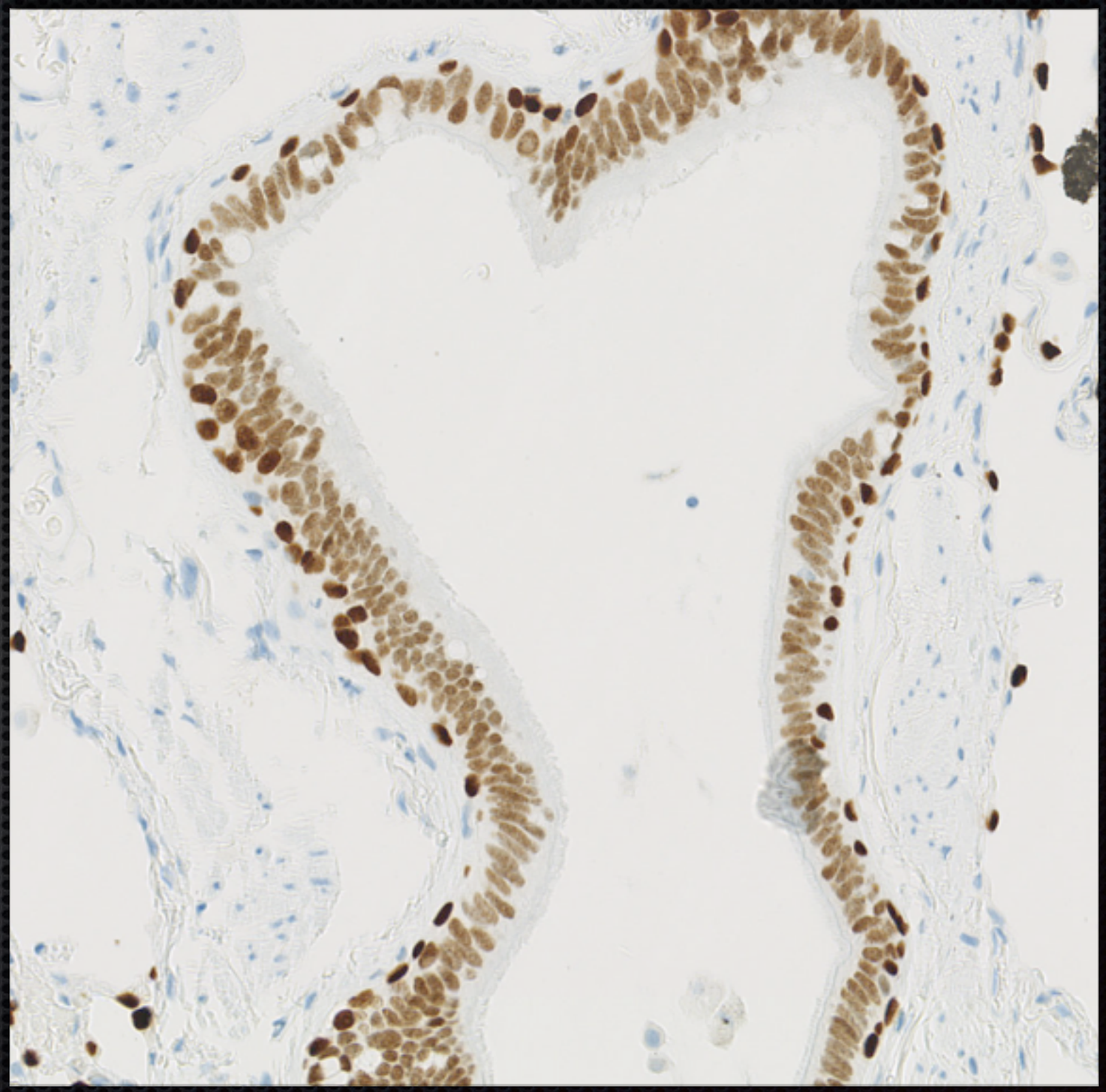
Lung tumours: Antibodies, protocols and controls

TTF1 / RUN 47 2016

Controls

Positive: Lung

* A moderate to strong nuclear staining reaction in the columnar epithelial cells of the terminal bronchioles
* A strong nuclear staining reaction in type II pneumocytes and basal epithelial cells of the terminal bronchioles



Negative: Colon

Lung tumours: Antibodies, protocols and controls

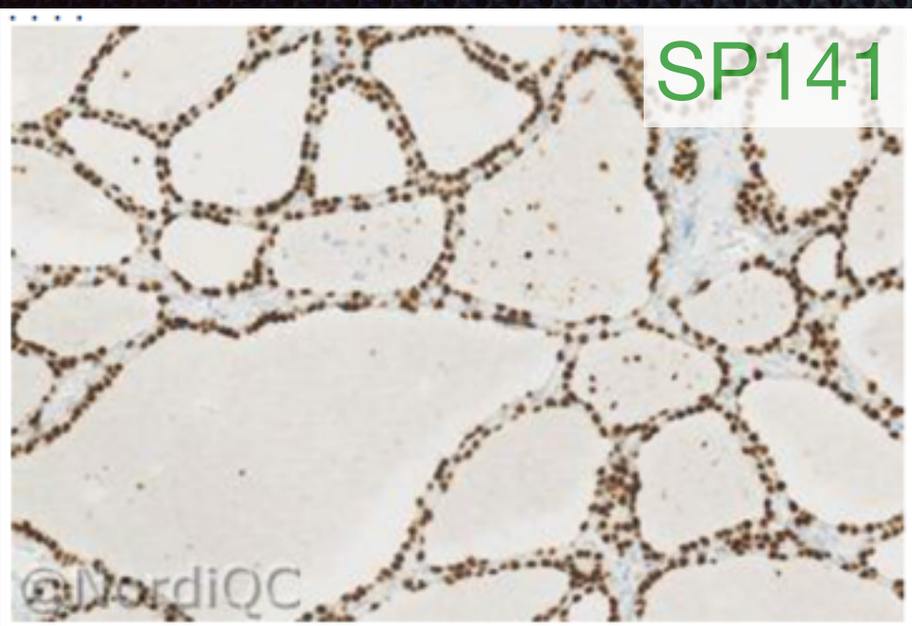


Fig. 1a
Optimal TTF1 staining of the thyroid gland using the rmAb clone SP141 (Ventana, RTU) optimally calibrated with HIER in an alkaline buffer (CC1) and performed on the BenchMark Ultra, Ventana. A strong nuclear staining reaction is seen in virtually all follicular epithelial cells. No background staining is seen.

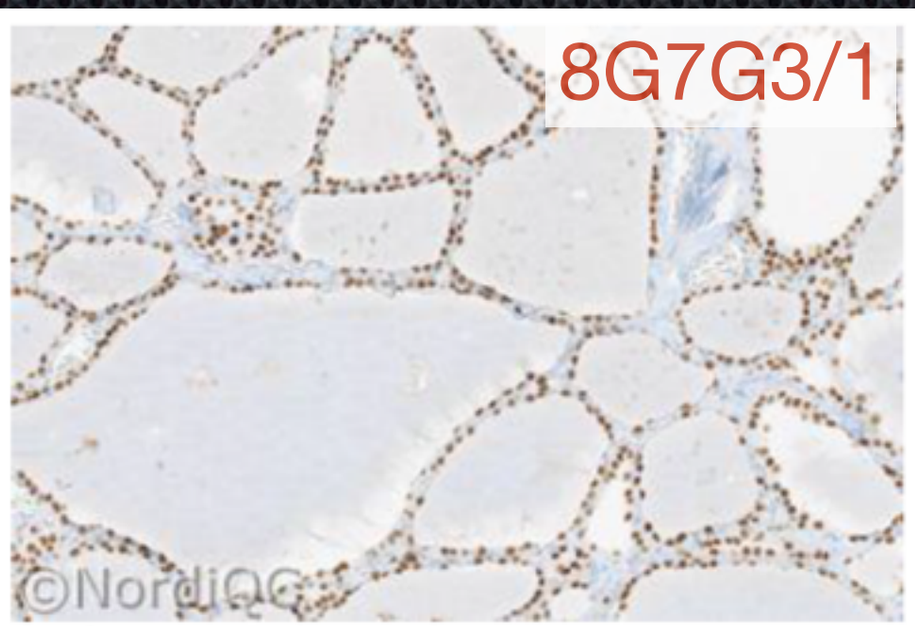


Fig. 1b
Insufficient TTF1 staining of the thyroid gland using the mAb clone 8G7G3/1 (Ventana, RTU) with HIER in an alkaline buffer (CC1) and performed on the BenchMark Ultra, Ventana. A moderate nuclear staining reaction is seen in the majority of follicular epithelial cells – same field as in Fig. 1a. Also compare with Figs. 2b, 3b and 4b – same protocol.

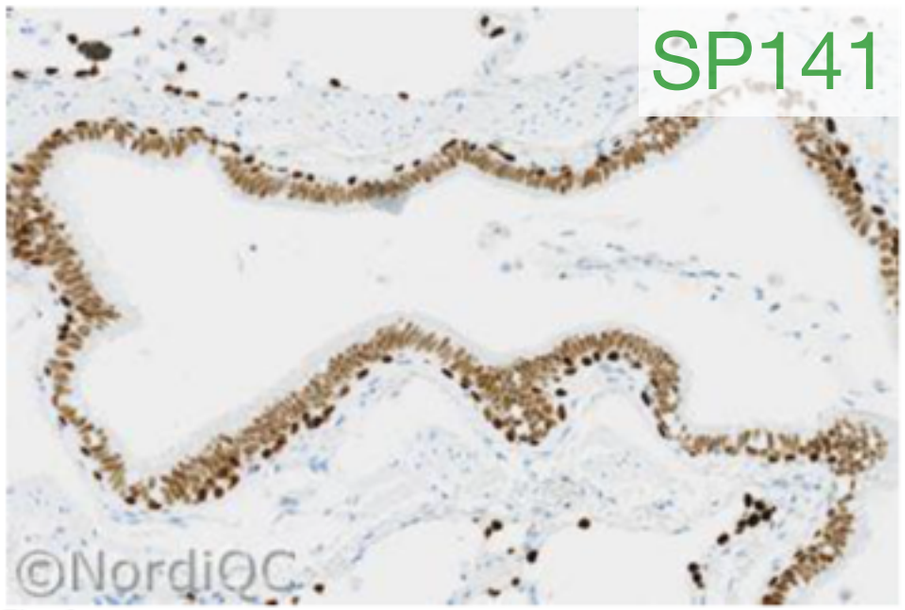


Fig. 2a
Optimal TTF1 staining of the lung using same protocol as in Fig. 1a. The type II pneumocytes and the basal epithelial cells lining the terminal bronchioles show a strong distinct nuclear staining reaction, whereas the columnar epithelial cells show a moderate nuclear staining reaction. No background staining is seen.

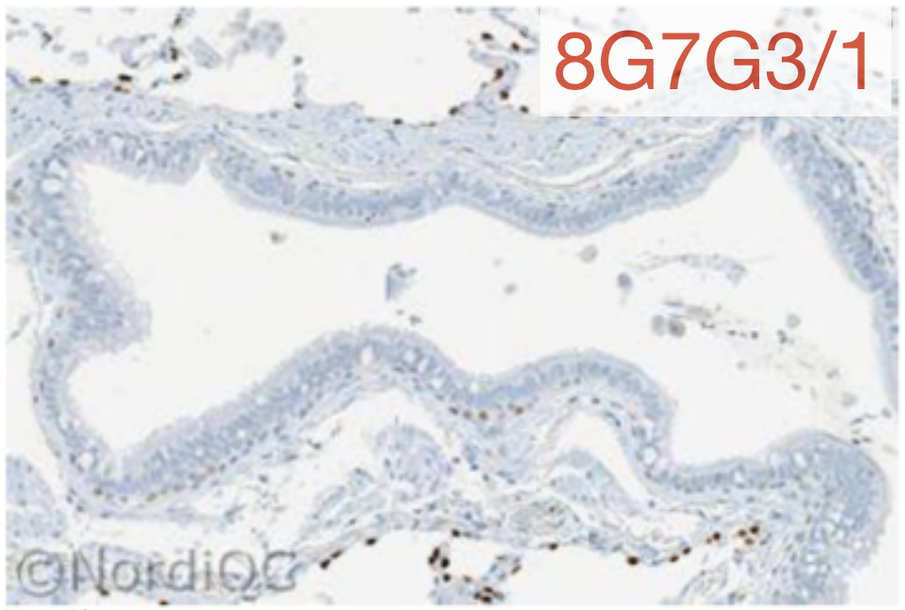


Fig. 2b.
Insufficient TTF1 staining of the lung using same protocol as in Fig. 1b. The type II pneumocytes and the basal epithelial cells lining the terminal bronchioles show only a weak to moderate positive nuclear staining reaction and no reaction is seen in the columnar epithelial cells - same field as in Fig. 2a.

Primary antibody with a too low sensitivity.

Lung tumours: Antibodies, protocols and controls

TTF1 / RUN 39 2013

NordiQC

SP141

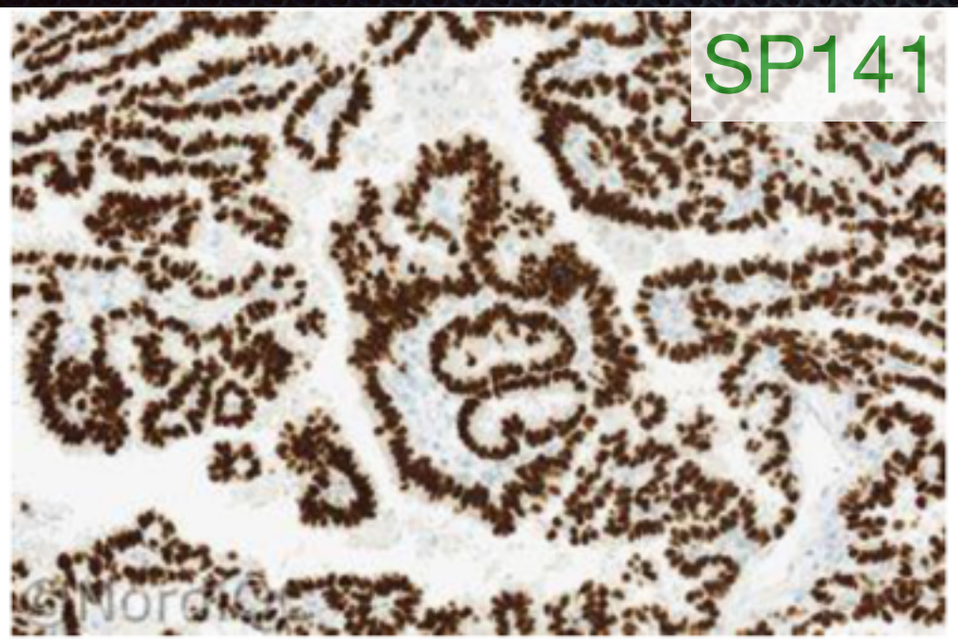


Fig. 3a
Optimal TTF1 staining of the lung adenocarcinoma no. 7 (high level expression of TTF1) using same protocol as in Figs. 1a & 2a. Virtually all the neoplastic cells show a strong and distinct nuclear staining reaction. No background staining is seen.

8G7G3/1

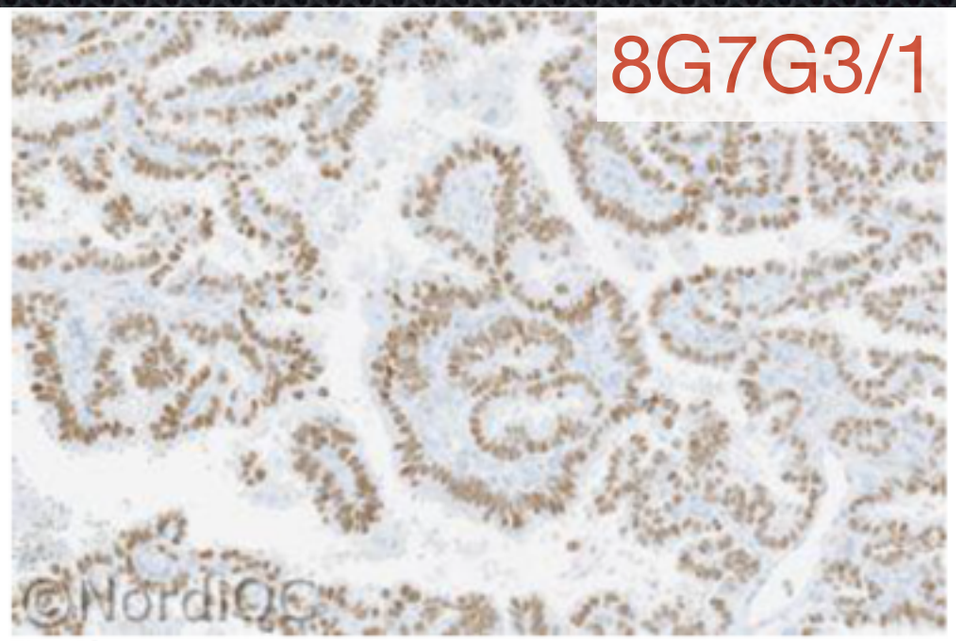


Fig. 3b
Insufficient TTF1 staining of the lung adenocarcinoma no. 7 using same protocol as in Figs. 1b & 2b. Despite a high level of TTF1 expression of the neoplastic cells only a moderate nuclear staining reaction is seen – same field as in Fig. 3a.

Primary antibody with a too low sensitivity.

SP141

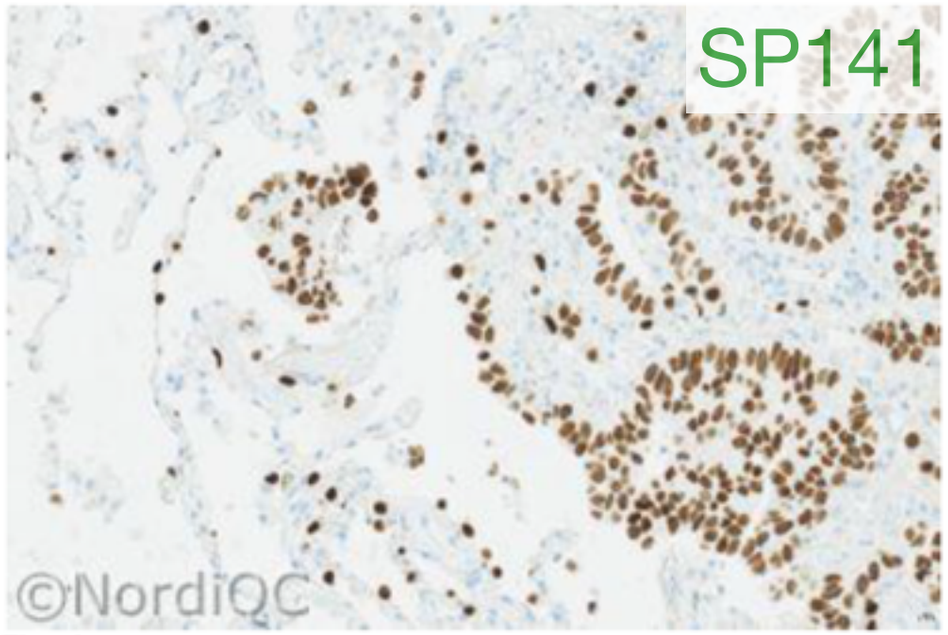


Fig. 4a
Optimal TTF1 staining of the lung adenocarcinoma no. 4 using same protocol as in Figs. 1a, 2a & 3a. Tumour (right side) with adjacent normal lung tissue. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. Strong reaction is also seen in all type II pneumocytes. No background staining is seen.

8G7G3/1

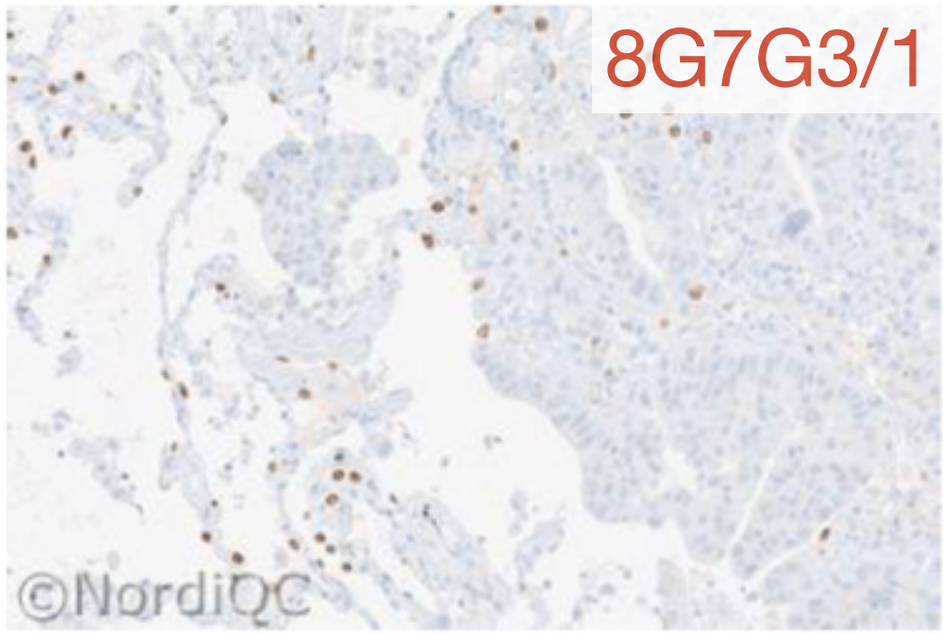
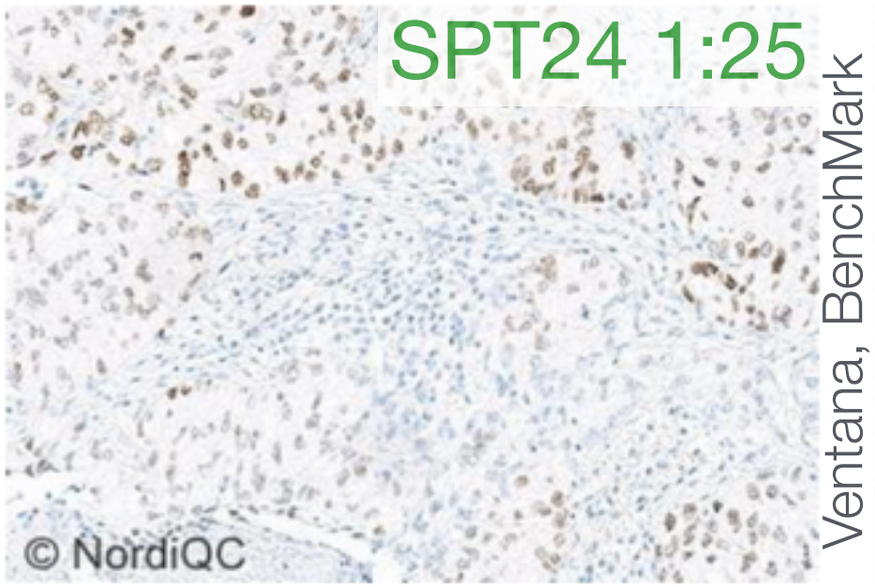


Fig. 4b
Insufficient TTF1 staining of the lung adenocarcinoma no. 4 using same protocol as in Figs. 1b, 2b & 3b. Despite a moderate positive staining reaction in the majority of type II pneumocytes - both in the normal tissue and within the tumour tissue - virtually all neoplastic cells are negative – same field as in Fig. 4a.

Lung tumours: Antibodies, protocols and controls

Primary antibody with a too low concentration



Ventana, BenchMark

Fig. 5a.
Optimal TTF1 staining of the lung adenocarcinoma no. 4 using mAb clone SPT24 diluted 1:25 with an incubation time of 32 min, HIER in CC1 for 64 min and performed at the BechMark Ultra instrument (Ventana), using the UltraView (2-step multimer) detection system. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. Compare with Fig 5b.

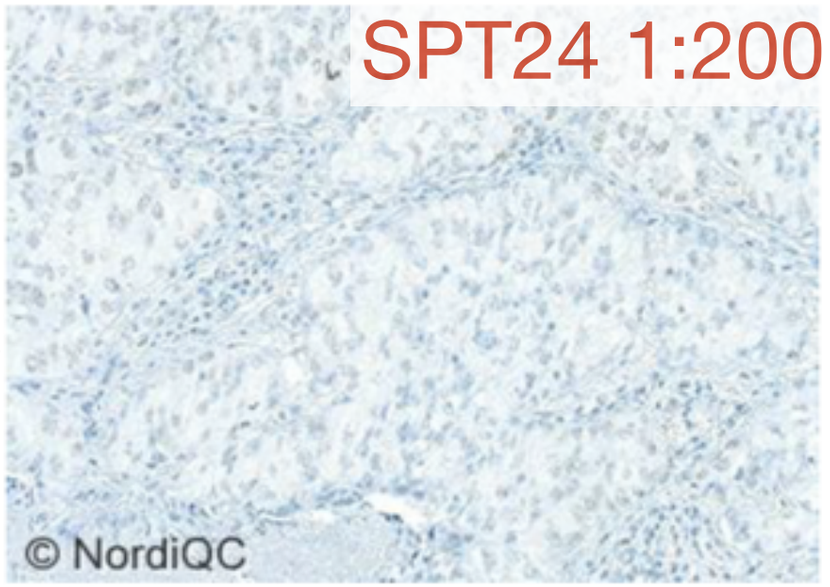
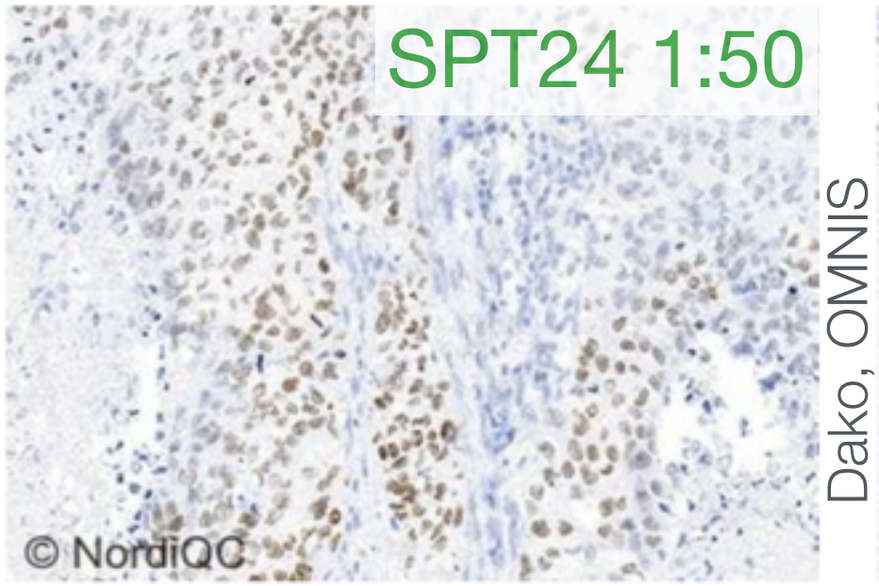


Fig. 5b.
Insufficient TTF1 staining of the lung adenocarcinoma no. 4. Same protocol as in Fig 5a. but with the use of **mAb clone SPT24 diluted 1:200**.
The lower concentration of the primary Ab results in a negative staining reaction of virtually all neoplastic cells – same field as in Fig. 5a.



Dako, OMNIS

Fig. 6a.
Optimal TTF1 staining of the lung adenocarcinoma no. 4 using mAb clone SPT24 diluted 1:50 with an incubation time of 20 min, HIER in TRS pH 9 for 30 min and performed at the OMNIS instrument (Dako), using the EnVision FLEX (2-step polymer) detection system. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction.

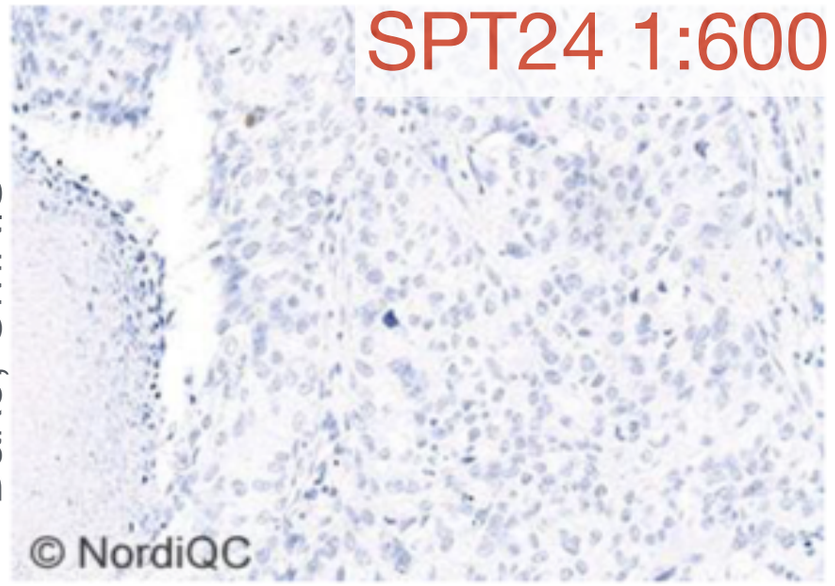


Fig. 6b.
Insufficient TTF1 staining of the lung adenocarcinoma no. 4. Same protocol as in Fig 6a. but with the use of **mAb clone SPT24 diluted 1:600**.
The lower concentration of the primary Ab results in a negative staining reaction of virtually all neoplastic cells – same field as in Fig. 6a.

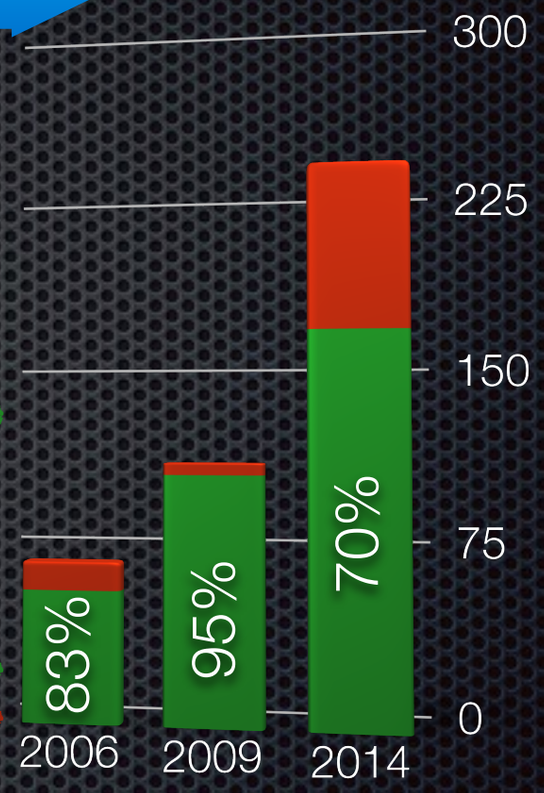
Lung tumours: Antibodies, protocols and controls

p63 / RUN 41 2014

Pass: 70 %

Table 1. **Antibodies and assessment marks for p63, run 41**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4A4**	38	BioCare Medical	21	25	27	2	61 %	67 %
	14	Dako						
	6	ImmunoLogic						
	5	Zeta Corporation						
	3	Santa Cruz						
	2	Zytomed Systems						
	1	BioLogo						
	1	BioGenex						
	1	BioSite						
	1	Bio SB						
	1	Minarini						
	1	NeoMarkers						
	1	Thermo Scientific						
mAb clone **DAK-p63**	26	Dako	13	9	4	0	85 %	95 %
mAb clone **7JUL**	14	Leica/Novocastra	0	0	10	4	0 %	-
rmAb clone **EP174**	1	Bio SB	0	0	1	0	-	-
mAb clone **SFI-6**	1	DCS Immunoline	0	0	0	1	-	-
Ab	1	Unknown	0	0	1	0	-	-
Ready-To-Use Abs								
mAb clone **4A4 790-4509**	74	Ventana	37	25	11	1	84 %	87 %
mAb clone **DAK-p63 IR662**	36	Dako	24	9	3	0	92 %	92 %
mAb clone **4A4 PM163**	3	BioCare	0	2	1	0	-	-
mAb clone **7JUL PA0103**	2	Leica/Novocastra	0	0	2	0	-	-
mAb clone **4A4 AM418**	1	BioGenex	0	0	1	0	-	-
mAb clone **4A4 PDM136**	1	DBS	0	0	0	1	-	-
mAb clone **4A4 MAD-000479QD**	1	Master Diagnostica SL	0	1	0	0	-	-
Total	236		95	71	61	9	-	
Proportion			40 %	30 %	26 %	4 %	70 %	



Lung tumours: Antibodies, protocols and controls

p63 / RUN 41 2014

Recommendable clones	Retrieval	Dilution range
mAb 4A4	HIER, High pH	1:50 - 1:600 or RTU
mAb DAK-p63	HIER, High pH	1:50 - 1:300 or RTU

Table 3. Proportion of optimal results for p63 using concentrated antibodies on the 3 main IHC systems*

Concentrated antibodies	Dako Autostainer Link / Classic		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **4A4**	2/17 (12%)**	-	14/26 (54%)	-	2/11 (18%)	0/1
mAb clone **DAK-p63**	1/9 (11%)	0/1	9/10 (90%)	-	-	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as recommended by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

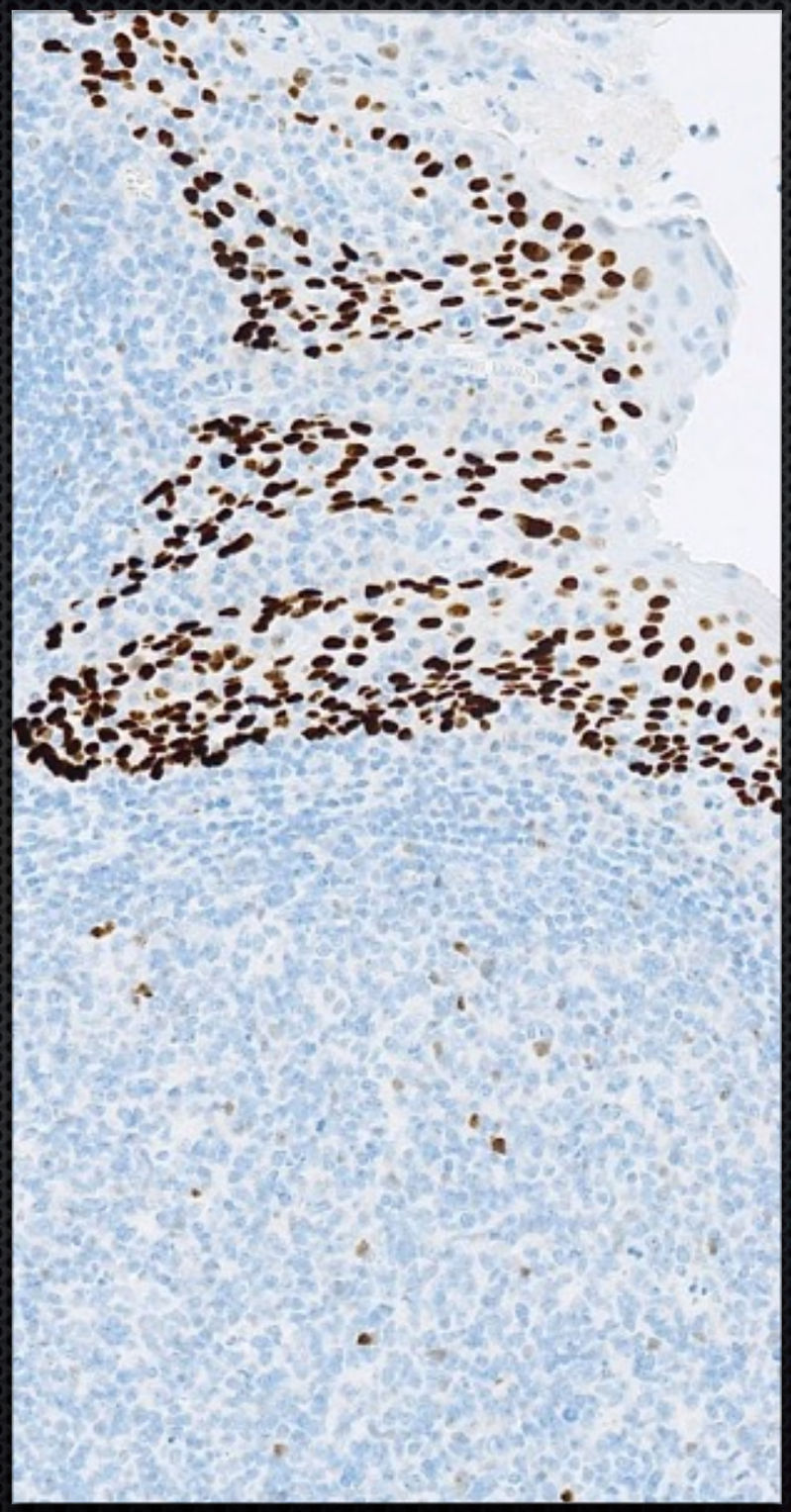
Lung tumours: Antibodies, protocols and controls

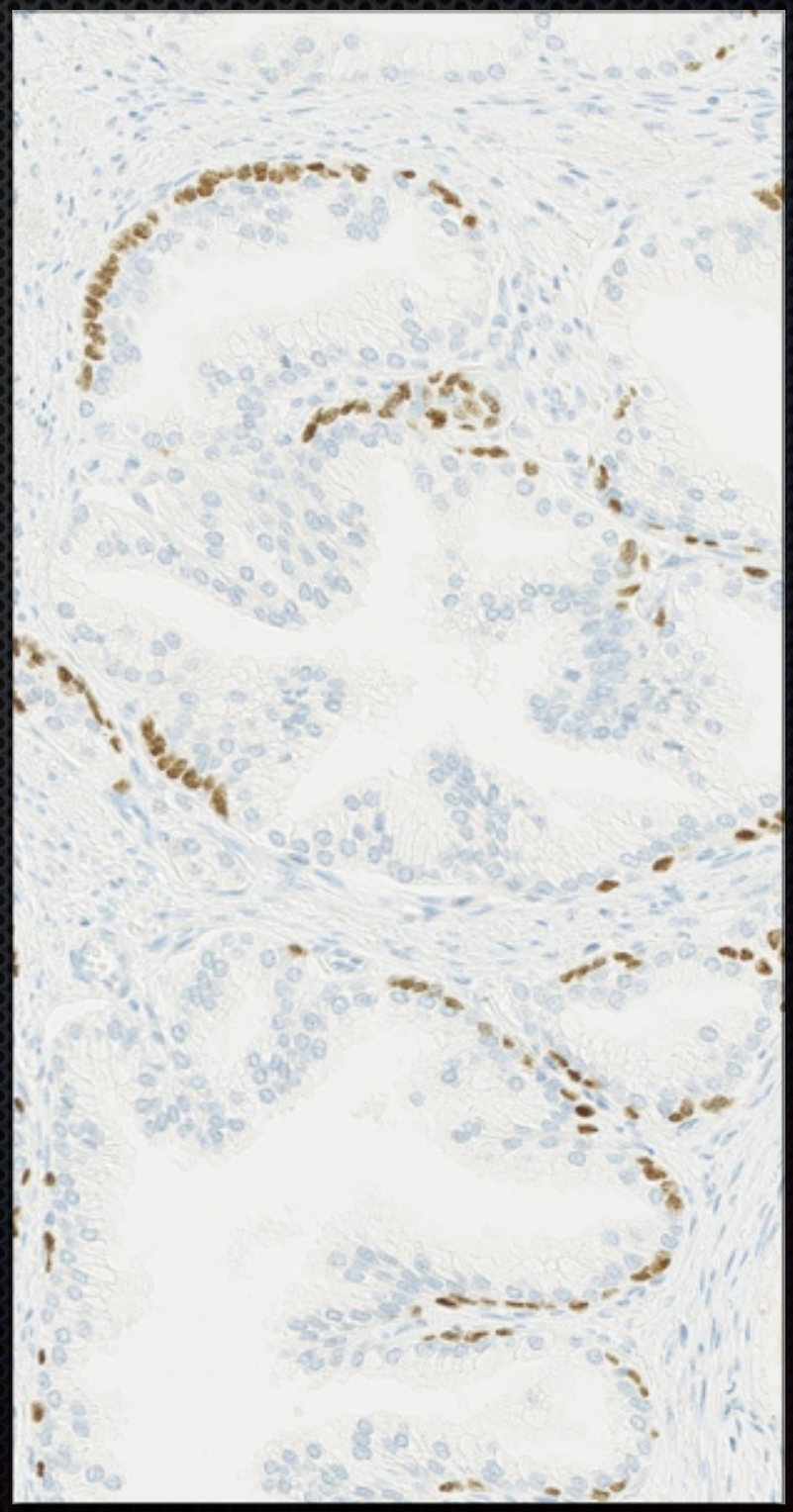
p63 / RUN 41 2014

Controls

Positive: Tonsil or prostate.

* Basal cells of prostate glands and squamous epithelial cells of tonsil must show a moderate to strong nuclear staining reaction.
* In the tonsil scattered lymphocytes must show a weak to moderate nuclear staining reaction.





Lung tumours: Antibodies, protocols and controls

NordiQC

Primary antibody with a too low sensitivity.

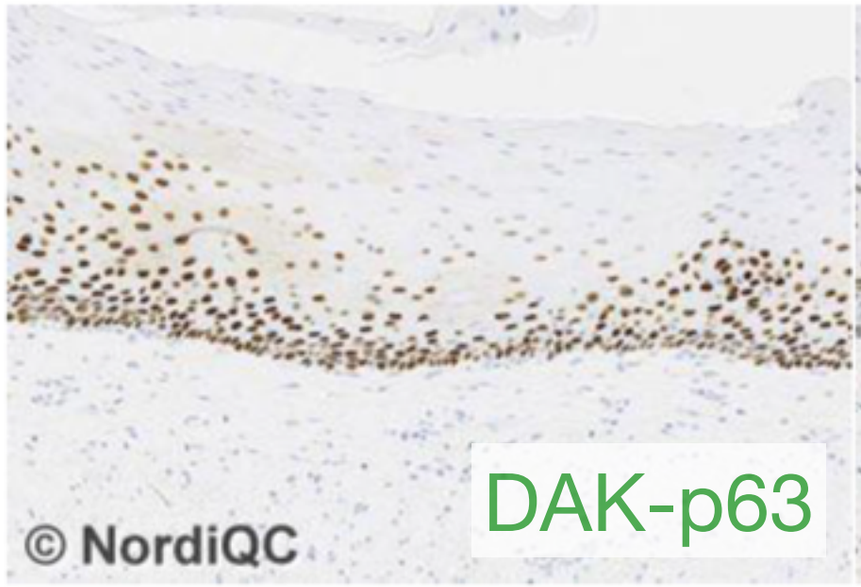


Fig. 1a
Optimal p63 staining of the esophagus using the mAb clone DAK-p63 (Dako RTU) with HIER in an alkaline buffer (TRS pH 9.0, Dako) and performed on the Dako Autostainer. A strong nuclear staining reaction is seen in the majority of the squamous epithelial cells in the esophagus. No background staining is seen. Same protocol used in Figs. 1a - 4a.

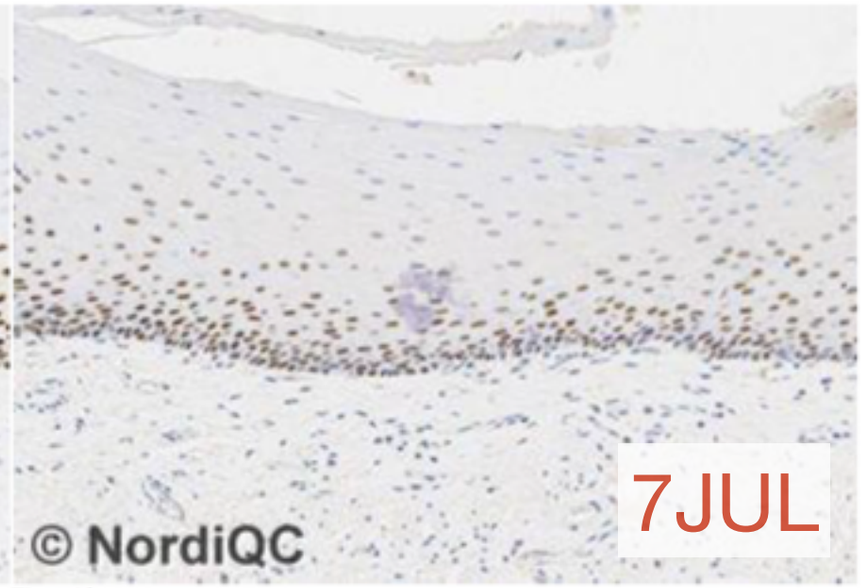


Fig. 1b
Insufficient p63 staining of the esophagus using the mAb clone 7JUL (Leica/Novocastra, 1:100) with HIER in an alkaline buffer (BERS2, Bond) and performed on the Bond III, Leica. A moderate nuclear staining reaction is seen in the majority of the squamous epithelial cells in the esophagus. Compare with Fig. 1a – same field. Also compare with Figs. 2b, 3b and 4b – same protocol.

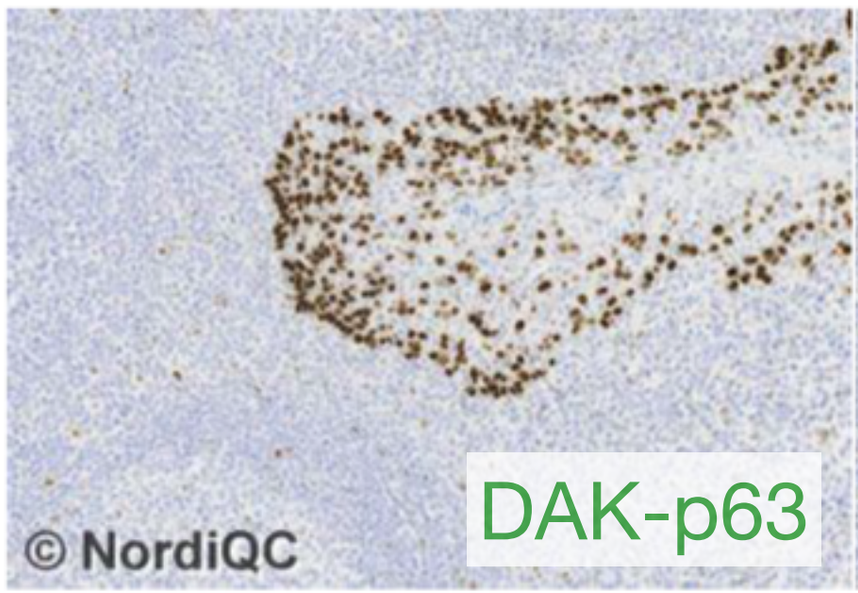


Fig. 2a
Optimal p63 staining of the tonsil using the same protocol as in Fig. 1a. A moderate to strong, distinct nuclear staining is seen in virtually all the squamous epithelial cells in the tonsil. In addition to the epithelial staining a weak but distinct nuclear reaction is present in scattered lymphocytes in the tonsil.

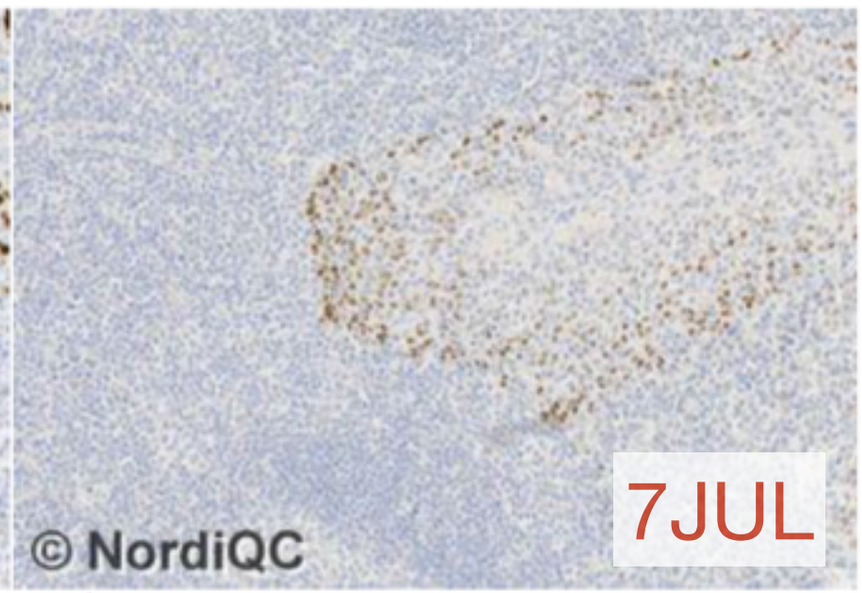


Fig. 2b
Insufficient p63 staining of the tonsil using the same protocol as in Fig. 1b. A weak to moderate, distinct nuclear staining is seen in the majority of the squamous epithelial cells in the tonsil. But in the insufficient protocol no staining is seen in lymphocytes. Compare with Fig. 2a. - same field.

Lung tumours: Antibodies, protocols and controls

Primary antibody with a too low sensitivity.

p63 / RUN 41 2014

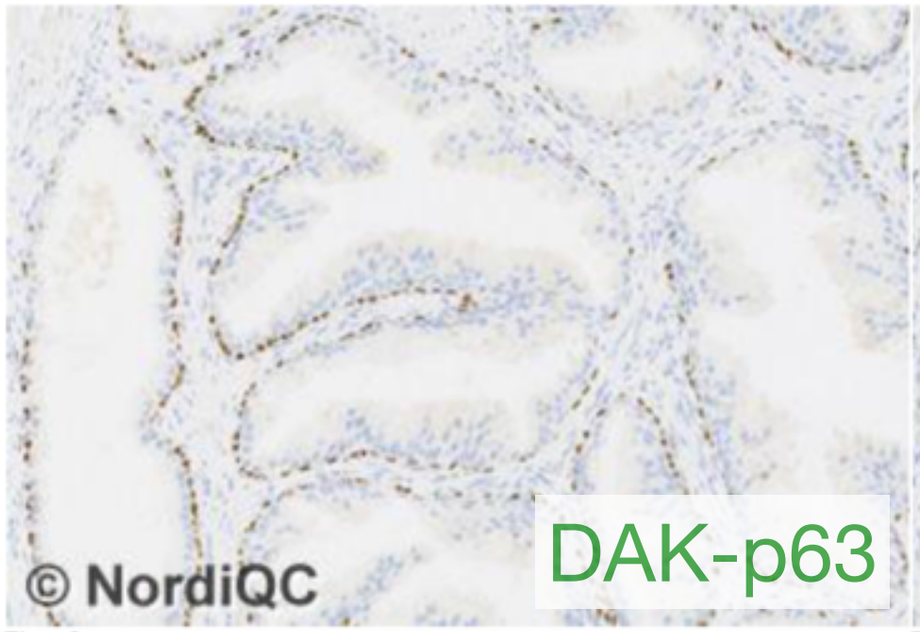


Fig. 3a
Optimal p63 staining in the prostate hyperplasia using the same protocol as in Figs. 1a & 2a. Virtually all the basal cells show a moderate to strong distinct nuclear staining reaction. No background staining is seen.

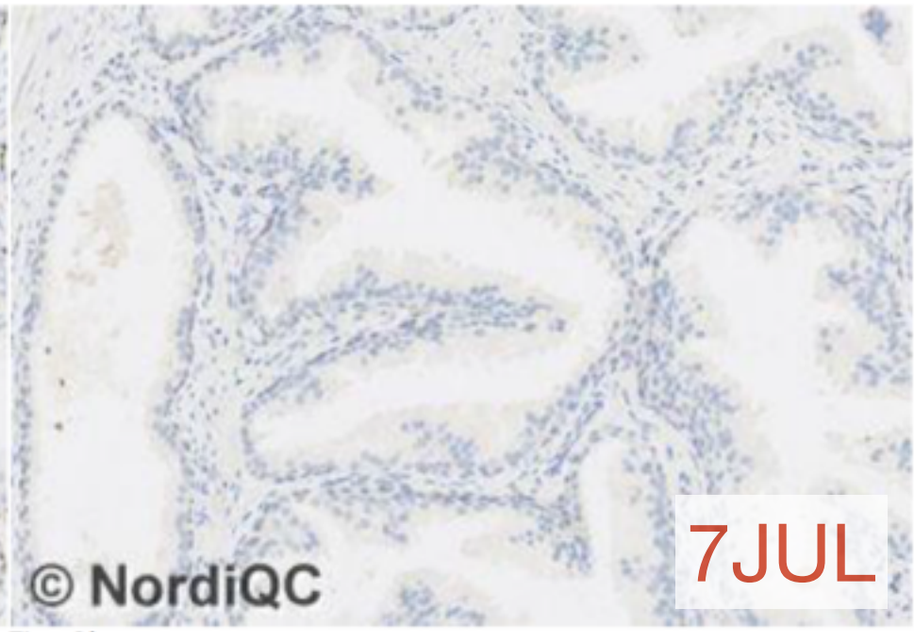


Fig. 3b
Insufficient p63 staining in the prostate hyperplasia using the same protocol as in Figs. 1b & 2b. Virtually all basal cells in the prostate hyperplasia are negative. Compare with Fig. 3a – same field.

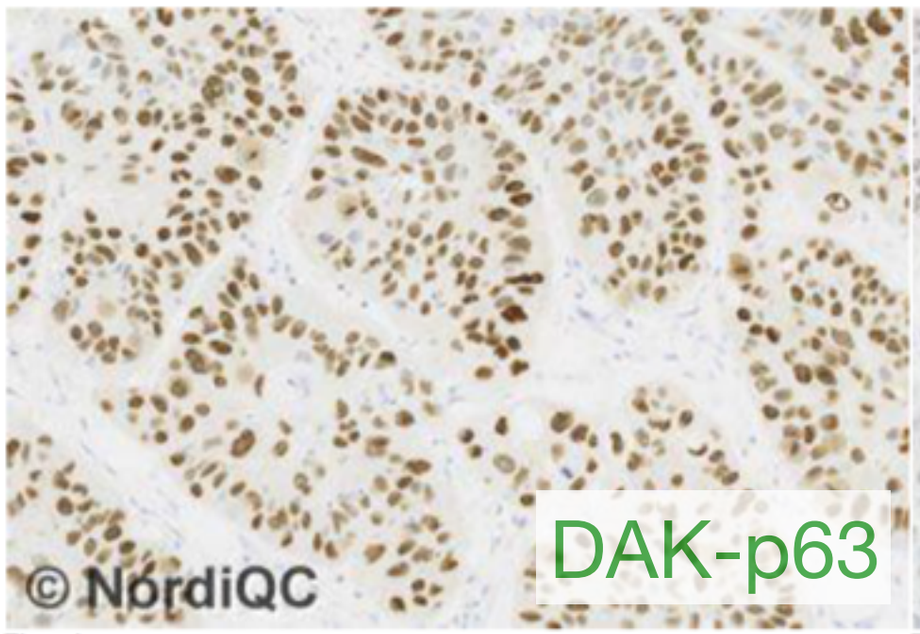


Fig. 4a
Optimal p63 staining of the lung squamous cell carcinoma using the same protocol as in Figs. 1a, 2a & 3a. Virtually all the neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen.

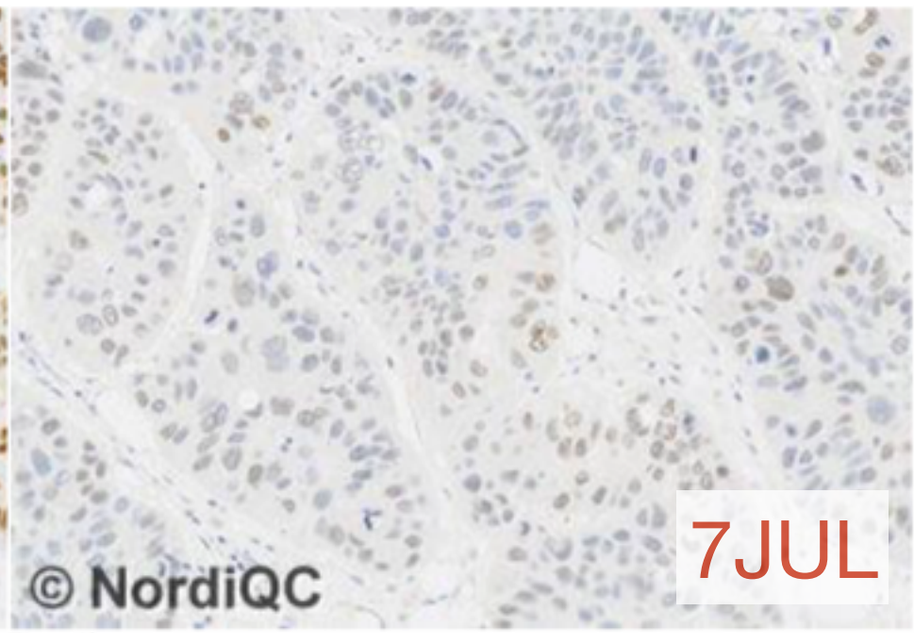


Fig. 4b
Insufficient P63 staining of the lung squamous cell carcinoma using the same protocol as in Figs. 1b, 2b & 3b. Only faint nuclear staining is seen and only in a minor fraction of the neoplastic cells. Compare with Fig. 4a – same field.

Lung tumours: Antibodies, protocols and controls

p63 / RUN 41 2014

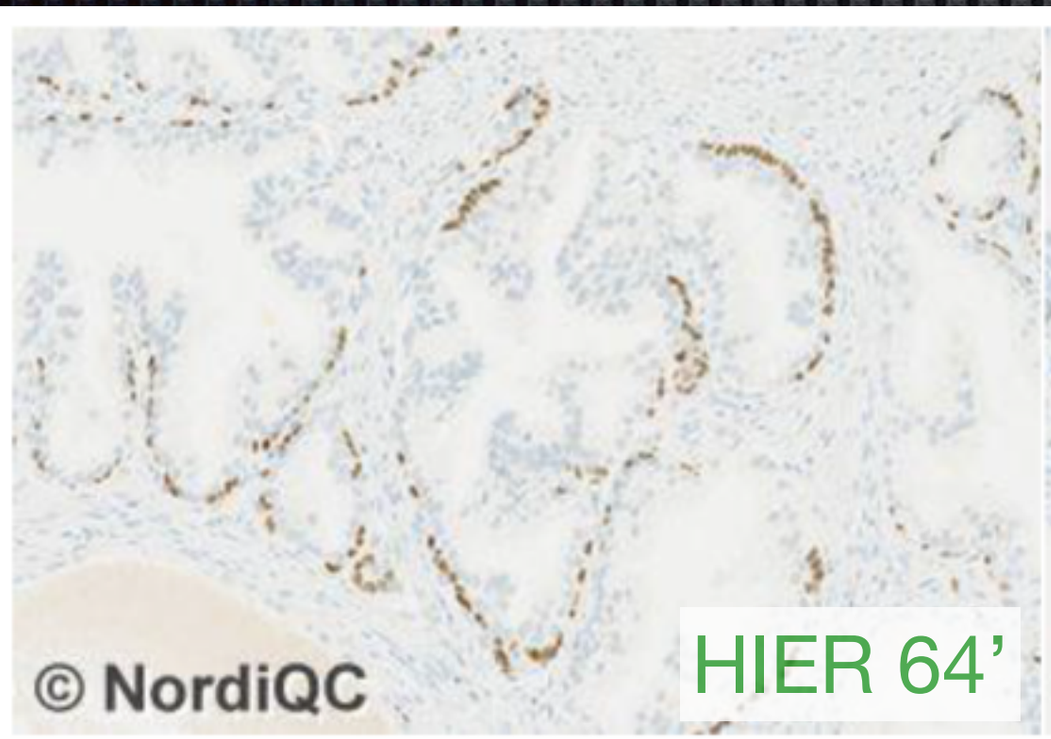


Fig. 5a
Optimal p63 staining of the prostate hyperplasia using the mAb clone 4A4 (Ventana, RTU) with HIER in CC1 (Ventana) for 64 min. Moderate to strong nuclear reaction is seen in virtually all basal cells. Efficient HIER pretreatment is essential to optimal P63 staining. Compare with Fig. 5b.

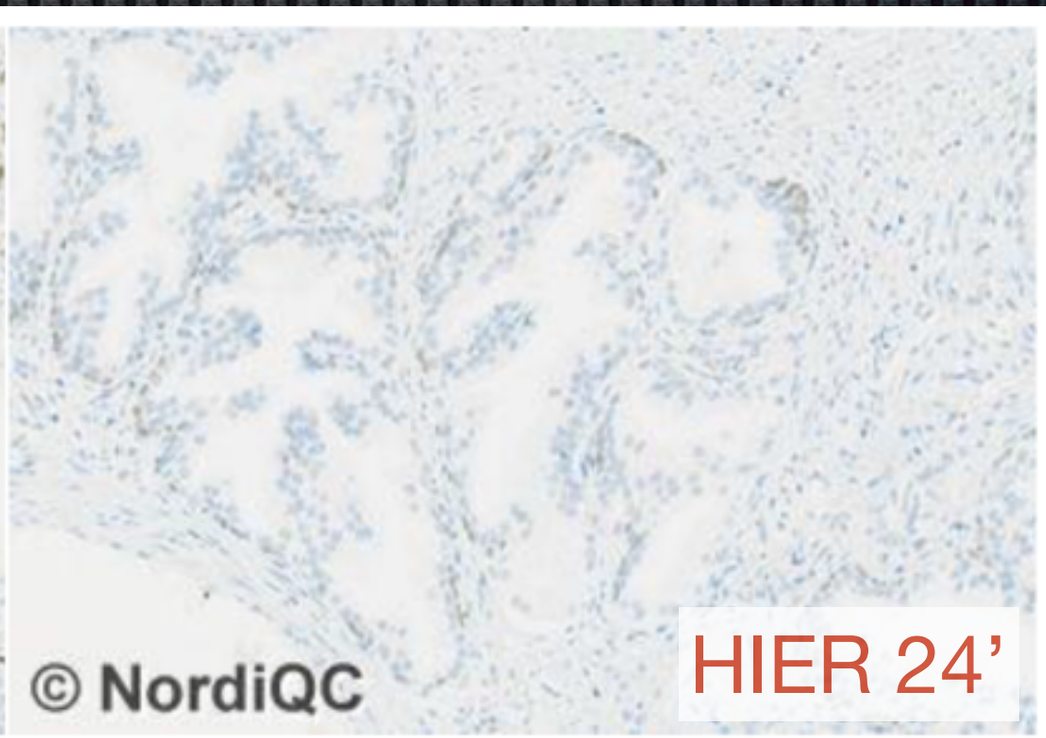
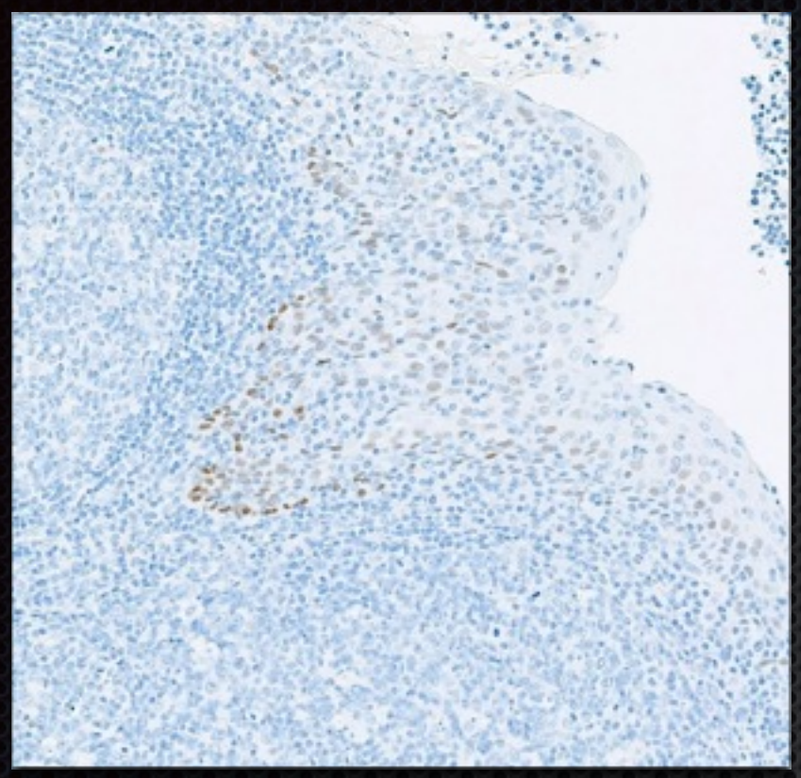


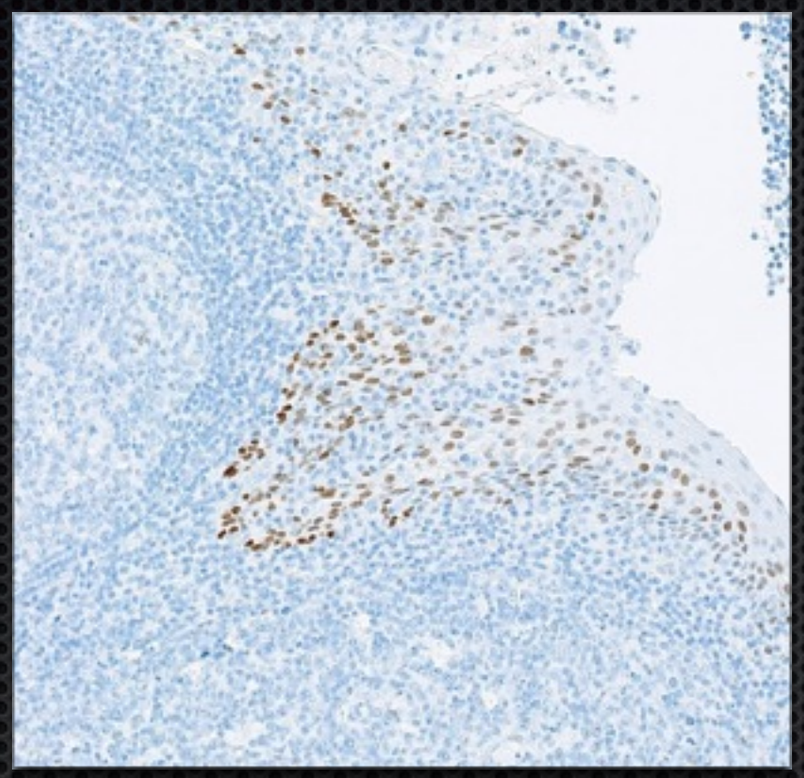
Fig. 5b
Insufficient p63 staining in the prostate hyperplasia using the mAb clone 4A4 (Ventana, RTU) in the same protocol as in Fig. 5a, except for the reduction in HIER pretreatment to 24 min compared to the 64 min i Fig 5a. Consequenctly a dramatic reduction in staining intensity is seen making the identification of the basal cell difficult. Compare with Fig. 5a – same field.

Insufficient HIER.

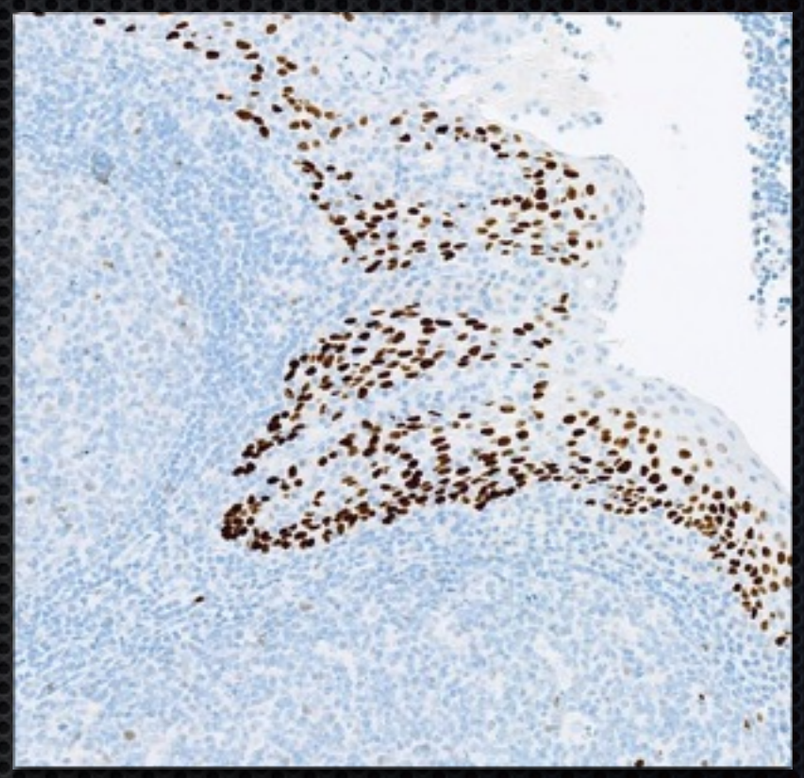
p63, 4A4 - OptiView (3-step) - Various HIER time



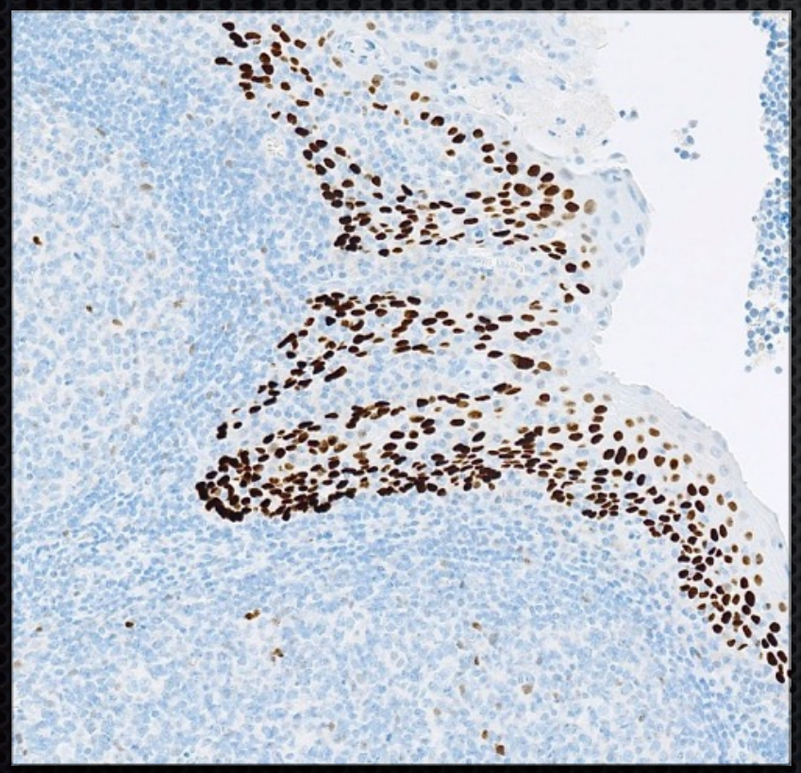
CC1_8_100°C



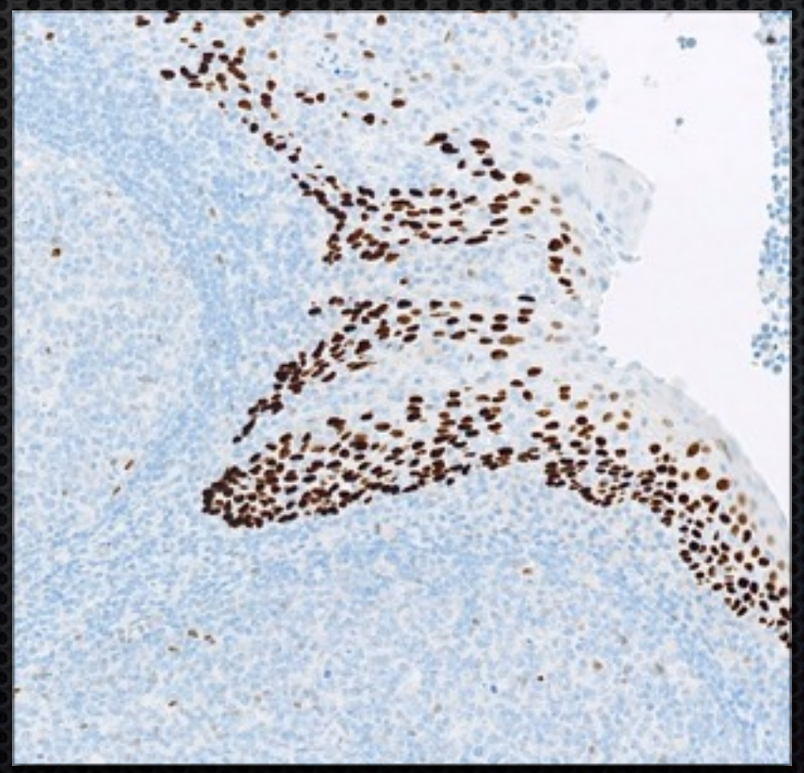
CC1_16_100°C



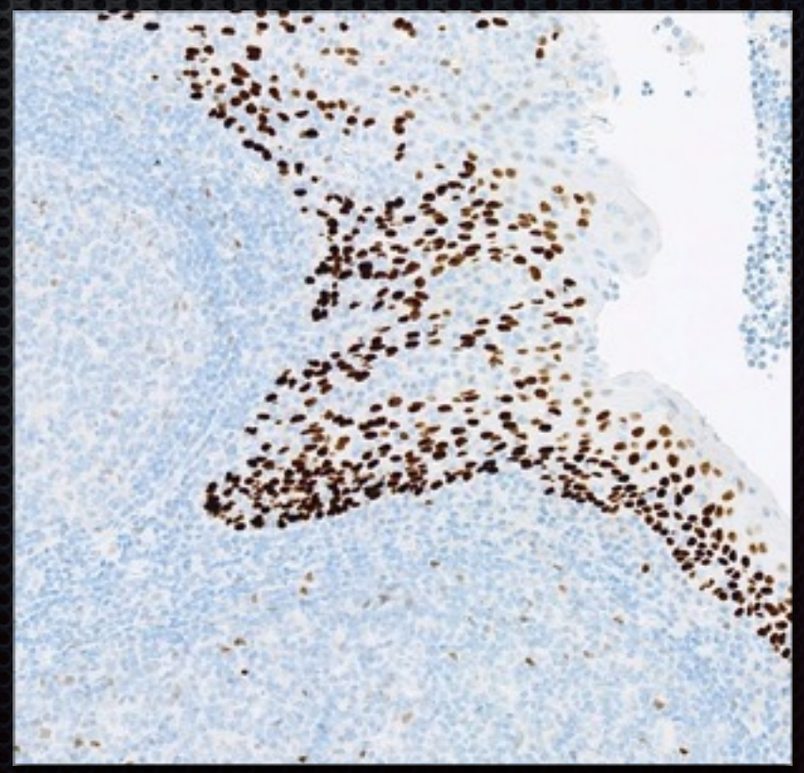
CC1_32_100°C



CC1_48_100°C

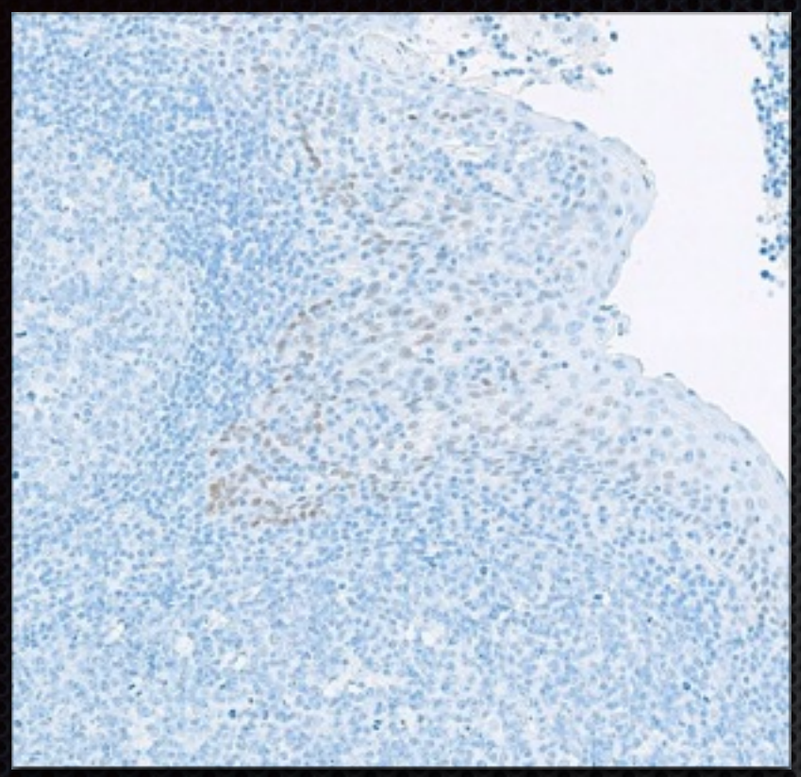


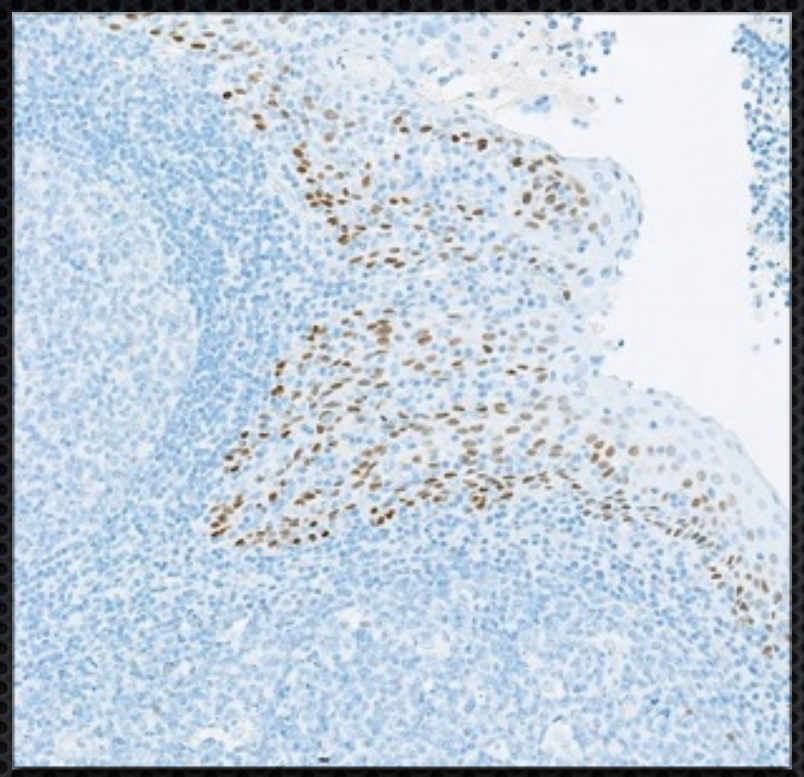
CC1_64_100°C

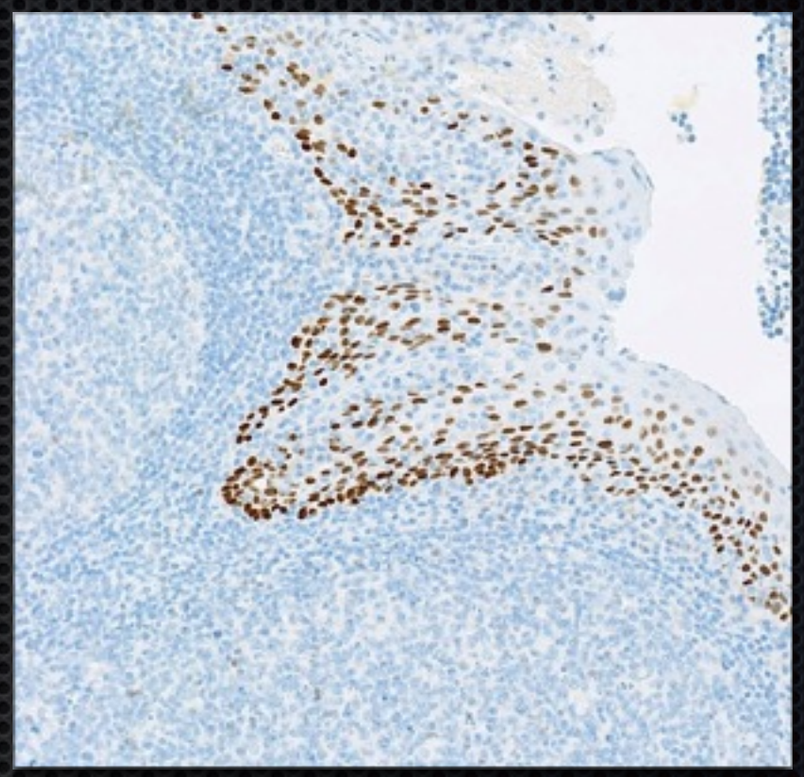


CC1_92_100°C

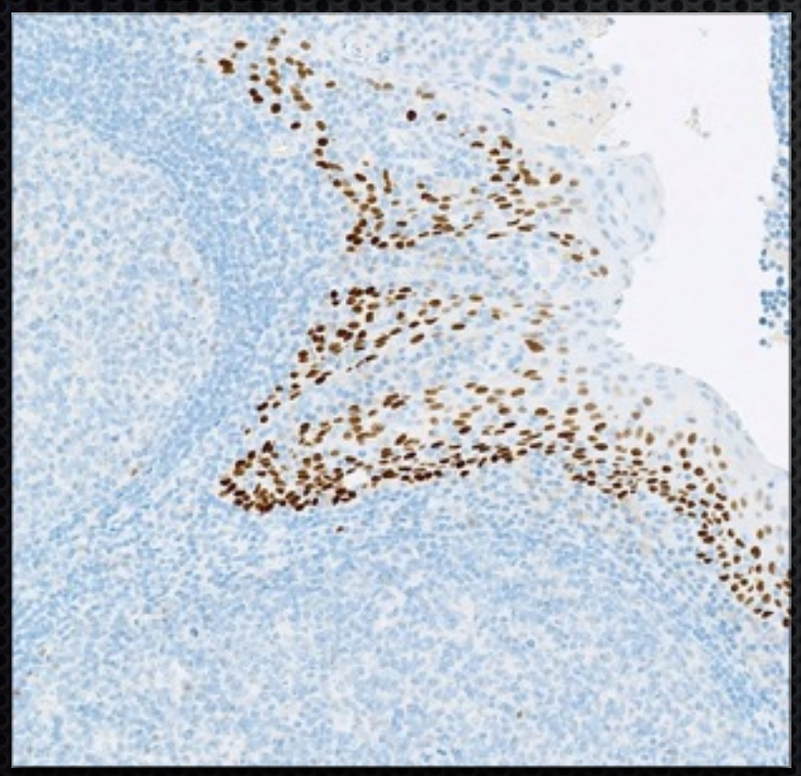
p63, 4A4 - UltraView (2-step) - Various HIER time



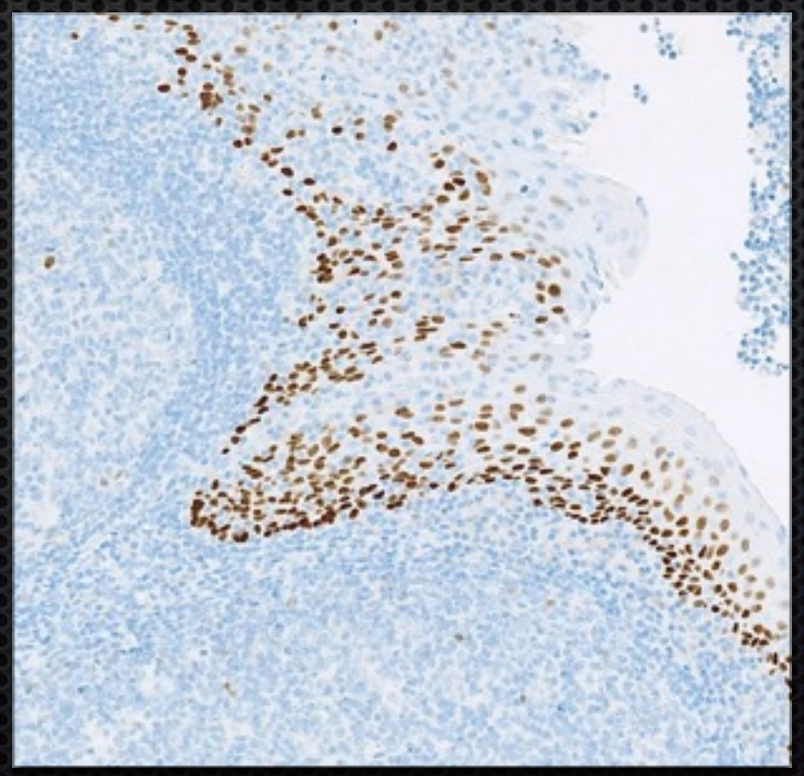




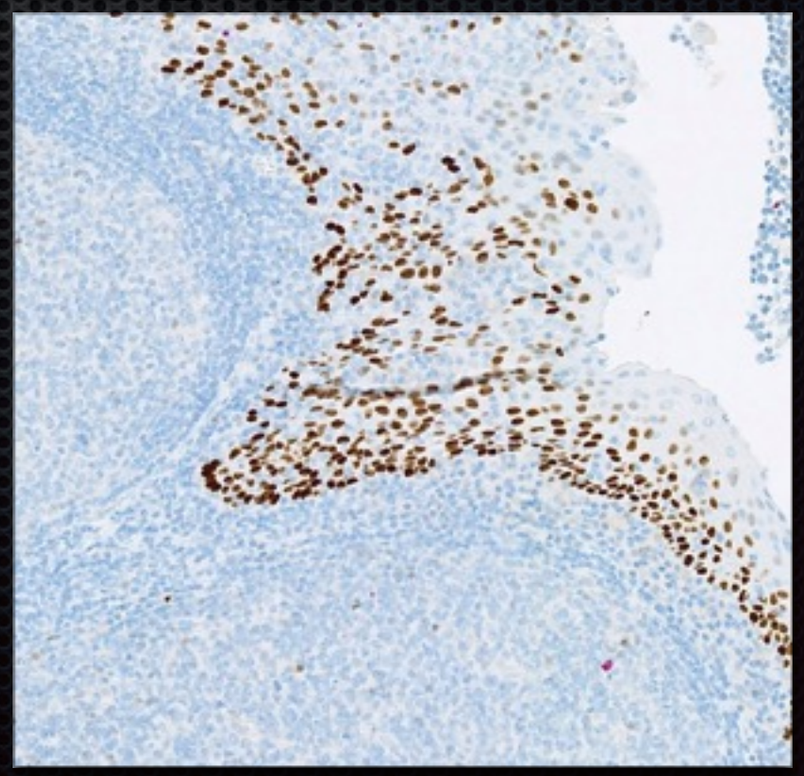
CC1_8_100°C



CC1_20_100°C



CC1_36_100°C

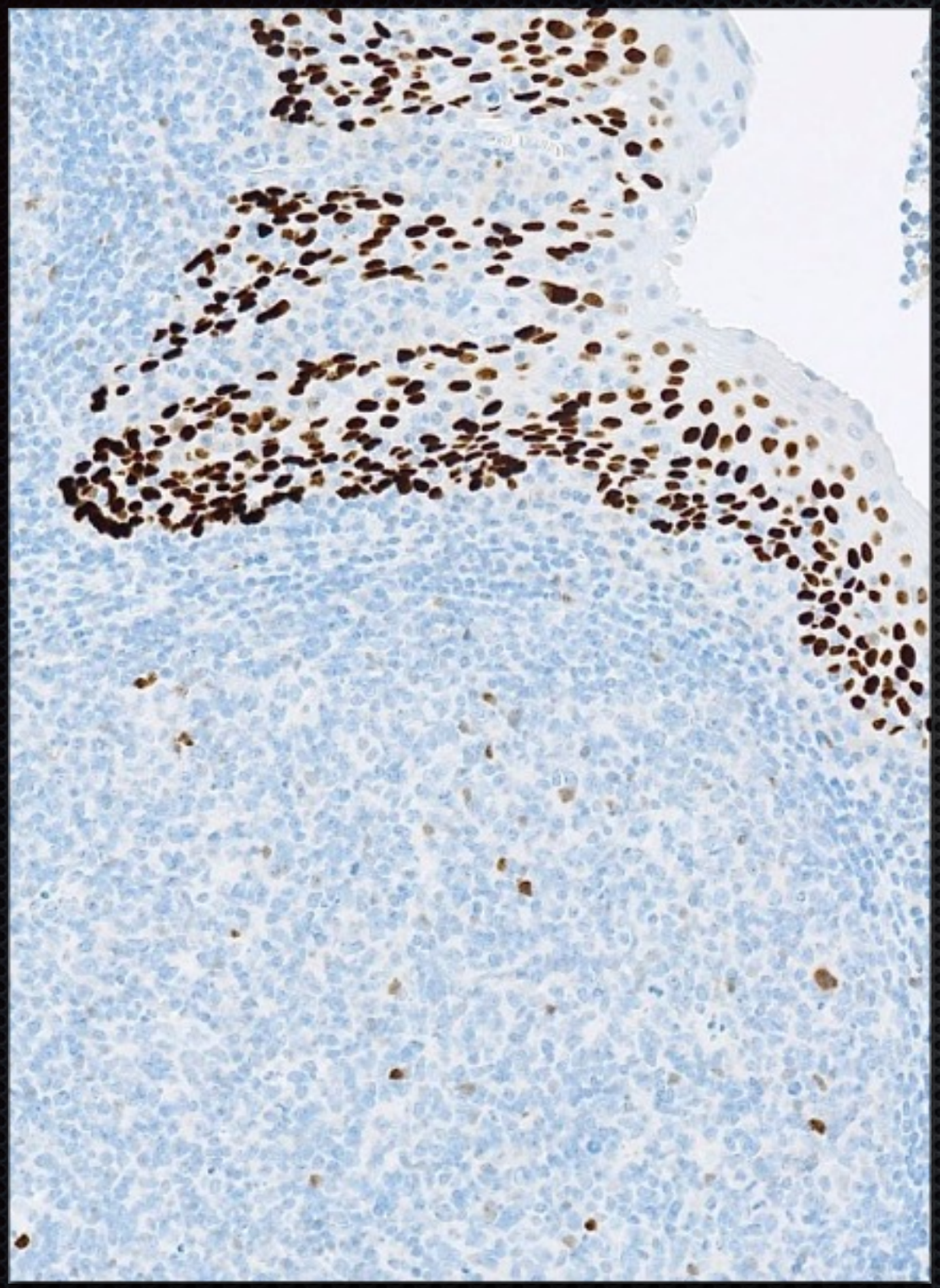


CC1_52_100°C

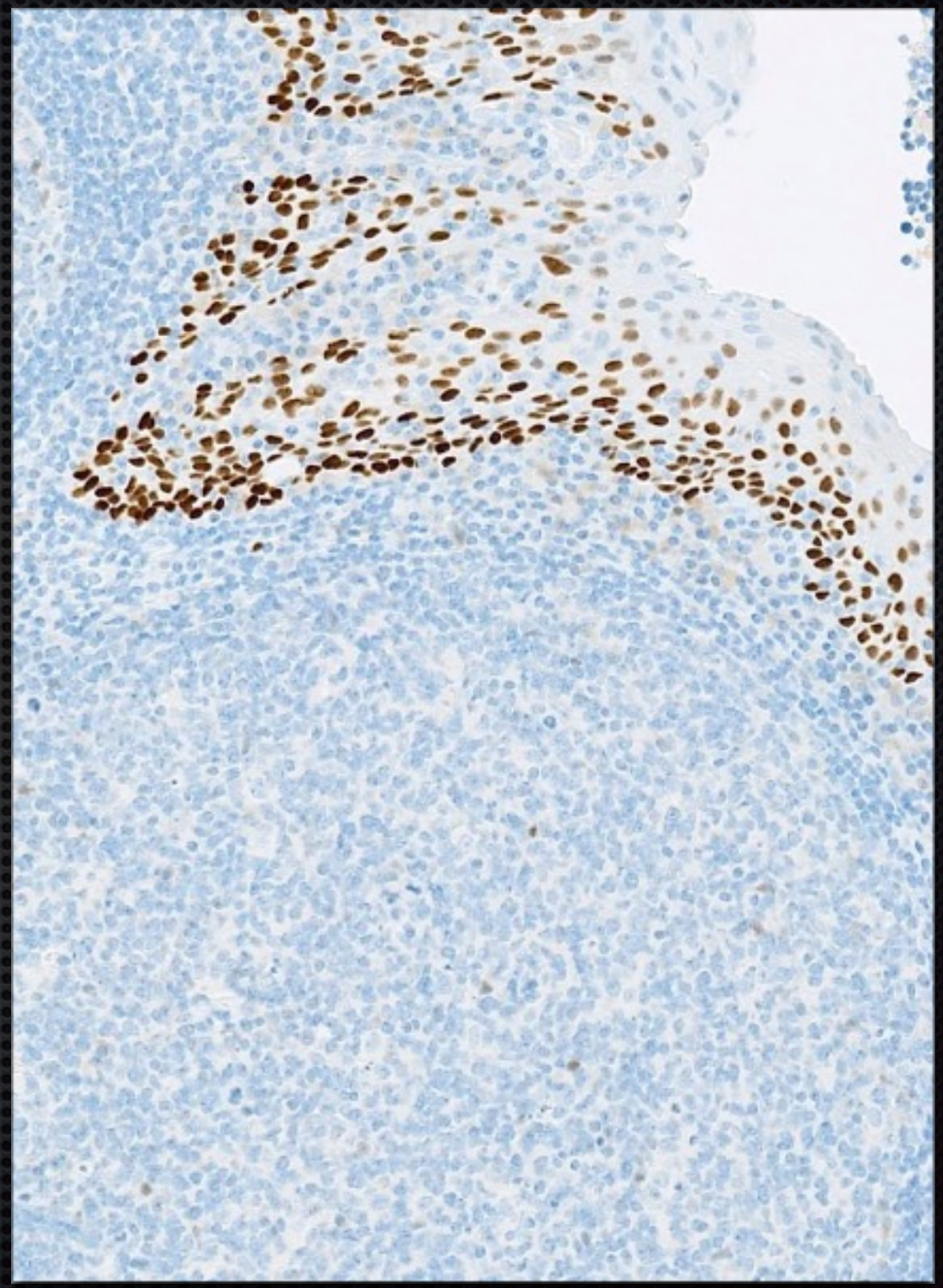
CC1_64_100°C

CC1_92_100°C

p63, 4A4 OptiView (3-step) vs UltraView (2-step)



OptiView - HIER CC1_48_100



UltraView - HIER CC1_52_100

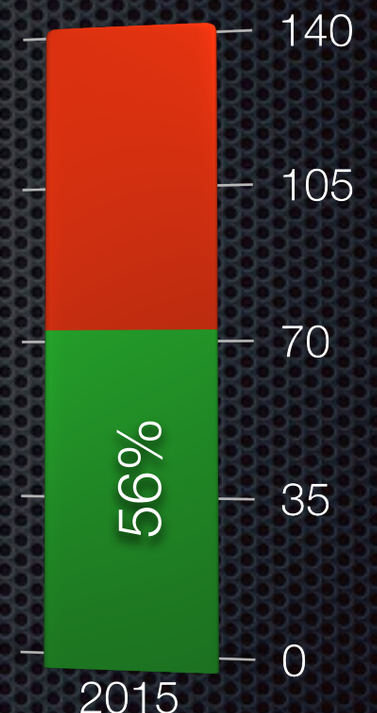
Lung tumours: Antibodies, protocols and controls

p40 / RUN 44 2015

Pass: 56 %

Table 1. **Antibodies and assessment marks for p40, run 44**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BC28**	49 3 1	Biocare Zytomed Nordic Biosite	26	17	10	0	81%	86%
rmAb clone **ZR8**	12 1	Immunologic Zeta Corporation	3	6	4	0	69%	80%
pAb **AC13030**	16	Biocare	0	3	11	2	19%	-
pAb **PC373**	9	Calbiochem, Merck	0	2	7	0	22%	-
pAb **RP163**	5 1 1	Diagnostic Biosystems Medac diagnostica ITK DIAGNOSTICS BV	0	2	4	1	29%	-
pAb **ab99513***	4	Abcam	0	0	4	0	-	-
pAb **PDR 055**	1 1	Diagnostic Biosystems ITK DIAGNOSTICS BV	0	1	0	1	-	-
pAb **RBK054**	2	Zytomed	0	1	1	0	-	-
pAb **ab166857**	1	Abcam	0	0	0	1	-	-
pAb **ab167612**	1	Abcam	0	0	0	1	-	-
pAb **BP4206**	1	ID Labs	0	1	0	0	-	-
pAb **ILP-3726**	1	Immunologic	0	0	1	0	-	-
pAb **Unknown**	1	Unknown	0	0	1	0	-	-
Ready-To-Use-antibodies								
mAb clone **BC28 API 3066**	6	Biocare	3	2	1	0	83%	100%
mAb clone **BC28 790-4950**	2	Ventana	1	1	0	0	-	-
pAb **API 3030**	3	Biocare	0	2	1	0	-	-
pAb **RAB-066**	3	Maixin	0	1	2	0	-	-
pAb **PDR 055**	2	ITK DIAGNOSTICS BV	0	0	2	0	-	-
mAb **MAD-000623QD**	2	Master Diagnostica	0	0	2	0	-	-
Total	129		33	39	51	6	-	
Proportion			26%	30%	40%	4%	56%	



Lung tumours: Antibodies, protocols and controls

p40 / RUN 44 2015

Recommendable clones	Retrieval	Dilution range
mAb BC28	HIER, High pH	1:25 - 1:100 or RTU
mAb ZR8	HIER, High pH	1:100 - 1:800

Table 3. **Proportion of optimal results for p40 for the most commonly used antibody as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **BC28**	7/15** (47%)	-	13/18 (72%)	-	1/3	-

Lung tumours: Antibodies, protocols and controls

p40 / RUN 44 2015

Controls

Positive: Placenta (LLOD)

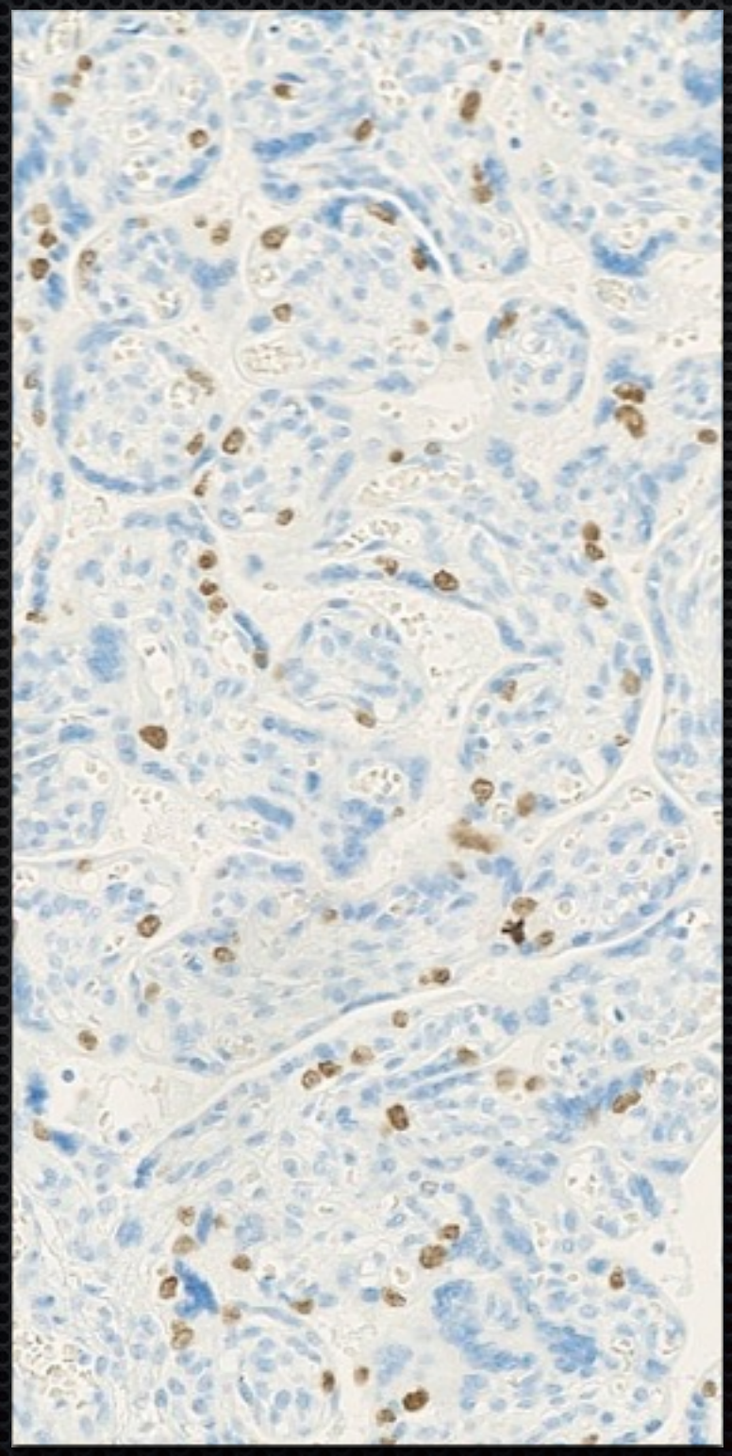
* Cytotrophoblasts must show an at least weak to moderate, distinct nuclear staining reaction.

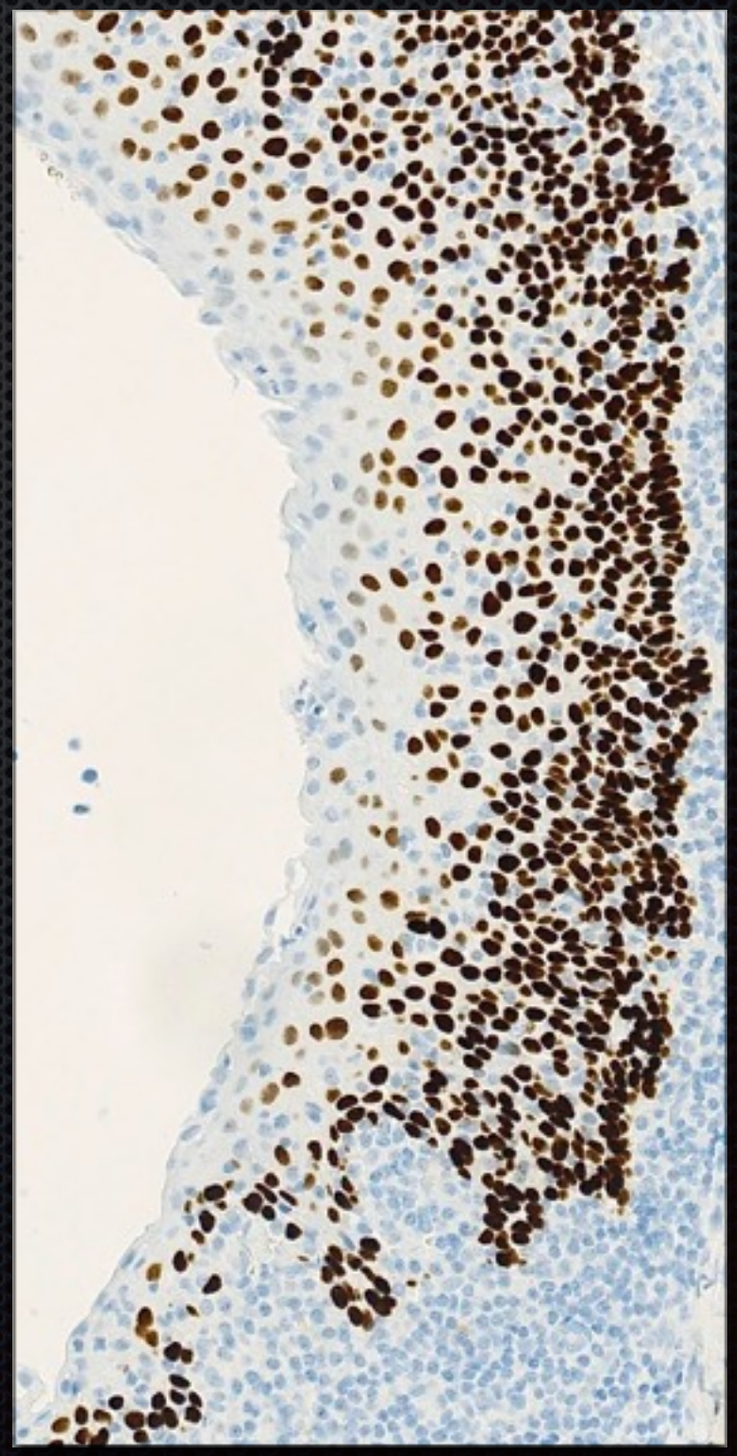
Positive: Tonsil

* Virtually all squamous epithelial cells must show a moderate to strong, distinct nuclear staining reaction.

Negative: Tonsil

* Lymphocyttes must be negative.





Lung tumours: Antibodies, protocols and controls

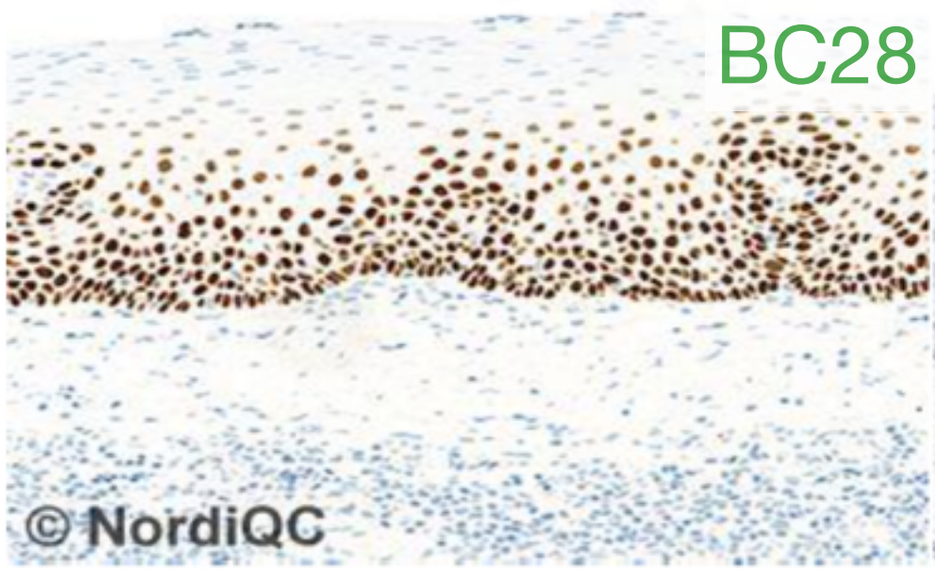


Fig. 1a (x200)
Optimal p40 staining of the tonsil using the mAb clone BC28 as a concentrate, optimally calibrated, HIER in an alkaline buffer (TRS pH 9.0, Dako), and a 3-step polymer based detection system (FLEX+, Dako). A moderate to strong nuclear staining reaction is seen in the majority of the squamous epithelial cells. No background staining is seen. Same protocol used in Figs. 1a - 4a.

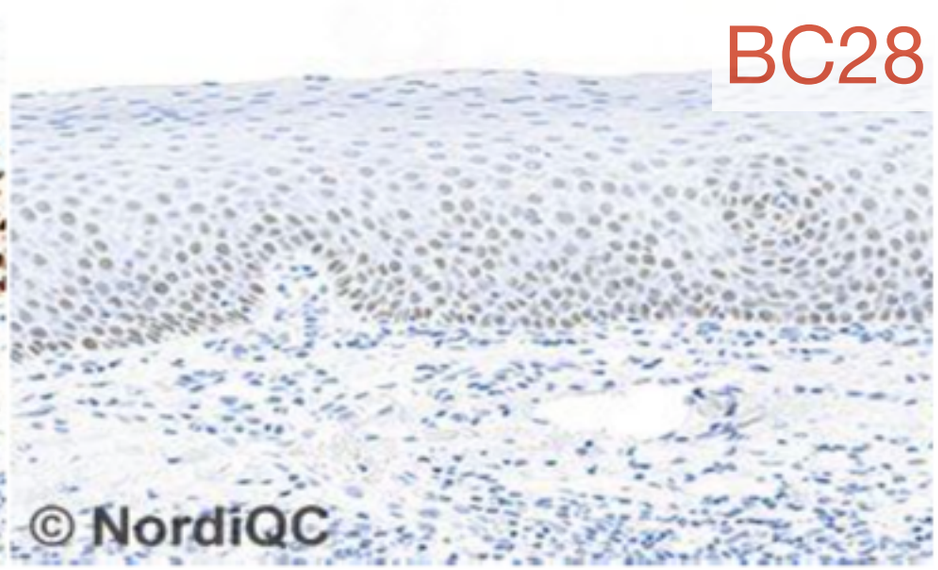


Fig. 1b (x200)
Insufficient p40 staining of the tonsil using the mAb clone BC28. The protocol provided an overall too low sensitivity most likely due to a combination of a too low concentration of the primary Ab and use of a 2-step polymer based detection system with a moderate sensitivity (FLEX, Dako)- compare with Fig. 1a (same field). The intensity and proportion of cells demonstrated is reduced. Also compare with Figs. 2b - 4b, same protocol.

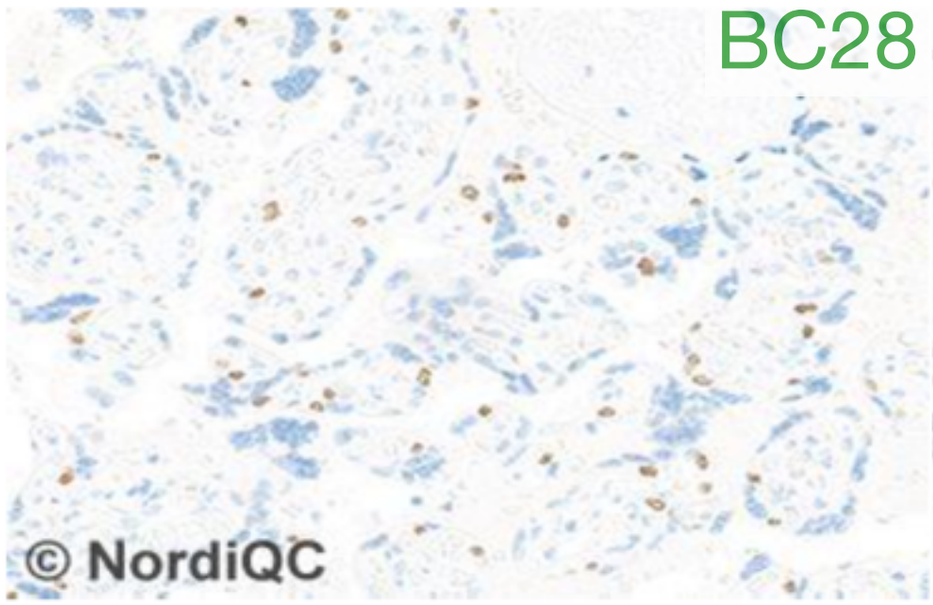


Fig. 2a (x200)
Optimal p40 staining of the placenta using same protocol as in Fig. 1a. Scattered cytothrophoblastic cells show a weak to moderate, distinct nuclear staining reaction.

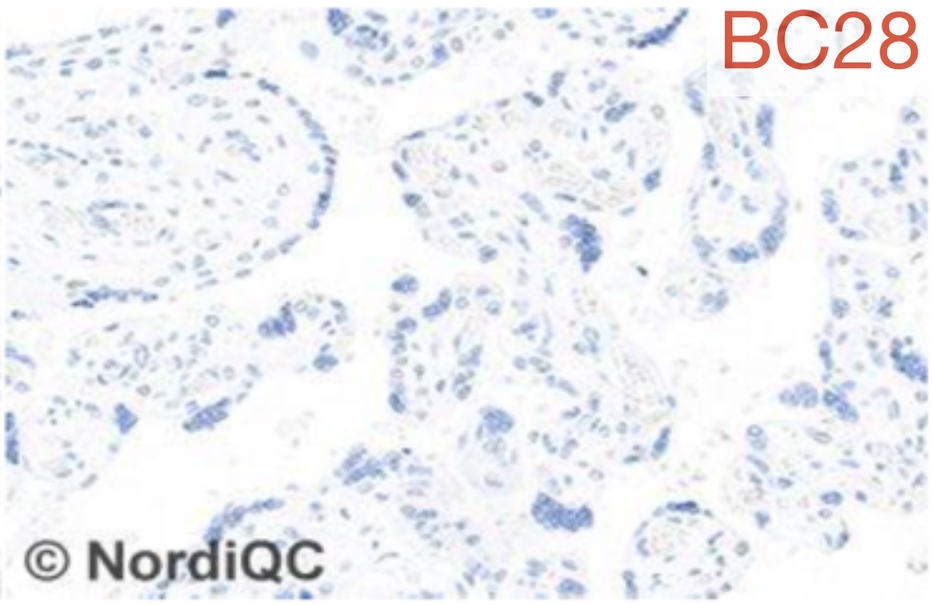


Fig. 2b (x200)
Insufficient p40 staining of the placenta using same protocol as in Fig. 1b. Virtually no staining reaction of cytothrophoblastsic cells is seen. Also compare with Figs. 3b and 4b, same protocol.

Combination of a too low concentration of the primary Ab and use of a less sensitive 2-step polymer based detection system!

Lung tumours: Antibodies, protocols and controls

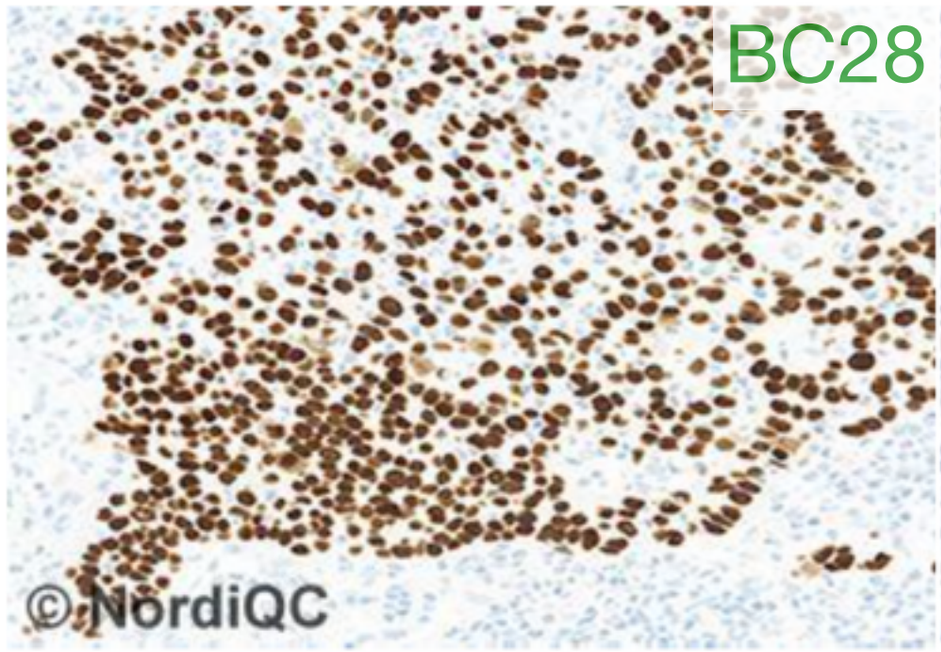


Fig. 3a (x200)
Optimal p40 staining of the lung squamous cell carcinoma using same protocol as in Figs. 1a & 2a.Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. No background staining is seen.

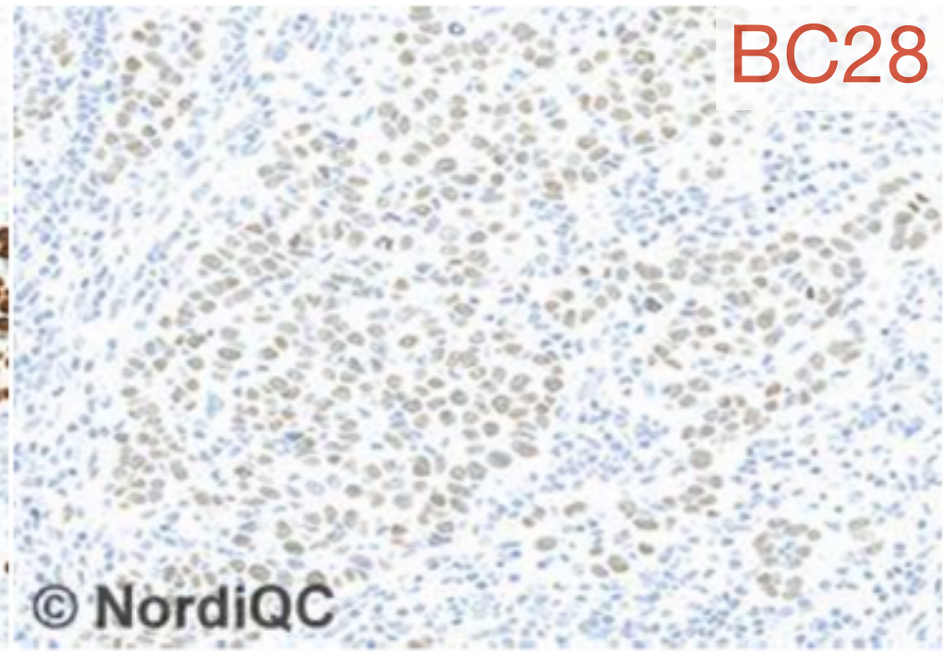


Fig. 3b (x200)
Insufficient p40 staining of the lung squamous cell carcinoma using same protocol as in Figs. 1b & 2b. The intensity and proportion of cells demonstrated is significantly reduced.

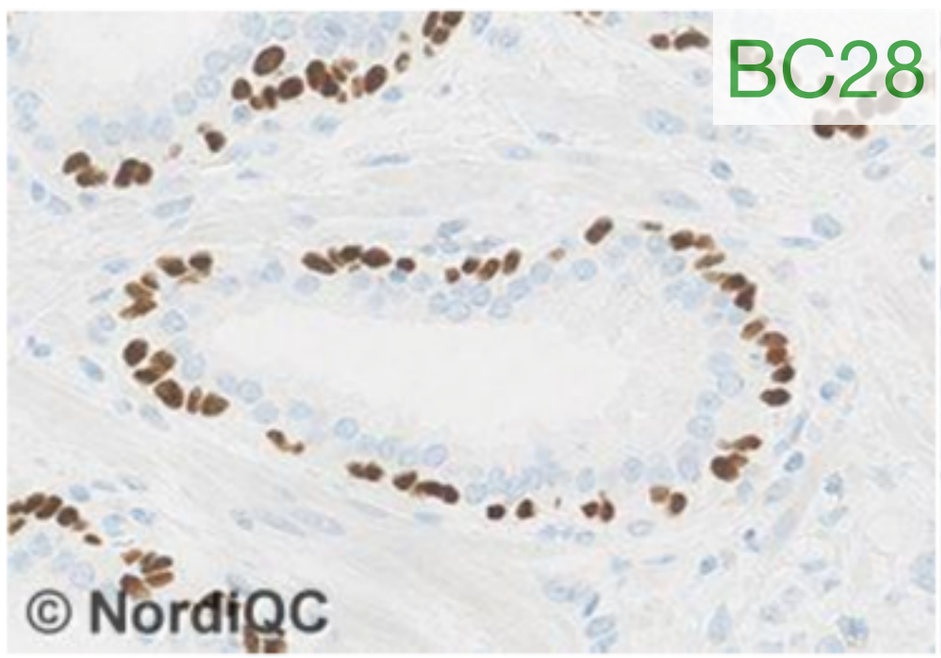


Fig. 4a (x400)
Optimal p40 staining of the prostate hyperplasia using same protocol as in Figs. 1a - 3a. The basal cells are distinctively demonstrated as a moderate to strong nuclear staining reaction is observed.

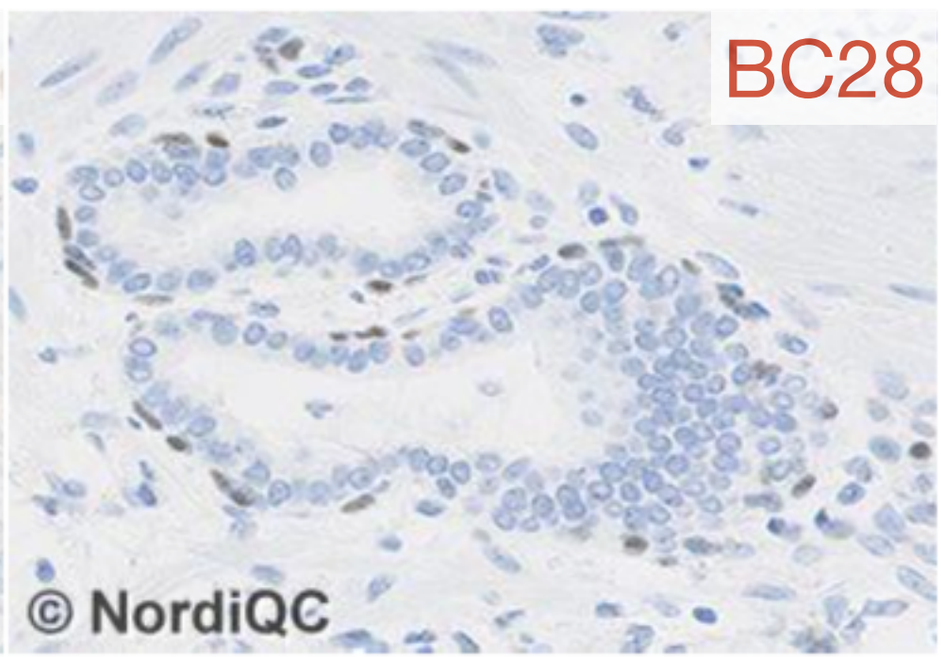


Fig. 4b (x400)
Insufficient p40 staining of the prostate hyperplasia using same protocol as in Figs. 1b – 3b. Only a weak and equivocal nuclear staining reaction in the basal cells is observed.

Combination of a too low concentration of the primary Ab and use of a less sensitive 2-step polymer based detection system!

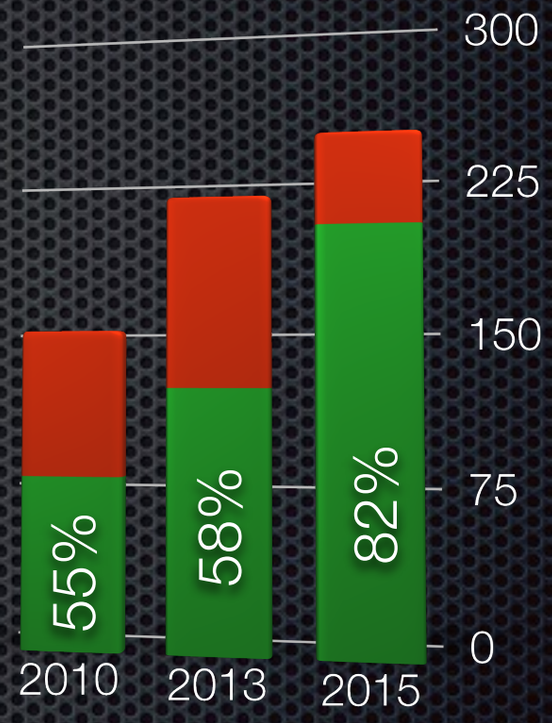
Lung tumours: Antibodies, protocols and controls

Synaptophysin / RUN 43 2015

Pass: 82 %

Table 1. **Antibodies and assessment marks for SYP, run 43**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **27G12**	69 2 1 1	Leica/Novocastra Biocare Monosan Genetech	28	35	6	4	86%	88%
mAb clone **BS15**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **DAK-SYNAP**	12	Dako	7	3	2	0	83%	100%
mAb clone **SNP88**	7	Biogenex	2	5	0	0	100%	100%
mAb clone **SY38***	3	Dako	0	2	1	0	-	-
rmAb clone **MRQ-40**	5 1	Cell Marque Monosan	3	2	1	0	83%	100%
rmAb clone **SP11**	10 2 1 1	Thermo/Neomarkers Spring Bioscience Abcam Immunologic	7	4	3	0	79%	83%
pAb **180130**	1	Immuno Diagnostics	0	0	1	0	-	-
pAb **RB-1461**	1	Thermo/Neomarkers	0	0	1	0	-	-
pAb **RBK011**	1	Zytomed	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **27G12 PA0299**	9	Leica/Novocastra	3	3	2	1	67%	100%
mAb clone **27G12 PM371**	1	Biocare	0	1	0	0	-	-
mAb clone **DAK-SYNAP IR660**	38	Dako	11	23	4	0	89%	90%
mAb clone **SNP88 AM363-5M**	2	Biogenex	0	1	1	0	-	-
mAb clone **SY38 IR/IS776***	5	Dako	0	2	2	1	-	-
rmAb **MRQ-40 760-4595**	31	Ventana/Cell Marque	23	7	1	0	97%	100%
rmAb clone **MRQ-40 336R**	1	Cell Marque	1	0	0	0	-	-
rmAb clone **SP11 790-4407**	33	Ventana	9	14	9	1	70%	81%
rmAb clone **SP11 KIT-0022**	1	Maixin	0	1	0	0	-	-
rmAb clone **SP11 MAD-000313QD**	2	Master Diagnostica	0	1	1	0	-	-
pAb **336A-78**	1	Cell Marque	0	1	0	0	-	-
Total	243		95	105	35	8	-	
Proportion			39%	43%	15%	3%	82%	



*SY38 discontinued from vendor

Lung tumours: Antibodies, protocols and controls

Synaptophysin / RUN 43 2015

The mAb clones 27G12, BS15, DAK-SYNAP and Snp88 and the rmAb clones SP11 and MRQ-40 could all be used to obtain an optimal staining reaction for SYP

Selected clones	Retrieval	Dilution range
mmAb 27G12	HIER, High pH	1:25 - 1:200 or RTU
mmAb DAK-SYNAP	HIER, High pH	1:50 - 1:100 or RTU
rmAb MRQ-40	HIER, High pH	1:50 - 1:300 or RTU
rmAb SP11	HIER, High pH	1:50 - 1:150 or RTU

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

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **27G12**	6/13** (46%)	0/2	15/28 (54%)	-	4/11 (36%)	0/1

Lung tumours: Antibodies, protocols and controls

Synaptophysin / RUN 43 2015

Controls

Positive/Negative: Colon.

* The axons of the Auerbach's and Meissner's plexus and the endocrine cells of the mucosa should be very strongly positive.
* The majority of goblet cells in the mucosa must show an at least weak to moderate cytoplasmic staining reaction.
* No staining must be seen in the smooth muscle cells.

Lung tumours: Antibodies, protocols and controls

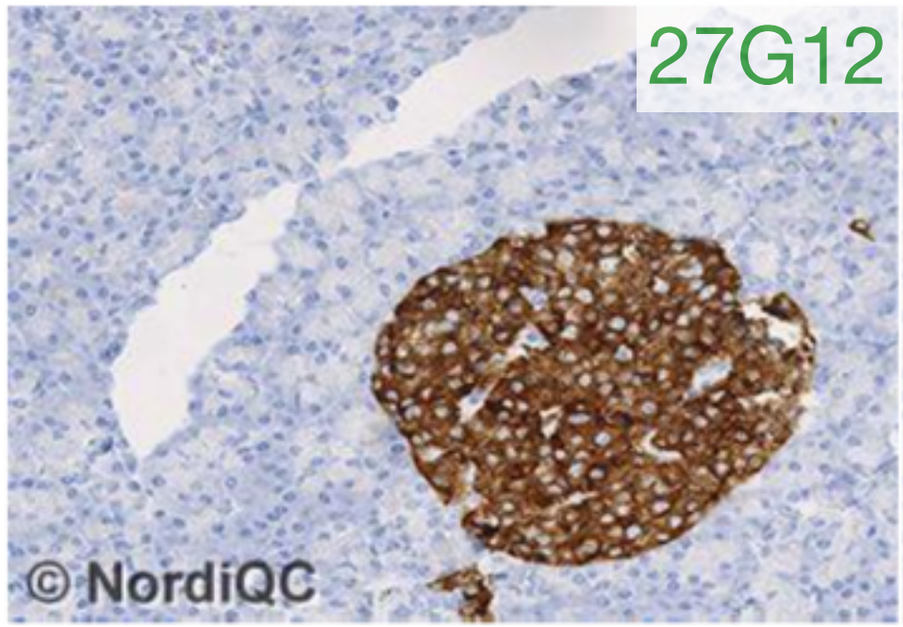


Fig. 1a
Optimal SYP staining of the pancreas using the mAb clone 27G12, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system. Virtually all endocrine islet cells show a strong and distinct cytoplasmic staining reaction and a high signal-to-noise ratio is observed. Also compare with Figs. 2a - 5a – same protocol.

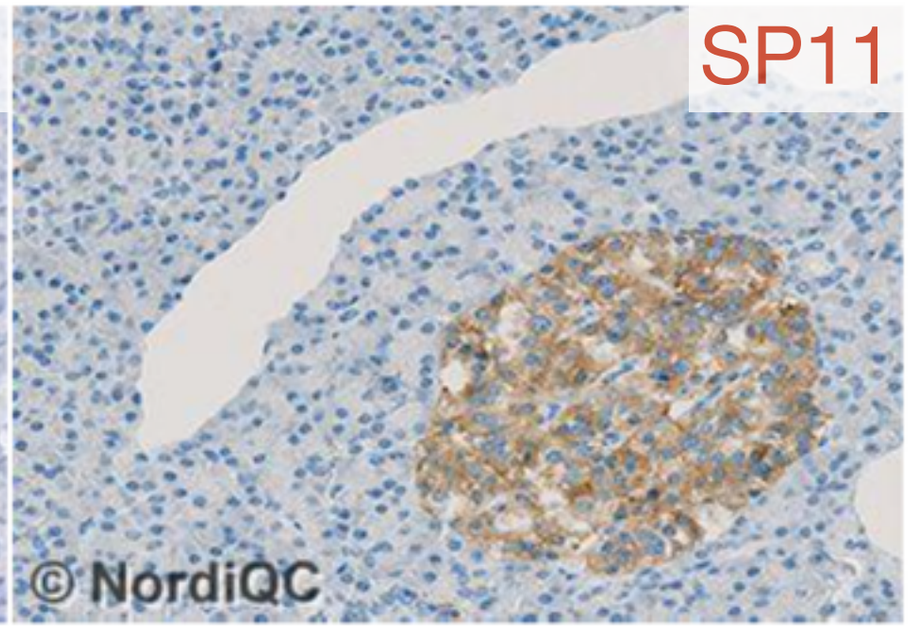


Fig. 1b
SYP staining of the pancreas using an insufficient protocol giving a too low sensitivity – same field as in Fig. 1a. The protocol was based on the rmAb clone SP11, HIER in an alkaline buffer. However the combination of a too low concentration of the primary Ab and use of a less sensitive 2-step multimer based detection system was less successful. The intensity of the endocrine cells is significantly reduced compared to the level expected and obtained in Fig. 1a. Also compare with Figs. 2b - 4b – same protocol.

The combination of a too low concentration of the primary Ab and use of a less sensitive 2-step multimer based detection system results in insufficient staining!

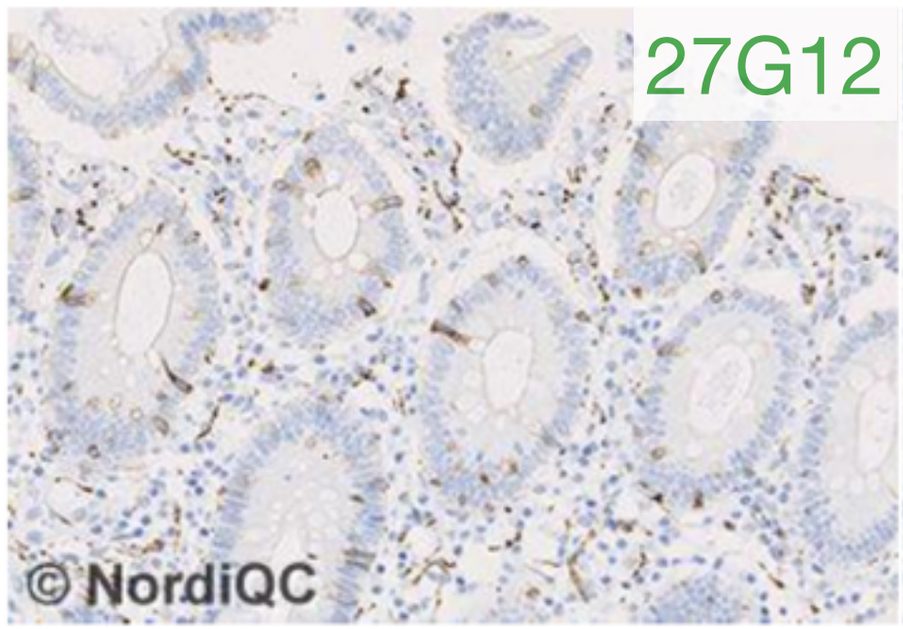


Fig. 2a
Optimal SYP staining of the colon using same protocol as

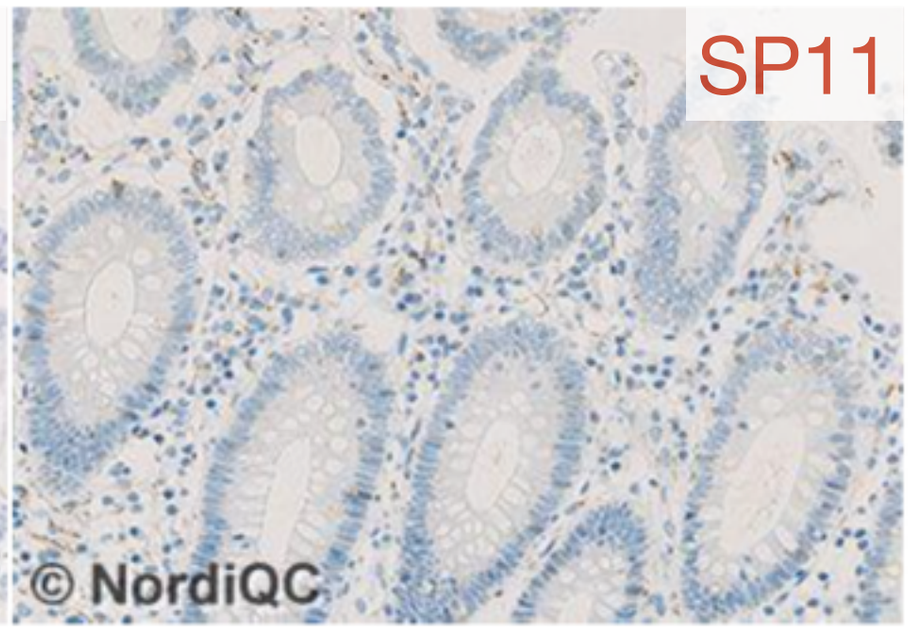


Fig. 2b
Insufficient SYP staining of the colon using same protocol

Lung tumours: Antibodies, protocols and controls

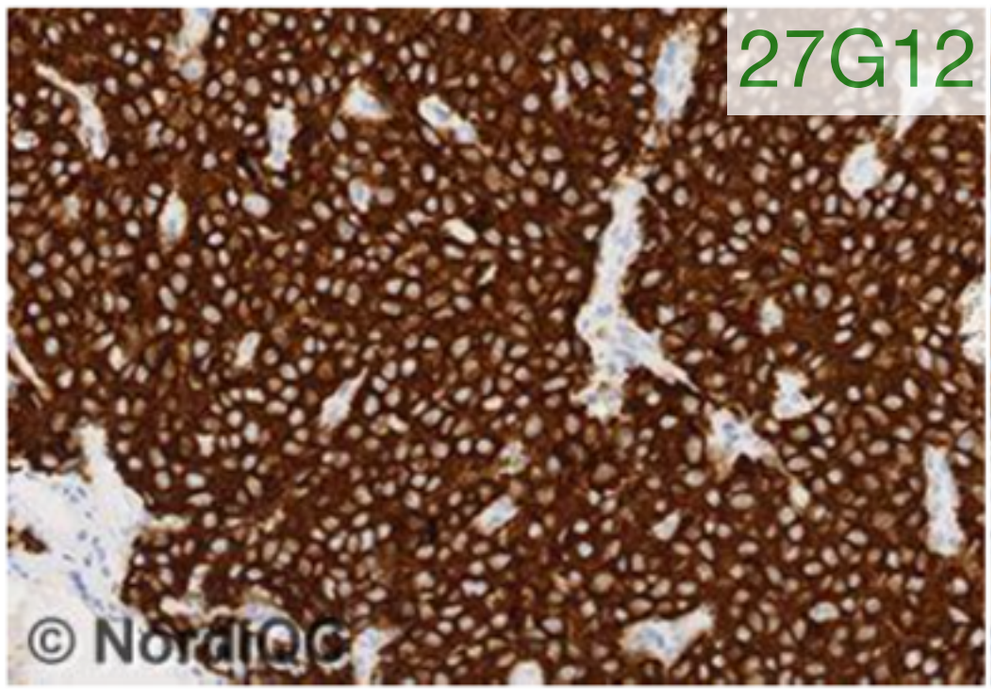


Fig. 3a
Optimal SYP staining of the intestinal neuroendocrine carcinoma using same protocol as in Figs. 1a. and 2a. Virtually all the neoplastic cells show a strong and distinct staining reaction. No background staining is seen.

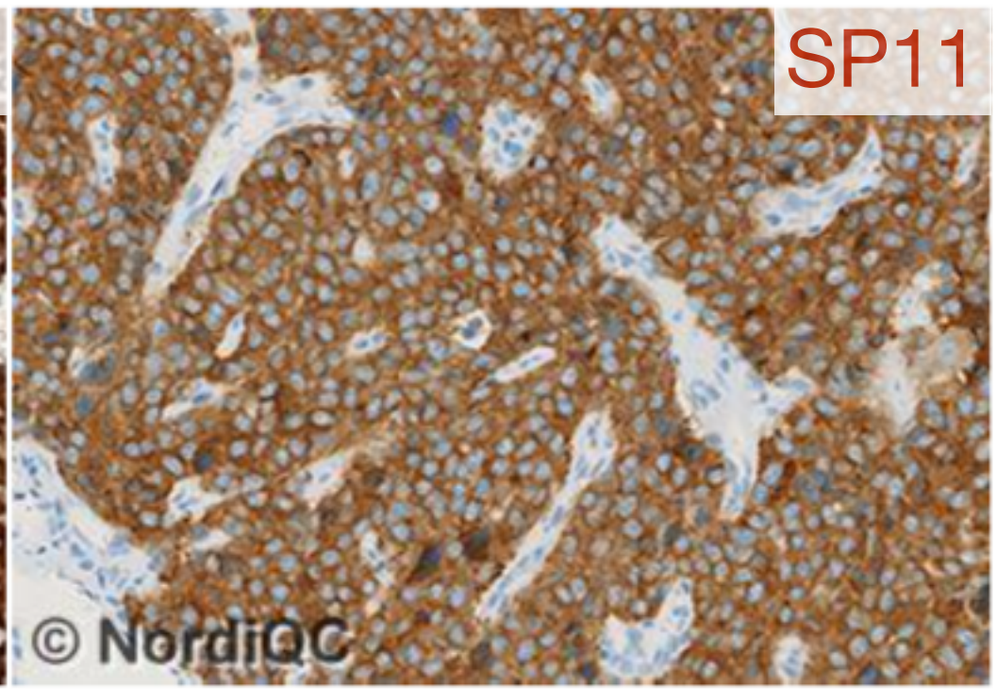


Fig. 3b
Staining for SYP of the intestinal neuroendocrine carcinoma using same insufficient protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The vast majority of the neoplastic cells are demonstrated. However also compare with Fig. 4b – same protocol.

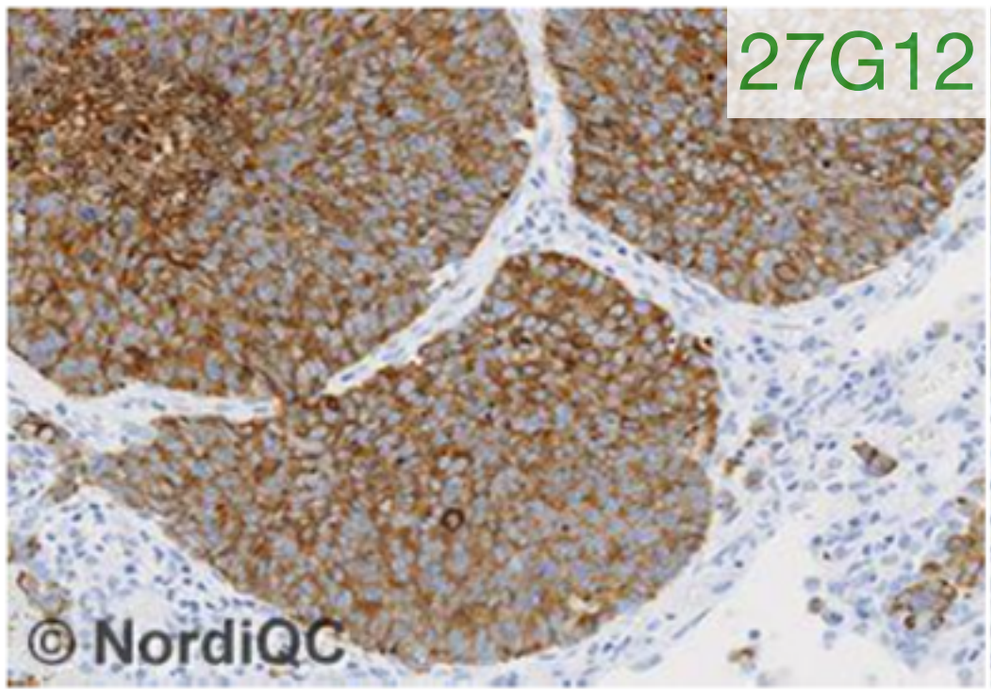


Fig. 4a
Optimal SYP staining of the SCLC using same protocol as

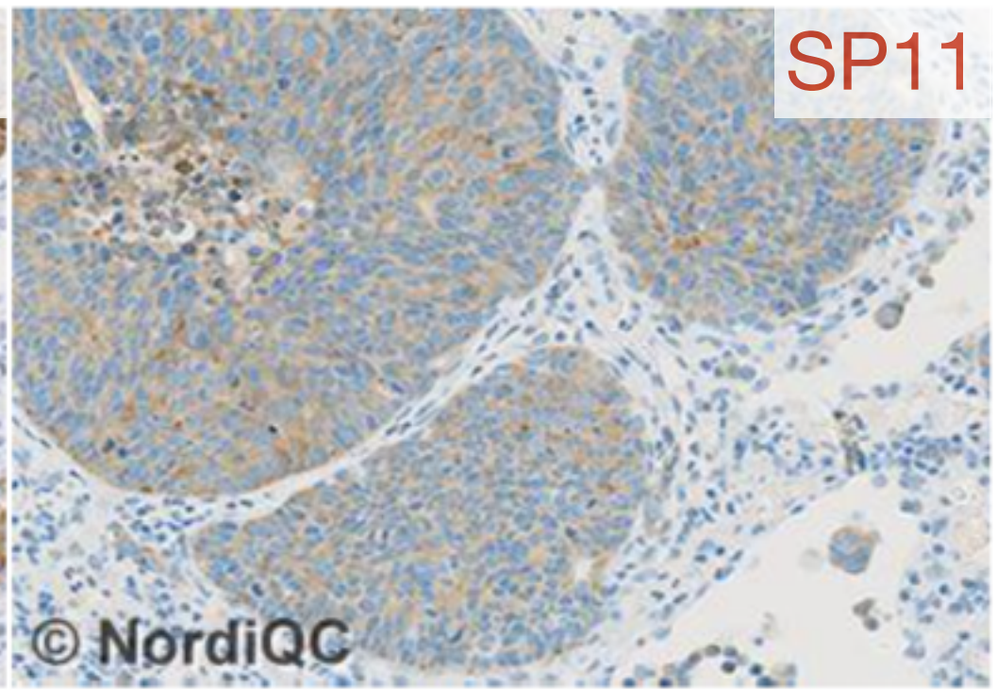


Fig. 4b
Insufficient SYP staining of the SCLC using same protocol

The combination of a too low concentration of the primary Ab and use of a less sensitive 2-step multimer based detection system results in insufficient staining!

Lung tumours: Antibodies, protocols and controls

NordiQC

Synaptophysin / RUN 43 2015

Aberrant staining

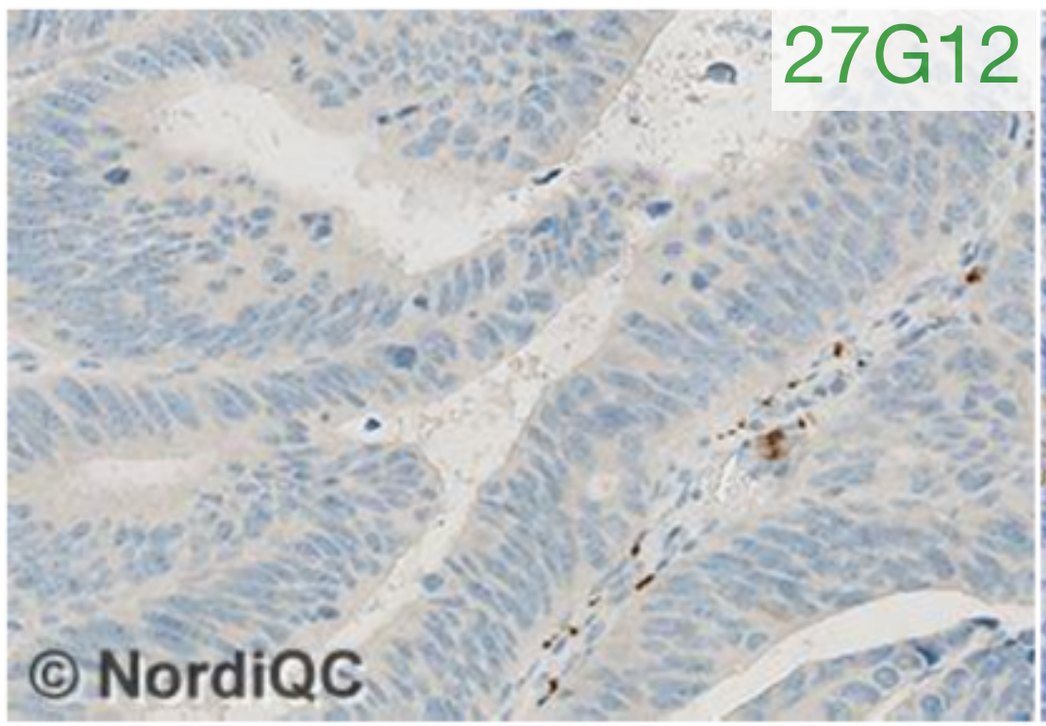


Fig. 5a
Optimal SYP staining of the colon adenocarcinoma using same protocol as in Figs. 1a – 4a. All neoplastic cells are negative and only peripheral nerves in the stromal compartment are positive. The protocol was based on the mAb clone 27G12, HIER in an alkaline buffer, a sensitive 3-step multimer based detection system and performed on the BenchMark ULTRA, Ventana. Virtually no background staining is seen.

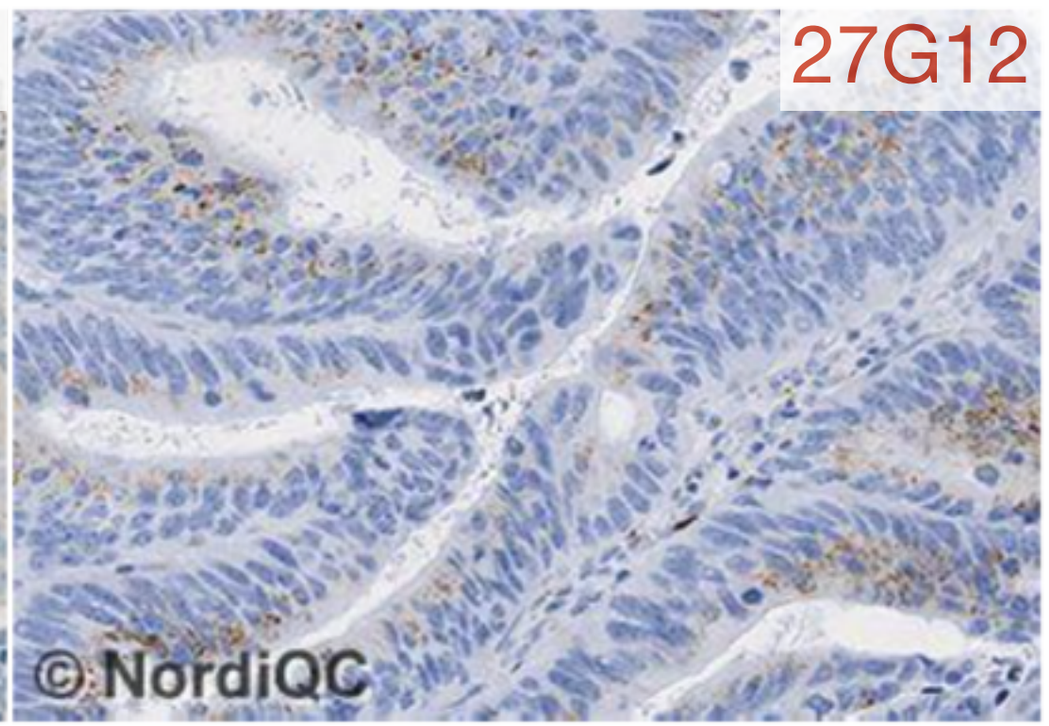
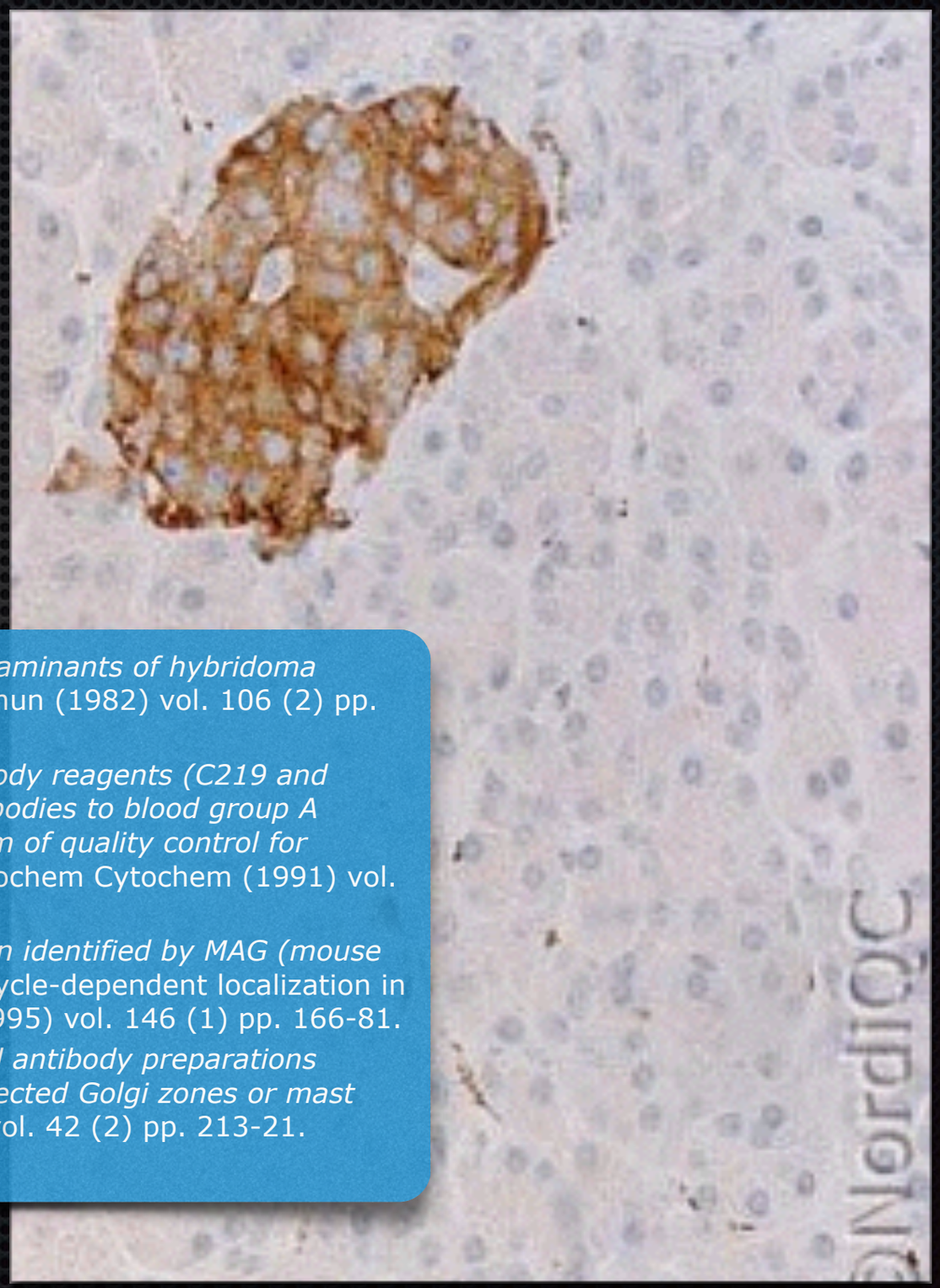


Fig. 5b
Staining for SYP of the colon adenocarcinoma – same field as in Fig. 5a. An aberrant granular cytoplasmic staining reaction is seen diffusely in the neoplastic cells. This pattern was occasionally observed, when the mAb clone 27G12 was applied on the Dako Autostainer and Biocare Intellipath platform with otherwise optimal settings based on HIER in an alkaline buffer and a 3-step polymer based detection system. The aberrant staining pattern for clone 27G12 was not observed on the BenchMark platform and different washing conditions and efficiency might cause the different staining pattern. As the staining reaction was not consistent on e.g. the Dako platform, a lot-to-lot variation might be a cofactor. If the aberrant cytoplasmic staining reaction was observed in > 30% of the neoplastic cells, the result was evaluated as borderline.

NordiQC

Lung tumours: Antibodies, protocols and controls

MouseAscitesGolgi reaction (Snp88)



Blood type A patient

Blood type 0 patient

Gooi et al. *Natural antibodies as contaminants of hybridoma products.* Biochem Biophys Res Commun (1982) vol. 106 (2) pp. 539-45.

Finstad et al. *Some monoclonal antibody reagents (C219 and JSB-1) to P-glycoprotein contain antibodies to blood group A carbohydrate determinants: a problem of quality control for immunohistochemical analysis.* J Histochem Cytochem (1991) vol. 39 (12) pp. 1603-10.

Kliman et al. *A mucin-like glycoprotein identified by MAG (mouse ascites Golgi) antibodies.* Menstrual cycle-dependent localization in human endometrium. Am J Pathol (1995) vol. 146 (1) pp. 166-81.

Spicer et al. *Some ascites monoclonal antibody preparations contain contaminants that bind to selected Golgi zones or mast cells.* J Histochem Cytochem (1994) vol. 42 (2) pp. 213-21.

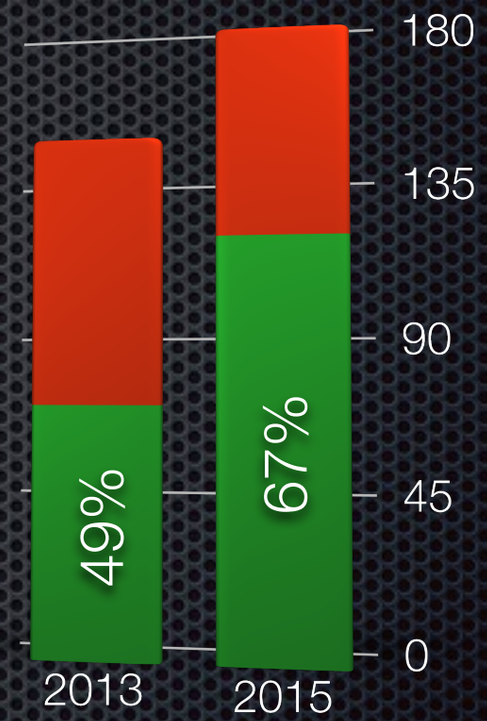
Lung tumours: Antibodies, protocols and controls

lu-ALK / RUN 45 2015

Pass: 67 %

Table 1. **Antibodies and assessment marks for lu-ALK, run 45**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5A4**	46 3 2 1 1 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam Biocare Zytomed	24	16	13	1	74%	81%
mAb clone **ALK1**	8	Dako	0	0	3	5	0%	-
mAb clone **OTI1A4**	5	ORIGENE	4	1	0	0	100%	100%
rmAb clone **D5F3**	21 1	Cell Signaling PrimeBioMed	18	2	1	1	91%	95%
rmAb clone **SP8**	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	3	Leica/Novocatra	0	1	2	0	-	-
mAb clone **5A4 API3041**	1	Biocare	1	0	0	0	-	-
mAb clone **5A4 MAB-0281**	1	Maixin	1	0	0	0	-	-
mAb **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	0	1	-	-
mAb **ALK1 IR641**	15	Dako	0	0	4	11	0%	-
mAb clone **ALK1 790/800-2918**	10	Ventana	0	1	6	3	10%	-
mAb clone **ALK1 204M-18**	1	Cell Marque	0	0	0	1	-	-
mAb clone **ALK1 GA641**	1	Dako	0	0	0	1	-	-
rmAb clone **D5F3 790-4794**	47	Ventana	41	4	2	0	96%	96%
rmAb clone **D5F3 790-4843** (CDx assay)	4	Ventana	3	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Total	176		93	25	33	25	-	
Proportion			53%	14%	19%	14%	67%	



Lung tumours: Antibodies, protocols and controls

lu-ALK / RUN 45 2015

Recommendable clones	Retrieval	Dilution range
mAb 5A4	HIER, High pH	1:10 - 1:50 or RTU
rmAb D5F3	HIER, High pH	1:50 - 1:250 or RTU
mAb OTI1A4	HIER, High pH	1:50 - 1:1000

Table 3. **Proportion of optimal results for lu-ALK for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **5A4**	6/9** (67%)	-	4/17 (24%)	-	6/6 (100%)	1/1
rmAb clone **D5F3**	7/8 (88%)	0/1	1/2	-	4/4	-

* 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)

Lung tumours: Antibodies, protocols and controls

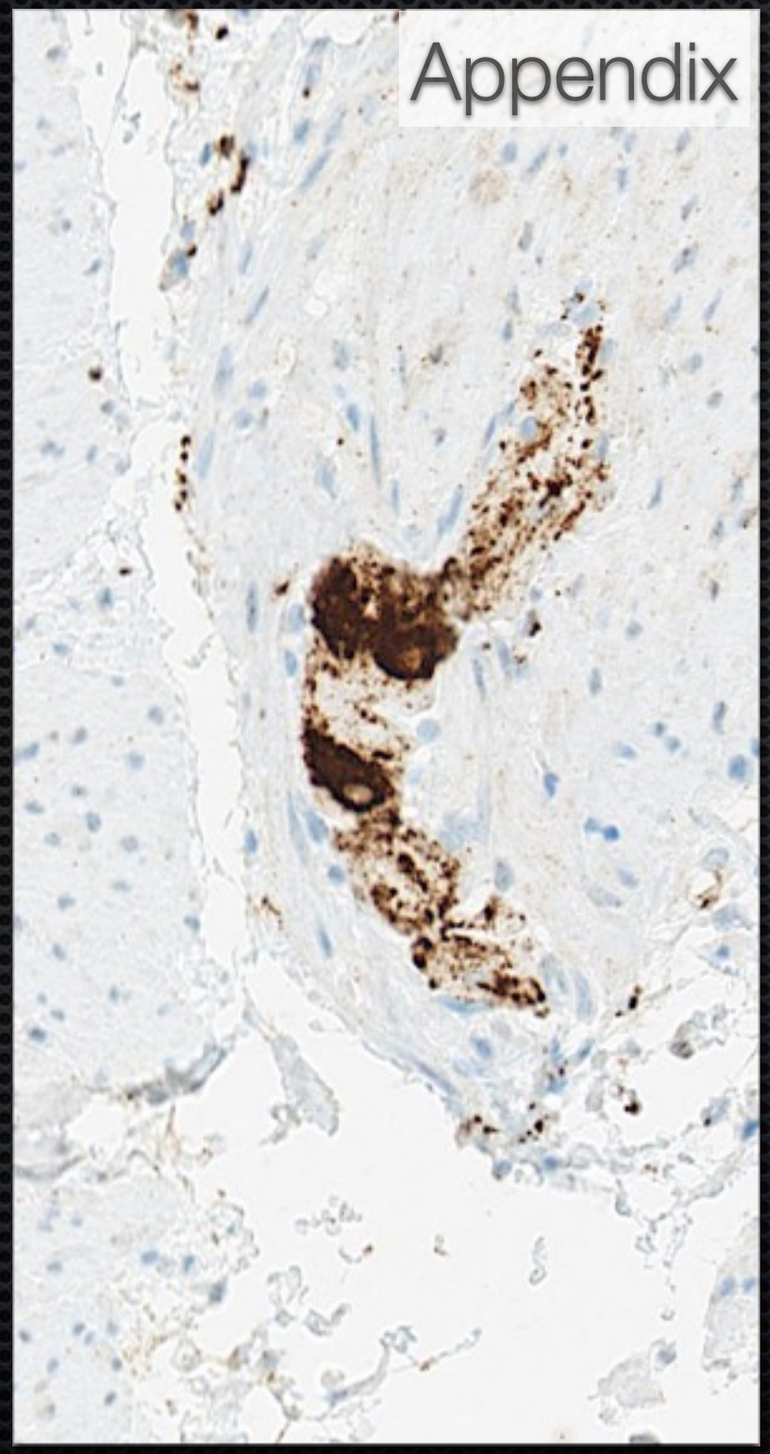
lu-ALK / RUN 45 2015

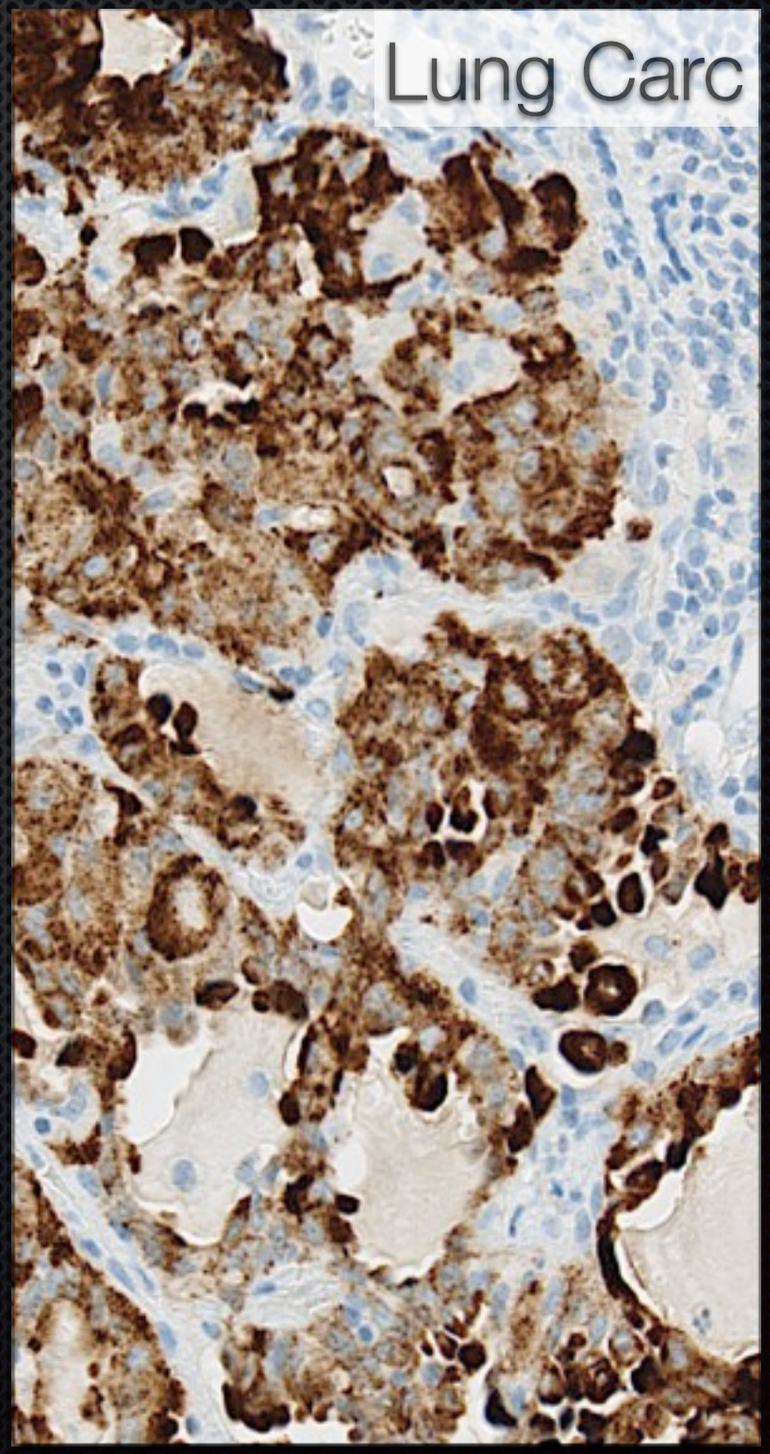
Controls

Positive: ALCL and lung adenocarcinoma with FISH verified ALK rearrangements and normal appendix.

* A weak to strong granular cytoplasmic staining reaction should be seen in the ganglion cells in appendix.

Negative: Lung cancer without ALK rearrangements





Lung tumours: Antibodies, protocols and controls

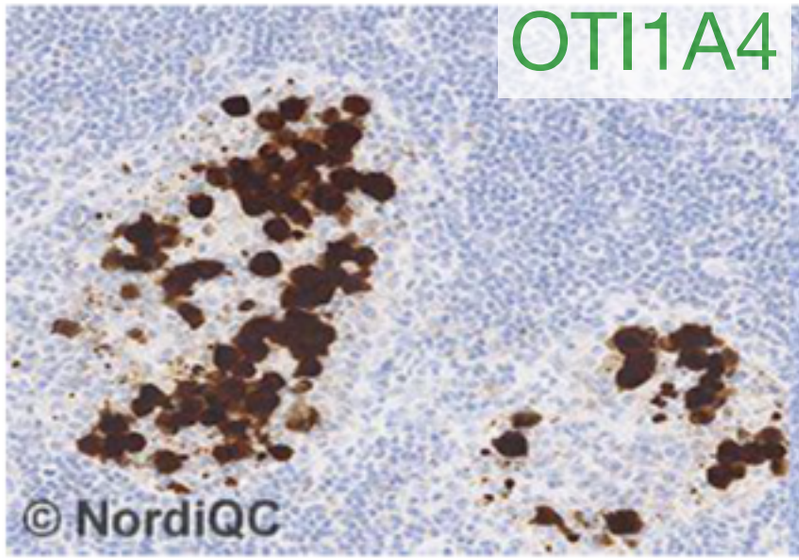


Fig. 1a
Optimal ALK staining of the ALCL with ALK rearrangement using the mAb clone OTI1A4 optimally calibrated, HIER in TRS High pH 9 (Dako), a 3-step polymer based detection system and performed on Omnis, Dako.
The neoplastic cells show an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided and no background staining is seen.
Also compare with Figs. 2a - 6a, same protocol.

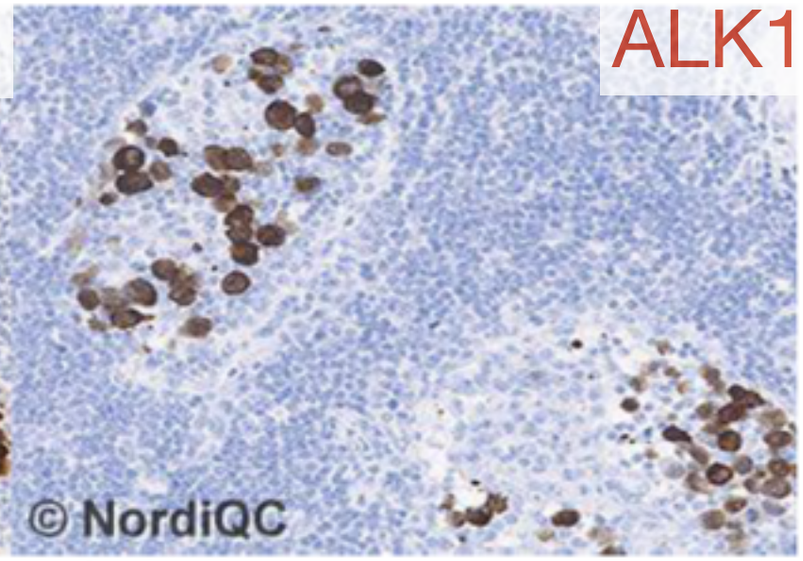


Fig. 1b
ALK staining of the ALCL with ALK rearrangement using an insufficient protocol providing a too low sensitivity for the demonstration of ALK rearrangement in lung adenocarcinoma - same field as in Fig. 1a.
The protocol was based on the mAb clone ALK1, HIER in an alkaline buffer, a 3-step polymer based detection system and performed on the Autostainer Link 48, Dako.
The neoplastic cells of the ALCL are demonstrated, however also compare with Figs. 2b – 5b, same protocol.

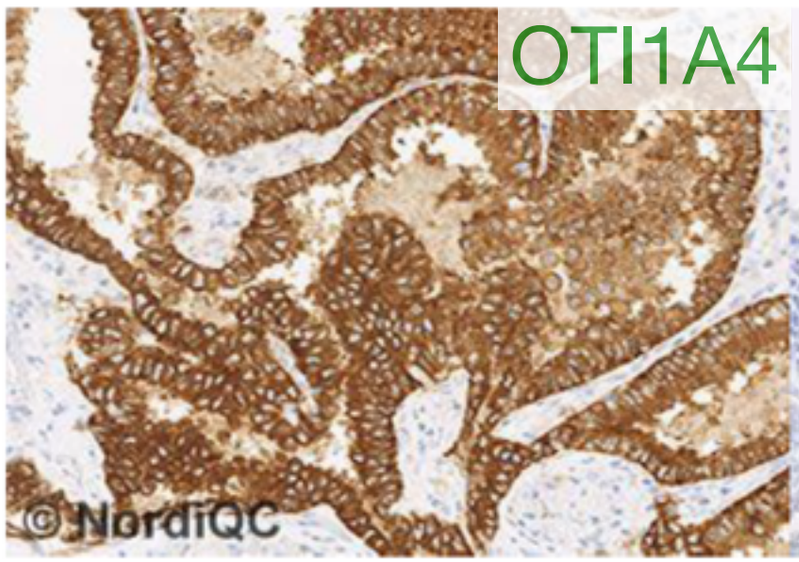


Fig. 2a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction.
No background staining is seen.

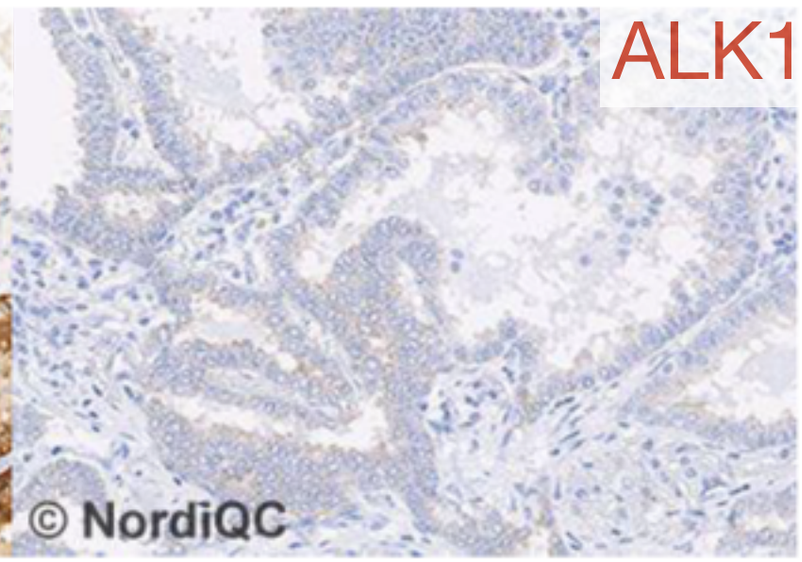


Fig. 2b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1b - same field as in Fig. 2a.
Only scattered neoplastic cells show a faint cytoplasmic staining reaction, while the vast majority is negative.

Less successful primary antibody: mAb clone ALK1

Lung tumours: Antibodies, protocols and controls

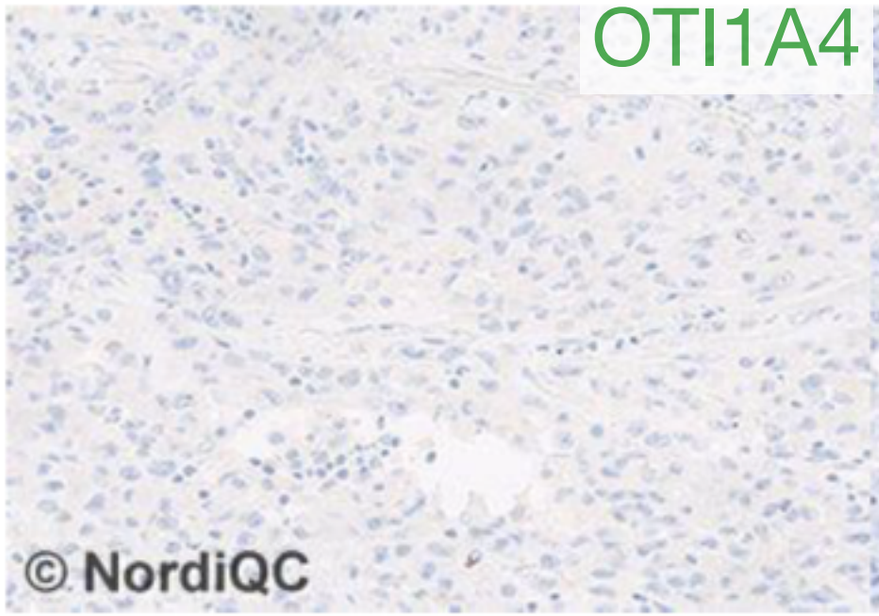


Fig. 3a
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 1a and 2a.
The neoplastic cells are all negative.

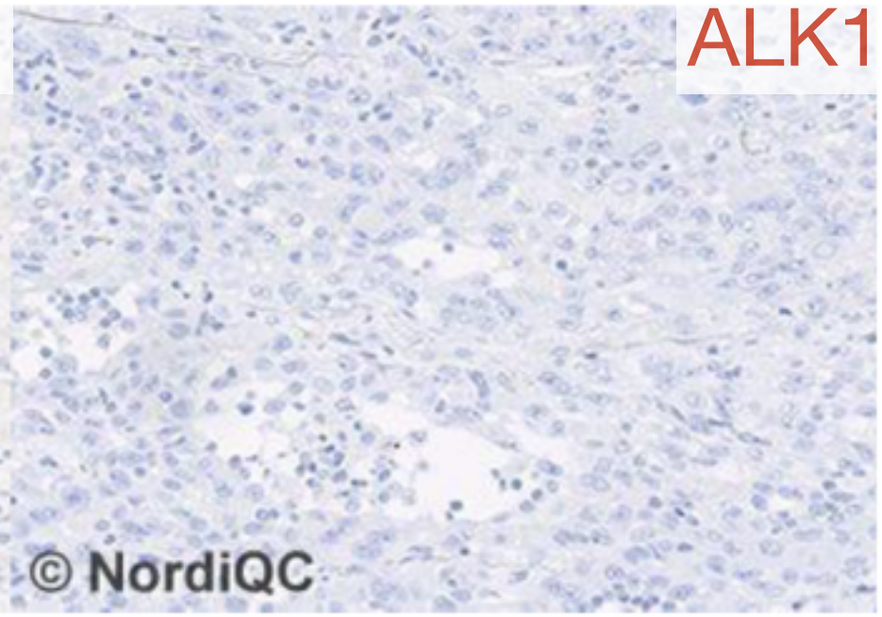


Fig. 3b
ALK staining of the lung adenocarcinoma without ALK rearrangement using same insufficient protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
The neoplastic cells are all negative.

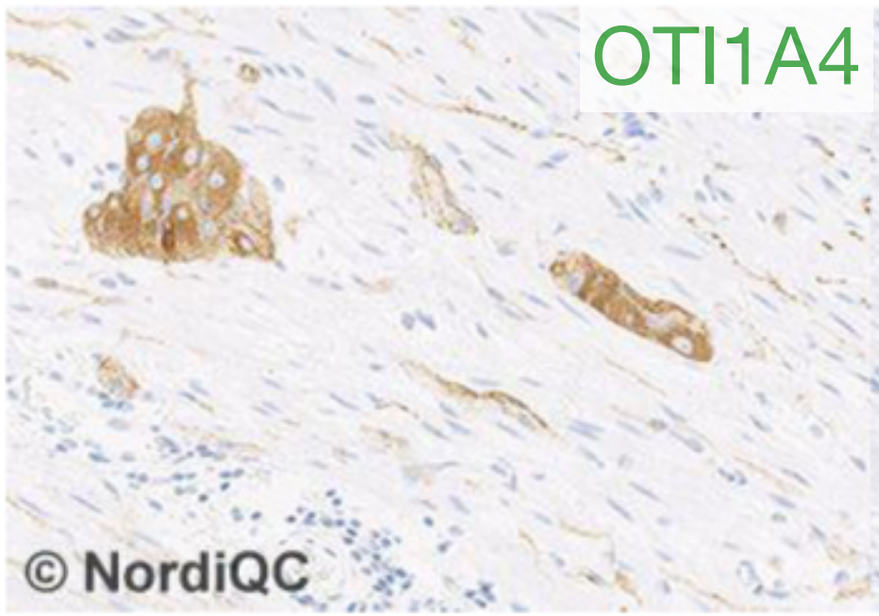


Fig. 4a
Optimal ALK staining of the appendix using same protocol as in Figs. 1a - 3a. The ganglion cells of the myenteric plexus show a moderate, distinct cytoplasmic staining reaction, while the axons show a weak to moderate staining reaction.

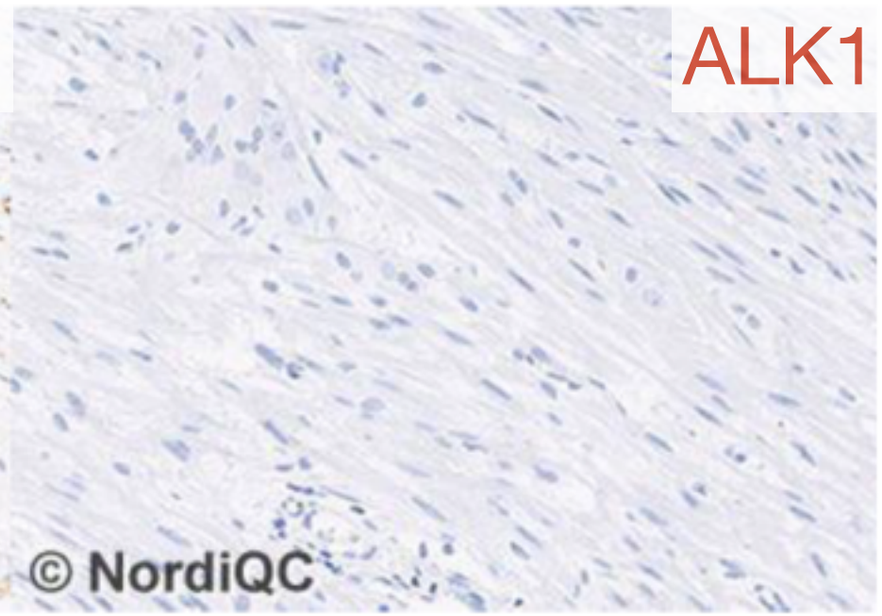


Fig. 4b
Insufficient ALK staining of the appendix using same protocol as in Figs. 1b - 3b. - same field as in Fig. 4a.
The ganglion cells and axons are unstained.

Less successful primary antibody: mAb clone ALK1

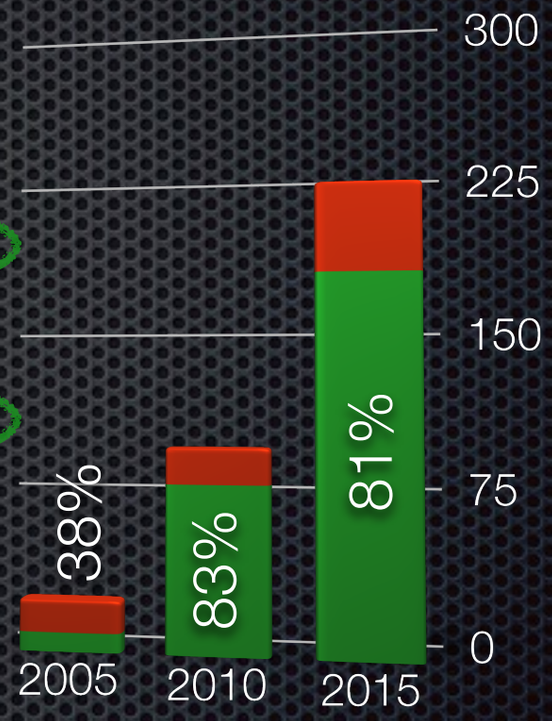
Lung tumours: Antibodies, protocols and controls

WT1 / RUN 43 2015

Pass: 81 %

Table 1. **Antibodies and assessment marks for WT1, run 43**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mmAb clone **6F-H2**	70 7 2 1 1 1 1 1	Dako Cell Marque Immunologic BioSB Genemed Novous Thermo Fisher Zeta	23	41	17	3	76%	81%
mmAb clone **WT49**	20 1	Leica/Novocastra Monosan	9	8	2	2	81%	88%
rmAb clone **EP122**	1	Epitomics	0	1	0	0	-	-
pAb, **C-19**	1	Santa Cruz	0	0	1	0	-	-
pAb, **RB-9267-P1**	1	Thermo Fisher	0	0	0	1	-	-
Ready-To-Use antibodies								
mmAb clone **6F-H2 IR055/IS055**	51	Dako	40	8	2	1	94%	100%
mmAb clone **6F-H2 760-4397**	45	Ventana/Cell Marque	4	33	5	3	82%	97%
mmAb clone **6F-H2 348M-98**	3	Cell Marque	0	2	1	0	-	-
mmAb clone **6F-H2 PM258**	1	BioCare	0	0	1	0	-	-
mmAb clone **6F-H2 MAD-005671QD**	1	Master Diagnostica	0	1	0	0	-	-
mmAb clone **6F-H2 MON-RTU1210**	1	Monosan	0	0	1	0	-	-
mmAb clone **WT49 PA0562**	8	Leica/Novocastra	5	2	1	0	88%	100%
mmAb clone **MX012 MAB-0678**	1	Maixin	0	1	0	0	-	-
rmAb clone **EP122 AN828-5M**	1	Biogenex	1	0	0	0	-	-
Total	220		82	97	31	10	-	
Proportion			37%	44%	14%	5%	81%	



Lung tumours: Antibodies, protocols and controls

WT1 / RUN 43 2015

Recommendable clones	Retrieval	Dilution range
mAb 6H-F2	HIER, High pH	1:30 - 1:400 or RTU
mAb WT49	HIER, High pH	1:10 - 1:60 or RTU
mAb 6H-F2	HIER, High pH + Prot.	1:200 - 1:250 or RTU

Table 3. **Proportion of optimal results for WT1 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic / Omnis		Ventana BenchMark XT / Ultra			Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC1 pH 8.5 + Protease 3	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **6F-H2**	9/13 (69%)**	-	3/38 (8%)	2/4	0/1	4/6 (67%)	-
mAb clone **WT49**	3/6 (50%)	-	2/4	-	-	4/7 (57%)	-

A cytoplasmic reaction in a variety of cells, e.g., endothelial cells, smooth muscle cells and plasma cells was expected and accepted for the mAb clone 6F-H2.

Lung tumours: Antibodies, protocols and controls

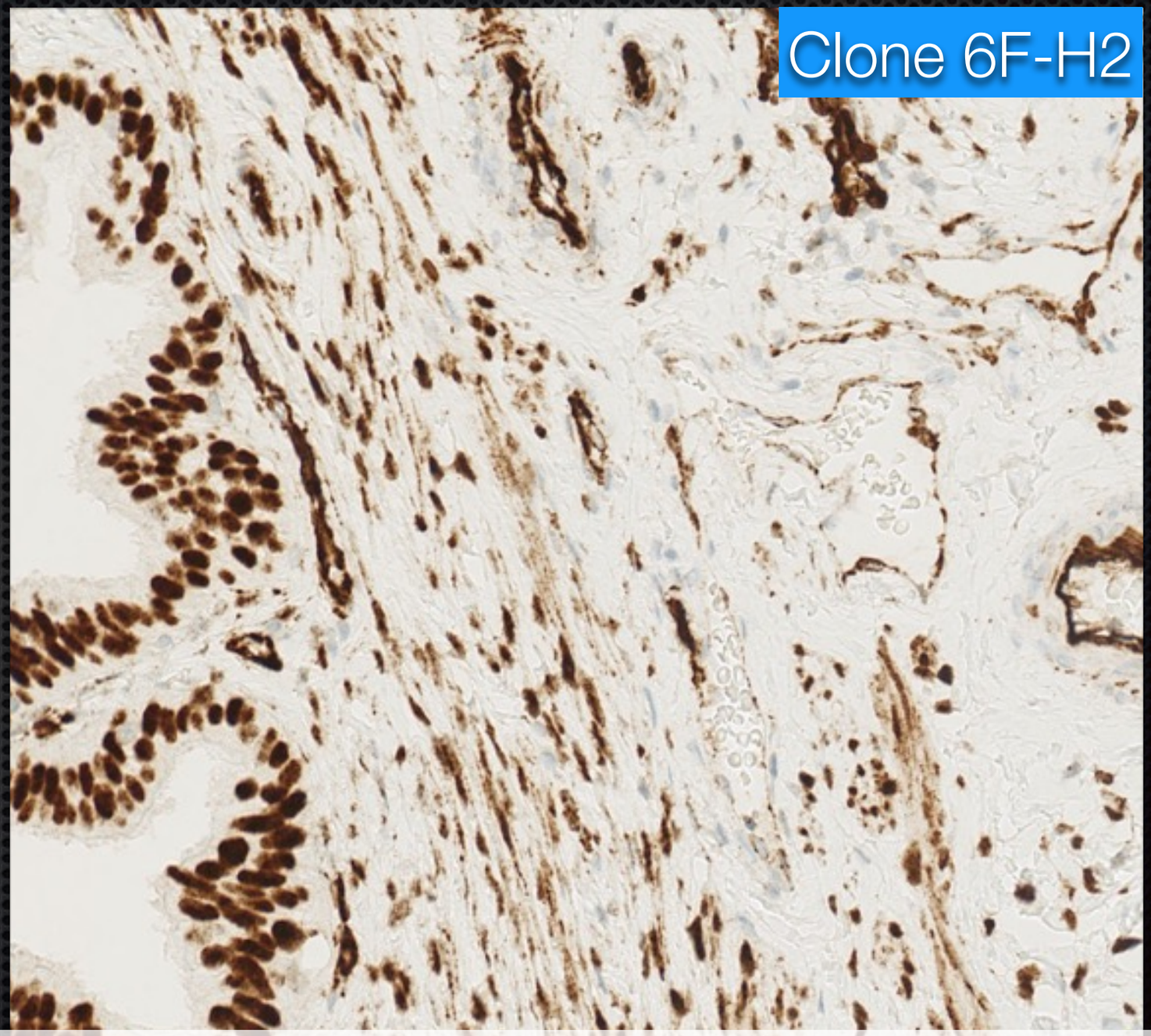
WT1 / RUN 43 2015

Controls

Positive: Fallopian tube

* Epithelial cells must show an as strong as possible nuclear reaction with only a minimal cytoplasmic reaction.

6H-F2 and WT49



A cytoplasmic reaction in a variety of cells, e.g., endothelial cells, smooth muscle cells and plasma cells was expected and accepted for the mAb clone 6F-H2.

Lung tumours: Antibodies, protocols and controls

WT1 / RUN 43 2015

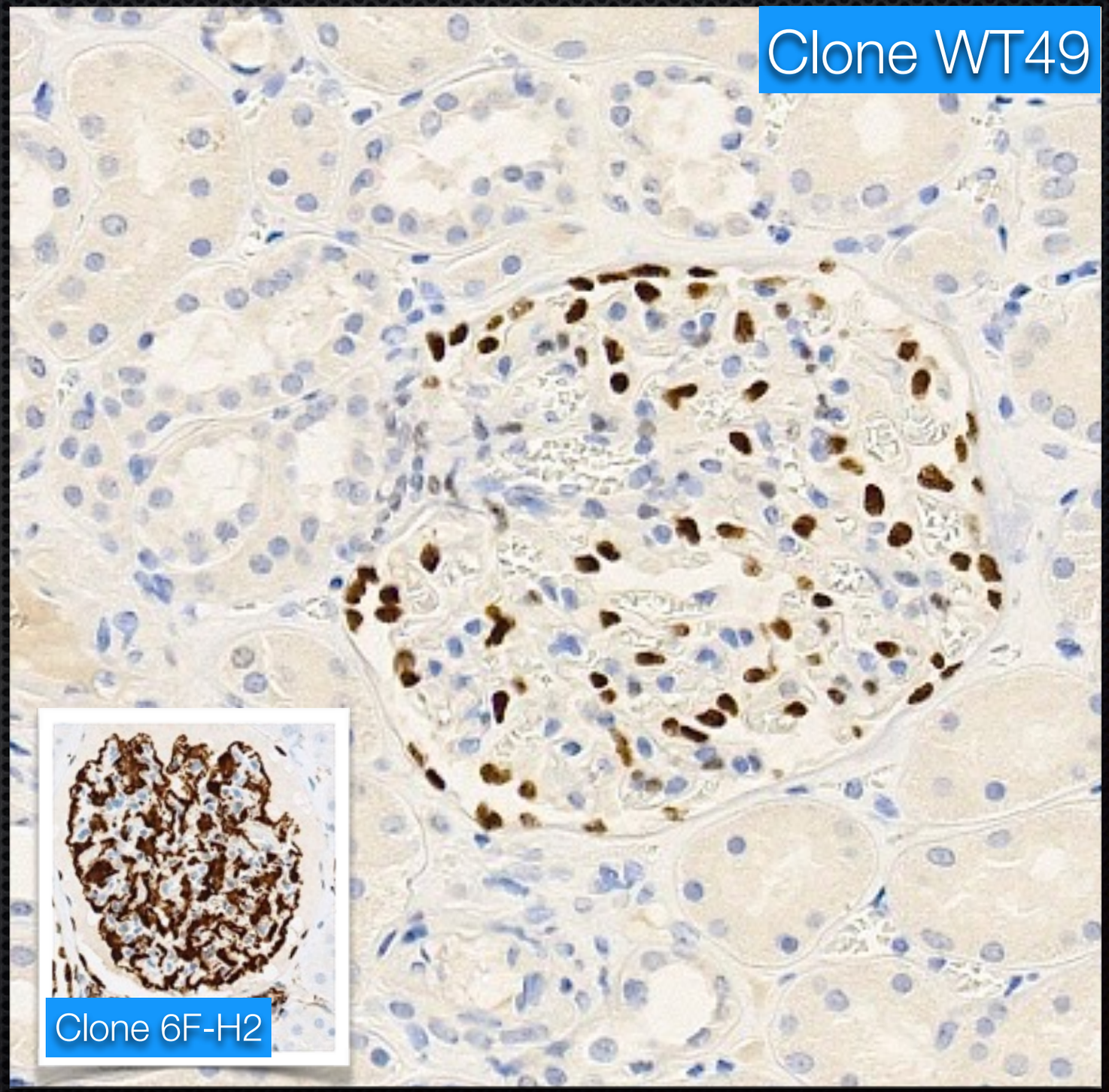
Controls

Positive: Kidney

* A moderate to strong nuclear staining must be seen in the parietal epithelial cells and podocytes of the Bowman capsule.

* The epithelial cells of the tubules should be negative

WT49



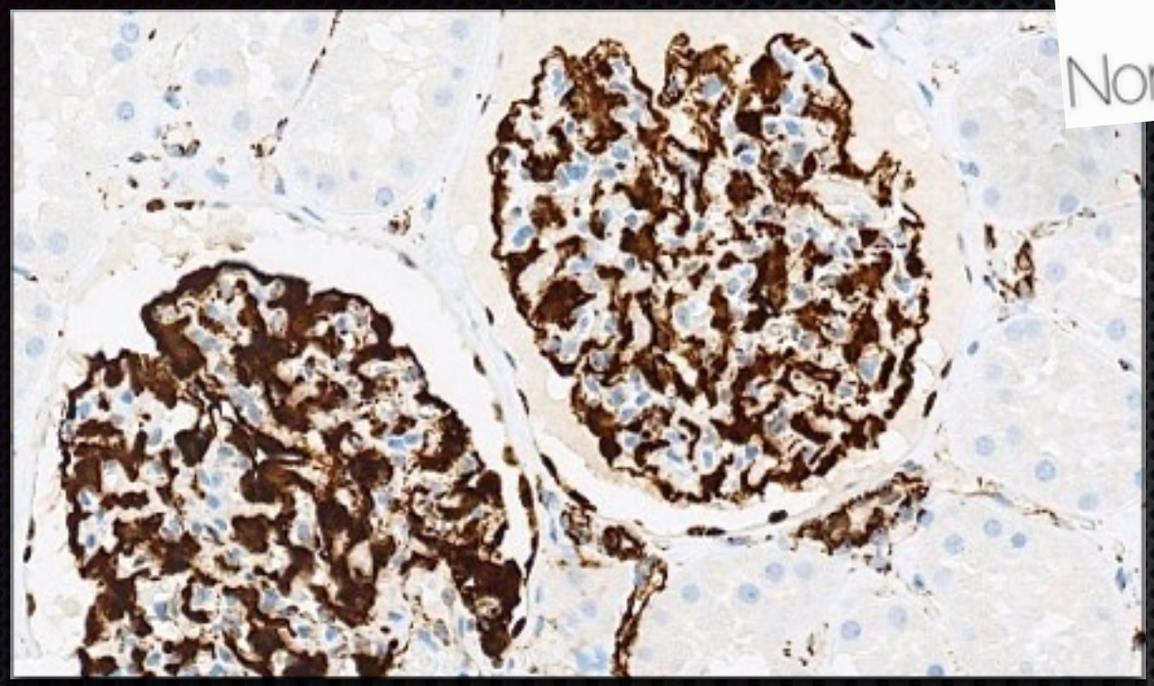
WT1, 6F-H2 and retrieval protocols

NordiQC

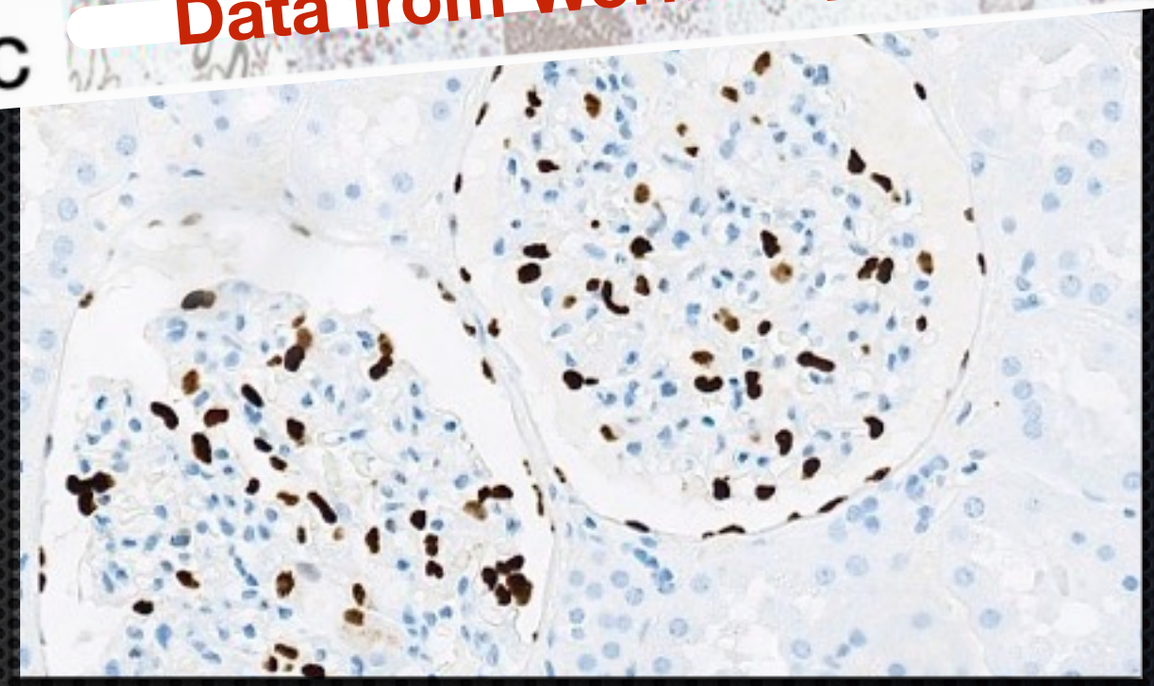
Data from Workshop 2014

Kidney

HIER (CC1_32_100)

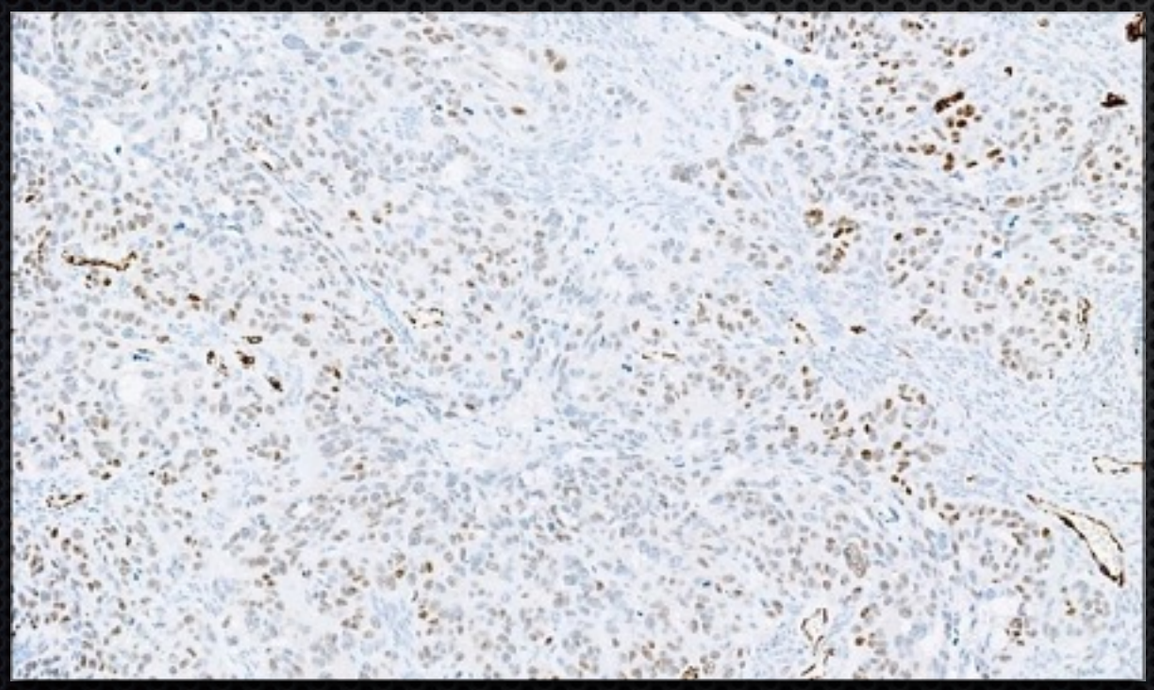


HIER + Prot (CC1_32_100/P3_4)

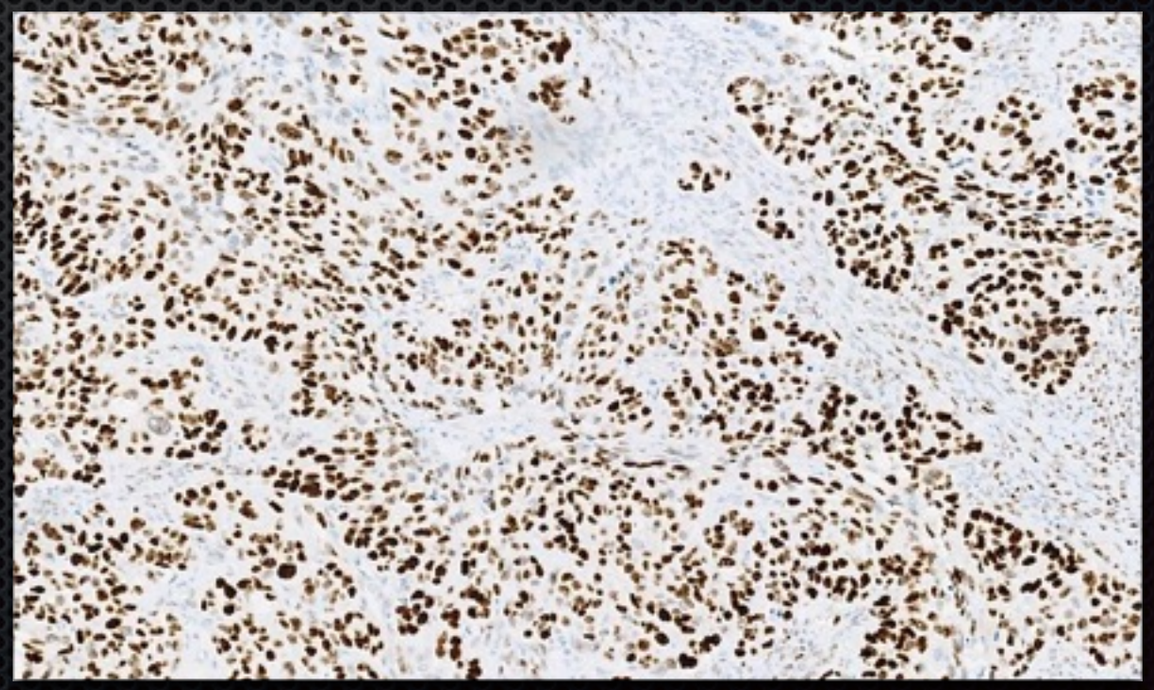


Ov. carcinoma

HIER (CC1_32_100)



HIER + Prot (CC1_32_100/P3_4)

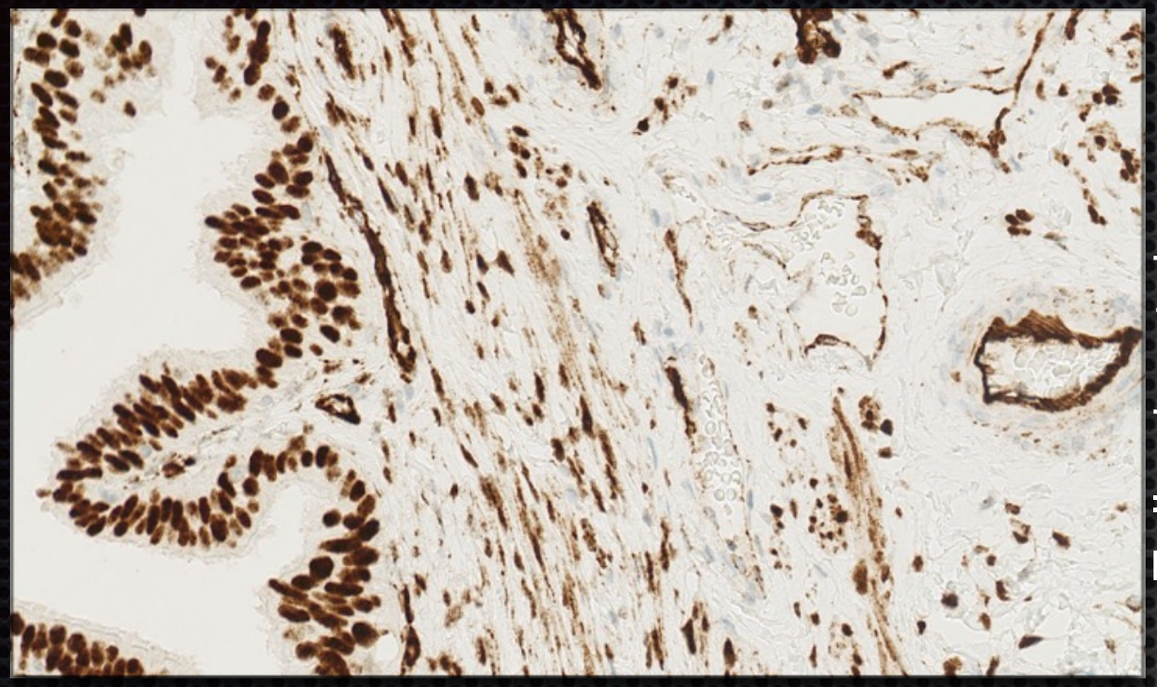


Lung tumours: Antibodies, protocols and controls

NordiQC

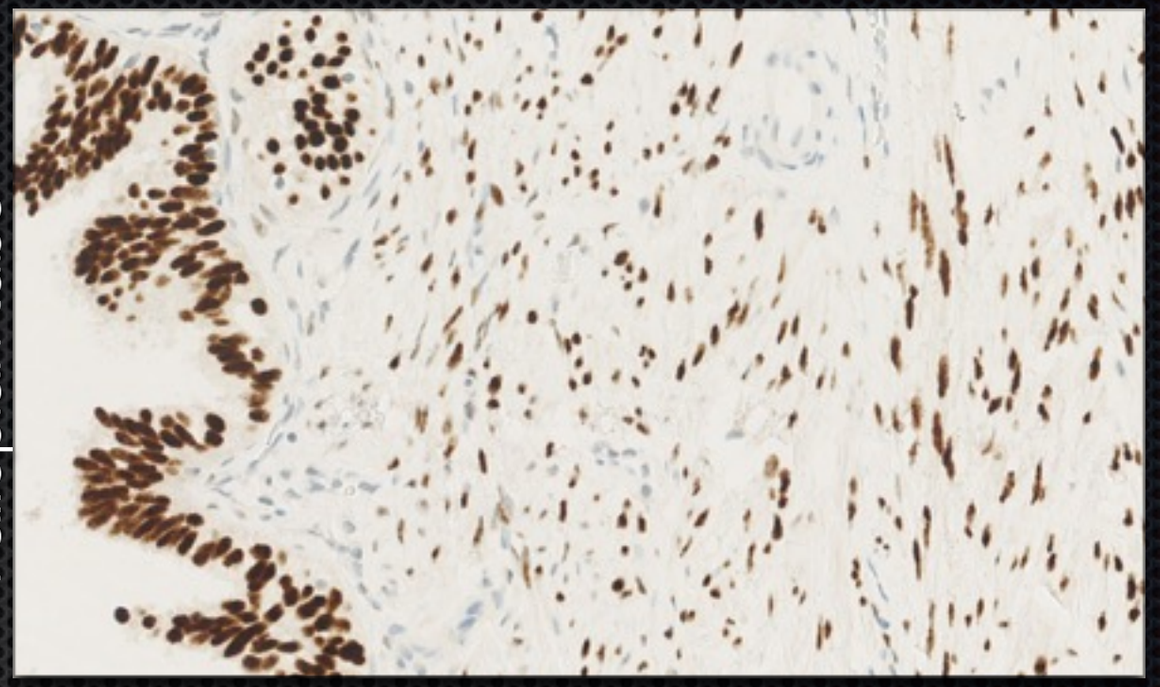
WT1 / RUN 43 2015

NordiQC

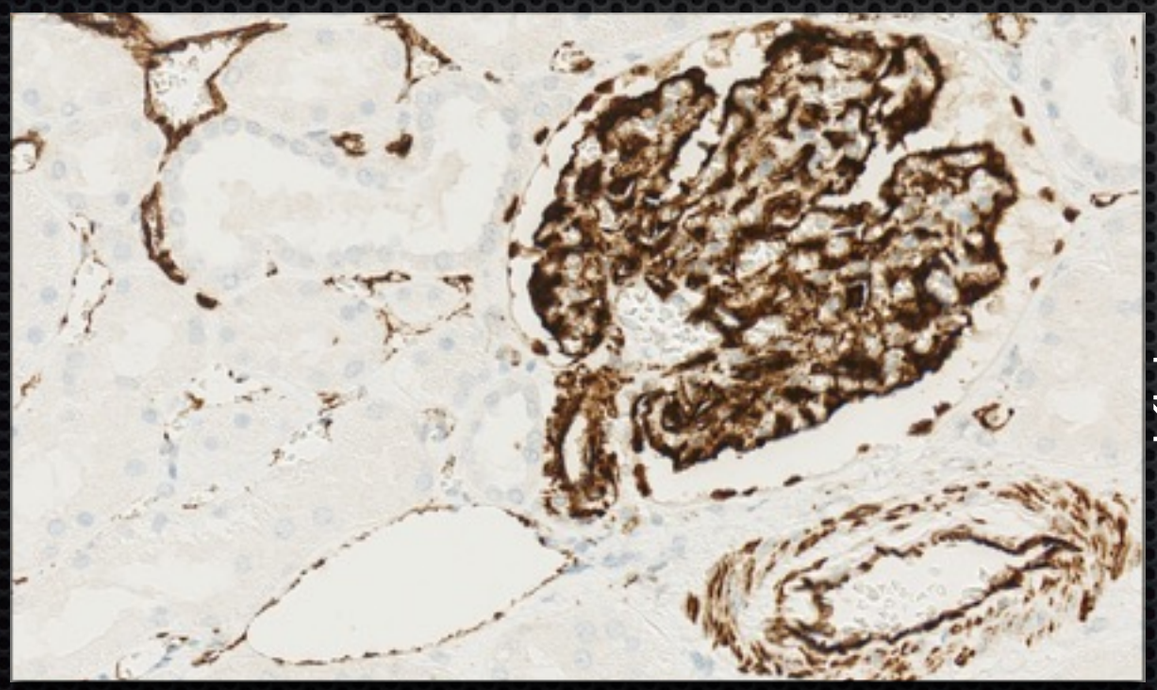


6F-H2 (HIER)

Fallopian tube

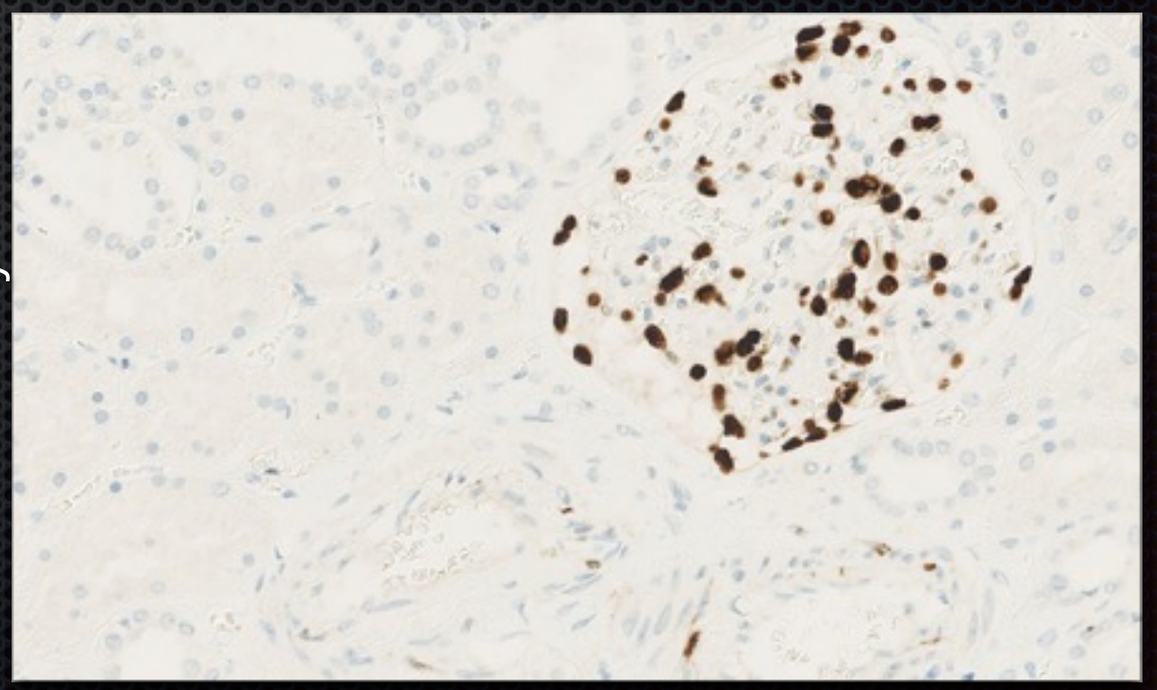


6F-H2 (HIER+P) or WT49 (HIER)



6F-H2 (HIER)

Kidney

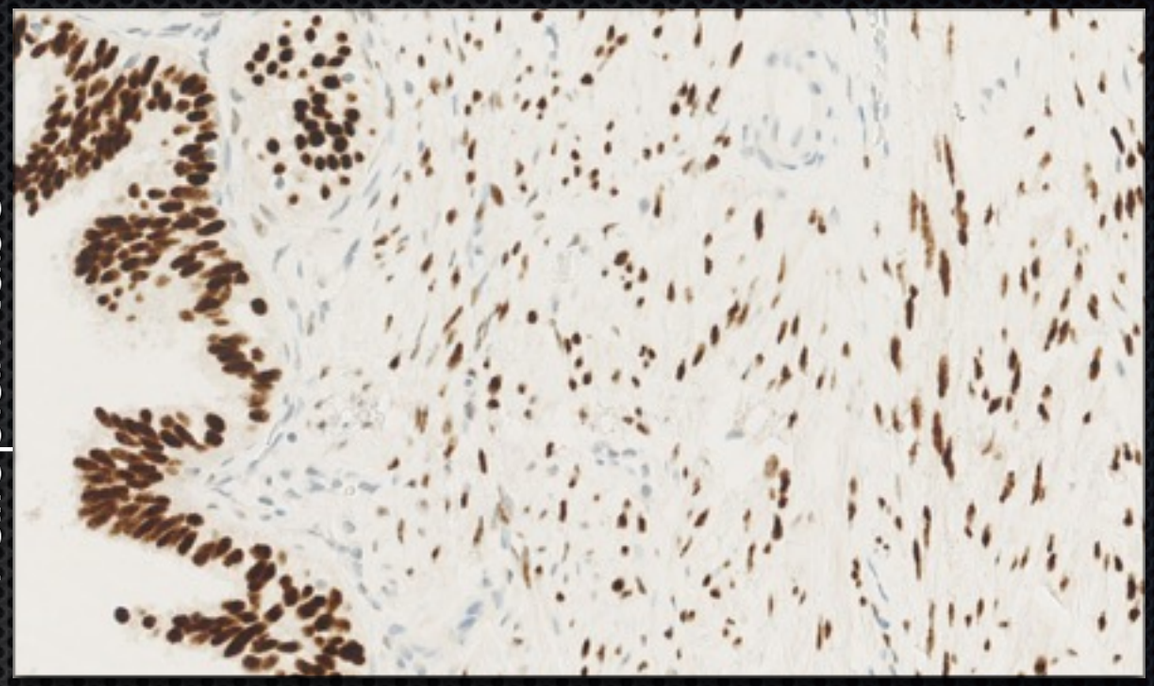


6F-H2 (HIER+P) or WT49 (HIER)

Lung tumours: Antibodies, protocols and controls

Positive: Fallopian tube

* Epithelial cells must show an as strong as possible nuclear reaction with only a minimal cytoplasmic reaction.

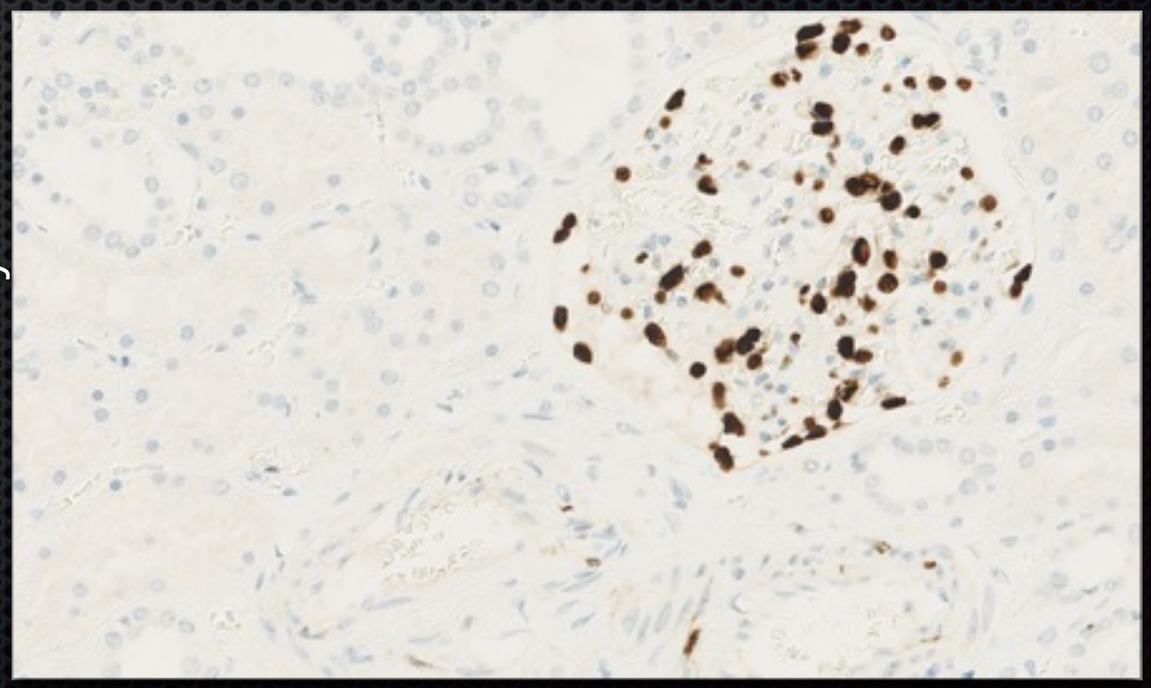


6F-H2 (HIER+P) or WT49 (HIER)

Positive: Kidney

* A moderate to strong nuclear staining must be seen in the parietal epithelial cells and podocytes of the Bowman capsule.

* The epithelial cells of the tubules should be negative



6F-H2 (HIER+P) or WT49 (HIER)

Optimizing protocols

Epitope retrieval “Test Battery”

BenchMark

No	Method				
1	No retrieval				
2	Protease 1 (36°C)	8 min			
3	CC2, pH6 (91°C)	32 min			
4	CC1, pH8,5 (100°C)	32 min			
5	CC1, pH8,5 (100°C)	48 min			
6	CC1, pH8,5 (100°C)	32 min	>>>>	Protease 3 (36°C)	4 min
7	Protease 3 (36°C)	4 min	>>>>	CC1, pH8,5 (100°C)	32 min
8	MW/TRS pH6.1	15 min		*Offline*	
9	Pepsin (0,4%/37°C)	20 min		*Offline*	
10	MW/Citrate pH2	10 min		*Offline*	

CC2: Citrate based buffer, pH6 CC1: Tris-EDTA based buffer pH8,5

Lung tumours: Antibodies, protocols and controls

WT1 / RUN 43 2015

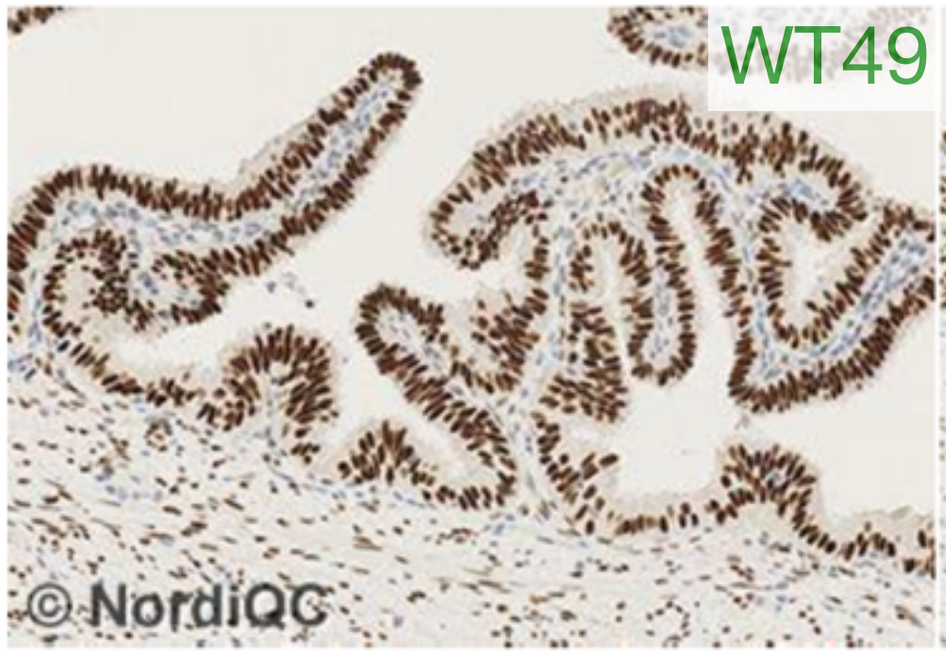


Fig. 1a
Optimal WT1 staining of the Fallopian tube using the mmAb clone WT49 (Leica) diluted 1:10 and with an incubation time of 25 min. after HIER in an alkaline buffer (BERS2, Leica) using a 3-step polymer system (Refine, Bond, Leica) and performed on the Bond III. A strong, distinct nuclear staining of virtually all epithelial cells and muscle cells is seen (same protocol used in Figs. 1a. - 4a.). Compare with Fig. 1b.

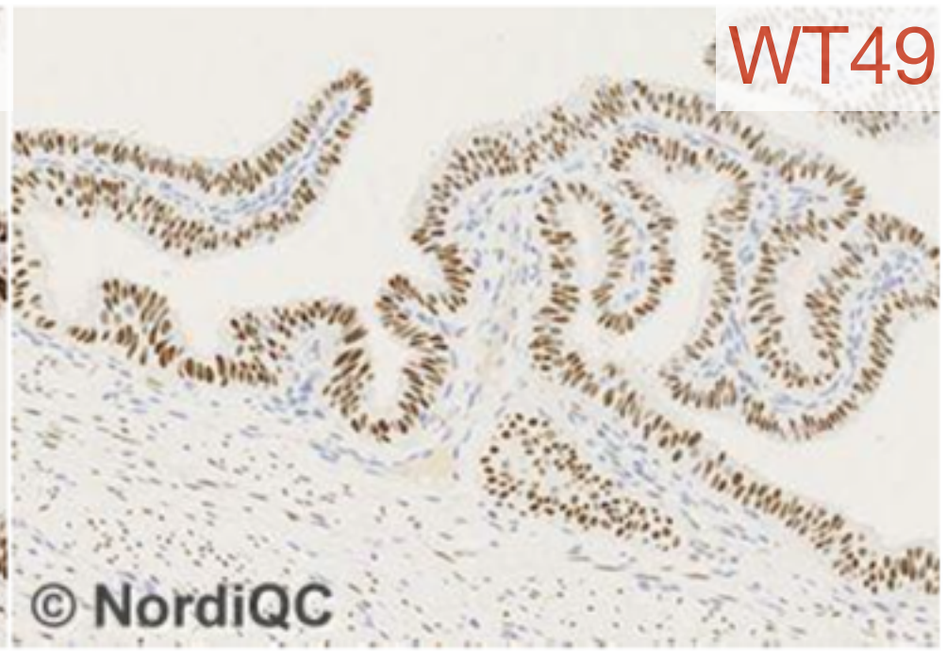


Fig. 1b
Insufficient WT1 staining of the Fallopian tube using the mmAb clone WT49 (Leica) diluted 1:25 and with an incubation time of 15 min. after HIER in an alkaline buffer (BERS2, Leica) using a 3-step polymer system (Refine, Bond, Leica) and performed on the Bond Max. The combination of a low titer and short incubation time results in insufficient staining. Only a moderate nuclear staining of the epithelial cells and muscle cells is seen.

The combination of a low concentration of the primary Ab and short incubation time results in insufficient staining.

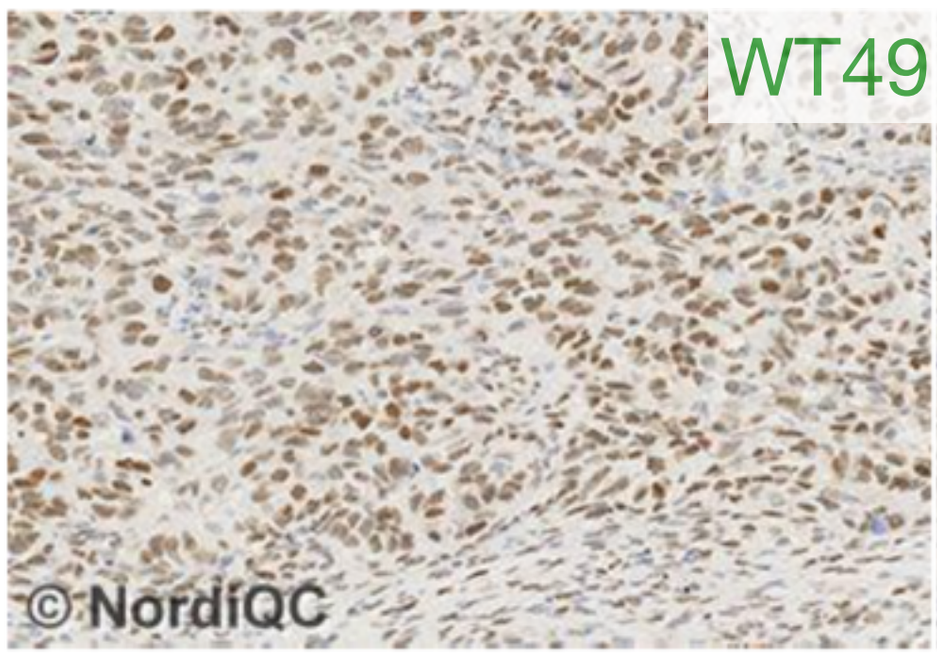


Fig. 4a
Optimal WT1 staining in the serous ovarian carcinoma

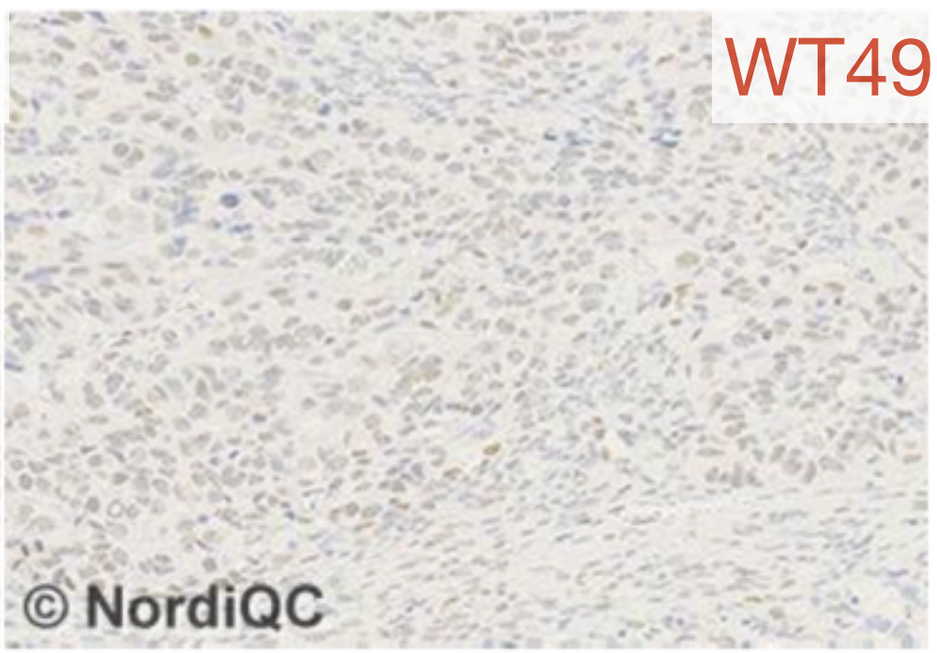
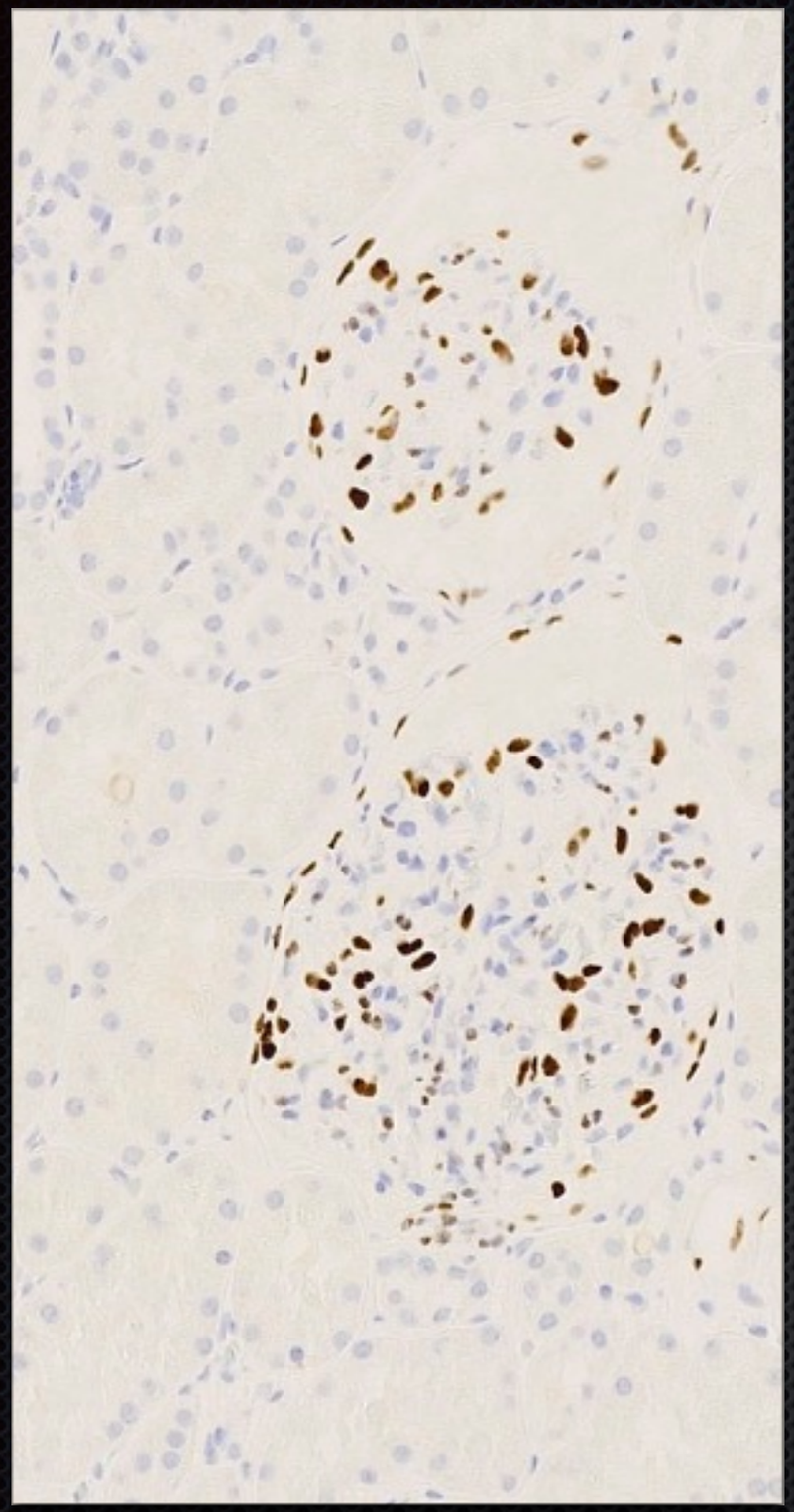
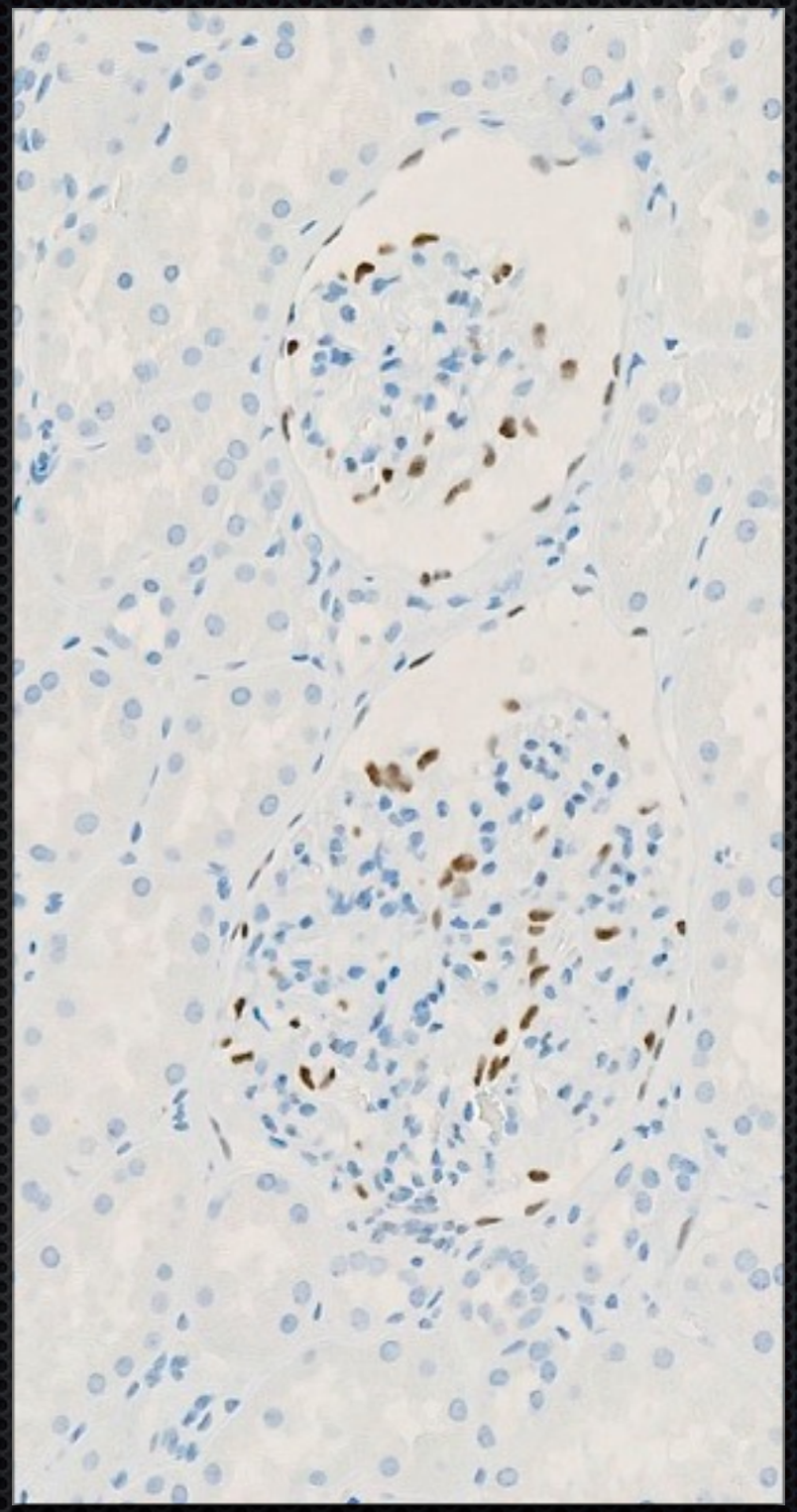


Fig. 4b
Insufficient WT1 staining in the serous ovarian carcinoma

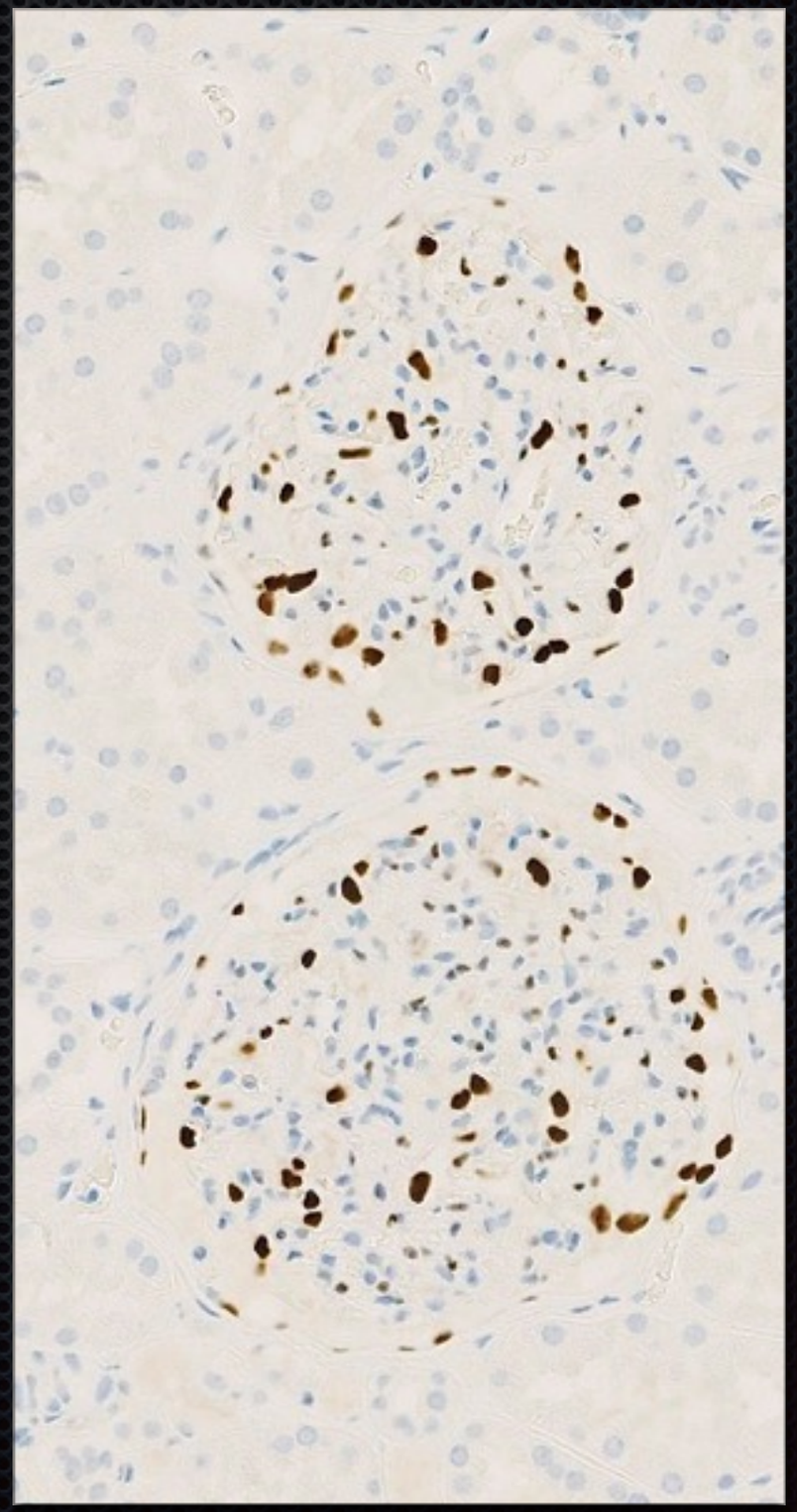
New WT-1 Ab, clone EP122 - kidney



EP122 / OMNIS

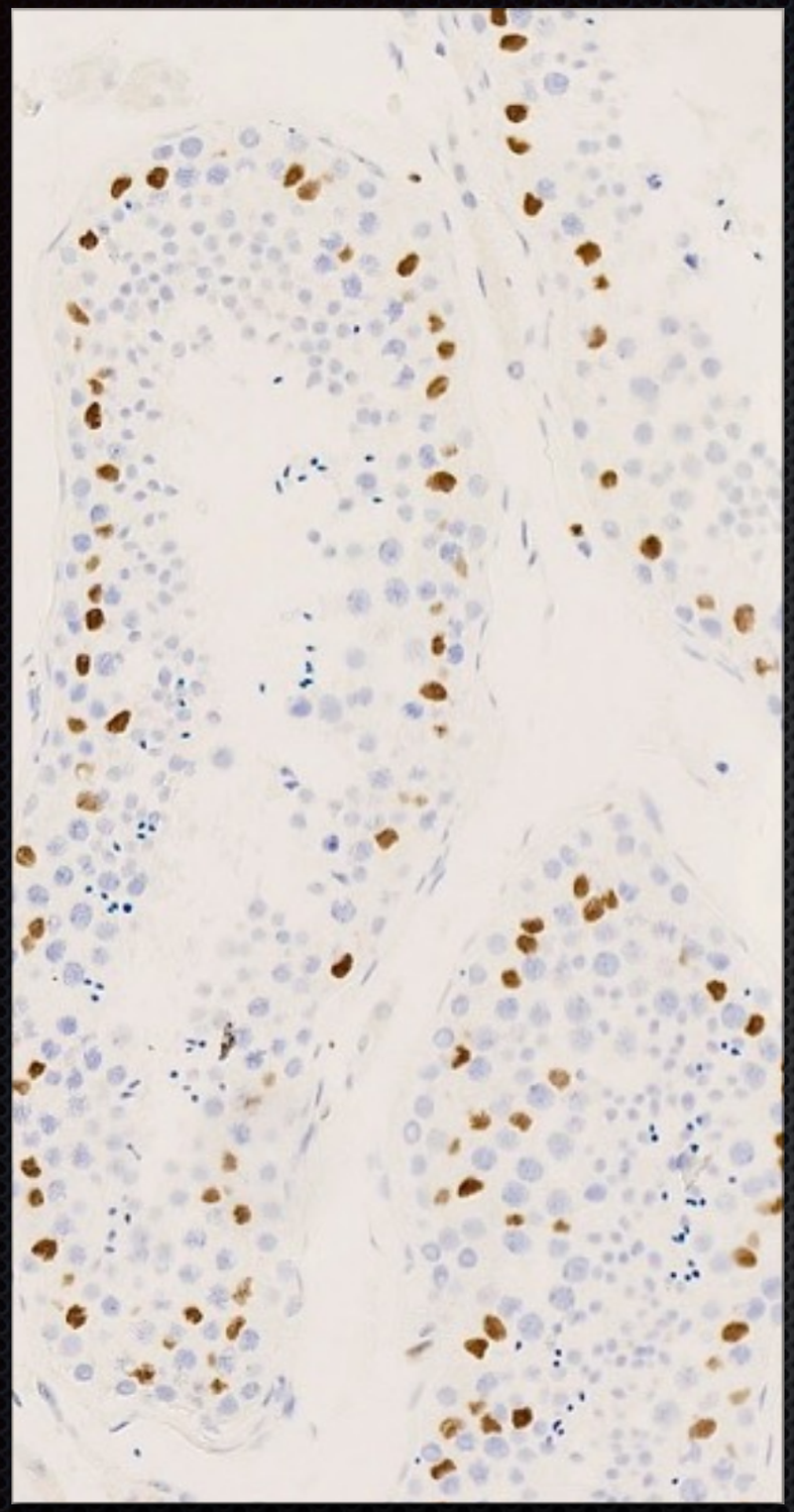


EP122 / BMU

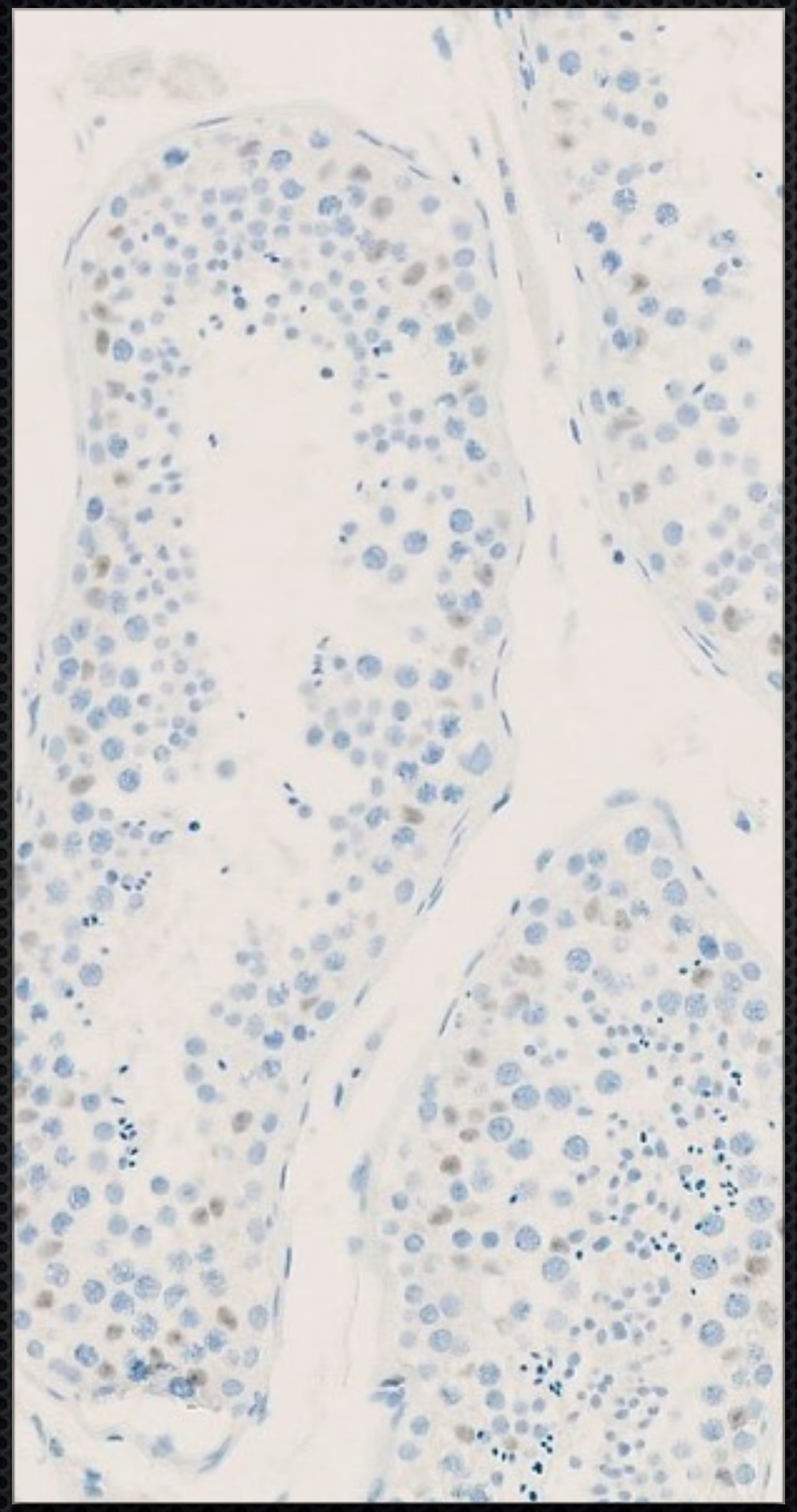


6F-H2 / BMU

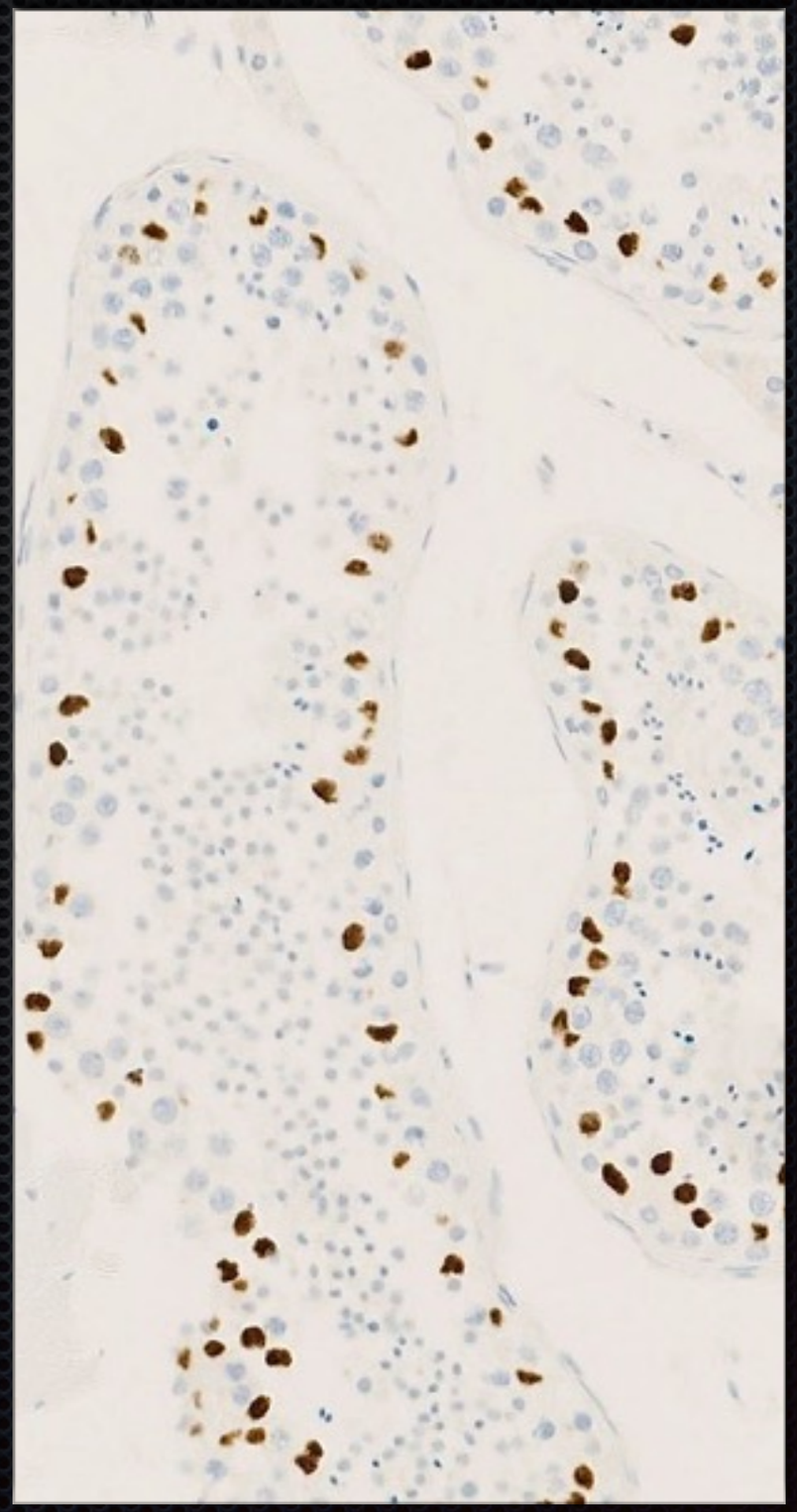
New WT-1 Ab, clone EP122 - testis



EP122 / OMNIS



EP122 / BMU



6F-H2 / BMU

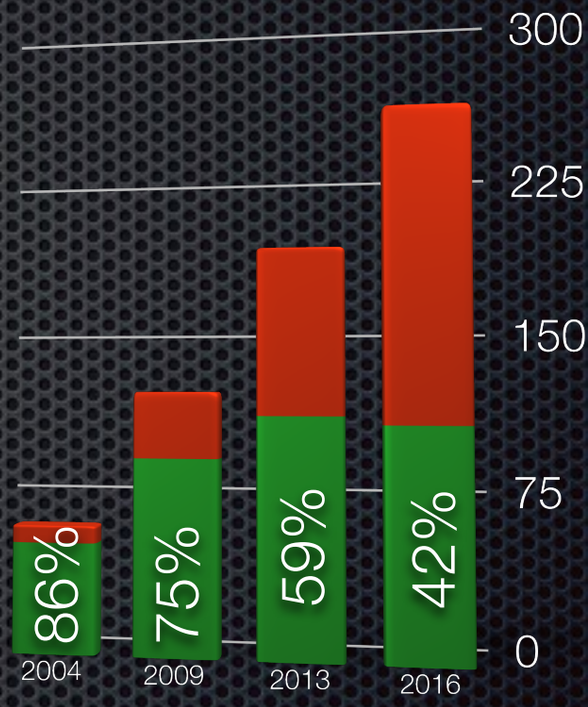
Lung tumours: Antibodies, protocols and controls

CEA / RUN 47 2016

Pass: 42 %

Table 1. **Antibodies and assessment marks for CEA, run 47**

Concentrated Antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **12-140-10**	3	Leica/Novocastra	0	0	0	3	-	-
mAb clone **CEA31**	9 1	Cell Marque BioSB	6	0	3	1	67%	75%
mAb **COL-1**	6 5 5 2 1 1	Thermo/Neomarkers Invitrogen/Zymed Biocare Immunologic Zytomed GeneTex	11	7	2	0	90%	94%
mAb **II-7**	85	Dako/Agilent	2	19	60	4	25%	58%
mAb **CEA88**	2	BioGenex	0	0	1	1	-	-
mAb **PARLAM 4**	1	Monosan	0	0	1	0	-	-
mAb **BS33**	1	Nordic Biosite	0	0	1	0		
Ready-To-Use Antibodies								
mAb clone **CEA31 760-4594**	53	Ventana/Cell Marque	22	26	5	0	91%	100%
mAb clone **CEA31 236M**	4	Cell Marque	1	2	1	0	-	-
mAb clone **COL-1 MAD-002095QD**	2	Master Diagnostica	0	0	1	1	-	-
mAb clone **COL-1 PM058**	1	Biocare	0	0	1	0	-	-
mAb clone **COL-1 Kit-0008**	1	Maixin	1	0	0	0	-	-
mAb clone **II-7 IR/IS622/GA622**	47	Dako/Agilent	0	6	40	1	13%	-
mAb clone **II-7 PA0004**	12	Leica	0	5	6	1	42%	-
mAb clone **TF3H8-1 760-2507**	13	Ventana/Roche	0	0	0	13	0%	-
Total	255		43	65	122	25	-	
Proportion			17%	25%	48%	10%	42%	



Lung tumours: Antibodies, protocols and controls

CEA / RUN 47 2016

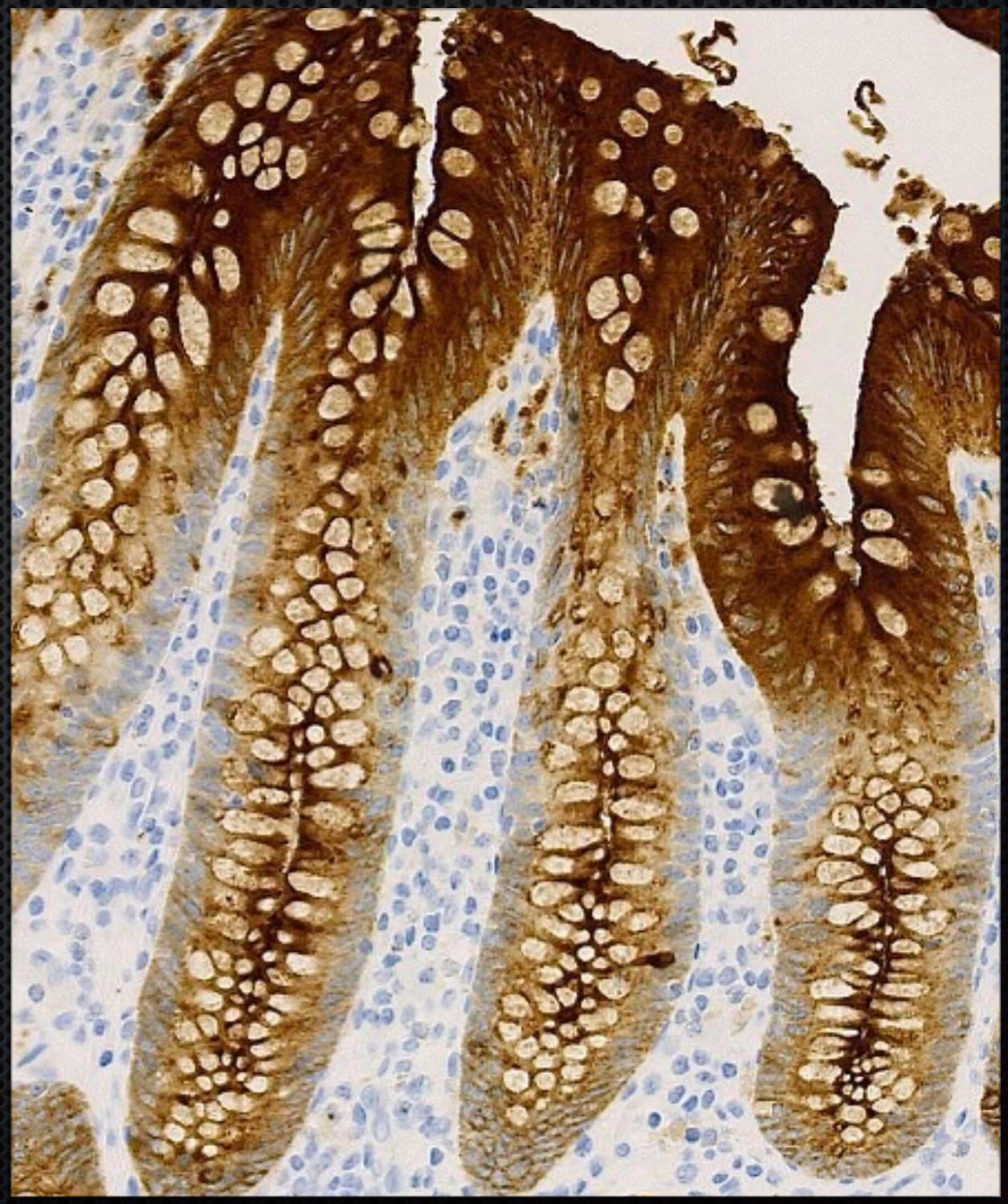
Controls

Positive: Appendix.

* The vast majority of the epithelial cells must show a moderate to strong cytoplasmic staining reaction.

Negative: Liver

* No cells must be positive.



Lung tumours: Antibodies, protocols and controls

CEA / RUN 47 2016

Recommendable clones	Retrieval	Dilution range
mAb CEA31	HIER, High pH	1:100 - 1:400 or RTU
mAb COL-1	HIER, High pH	1:100 - 1:400

Table 3. **Optimal results for CEA for the three most commonly used concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic / OMNIS**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **II-7**	1/17** (6%)	0/2	0/35 (0%)	-	1/10 (10%)	0/4 (0%)
mAb clone **COL-1**	1/2	-	8/13 (62%)	-	1/1	-
mAb clone **CEA31**	3/3	-	3/6 (50%)	-	-	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.

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

Lung tumours: Antibodies, protocols and controls

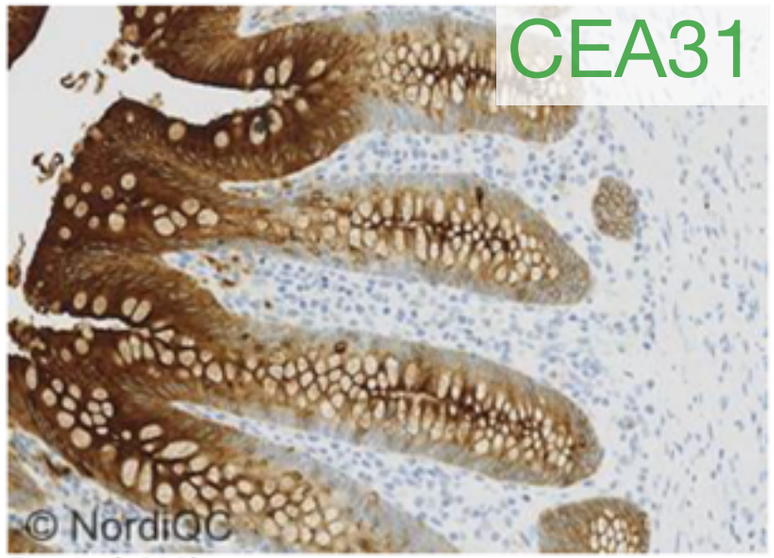


Fig. 1a (x200)
Optimal CEA staining of the appendix using the **mAb clone CEA31** diluted 1:100 and with an incubation time of 30 min. after HIER in an alkaline buffer (TRS pH 9, Dako). Staining was performed on the Dako Omnis using a 3-step polymer system (EnVision Flex+). A weak to moderate staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction.
Also compare with Figs. 2a – 4a, same protocol. No background staining is seen.

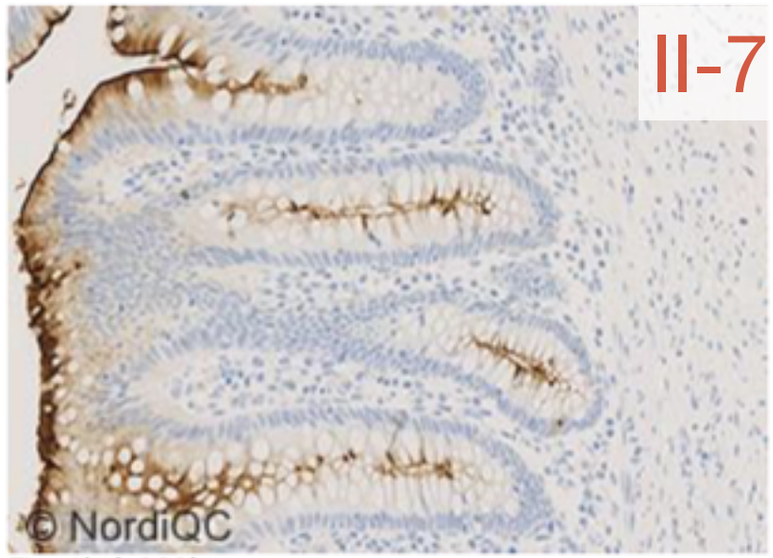


Fig. 1b (x200)
Insufficient CEA staining of the appendix using the **mAb clone II-7** in a RTU format (Dako GA622) with an incubation time of 25 min. after HIER in an alkaline buffer (TRS pH 9, Dako). Staining was performed on the Dako Omnis using a 3-step polymer system (EnVision Flex+). In spite of very similar protocol settings the "clone II-7"-protocol only demonstrates the glycocalyx distinctively, while the cytoplasmic compartment in the vast majority of epithelial cells is unstained - same field as in Fig. 1a. Also compare with Figs. 2b - 4b, same protocol.

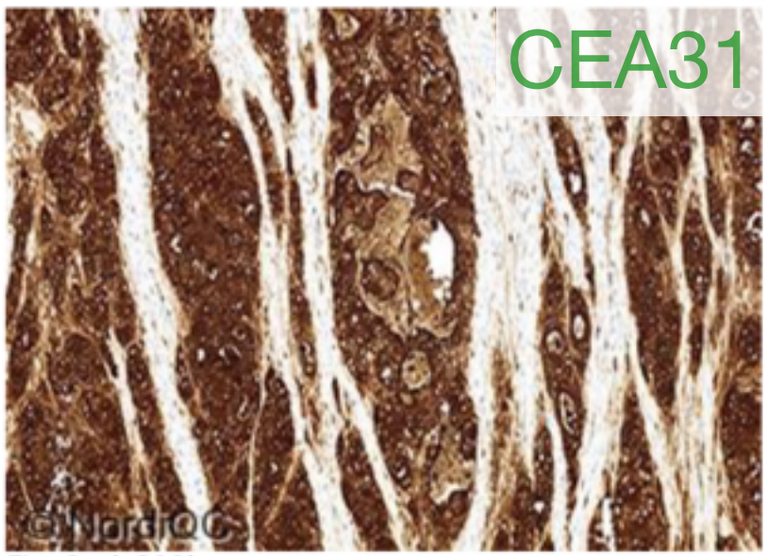


Fig. 2a (x200)
Optimal CEA staining of the colon adenocarcinoma with high level CEA expression using same protocol as in Fig. 1a. Virtually all the neoplastic cells show a strong and distinct cytoplasmic staining reaction. Weak background staining in the vicinity of the neoplastic cells, due to diffusion of antigen, is seen and accepted.

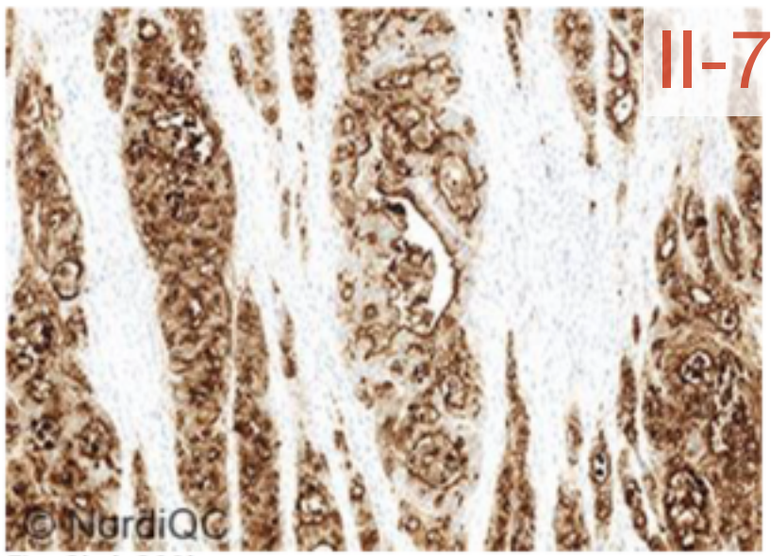


Fig. 2b (x200)
CEA staining of the colon adenocarcinoma with high level CEA expression using same insufficient protocol as in Fig. 1b – same field as in Fig. 2a. The intensity of the neoplastic cells demonstrated is reduced compared to the level expected and obtained in Fig. 2a.

Less successful primary antibody: mAb clone II-7

Lung tumours: Antibodies, protocols and controls

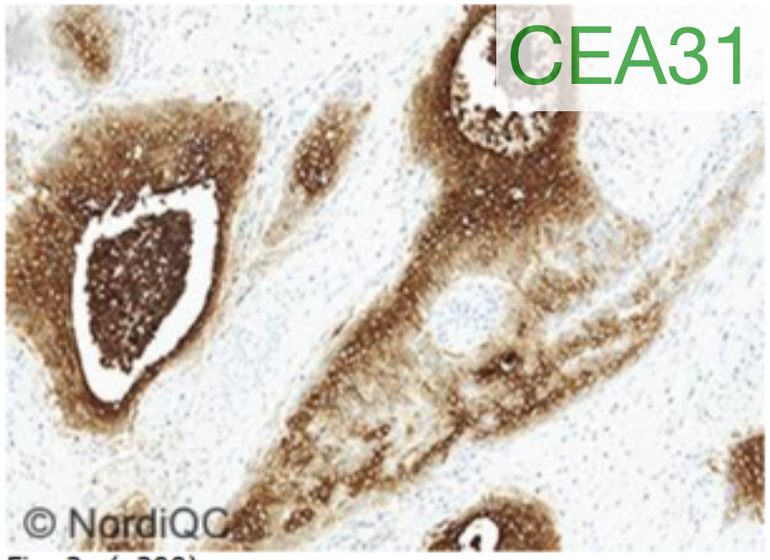


Fig. 3a (x200)
Optimal CEA staining of the urothelial carcinoma, tissue core no. 4, using same protocol as in Figs. 1a and 2a. The majority of the neoplastic cells show a strong and distinct staining reaction. No background staining is seen.

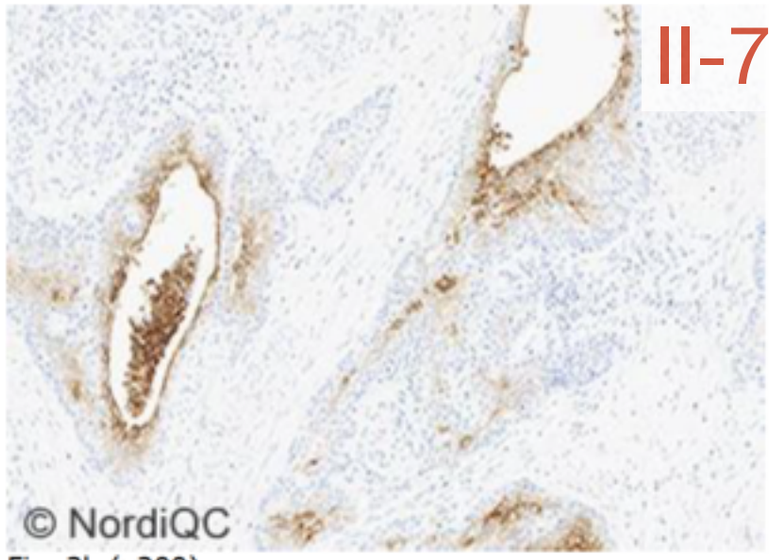


Fig. 3b (x200)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 4, using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a. The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 3a.

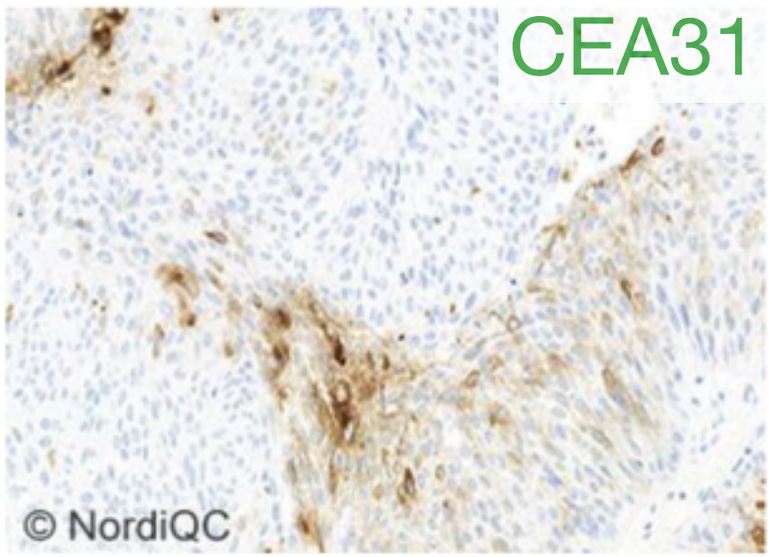


Fig. 4a (x200)
Optimal CEA staining of the urothelial carcinoma, tissue core no. 5, with low level CEA expression using same protocol as in Figs. 1a - 3a. Focally the neoplastic cells show a moderate to strong and distinct staining reaction. No background staining is seen.

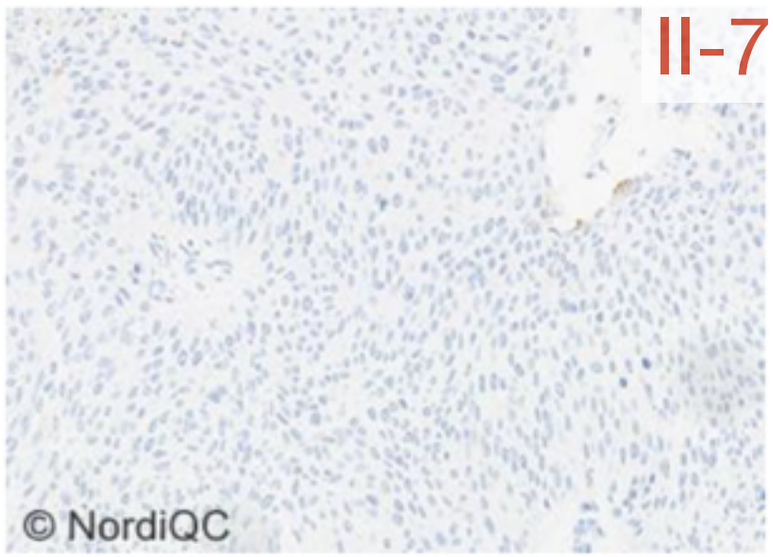


Fig. 4b (x200)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 5, with low level CEA expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
The neoplastic cells show no staining reaction and a false negative result of the tumour is seen.

Less successful primary antibody: mAb clone II-7

Lung tumours: Antibodies, protocols and controls

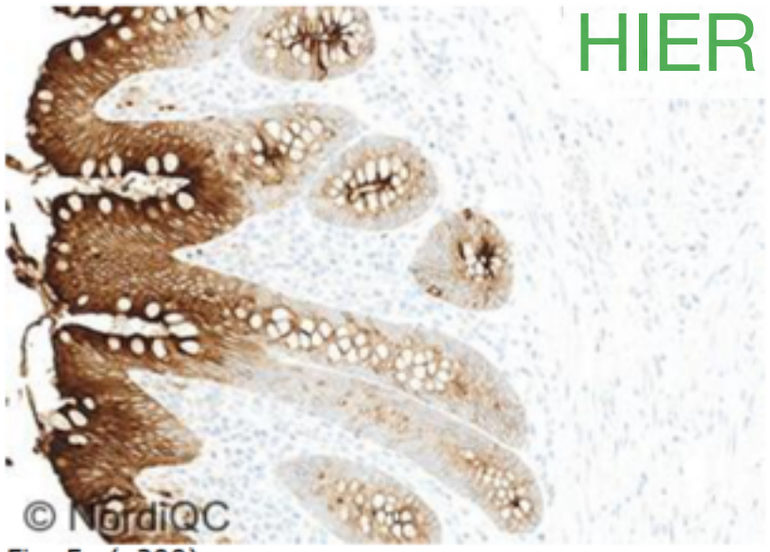


Fig. 5a (x200)
Optimal CEA staining of the appendix using the mAb clone CEA31 diluted 1:400 and with an incubation time of 30 min. after **HIER** in an alkaline buffer (CC1, Ventana). Staining was performed on the Ventana BenchMark using a 3-step multimer system (OptiView)
A weak to moderate staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction. Compare also to Fig. 6a, same protocol.

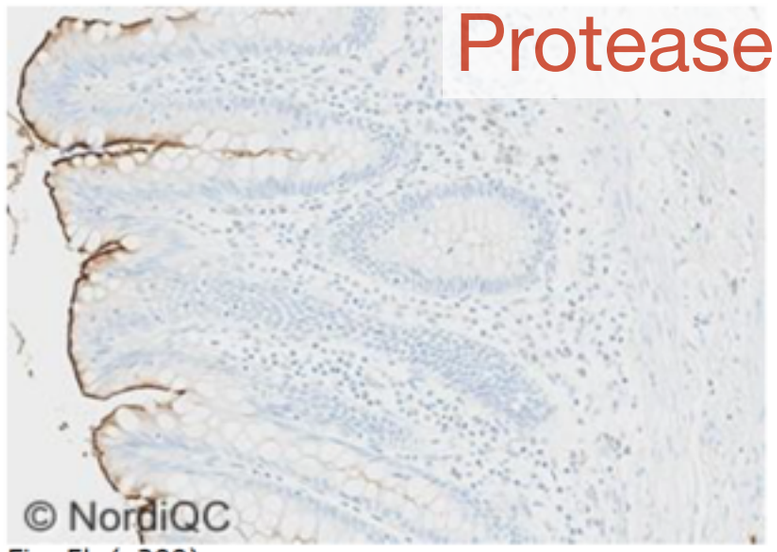


Fig. 5b (x200)
Insufficient CEA staining of the appendix using the mAb clone CEA31 with similar protocol settings as used in Fig. 5a. Only difference was the use of proteolytic pretreatment (Protease 1, Ventana for 8 min.) instead of HIER. Proteolytic pre-treatment results in a drastic reduction in staining intensity. Only the glycocalyx is distinctively demonstrated, while the cytoplasmic compartment of the epithelial cells is unstained - same field as in Fig. 5a. Compare also to Fig. 6b, same protocol.

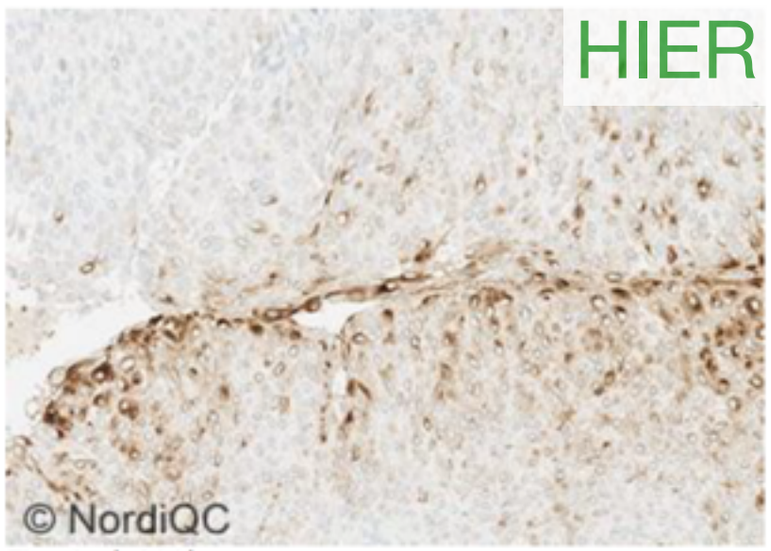


Fig. 6a (x200)
Optimal CEA staining of the urothelial carcinoma, tissue core no. 5, with low level CEA expression using same protocol as in Fig 5a. Focally the neoplastic cells show a moderate to strong and distinct staining reaction.

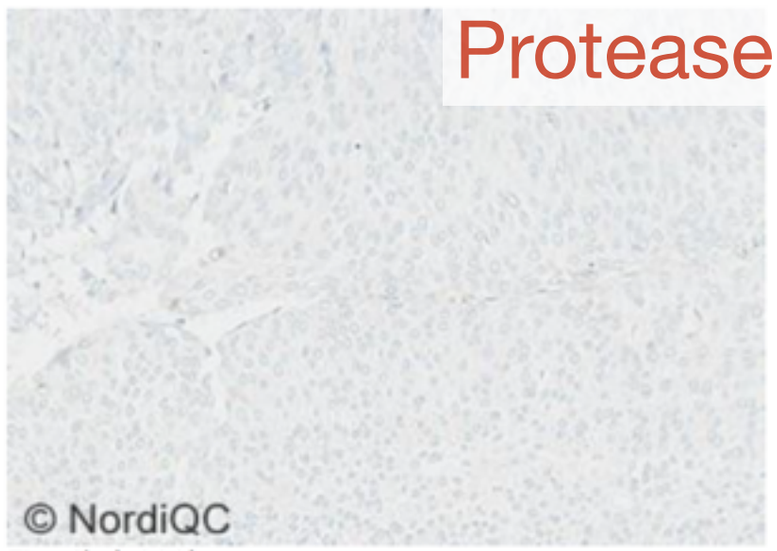


Fig. 6b (x200)
Insufficient CEA staining of the urothelial carcinoma, tissue core no. 5, with low level CEA expression using same protocol as in Fig. 5b – same field as in Fig. 6a. The neoplastic cells show no staining reaction and a false negative result in this tumour is seen.

Inappropriate retrieval - use of proteolysis

Lung tumours: Antibodies, protocols and controls

Inappropriate antibody - NCA and BGP cross reaction

CEA / RUN 47 2016

CEA31

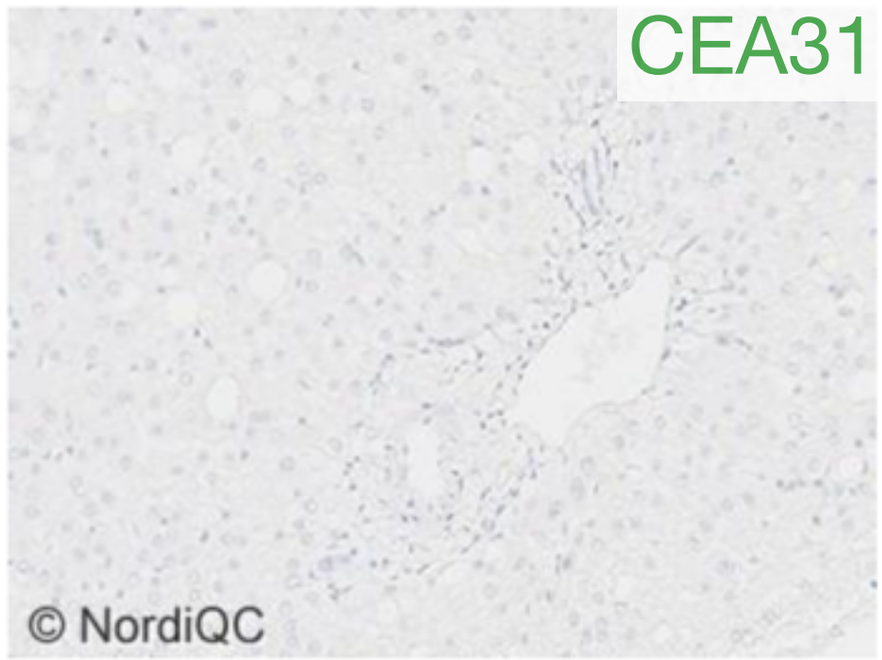


Fig. 7a (x200)
Optimal CEA staining of the liver using same protocol as in Figs. 5a and 6a based on the **mAb clone CEA31**. No staining reaction is seen in the Kupffer cells, leucocytes and the bile canaliculi. No background staining is seen.

TF3H8-1

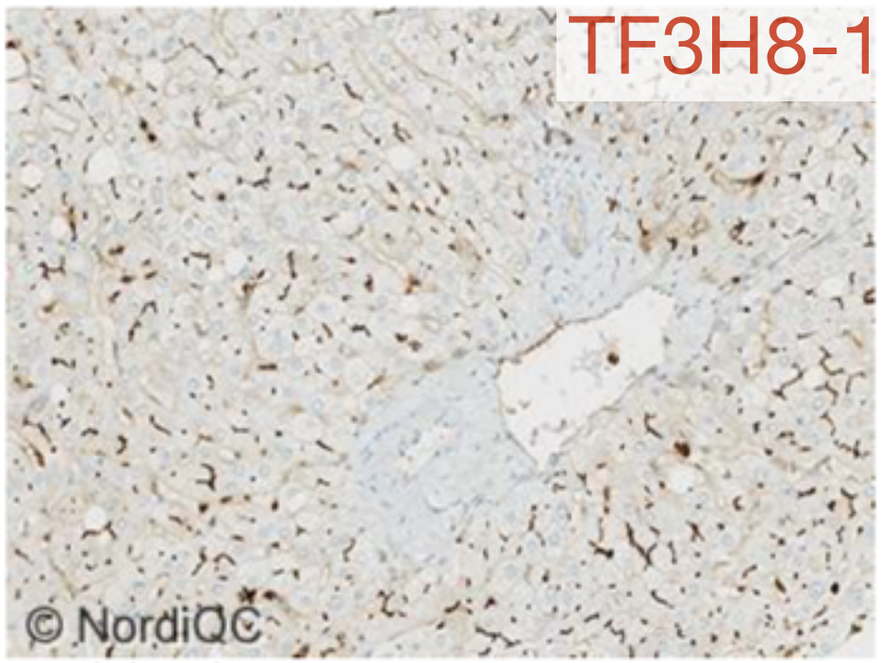


Fig. 7b (x200)
Insufficient CEA staining of the liver using the **mAb clone TF3H8-1.** Both the Kupffer cells, leucocytes and bile canaliculi are stained due to a cross reaction of the Ab to NCA (CEACAM6) and BGP (CEACAM1) – same field as in Fig. 7a.

Lung tumours: Antibodies, protocols and controls

Target	High scoring clones*	Low scoring clones*
Napsin A	mmAb: IP64 and MRQ-60	pAb: 760-4446 and 352A-7x
TTF1	mmAb: SPT24 and SP141	mmAb: 8G7G3/1
p63	mmAb: DAK-p63 and 4A4	mmAb: 7JUL
p40	mmAb: BC28 and rmAb: ZR8	Many pAbs
SYP	mmAb: 27G12, rmAb MRQ-40 and DAK-SYNAP	mmAb: SY38
Iu-ALK	rmAb: D5F3, mmAb: 5A4	mmAb: ALK1
WT1	mmAb: WT49 and 6F-H2	
CEA	mmAb: CEA31 and COL-1	mmAb: TF3H8-1 and II-7
CGA	pAb: A0430§ / IR502§, mmAb: LK2H10	rmAb: SP12, mmAb DAK-A3
Calretinin	rmAb: SP65, pAb 18-0211	rmAb: SP11
Podoplanin	mmAb: D2-40	mmAb: D2-40 #
CD56	rmAb: MRQ-42, mmAb: CD564 and 123C3	mmAb: 123C3 #

Ventana platform § Products discontinued * on the basis of the assessments in NordiQC

Thank you for your attention!

Coffee…..

Extra material:

Target	High scoring clones*	Low scoring clones*
CGA	pAb: A0430§ / IR502§, mmAb: LK2H10	rmAb: SP12, mmAb DAK-A3
Calretinin	rmAb: SP65, pAb 18-0211	rmAb: SP11
Podoplanin	mmAb: D2-40	mmAb: D2-40 #
CD56	rmAb: MRQ-42, mmAb: CD564 and 123C3	mmAb: 123C3 #

Ventana platform

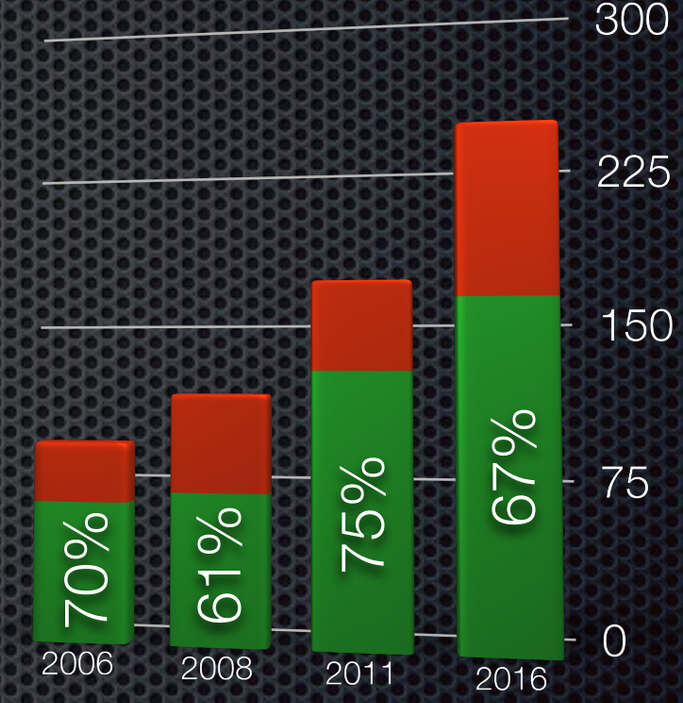
* on the basis of the assessments in NordiQC

Chromogranin A / RUN 46 2016

Pass: 67 %

Table 1. **Antibodies and assessment marks for CGA, run 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5H7**	4	Leica/Novocastra	0	0	3	1	-	-
mAb clone **DAK-A3**	36	Dako/Agilent	0	2	17	17	6%	-
mAb clone **LK2H10**	22 18 6 3 2 2 1 1 1 1	Thermo/Neomarkers Cell Marque Immulologic Biogenex Millipore Zytomed Abcam A.Menarini Diagnostic Biosystems Europroxima Monosan Unknown	24	31	0	4	93%	98%
mAb clone **PHE5**	1	Unknown	0	0	1	0	-	-
mAb clones **LK2H10+PHE5**	6 5	Thermo/Neomarkers Biocare	3	8	0	0	100%	100%
~~rmAb clone EP38~~	1	Epitomics	0	1	0	0		
rmAb clone **SP12**	1 1	Master Diagnostica Thermo/NeoMarkers	0	0	0	2	-	-
pAb **A0430***	38	Dako/Agilent	8	17	8	5	66%	-
pAb **NB120-17064**	1	Novus Biologicals	0	1	0	0	-	-
pAb **RB-9003**	1	Thermo/NeoMarkers	0	1	0	0		
Ready-To-Use antibodies								
mAb clone **5H7 PA0430**	6	Leica/Novocastra	0	0	2	4	-	-
mAb clone **LK2H10 760-2519**	69	Ventana/Roche	27	28	6	8	80%	96%
mAb clone **LK2H10 E001**	3	Linaris	0	3	0	0	-	-
mAb **LK2H10 AM126-5M**	1	Biogenex	0	0	1	0	-	-
mAb **LK2H10 238M-90**	1	Cell Marque	1	0	0	0	-	-
mAb clone **LK2H10 MAD-000616QD**	2	Master Diagnostica	1	1	0	0	-	-
mAb clones **LK2H10+PHE5 PM010**	2	Biocare	1	1	0	0	-	-
mAb clones **LK2H10+PHE5 BSB5345**	1	Bio SB	0	1	0	0	-	-
mAb clones **LK2H10+PHE5 MAB-0202**	1	Maixin	1	0	0	0		
pAb **IR502***	2	Dako	0	1	1	0		
Total	242		66	96	39	41	-	
Proportion			27%	40%	16%	17%	67%	



** Discontinued products*

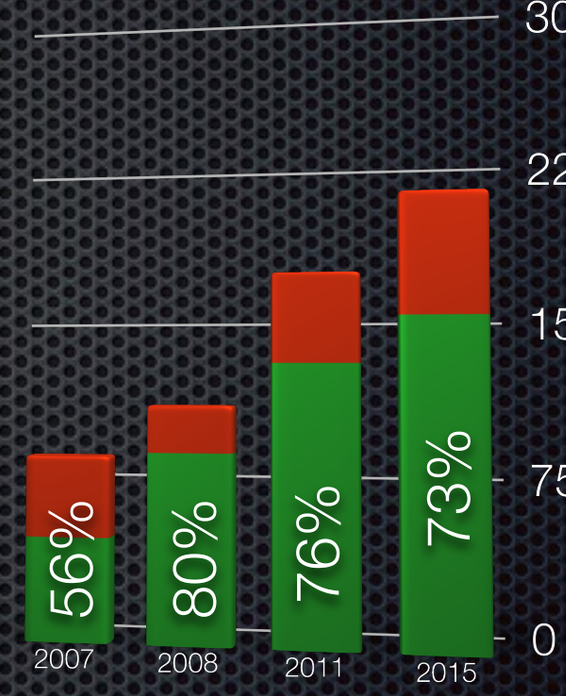
Lung tumours: Antibodies, protocols and controls

Calretinin / RUN 45 2015

Pass: 73 %

Table 1: **Antibodies and assessment marks for CR, run 45**

	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mmAb clone **2E7**	1	Immunologic	1	0	0	0	-	-
mmAb clone **5A5**	21 1	Leica/Novocastra Immunologic	3	10	8	1	59%	56%
mmAb clone **CAL6**	6 1	Leica/Novocastra Monosan	4	1	0	2	71%	-
mmAb clone **DAK-Calret 1**	35	Dako	10	13	9	3	66%	87%
rmAb clone **SP13**	3 1 2	Thermo/Neomarkers Spring Bioscience Cell Marque	1	2	2	1	50%	-
pAb **18-0211**	16	Invitrogen/Zymed	2	8	6	0	63%	-
pAb **232A**	5	Cell Marque	0	1	2	2	20%	-
pAb **61-0006**	1	Genemed	0	1	0	0	-	-
pAb **7699/3H**	1	Swant	0	0	0	1	-	-
pAb **RBK003**	1	Zytomed	0	0	1	0	-	-
Ready-To-Use antibodies								
mmAb clone **CAL6 PA0346**	8	Leica/Novocastra	2	3	2	1	63%	-
mmAb clone **DAK-Calret 1 IS/IR627**	38	Dako	9	17	10	2	68%	79%
rmAb **SP13 RMA-0524**	1	Maixin	1	0	0	0	-	-
rmAb **SP13 232R-18**	1	Cell Marque	0	1	0	0	-	-
rmAb **SP13 MAD-000315QD**	1	Master Diagnostica	0	1	0	0	-	-
rmAb clone **SP65 790-4467**	64	Ventana	52	8	2	2	94%	94%
pAb **232A-78**	1	Cell Marque	0	1	0	0	-	-
pAb **PP092**	1	BioCare	0	1	0	0	-	-
Total	210		85	68	42	15	-	
Proportion			41%	32%	20%	7%	73%	



Lung tumours: Antibodies, protocols and controls

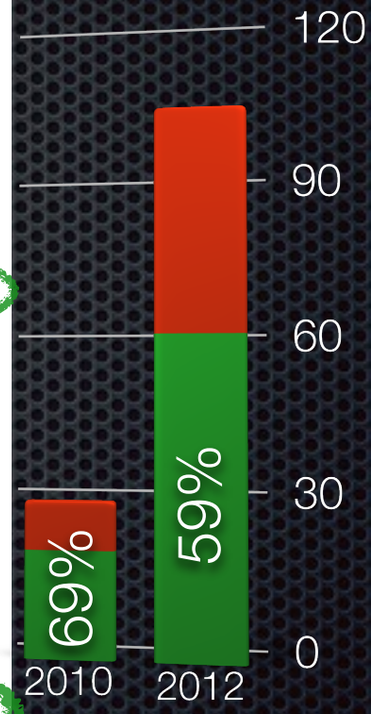
Podoplanin / RUN 36 2012

Pass: 59 %

Table 1. Abs and assessment marks for Podop, run 36

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **D2-40**	48 3 2 1 1 1	Dako Signet Biocare Cell Marque Immunologic Zytomed	10	20	22	4	54 %	56 %
mAb clone **AB3**	1	AngioBio	0	1	0	0	-	-
mAb clone **18H51**	1	Acris	0	0	0	1	-	-
rmAb clone **EP215**	1	Epitomics	0	0	1	0	-	-
Ready-To-Use Abs								
mAb clone **D2-40 IS/IR072**	15	Dako	11	4	0	0	100 %	100 %
mAb clone **D2-40 N1607**	3	Dako	0	3	0	0	-	-
mAb clone **D2-40 760-4395**	21	Ventana/Cell Marque	0	8	13	0	38 %	-
mAb clone **D2-40 322M-17/18**	2	Cell Marque	0	1	1	0	-	-
mAb clone **D2-40 MON-RTU1092**	1	Monosan	0	1	0	0	-	-
mAb clone **D2-40 MAD-000402QD**	1	Master Diagnostica	0	1	0	0	-	-
Total	102		21	39	37	5	-	
Proportion			21 %	38 %	36 %	5 %	59 %	

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



Lung tumours: Antibodies, protocols and controls

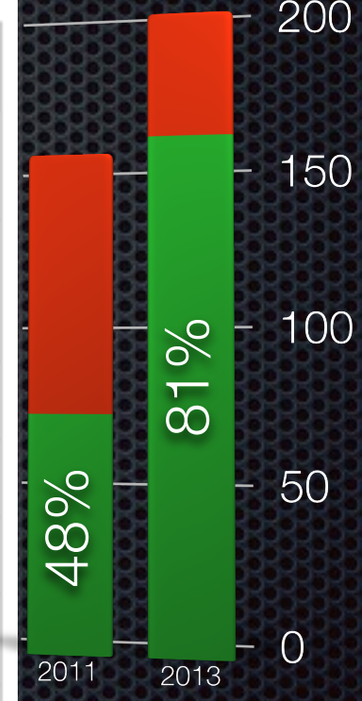
CD56 / RUN 37 2013

Pass: 81 %

Table 1. Abs and assessment marks for CD56, run 37

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1B6**	42 1 1	Novocastra/Leica Linaris Vector Lab.	16	17	10	1	75 %	77 %
mAb clone **123C3** *	18 4 2 1	Dako Monosan Invitrogen Spring Bioscience	10	10	3	2	80 %	100 %
rmAb clone **MRQ-42**	21 1	Cell Marque Immunologic	21	1	0	0	100 %	100 %
mAb clone **123C3.D5** *	18 1	NeoMarkers/Thermo Immunologic	5	6	5	3	58 %	100 %
mAb clone **CD564**	8 1	Novocastra/Leica Monosan	5	4	0	0	100 %	100 %
mAb clone **56C04**	2	NeoMarkers/Thermo	1	1	0	0	-	-
rmAb clone **RCD56**	1	Zytomed System	0	0	1	0	-	-
Ready-To-Use Abs								
mAb clone **123C3, IR628**	34	Dako	16	13	3	2	85 %	88 %
rmAb clone **MRQ-42 760-4596**	16	Ventana	14	2	0	0	100 %	100 %
mAb clone **123C3, 790-4465** *	9	Ventana	2	1	6	0	33 %	-
mAb, clone **CD564, PA0191**	6	Novocastra/Leica	3	3	0	0	100 %	100 %
mAb, clone **1B6**	4	Novocastra/Leica	0	2	0	2	-	-
mAb, clone **123C3.D5, Mon-RTU1049**	1	Monosan	0	1	0	0	-	-
mAb clone **BC56C04, PM164**	2	Biocare	0	2	0	0	-	-
rmAb clone **MRQ-42, 156R-97**	1	Cell Marque	1	0	0	0	-	-
mAb clone **56C04, MAD-000218QD**	1	Master Diagnostica	1	0	0	0	-	-
Total	196		95	63	28	10	-	-
Proportion			49 %	32 %	14 %	5 %	81 %	-

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



* Poor performance of clone 123C3 and 123C3.D5 on the Ventana BenchMark platform.

Lung tumours: Antibodies, protocols and controls

CEA / RUN 37 2013

Recommendable clones	Retrieval	Dilution range
mAb CEA31	HIER, High pH (CC1)	1:400 or RTU
mAb COL-1	HIER, High pH	1:100 - 1:500
mAb II-7	HIER, High pH	1:30 - 1:320 or RTU *

Table 2. Optimal results for CEA using concentrated antibodies on the 3 main IHC systems*

Concentrated antibodies	Dako Autostainer Link / Classic		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **II-7**	**20 %** 5/25*	-	**0 %** 0/35 !?	**0 %** 0/1	**50 %** 3/6	**0 %** 0/2
mAb clone **COL-1**	**100 %** 1/1	-	**89 %** 8/9	-	**100 %** 1/1	**50 %** 1/2

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms. ** (number of optimal results/number of laboratories using this buffer)

* Poor performance of the mAb clone II-7 on the Ventana BenchMark platform.

Lung tumours: Antibodies, protocols and controls

CEA / RUN 37 2013

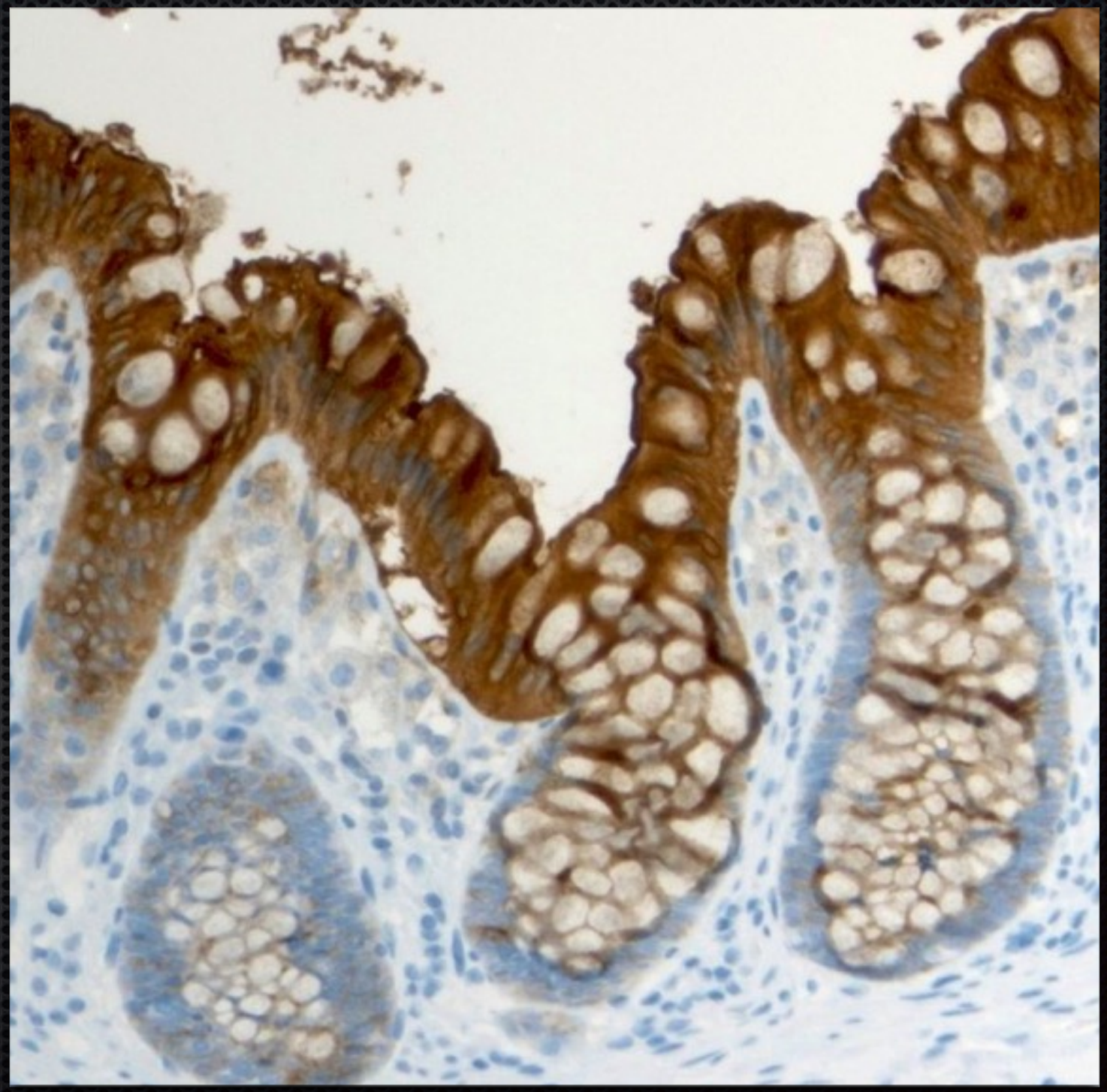
Controls

Positive: Appendix.

* The vast majority of the epithelial cells must show a moderate to strong cytoplasmic staining reaction.

Negative: Liver

* No cells must be positive.



Lung tumours: Antibodies, protocols and controls

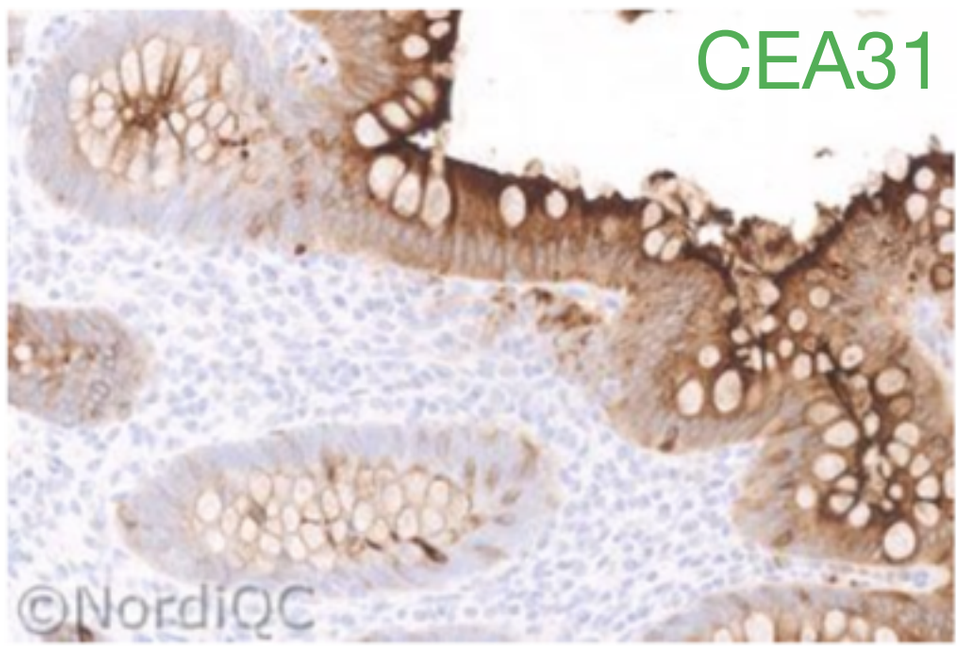


Fig. 1a. Optimal CEA staining of the appendix using the mAb clone CEA31 optimally calibrated and with HIER. A weak to moderate staining reaction is seen in the vast majority of the luminal epithelial cells of the appendix, whereas the glycocalyx show an intense staining reaction.
Also compare with Figs. 2a – 4a, same protocol.

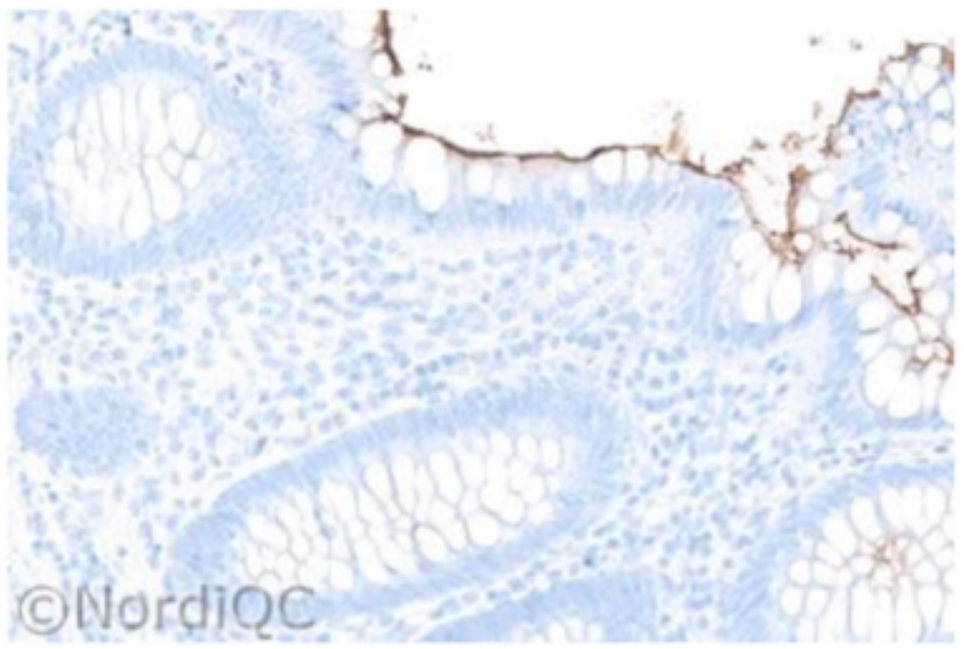


Fig. 1b. Insufficient CEA staining of the appendix using the mAb clone II-7 with a less successful protocol – insufficient HIER and too diluted Ab. Only the glycocalyx is distinctively demonstrated, while the cytoplasmic compartment of the epithelial cells is unstained - same field as in Fig. 1a.
Also compare with Figs. 2b & 3b, same protocol.

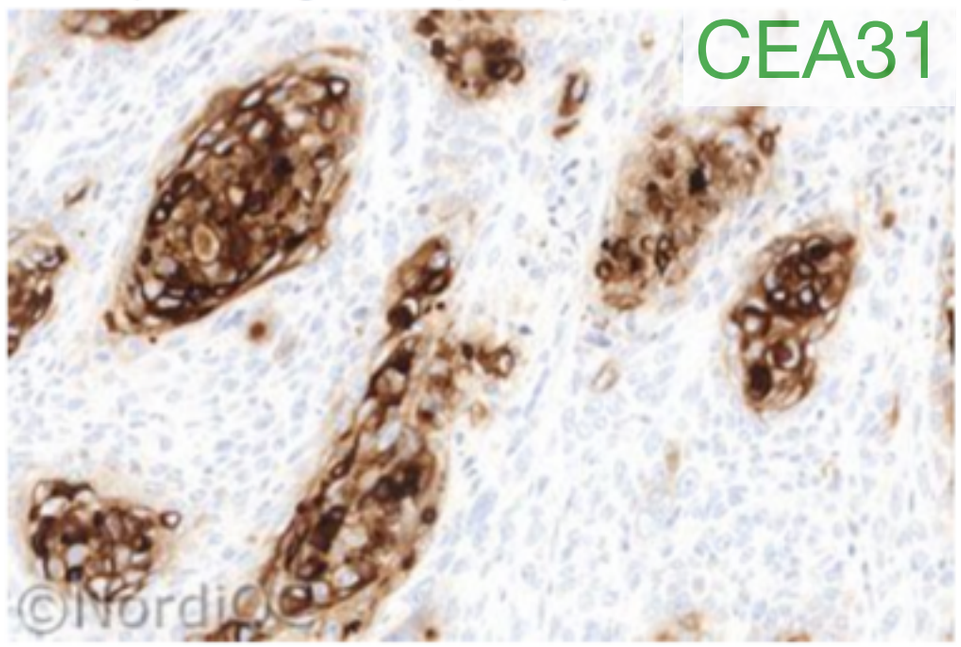


Fig. 2a. Optimal CEA staining of the colon adenocarcinoma, tissue core no. 4 using same protocol as in Fig. 1a.
Virtually all the neoplastic cells show a strong and distinct cytoplasmic staining reaction. No background staining is seen.

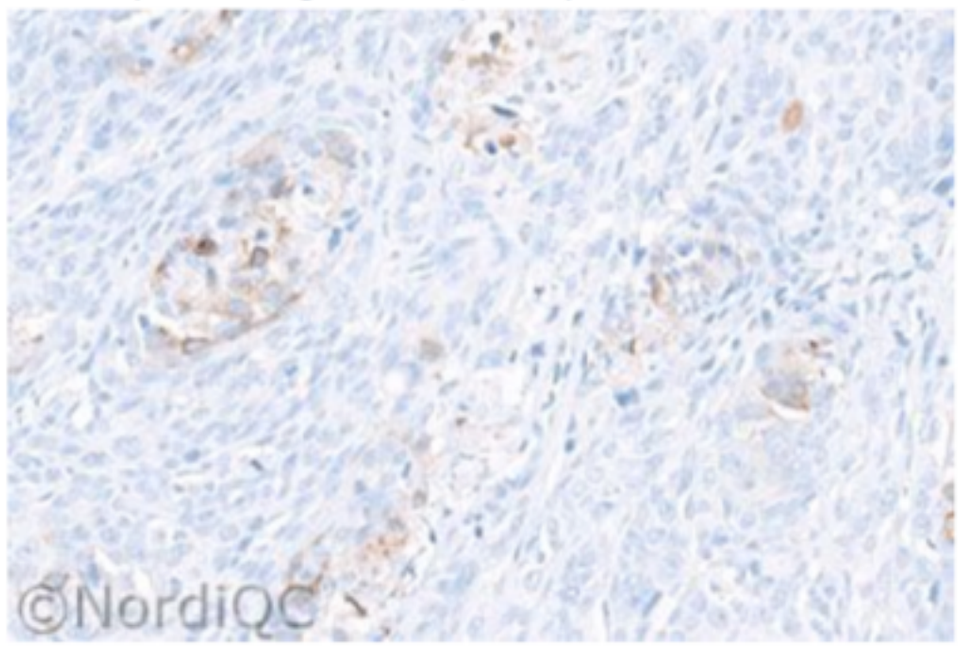


Fig. 2b. Insufficient CEA staining of the colon adenocarcinoma, tissue core no. 4 using same protocol as in Fig. 1b. – same field as in Fig. 2a. The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 2a.

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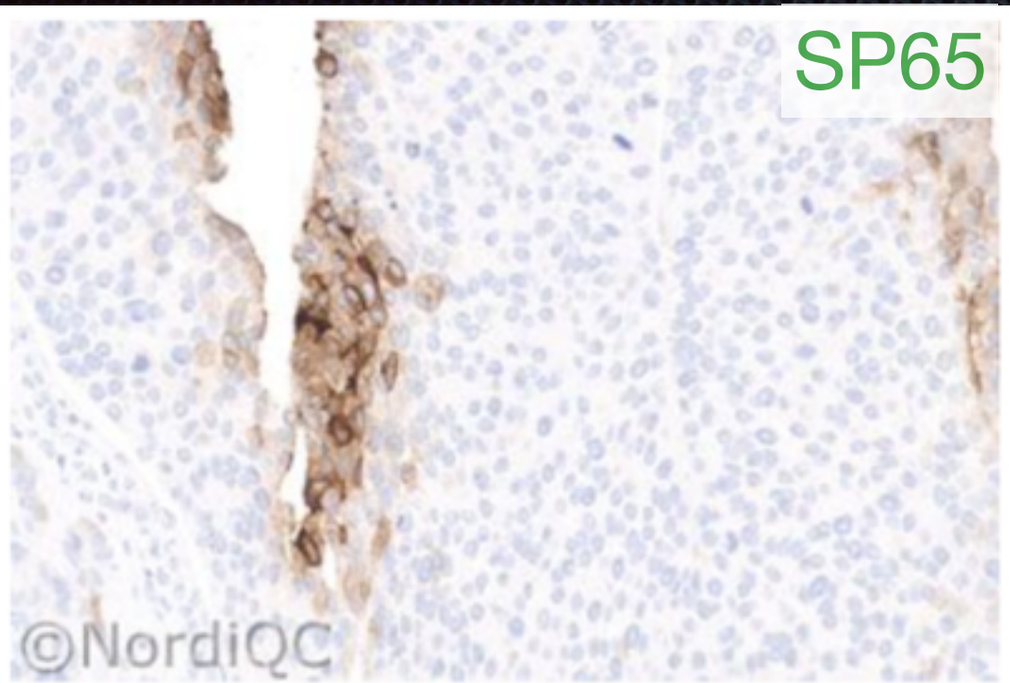

Fig. 3a. Optimal CEA staining of the urothelial carcinoma using same protocol as in Figs. 1a & 2a. Focally the neoplastic cells show a strong and distinct staining reaction.
No background staining is seen.

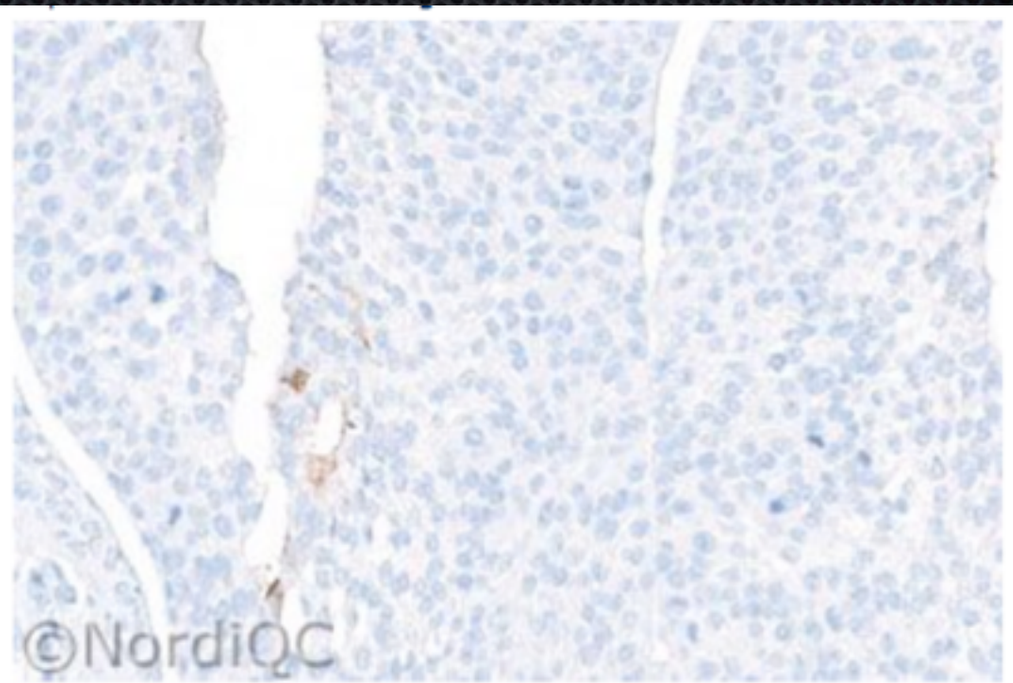

Fig. 3b. Insufficient CEA staining of the urothelial carcinoma using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a. Only dispersed neoplastic cells show a weak or equivocal staining reaction.

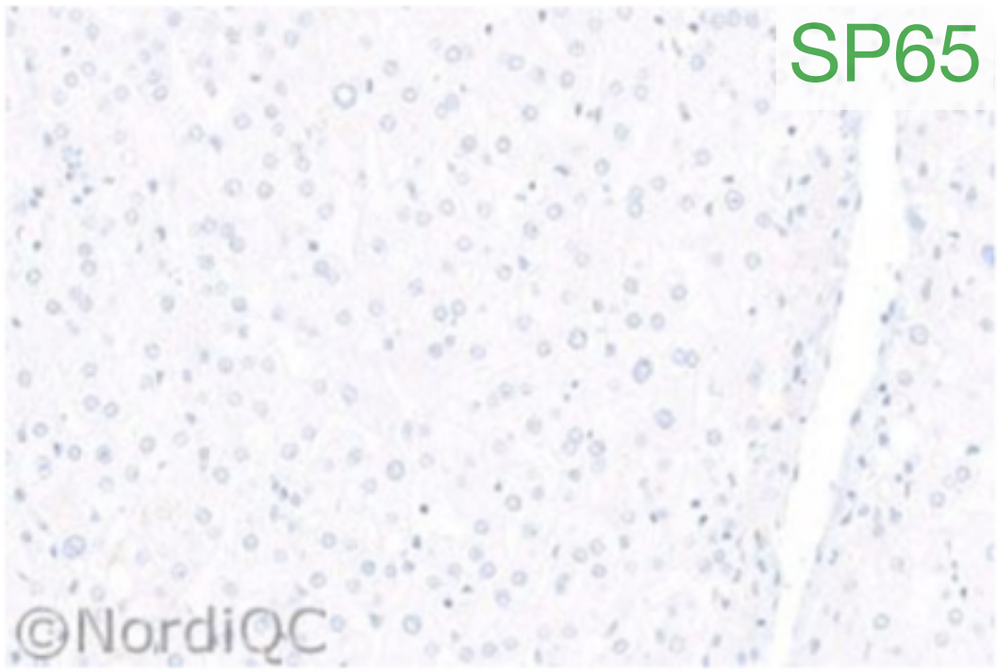

Fig. 4a. Optimal CEA staining of the liver using same protocol as in Figs. 1a - 3a based on the mAb clone CEA31. No staining reaction is seen in the Kupffer cells, leucocytes or bile canaliculi.

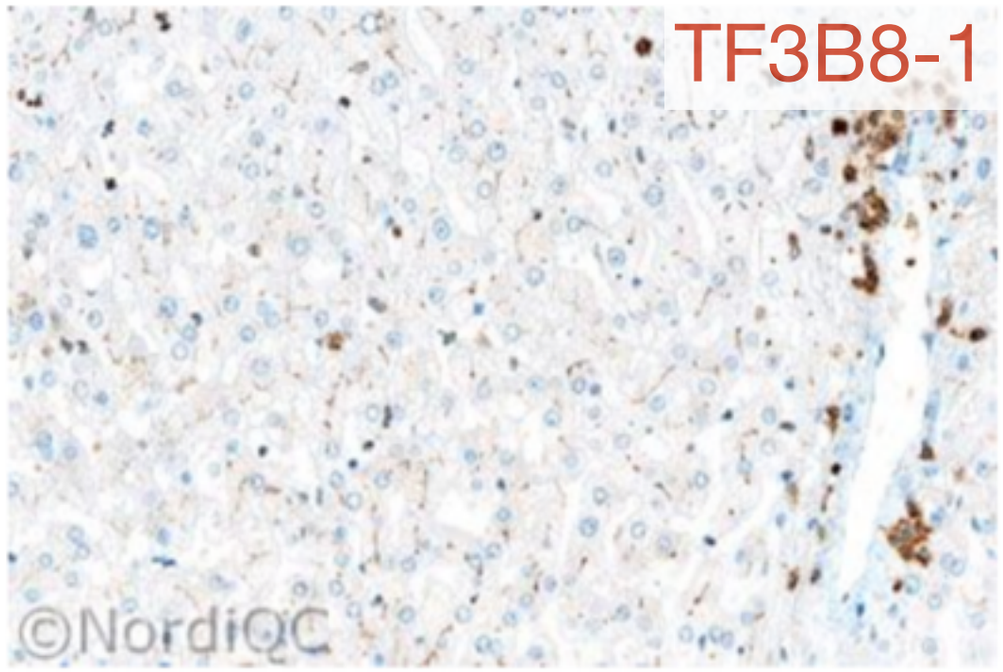

Fig. 4b. Insufficient CEA staining of the liver using the mAb clone TF3H8-1. Both the Kupffer cells, leucocytes and bile canaliculi are stained due to a cross reaction of the Ab to NCA (CEACAM6) and BGP (CEACAM1) – same field as in Fig. 4a.