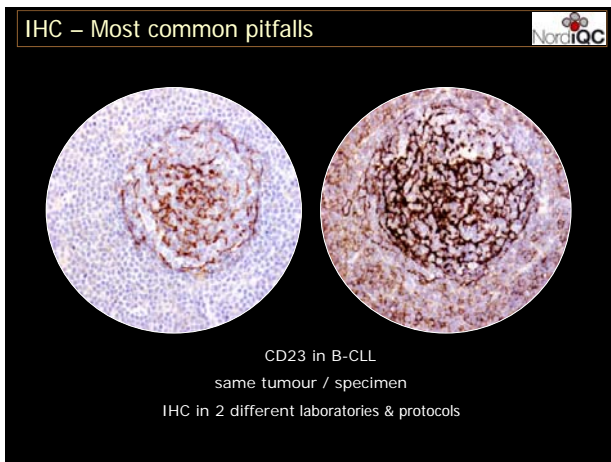
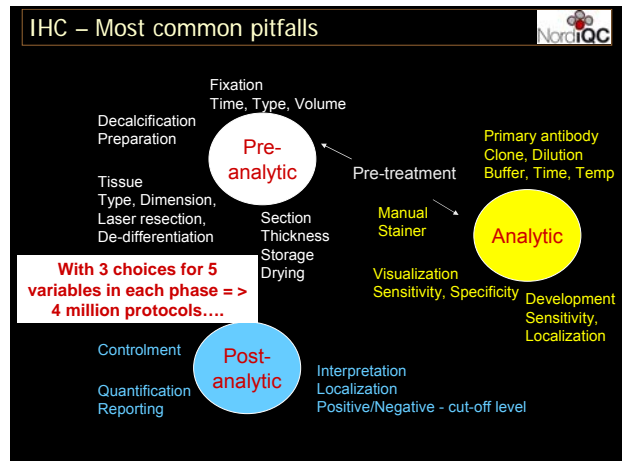


IHC – Most common pitfalls

CIQC/CAP-ACP Seminar
Diagnostic Immunohistochemistry
July 08-09, 2010

Most common laboratory pitfalls in immunohistochemistry

Søren Nielsen
Scheme Manager
NordiQC
Aalborg Hospital, Denmark



- IHC – Most common pitfalls
- The basal fundament for a technical optimal IHC performance:
- Appropriate tissue fixation and processing
 - Appropriate and efficient epitope retrieval
 - Appropriate choice of antibody/clone
 - Robust, specific & sensitive detection system
 - Appropriate choice of control material

- IHC – Most common pitfalls
- Appropriate **tissue fixation** and processing
 - Problem 1: Too short fixation in NBF
 - Problem 2: Delayed fixation in NBF
 - Too long fixation in NBF is not a problem !!!
 - Appropriate tissue fixation and **processing**
 - Problem 1: Aggressive decalcification
 - Problem 2: Deviation from SOP – e.g. section baking
- False negative or false positive*

IHC – Most common pitfalls

Arch Pathol Lab Med—Vol 131, January 2007

American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

Antonio C. Wolff, M. Elizabeth H. Hammond, Jared N. Schwartz, Karen L. Hagen, D. Craig Allred, Richard J. Coe, Mitchell Dowsett, Patrick E. Fitzgibbon, Widad M. Hanna, Amy Langer, Lisa M. McShane, Soominjung Park, Mark D. Pegram, Edith A. Perez, Michael F. Press, Anthony Rhodes, Catherine Steeghs, Shadi E. Toubi, Raymond Etkin, Gail H. Vance, Marc van de Vijver, Thomas M. Wheeler, Daniel F. Hayes

6 - 48h

Appl Immunohistochem Mol Morphol • Volume 16, Number 6, December 2008

Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry

Hadi Yazji, MD,* Clive R. Taylor, MA, MD, D.Phil,† Neal S. Goldstein, MD,‡ David J. Dabbs, MD,§ Elizabeth H. Hammond, MD,|| Bryan Hewlett, ART (CSMLS), MLT (CMLTO),* Alton D. Floyd, PhD,† Todd S. Barry, MD,|| Alvin W. Martin, MD,** Samir Bashe, MD,†† Frederick Buchner, MD,†† Richard W. Cartun, MD,‡‡ Richard N. Eisen, MD,§§ Paul E. Swanson, MD,||| Stephen M. Hewitt, MD, PhD,** Mogen Vysberg, MD,|||| and David G. Hicks, MD**** and Members of the Standardization Ad-Hoc Consensus Committee

8 - 72h

IHC – Most common pitfalls

Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma.
Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F
Am J Clin Pathol. 2003 Jul;120(1):86-92

Figure 1 Fixation, 3 h; antigen retrieval, 40 min. **Figure 2** Fixation, 6 h; antigen retrieval, 40 min. **Figure 3** Fixation, 12 h; antigen retrieval, 40 min.

Table 1
Formalin Fixation Times and Estrogen Receptor Staining With Standard, 40 Minutes of Antigen Retrieval Pretreatment

Formalin Fixation Time (Range)	Mean Q Score (Range)	Mean Difference in Q Score (Range)*	P†
3 h	2.45 (0-6)	4.35 (1-7)	<.001
6 h	6.75 (2-7)	1.14 (0-4)	<.001
8 h	6.70 (5-7)	0.04 (0-1)	.791
10 h	6.70 (5-7)	0.05 (0-1)	.791
12 h	6.70 (5-7)	0.04 (0-1)	1.000
1 d	6.70 (5-7)	0.04 (0-1)	1.000
2 d	6.70 (5-7)	0.04 (0-1)	.625
7 d	6.60 (5-7)	0.12 (0-1)	—

*Pair maximum minus blank.
†Compared with reference block fixed for a longer period.

Tissue sections of 24 ER-positive, invasive breast carcinomas were fixed for 3, 6, 8, and 12 hours and 1, 2, and 7 days. ER values were quantified using the Q score (0-7).
"The minimum formalin fixation time for reliable immunohistochemical ER results is 6 to 8 hours in our laboratory, regardless of the type or size of specimen (core biopsy or resection)".

IHC – Most common pitfalls

HUMAN PATHOLOGY Volume 33, No. 7 (July 2002)
Inadequate Formalin Fixation Decreases Reliability of p27^{Kip1} Immunohistochemical Staining: Probing Optimal Fixation Time Using High-density Tissue Microarrays
ANGELO M. DE MARZO, MD, PhD, HELEN H. FEDOR, BS, WESELY R. GAGE, AND MARK A. RUBIN, MD

Figure 2. Fraction of strongly staining prostate epithelium in TMA spots in reaction to time of fixation.

"0" day Fixation
Fixed in toto 4 hours
- sliced and processed

IHC – Most common pitfalls

CD4 – SP35

IHC – Most common pitfalls

Delayed fixation.....
Kappa; Burkitt's Lymphoma

Lambda; Burkitt's Lymphoma

By courtesy Stavanger IHC

IHC – Most common pitfalls


Aggressive decalcification

Prostate – Ki67, rmAb clone 30.9
10 % NBF 24h → 24h 10 % form. acid 10 % NBF + 10 % form. acid 24h

IHC – Most common pitfalls


Immunocytochemistry Volume 8 Issue 3 (Fall 76) Technical Article
SOP deviation
EXCESSIVE SECTION DRYING OF BREAST CANCER TISSUE PRIOR TO DEPARAFFINISATION AND ANTIGEN RETRIEVAL CAUSES A LOSS IN HER2-IMMUNO-REACTIVITY
Bent Lundgaard Hansen, Henrik Winther and Kristian Møller
DOI: 10.1080/09637460600600000
Correspondence: kml@kks.ku.dk

60°C 1h. HER-2: 3+ 80°C 16h. HER-2: 1+

IHC – Most common pitfalls 

- Appropriate and efficient epitope retrieval
 - Problem 1: Too short efficient HIER period
 - Problem 2: Use of a non-alkaline HIER buffer
 - Problem 3: Wrong epitope retrieval type

False negative or false positive

IHC – Most common pitfalls 

Heat Induced Epitope Retrieval


Optimized temperature-time-pH-buffer system

'Heating condition' = temperature × time:

121°C/1 min 100°C/20 min 95°C/40 min 60°C/24 h.

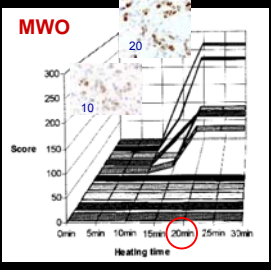
Device:
 Water bath
 MWO
 Pressure cooker
 Pressure cooker & MWO
 Autoclave
 Steam

Considerations:
 Efficiency
 Standardization
 Tissue damage
 Performance

IHC – Most common pitfalls 

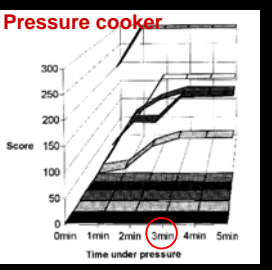
Ref. A.J. Balaton et al. Appl. Immunohistochem. 4(4):259-263,1996

MWO




Domestic
Polar Patent
Milestone


Pressure cooker



Prestige
Pascal

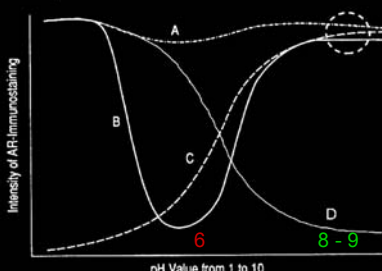
IHC – Most common pitfalls 

CD23 rmAb SP23	Ton 6h 10 % NBF	Ton 24h 10 % NBF	Ton 168h 10 % NBF
HIER CD1 short: 8 min			
HIER CD1 stand: 60 min			


IHC – Most common pitfalls 

Intensity of AIF-Immunostaining

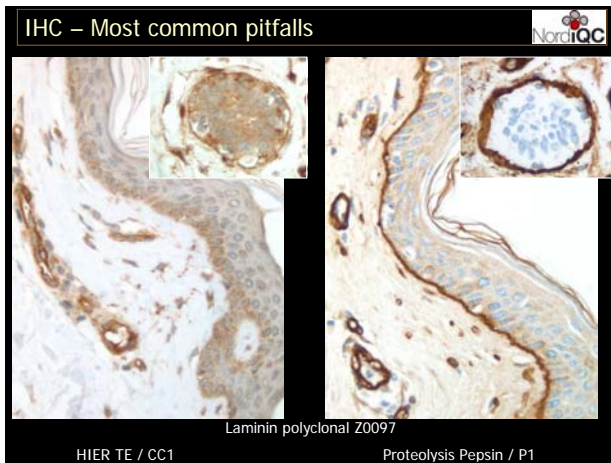
A: CD20, cl. L26
 B: Ki67, cl. MIB1
 C: HMB45
 (D: MOC31)



Modified from: Shi et al. J Histochem Cytochem 1995 43:193-201

IHC – Most common pitfalls 

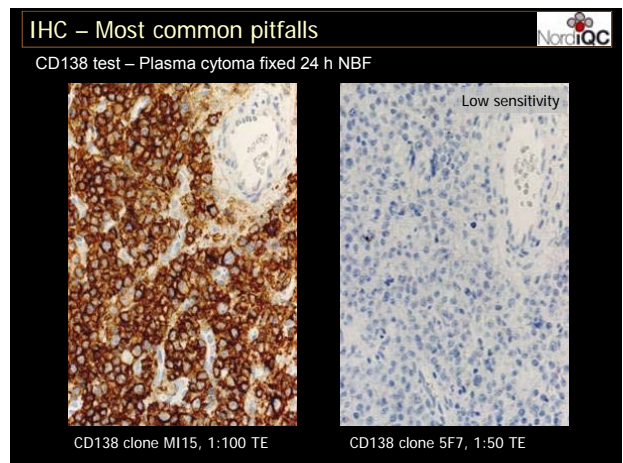
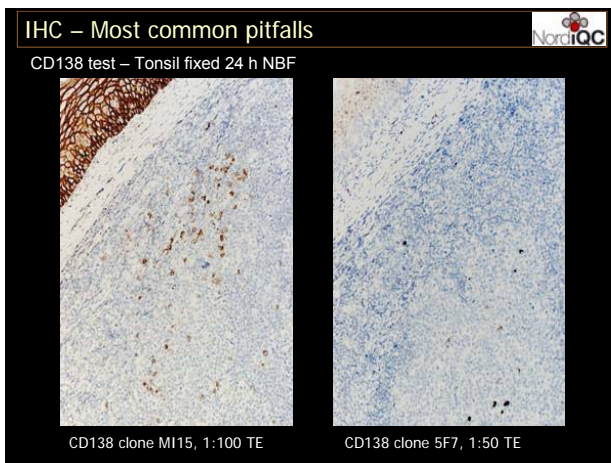
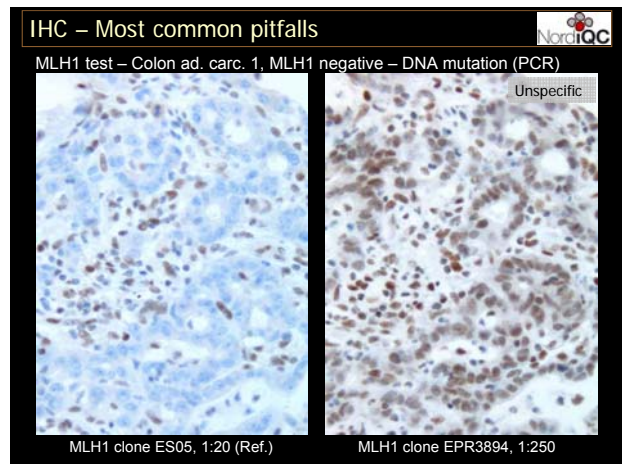
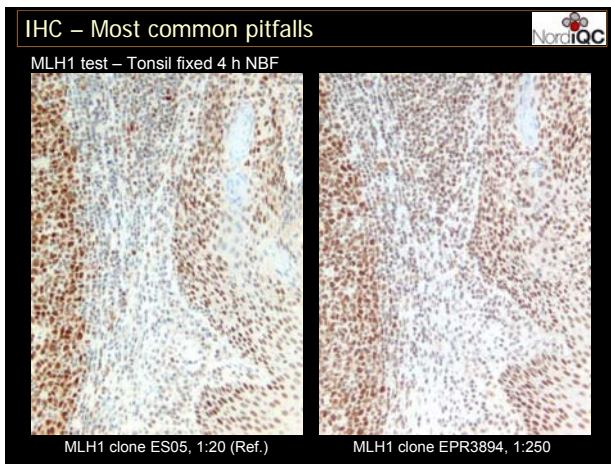
Tonsil 24 h NBF HIER Pascal PC			
TE pH 9	CD3 PS1	CD19 LE-CD19	PMS2 A16-4
CI pH 6			



IHC – Most common pitfalls

- Appropriate choice of Ab / clone
 - Problem 1: Provides low sensitivity
 - Problem 2: Provides low specificity
 - Problem 3: Selective to IHC platform

False negative or false positive



IHC – Most common pitfalls

Antigen	Clone	High expressor	Low expressor	Non expressor
CD5	CD5/54/F6	√	FN	–
CD15	C3D-1	√	FN	–
CD23	MHM6	√	FN	–
CD31	1A10	FN	FN	–
CD79a	HM57	(√)	FN	FP
CD138	5F7	(√)	FN	–
CEA	TF-3H8-1	√	√	FP
CGA	DAK A3	√	FN	–
CK-LMW	CAM 5.2	√	FN	–
CyD1	DCS6	(√)	(√)	FP
PR	SP2	√	√	FP
SYP	SY38	√	FN	–
TTF1	8G7G4/1	√	FN	FP

IHC – Most common pitfalls

Antigen	Clone	XT / Ultra	Autostainer	Bond-max
CD4	1F6, 4B12	FN (3%+H2O2)	√	√
CD4	SP35	√	√	√
CD5	4C7	FP	√	√
CD5	SP19	√	√	√
CD79a	JCB117	Weak	√	√
CD79a	SP18	Weak	√	√
ASMA	1A4	(√) Weak	√	√
BSAP	24	FN	√	- Weak
BSAP	SP34	√	√	√
BCL6	PG-B6p	FN (3%+H2O2)	√	√
BCL6	GI191E/A8	√	√	√

"IHC-Platform" depending markers

IHC – Most common pitfalls

ORIGINAL ARTICLE

Am J Surg Pathol • Volume 33, Number 3, March 2009

Identification of the Most Sensitive and Robust Immunohistochemical Markers in Different Categories of Ovarian Sex Cord-stromal Tumors

Chengquan Zhao, MD,*† Tuyenhoa N. Vinh, MD,† Kim McManus, HT (ASCP), QIHC,* David Dabbs, MD,* Ross Barner, MD,† and Russell Yang, MD,‡§

TABLE 1. Details for Antibodies Used in This Study

Antigen	Clone	Dilution	Source	Pretreatment	Detection Method and Chromogen	Autostainer
WT1	6F-8I2	Prediluted	Cell Marque; Hot Springs, AR	Protease 14 min, plus CC1 mild	Amplification kit, 1 VIEW DAB (Ventana Medical, Tucson, AZ)	BenchMark XT (Ventana Medical, Tucson, AZ)
SF-1	N1665	1:100	R&D Systems; Minneapolis, MN	TRIS/HL60 Steamer 30 min	Envision + (Dako; Carpinteria, CA)	Dako; Carpinteria, CA
MART-1; melan-A	A103	Prediluted	Ventana Medical, Tucson, AZ	CC1 standard	Enhanced V-Rol (Ventana Medical, Tucson, AZ)	BenchMark XT (Ventana Medical, Tucson, AZ)
Inhibin	R1	1:10	Dako; Carpinteria, CA	CC1 standard	1 VIEW DAB (Ventana Medical, Tucson, AZ)	BenchMark XT (Ventana Medical, Tucson, AZ)
Caldesin	Rabbit polyclonal	Prediluted	Ventana Medical, Tucson, AZ	CC1 mild	1 VIEW DAB (Ventana Medical, Tucson, AZ)	BenchMark XT (Ventana Medical, Tucson, AZ)
CD99	HH6-1.1	Prediluted	Ventana Medical, Tucson, AZ	CC2 standard	1 VIEW DAB (Ventana Medical, Tucson, AZ)	BenchMark XT (Ventana Medical, Tucson, AZ)

*Target retrieval solution (Dako, Carpinteria, CA).

IHC – Most common pitfalls

SF-1 test – mAb clone N1665 R&D systems

1:100, PC TE pH 9, EnV+, AS+ 1:100, CC1 pH 8.5, Ul.W., Ultra

IHC – Most common pitfalls

- Robust, specific & sensitive detection system
 - Problem 1: Use of biotin based detection systems
 - Problem 2: Use of detection systems with low sensitivity

False negative or false positive

IHC – Most common pitfalls

Histopathology 1997, 30, 518-522

Immunohistochemical staining of hepatocellular carcinoma with monoclonal antibody against inhibin

W.G. McCLUGGAGE, P. MAXWELL, A. PATTERSON & J.M. SLOAN*
Department of Pathology, Royal Group of Hospitals Trust, The Queen's University of Belfast, Belfast, UK

UROLOGIC ONCOLOGY

Original article
Caveolin expression in adult renal tumors

Rafael Carrion*, Beale E. Morgan*, Myron Tannenbaum*, Raoul Salup*, Michael B. Morgan*

Available online at www.sciencedirect.com

Pathology - Research and Practice 201 (2005) 801-808

PATHOLOGY RESEARCH AND PRACTICE

ORIGINAL ARTICLE
Expression of CD3 antigens in renal tubule epithelium and renal oncocytomas

Joseph Alroy*, Angelo A. Ucci*, Gino Azaboullian*, Barbara F. Banner*, John C. Chevilly*

Biotin based detection systems should not be used for IHC....!

IHC – Most common pitfalls

- EnVision
- UltraView
- UltraVision LP
- UltraVision One
- Refine
- ImPress
- Super Sensitive
- Super Picture
- Hi Def
- Quanto
- Advance
- MACH 3
-

The choice of a polymer / multimer based system

IHC – Most common pitfalls

Complexity		Quanto Hi Def. EnV.FI.+ Refine Super Sens. Ultra Vis. LP UltraView+amp		
	Ult.Vis. ONE	EnV. FI. UltraView		
	Sensitivity			
	1:25	1:50	1:150	1:500

IHC – Most common pitfalls

CD4 SP35 RTU – 32 min in primary, HIER CC1 standard:

3-step Multimer system (UltraView + Amplification) Tonsil & T-ALL

2-step Multimer system (UltraView)

IHC – Most common pitfalls

- Appropriate choice of control material
 - Problem 1: What normal tissue ?
 - Problem 2: How shall the reaction pattern be ?
 - Problem 3: Identification of critical staining quality indicators

Begin at the beginning,' the King said gravely, 'and go on till you come to the end: then stop.'

Alice in Wonderland

For IHC: *begin at the end, tune in your protocol: then stop.*

IHC – Most common pitfalls

NordiQC run 22 Chromogranin A

Fig. 1a. Optimal staining for CGA using the pAb A0430 with HIER. Left, appendix: Both the axons of the peripheral nerves and the ganglion cells in muscularis propria show a distinct granular reaction, while the smooth muscle cells are negative. Right, pancreas: Virtually all the endocrine cells of the Langerhans islet show a distinct staining.

Fig. 1b. CGA staining using an insufficient protocol (mAb D04-03) – same field as in Fig. 1a. Left, appendix: The peripheral nerves are demonstrated, but the number of positive cells is reduced and show a weaker reaction compared to the reaction in Fig. 1a. Right, pancreas: Only the glucagon producing cells show a strong and distinct staining. Also compare with Fig. 1c & 1d – same antibody.

Fig. 1c. Optimal CGA staining of the medullary thyroid carcinoma using same protocol as in Fig. 1a. The majority of the neoplastic cells show a distinct cytoplasmic staining with a scattered dot-like reaction.


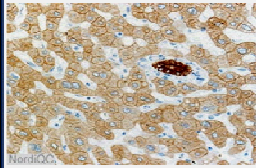
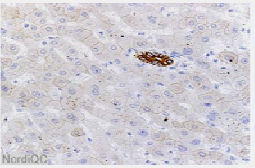
Fig. 1d. Insufficient CGA staining of the medullary thyroid carcinoma using same protocol as in Fig. 1b. Only scattered cells show a weak dot like staining. Same field as in Fig. 1c.

IHC – Most common pitfalls

MB K1	APPENDIX	TONSIL	PANCREAS	HEPAR
ASMA Anti-smooth muscle actin (Cytopl)	Smooth muscle cells in vessels and in muscle layers. Myofibroblasts lining the epithelial surface	Smooth muscle cells in vessels	Smooth muscle cells in vessels	Smooth muscle cells in the liver sinusoids
B-CATENIN Beta-catenin (Membrane)	Membranes of columnar epithelial cells. Endothelial and follicular dendritic cells	Membranes of squamous epithelial cells	Membranes of acinar epithelial cells (ducts) and endocrine cells	Hepatocytes - weak membranous
BCL2 Bcl2-oncoprotein (Cytopl)	A weak to moderate staining in the epithelial cells in the basal crypts	All peripheral lymphocytes and T-cells in germinal centres - Germinal center B-cells are negative	Weak reaction of the epithelial cells.	Weak reaction of the epithelial cells in bile ducts.
BCL6 Bcl6-protein (Nuclear)	Germinal center B-cells	Germinal center B-cells and basal squamous cells		
CD2 (Membrane)	All T-cells - Scattered intraepithelial T-cells	All T-cells - Scattered T-cells in germinal centres	T-cells	T-cells
CD3 (Membrane)	All T-cells - Scattered intraepithelial T-cells	All T-cells - Scattered T-cells in germinal centres	T-cells	T-cells
CD4 (Membrane)	80 - 80 % of T-cells	80 - 80 % of T-cells and germinal center macrophages	T-cells	Kupfer cells and sinusoidal endothelial cells
CD5 (Membrane)	All T-cells - Scattered intraepithelial T-cells	All T-cells - Scattered B-cells in the mantle zone must show a weak membranous staining	T-cells	T-cells

= CSQI
Critical Staining Quality Indicator

IHC – Most common pitfalls

CK LMW & Pan

CSQI:
Livercells

Fig. 1a. Optimal staining for CK Pan of the liver. The majority of the hepatocytes show a distinct, moderate, preferentially intracytoplasmic reactions. The bile duct is virtually unstained.

Fig. 1b. Inadequate staining for CK Pan of the liver using an insufficient protocol (same field as in Fig. 1a.). Only the bile duct is stained, while the hepatocytes are virtually unstained. The antibody is too dilute.

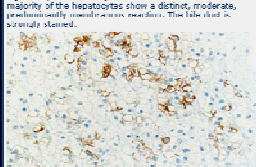
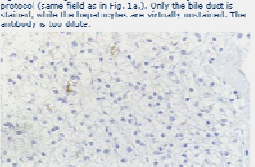



Fig. 2a. Optimal staining for CK Pan of the small cell carcinoma. About half of the neoplastic cells are moderately or strongly stained (same protocol as in Fig. 1a.).

Fig. 2b. Inadequate staining for CK Pan of the small cell carcinoma (same field as in Fig. 2a.). Only scattered neoplastic cells are weakly positive, while the majority are unstained.

IHC – Most common pitfalls





*Begin at the beginning,' the King said
gravely, and go on till you come to
the end: then stop.'*

Alice in Wonderland

For IHC: *begin at the end, tune in
your protocol: then stop.*

IHC is a challenge, technical complex but not mission impossible and rests on 5 legs

- Use proper controls
- Use a robust and specific detection system
- Use efficient HIER
- Use Ab clones, optimal for the IHC platform
- Harmonize and standardize tissue processing